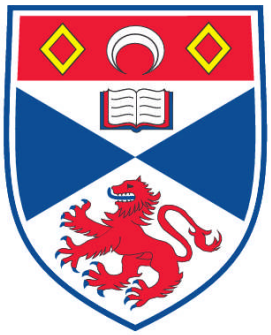
# INFLUENZA A VIRUSES AND PI3K SIGNALLING

**Benjamin G. Hale**

**A Thesis Submitted for the Degree of PhD**
**at the**
**University of St. Andrews**



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# Influenza A viruses and PI3K signalling

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A thesis submitted for the degree of Doctor of Philosophy in Molecular Virology

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*Abstract*

The influenza A virus non-structural (NS1) protein is multifunctional, and during virus-infection NS1 interacts with several factors in order to manipulate host-cell processes. This study reports that NS1 binds directly to p85β, a regulatory subunit of phosphoinositide 3-kinase (PI3K), but not to the related p85α. Expression of NS1 was sufficient to activate PI3K and cause the phosphorylation of a downstream mediator of PI3K signalling, Akt. However, in virus-infected MDCK cells, the kinetics of Akt phosphorylation did not correlate with NS1 expression, and suggested that negative-regulation of this signalling pathway occurs subsequent to ~8h post-infection.

Mapping studies showed that the NS1:p85β interaction is primarily mediated by the NS1 C-terminal domain and the p85β inter-SH2 (Src homology 2) domain. Additionally, the highly conserved tyrosine at residue 89 (Y89) of NS1 was found to be important for binding and activating PI3K in a phosphorylation-independent manner. The inter-SH2 domain of p85β is a coiled-coil structure that acts as a scaffold for the p110 catalytic subunit of PI3K. As NS1 does not displace p110 from the inter-SH2 domain, a model is proposed whereby NS1 forms an active heterotrimeric complex with PI3K, and disrupts the ability of p85β to control p110 function.

Biological studies revealed that a mutant influenza A virus (Udorn/72) expressing NS1 with phenylalanine substituted for tyrosine-89 (Y89F) exhibited a small-plaque phenotype, and grew more slowly in MDCK cells than wild-type virus. Unexpectedly, another mutant influenza A virus strain (WSN/33) expressing NS1-Y89F was not attenuated in MDCK cells, yet appeared to be less pathogenic than wild-type *in vivo*.

Overall, these data indicate a role for NS1-mediated PI3K activation in efficient influenza A virus replication. The potential application of this work to the design of novel anti-influenza drugs and vaccine production is discussed.

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I, **Benjamin G. Hale**, hereby certify that this thesis (which is approximately **57,500** words in length) has been written by me, that it is the record of work carried out by me (unless where stated), and that it has not been submitted in any previous application for a higher degree.

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| | |
|---|---|
| **% (v/v)** | percentage concentration (volume per volume). |
| **% (w/v)** | percentage concentration (weight per volume). |
| **°C** | degrees Celsius (temperature). |
| **2'5'-OAS** | 2'5'-oligoadenylate synthetase. |
| **5'UTR** | 5' untranslated region. |
| **aa** | amino acid. |
| **ABD** | adapter-binding domain (p110 subunit of PI3K). |
| **AdV** | adenovirus. |
| **ADP** | adenosine diphosphate. |
| **Akt** | protein kinase B (PKB). |
| **ATP** | adenosine triphosphate. |
| **AP** | alkaline phosphatase. |
| **BH** | B-cell receptor homology. |
| **bp** | base-pair. |
| **BSA** | bovine serum albumin. |
| **CARD** | caspase recruitment domain. |
| **cm** | centimetre. |
| **CPE** | cytopathic effect. |
| **CPI-** | canine isolate of PIV5. |
| **CPSF30** | 30 kDa subunit of the cleavage and polyadenylation specificity factor. |
| **cRNA** | anti-genomic RNA. |
| **DAPI** | 4', 6-diamidino-2-phenylindole. |
| **DCs** | dendritic cells. |
| **DMEM** | Dulbecco's modified Eagle's medium. |
| **DMSO** | dimethyl sulphoxide. |
| **dNTP** | deoxyribonucleotide triphosphate. |
| **DNA** | 2' de-oxyribonucleic acid. |
| **DTT** | dithiothreitol. |
| **dsRNA** | double-stranded RNA. |
| **EBV** | Epstein-Barr virus. |
| **ECL** | enhanced chemiluminescence. |
| **EDTA** | ethylene diamine tetra-acetic acid. |
| **EGF** | epidermal growth factor. |
| **EGTA** | ethylene glycol tetra-acetic acid. |
| **eIF2α** | eukaryotic translation initiation factor 2α. |
| **eIF4GI** | eukaryotic translation initiation factor 4G. |
| **ELISA** | enzyme-linked immunosorbant assay. |
| **EMCV** | encephalomyocarditis virus. |
| **ENaC** | epithelial sodium channel. |
| **ER** | endoplasmic reticulum. |
| **FBS** | foetal bovine serum. |
| **FITC** | Fluorescein isothiocyanate. |
| **FKHR** | Forkhead transcription factor (FOXO). |
| **g** | gram. |
| **GFP** | green fluorescent protein. |
| **GSK** | glycogen synthase kinase. |
| **GST** | glutathione-S-transferase. |

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| **HA** | influenza A virus haemagglutinin. |
| **HBV** | hepatitis B virus. |
| **HCV** | hepatitis C virus. |
| **HEPES** | N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid. |
| **HRP** | horse-radish peroxidase. |
| **IFN** | interferon. |
| **IgG** | immunoglobulin G. |
| **IL** | interleukin. |
| **IPTG** | isopropyl-β-D-thiogalactopyranoside. |
| **IRF-3** | interferon regulatory factor 3. |
| **IRS-4** | insulin receptor substrate 4. |
| **kDa** | kilodalton. |
| **M** | molar concentration (moles per litre). |
| **mAb** | monoclonal antibody. |
| **mda5** | melanoma differentiation-associated protein-5. |
| **MCS** | multiple cloning site. |
| **MDCK** | Madin-Darby canine kidney. |
| **mg** | milli-gram. |
| **MHC** | major histocompatibility complex. |
| **mins** | minutes. |
| **ml** | milli-litre. |
| **mM** | milli-molar. |
| **MOI** | multiplicity of infection. |
| **mRNA** | messenger RNA. |
| **MW** | molecular weight. |
| **NA** | influenza A virus neuraminidase. |
| **NEP** | nuclear export protein. |
| **NLS** | nuclear localisation signal. |
| **ng** | nano-gram. |
| **Ni-NTA** | nickel-nitrilotriacetic acid. |
| **nm** | nanometre. |
| **NoLS** | nucleolar localisation signal. |
| **NP** | influenza A virus nucleocapsid protein. |
| **NS1** | influenza A virus non-structural protein. |
| **OD** | optical density. |
| **ORF** | open reading frame. |
| **PAB** | poly(A) binding protein (I or II). |
| **pAb** | polyclonal antibody. |
| **PAMP** | pathogen-associated molecular pattern. |
| **PARP** | poly-(ADP-ribose) polymerase. |
| **PBS** | phosphate-buffered saline. |
| **PCR** | polymerase chain reaction. |
| **PDK1** | phosphoinositide-dependent kinase-1. |
| **PDZ** | postsynaptic density; discs large; zonula occludens-1. |
| **PFU** | plaque forming units. |
| **PH** | pleckstrin homology. |
| **pH** | $-\log_{10}[H^+]$. |

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| **p.i.** | post-infection. |
| **PI3K** | phosphoinositide 3-kinase. |
| **PIV5** | parainfluenza virus 5 (formerly known as SV5; simian virus 5). |
| **PKB** | protein kinase B (Akt). |
| **PKR** | double-stranded RNA activated protein kinase. |
| **PMSF** | phenylmethylsulphonyl fluoride. |
| **poly I:C** | polyriboinosinic-polyribocytidylic acid. |
| **PR8** | influenza A virus strain A/Puerto Rico/8/34. |
| **PtdIns** | phosphatidylinositol. |
| **PTEN** | phosphatase and tensin homologue deleted on chromosome 10. |
| **PVDF** | polyvinylidene difluoride. |
| **RIG-I** | retinoic acid-inducible gene product I. |
| **RISC** | RNA-induced silencing complex. |
| **RLU** | relative light units. |
| **RNA** | ribonucleic acid. |
| **RNAi** | RNA interference. |
| **RNP** | ribonucleoprotein complex. |
| **rpm** | revolutions per minute. |
| **RSV** | respiratory syncytial virus. |
| **SDS-PAGE** | sodium dodecyl sulphate polyacrylamide gel electrophoresis. |
| **SeV** | Sendai virus. |
| ***Sf9*** | *Spodoptera frugiperda* cell-line. |
| **SGK1** | serum- and glucocorticoid- induced kinase-1. |
| **SH2** | Src homology 2. |
| **SH3** | Src homology 3. |
| **SHIP** | SH2 domain-containing inositol 5' phosphatase. |
| **siRNA** | short-interfering RNA. |
| **STAT1** | signal transducer and activator of transcription 1. |
| **TEV** | tobacco etch virus. |
| **TLR** | Toll-like receptor. |
| **TNFα** | tumour necrosis factor α. |
| **TPCK** | L-1-tosylamido-2-phenylethylchloromethylketone. |
| **Tris** | tris-hydroxymethyl-aminomethane. |
| **Ud** | influenza A virus strain A/Udorn/72. |
| **UV** | ultra-violet. |
| **V** | volts. |
| **V5** | 14aa peptide tag derived from the PIV5 V/P proteins. |
| **Vic** | influenza A virus strain A/Victoria/3/75. |
| **vRNA** | genomic viral RNA. |
| **WSN** | influenza A virus strain A/WSN/33. |
| **WT** | wild-type. |

Chapter 1

# Introduction

## 1.1 Influenza A viruses.

### *1.1.1 Influenza and the influenza A virus.*

In humans, influenza is usually an acute, yet highly contagious, respiratory illness that mainly affects the upper respiratory tract (i.e. the nose, throat and bronchi) [reviewed in (WHO, 2003, Wright & Webster, 2001)]. Infection is often characterised by the sudden onset of fever, muscle pain, headache, and severe malaise, together with a non-productive cough, sore throat, and nasal inflammation [reviewed in (Wright & Webster, 2001)]. In temperate climates, annual epidemics (seasonal influenza; increased general incidence) occur mostly during the winter, and seriously affect an estimated 3-5 million people worldwide [reviewed in (Subbarao *et al*., 2006, WHO, 2003)]. Although most people recover within 1-2 weeks without requiring medical treatment, infections in the young, elderly, or chronically ill can sometimes result in pneumonia, hospitalisation, and death [reviewed in (Wright & Webster, 2001)]. Indeed, seasonal influenza still accounts for 250,000-500,000 deaths every year around the world (WHO, 2003).

Occasionally, influenza affects 20-40% of the world's population in a single year (Subbarao *et al*., 2006, Taubenberger & Palese, 2006). Such unpredictable influenza "pandemics" (global increases in incidence) occurred three times in the 20$^{th}$ century: 1918-1919, 1957-1958, and 1968 [reviewed in (Taubenberger & Palese, 2006, Wright & Webster, 2001)]. The 1918 ("Spanish flu") pandemic was the most severe, and is estimated to have clinically affected ~500 million people (approximately one-third of the world's population), and caused >40 million deaths [reviewed in (Taubenberger & Palese, 2006)]. The pandemics of 1957 ("Asian flu") and 1968 ("Hong Kong flu") had much lower, yet still significant, mortality rates (Subbarao *et al*., 2006, Wright &

Webster, 2001). A characteristic of all these pandemics (although most dramatically with the "Spanish flu") is their association with severe disease progression even in young healthy adults [reviewed in (Taubenberger & Palese, 2006)]. Since 1997, human infections caused by highly-pathogenic avian influenza have lead many to believe that another global pandemic is inevitable in the near future [reviewed in (Webster, 2006)].

The major causative agent of influenza is the influenza A virus, which was first isolated from humans in 1933 by Wilson Smith, Sir Christopher Andrewes, and Sir Patrick Laidlaw, whilst working together at the National Institute for Medical Research (NIMR), London, U.K. (Smith *et al*., 1933) (**Fig. 1.1**). However, Richard Shope (U.S.A.) had previously shown that swine influenza could be transmitted by filtered mucus, giving the first indication that the causative agent might be a virus (Shope & Lewis, 1931).

### *1.1.2 Nomenclature.*

The nomenclature given to individual influenza A virus strains usually includes reference to the host of origin (except in humans), geographic location of first isolation, strain number, and year of isolation. Additionally, the antigenic description (subtype) of the two viral surface glycoproteins (see below - haemagglutinin, HA; and neuraminidase, NA) is given in parentheses [reviewed in (Wright & Webster, 2001)]. For example, A/Duck/Ukraine/1/63 (H3N8) is a type A influenza virus strain first isolated from a duck in the Ukraine in 1963. Of the 16 antigenically distinct HA subtypes (H1-H16) and 9 antigenically distinct NA subtypes (N1-N9), this virus has H3 and N8. Similarly, the lack of a designated species in A/Singapore/1/57 (H2N2) indicates that this virus was isolated from a human in Singapore in 1957 (Wright & Webster, 2001).

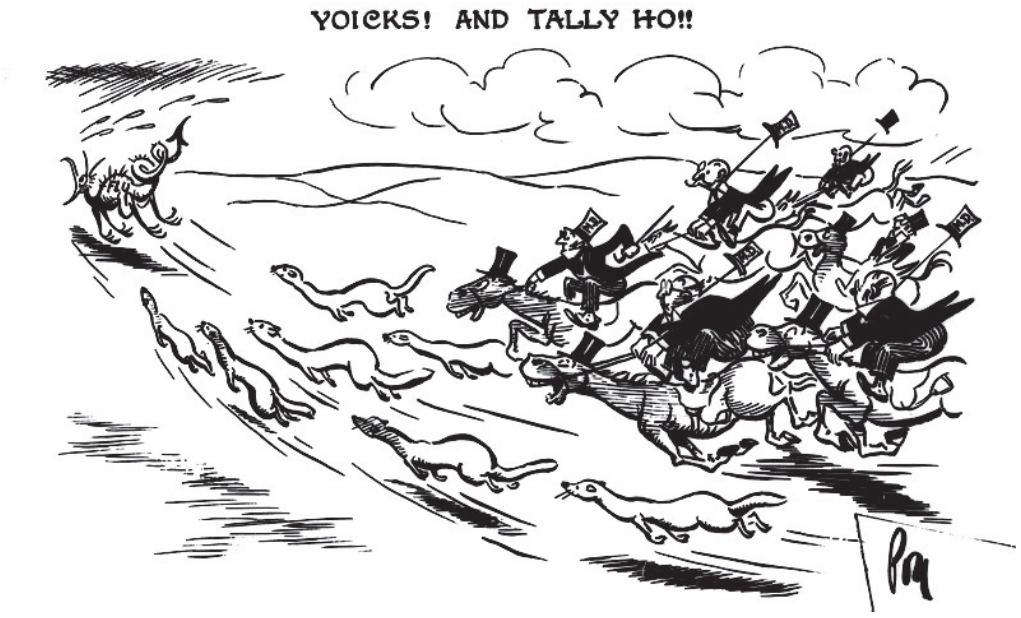


**Fig. 1.1. Cartoon from the London Evening Standard (1934).** Researchers from the National Institute for Medical Research (NIMR) are depicted on horseback "hunting down" the newly isolated human influenza A virus. The pack-dogs are represented by ferrets, which were used as the animal model for research. *Courtesy of Prof. Willie Russell, University of St. Andrews, U.K.*

### *1.1.3 Virion structure.*

Influenza A viruses are classified as members of the *Orthomyxoviridae* family, which also includes the influenza B virus, influenza C virus, and thogotovirus genera (Klenk *et al*., 2004, Lamb & Krug, 2001). The *Orthomyxoviridae* are enveloped viruses containing a segmented, single-stranded, negative-sense RNA genome (i.e. the genome is complementary to mRNA, which by convention is termed positive-sense) (Klenk *et al*., 2004). Influenza A viruses can be distinguished from influenza B and C viruses by antigenic differences, morphological features, protein-encoding mechanisms, number of RNA segments, and sequence variability in their surface glycoproteins [reviewed in (Lamb & Krug, 2001)]. Additionally, influenza A viruses naturally infect a variety of animal species, including birds, humans, pigs, horses, seals, cats, and dogs (IDSA, 2007, Wright & Webster, 2001), whilst influenza B and C viruses have so far been isolated predominantly (but not exclusively) from humans [reviewed in (Wright & Webster, 2001)].

Influenza A virus particles (virions) have a typical diameter of 80-160 nm, and are pleomorphic in shape, a characteristic dependent upon the viral strain, as well as the cell-type used for propagation (Lamb & Krug, 2001). Virions consist of a lipid envelope (derived from the host plasma membrane) out of which protrude two surface glycoproteins: haemagglutinin (HA) and neuraminidase (NA). Embedded within the lipid envelope bi-layer is the viral integral membrane protein, M2, whilst the viral matrix protein (M1) lies underneath the envelope and structurally supports the particle. Contained within this matrix shell is the segmented viral RNA genome, which (for influenza A virus) consists of eight strands. Each strand is wrapped-up in multiple copies of the nucleocapsid protein (NP), and is associated with three proteins responsible for transcription and replication: PB1, PB2, and PA (the viral polymerase

complex). Each NP-encapsidated RNA segment, together with its heterotrimeric polymerase, constitutes a viral ribonucleoprotein complex (RNP) (**Fig. 1.4A**). A schematic representation of the influenza A virus particle, together with its RNA segments and encoded proteins, is shown in **Fig. 1.2**.

Although the influenza A virus genome consists of only 8 single-stranded, negative-sense RNA segments, up to 11 viral proteins are produced during infection [reviewed in (Garcia-Sastre, 2005, Lamb & Krug, 2001)]: segment 1, PB2; segment 2, PB1 and PB1-F2; segment 3, PA; segment 4, HA; segment 5, NP; segment 6, NA; segment 7, M1 and M2; segment 8, NS1 and NEP). The varied roles of these proteins are briefly outlined below in the context of the virus replication cycle (summarised in **Fig. 1.3**). Interestingly, a putative additional ORF in the positive-sense of segment 8 has recently been identified (Zhirnov *et al*., 2007). This ORF could encode a hypothetical negative-sense protein (NSP) of ~25 kDa, and might share some homology with a mitochondrial transmembrane proton channel (Zhirnov *et al*., 2007). However, the actual expression of NSP in influenza A virus-infected cells has yet to be demonstrated.

### *1.1.4 Replication cycle – virus entry.*

Virus particles bind to the apical surfaces of polarized epithelial cells via an interaction between the viral HA spike glycoprotein and sialic acid residues linked to the cell membrane [reviewed in (Skehel & Wiley, 2000, Whittaker & Digard, 2006)]. Receptor-mediated endocytosis causes internalisation of the whole virus particle into intracellular pre-lysosomal, low pH vesicles [reviewed in (Whittaker & Digard, 2006)]. Such internalisation at the cell periphery requires the host GTPase dynamin (Roy *et al*., 2000), and involves actin rearrangements (Lakadamyali *et al*., 2003). The virus particle is then rapidly trafficked through both early- and late- endosomes (of increasingly

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**Fig. 1.2. Schematic representation of the influenza A virus particle.** The 8 single-stranded negative-sense RNA segments are encapsidated by nucleoprotein (NP) and are each associated with a single polymerase complex (consisting PB1, PB2, and PA). These ribonucleoproteins (RNPs) interact with the M1 protein (which forms a matrix underneath the host-derived lipid bi-layer), and probably the NEP (NS2) protein. The haemagglutinin (HA) and neuraminidase (NA) glycoproteins, together with the M2 ion channel, span the membrane and likely form structural contacts with M1. NS1 and PB1-F2 are only found in infected cells, therefore they are not represented on the diagram. The proteins encoded by each genomic segment are indicated. *Diagram modified from Lamb & Krug, 2001.*

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**Fig. 1.3. Schematic representation of the influenza A virus replication cycle.** See text for details. *Diagram reproduced from Lamb & Krug, 2001.*

acidic pH, regulated by endosomal proton pumps) in a dynein-dependent manner to the perinuclear region (Lakadamyali *et al*., 2003, Lamb & Krug, 2001, Whittaker & Digard, 2006). A number of cellular kinases have also been implicated in this process, including protein kinase C (Sieczkarski *et al*., 2003), and phosphoinositide 3-kinase (Ehrhardt *et al*., 2006).

In order to release the viral genomic segments into the host-cell cytoplasm, the viral membrane and that of the late-endosome must first fuse. Fusion is mediated by a low-pH-induced conformational change in the viral HA protein, which usually requires an optimum pH of ~5.0-5.5, but can be temperature dependent [reviewed in (Skehel & Wiley, 2000)]. Mature endosomal compartments have an acidic pH of between 4.5 and 5.5, therefore localisation of influenza A virus particles into these specific vesicles is the physiological trigger for membrane fusion, and acts to direct virion release to the perinuclear region [reviewed in (Lamb & Krug, 2001, Whittaker & Digard, 2006)]. This sorting event alone is insufficient to deliver the individual viral genomic segments into the cytoplasm. However, the low-pH-activated ion channel activity of the viral M2 protein permits the flow of protons from the lumen of the endosomal compartment into the virion (Pinto *et al*., 1992), thus acidifying the virion interior and consequently disrupting protein:protein interactions between the viral RNPs and M1, as well as HA and M1 (Bui *et al*., 1996, Lamb & Pinto, 2006). Dissociation of these interactions allows full egress of viral RNPs from the disassembled virion into the cytoplasm subsequent to membrane fusion [reviewed in (Lamb & Pinto, 2006)]. Regulation of virus uncoating/RNP release by ensuring prior translocation to low-pH endosomes near the perinuclear region efficiently targets RNPs to the site of transcription and replication (i.e. the cell nucleus, see below). This mechanism also potentially

minimises the contact time of "naked" RNPs with the cytoplasm, which may be beneficial to the virus for evading some host-cell viral sensors.

### *1.1.5 Replication cycle – RNP nuclear import.*

Unlike most RNA viruses, transcription and replication of the influenza A virus genome occurs in the nucleus of the infected cell (Herz *et al*., 1981, Jackson *et al*., 1982). It is not entirely clear why this should occur, although specific transcriptional requirements of the virus have been postulated to be determinants: initiation of viral mRNA synthesis requires the capped 5' ends of host mRNAs to act as primers (Plotch *et al*., 1981), and two viral mRNAs require splicing [reviewed in (Lamb & Krug, 2001)]. Thus, it has been suggested that localising within the nucleus may allow close contact between viral replication centres and high concentrations of essential host factors, such as cellular mRNAs (their export is blocked by the viral NS1 protein, see below), and the cellular splicing machinery (Boulo *et al*., 2007, Lamb & Krug, 2001). However, viruses within the *Bunyaviridae* and *Arenaviridae* families (which possess segmented, negative-sense, RNA genomes) also require host-derived 5' cap structures to initiate mRNA transcription, yet these viruses replicate in the cytoplasm [reviewed in (Buchmeier *et al*., 2001, Schmaljohn & Hooper, 2001)]. Thus, as mRNA splicing is a common protein-encoding mechanism among all members of the *Orthomyxoviridae* family, it seems likely that the nuclear localisation of their replication is due to the requirement for splicing. In this regard, it is interesting to note that viruses of the *Bunyaviridae* and the *Arenaviridae* do not produce spliced mRNAs (and undergo cytoplasmic replication), whilst Borna disease virus (BDV; *Bornaviridae*), which possesses a non-segmented, negative-sense RNA genome, does produce spliced mRNAs and replicates in the cell nucleus (Carlos de la Torre, 2001).

After uncoating, all viral RNPs are actively transported into the nucleus [reviewed in (Whittaker & Digard, 2006)]. NP and the three polymerase subunits all contain nuclear localisation signals (NLSs) (Davey *et al*., 1985, Mukaigawa & Nayak, 1991, Nath & Nayak, 1990, Nieto *et al*., 1992). NP has multiple NLSs, and is both necessary and sufficient for mediating the nuclear import of each viral RNP segment, probably via its interaction with the cellular importin-α/β family (O'Neill *et al*., 1995, O'Neill & Palese, 1995, Whittaker & Digard, 2006). Within the nucleus, RNPs strongly associate with insoluble "nuclear matrix" or chromatin components (Bui *et al*., 2000, Whittaker & Digard, 2006).

### *1.1.6 Replication cycle – viral mRNA synthesis.*

Influenza A virus RNA synthesis requires four proteins, which together with viral RNA, make up the functional RNP: NP, PA, PB1, and PB2 (Huang *et al*., 1990, Portela & Digard, 2002) (**Fig. 1.4A**). Genomic viral RNA (vRNA) has conserved sequences at the 5' and 3' termini that share some sequence complementarity (Desselberger *et al*., 1980, Robertson, 1979, Skehel & Hay, 1978), and thus cause base-pairing leading to the proposed formation of RNA structures, such as panhandles (Hsu *et al*., 1987), forks (Fodor *et al*., 1995), or corkscrews (Flick *et al*., 1996). Each vRNA is encapsidated by NP, and is associated with a single heterotrimeric polymerase complex [reviewed in (Elton *et al*., 2006)]. The incoming RNP (i.e. from the virion) is all that is required to transcribe mRNA, as long as an appropriate 5' host-derived capped RNA (to act as a primer, see below) is available (Bouloy *et al*., 1978, Krug *et al*., 1979, Plotch & Krug, 1977).

During mRNA synthesis (**Fig. 1.4B**), the polymerase (via PB1) binds to the 5' end of the vRNA segment and thereby stimulates the cap-binding activity of PB2, which

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**Fig. 1.4. Structure and function of the influenza A virus RNP.** (**A**) **Cartoon model of a single RNP.** The single-stranded vRNA (black line) is coiled into a panhandle due to complementary base-pairing between the 5` and 3` ends, and is encapsidated by nucleoprotein (NP; blue spheres). The heterotrimeric polymerase complex (PB1, PB2, and PA) binds to the RNP at the site of RNA duplexing. (**B**) **Model of mRNA synthesis.** Interaction of the polymerase complex with the 5` end of vRNA stimulates binding to host-cell mRNAs. Endonuclease activity is stimulated by binding of the 3` end of vRNA to the polymerase, such that a short, capped, host-derived RNA primer (blue line) is generated, which acts to initiate transcription and viral mRNA synthesis (green line). Termination of transcription (and addition of the poly(A) tail) occurs via a steric block to polymerase progression and a "stuttering" mechanism. (**C**) **Possible mechanisms of cRNA synthesis (primer-independent transcription).** (**i**) Initiation is promoted by NP "modifying the polymerase" by a protein:protein interaction. (**ii**) Initiation is promoted by NP disrupting the panhandle structure. For cRNA (and subsequent vRNA) synthesis, soluble NP is required in order to encapsidate newly transcribed RNA. The polymerase must "read-through" the poly(A) signal so as to produce a full-length genome copy. *Diagrams modified from Portela & Digard, 2002.*

allows the polymerase to bind host-cell mRNAs produced as RNA polymerase II transcripts (Elton *et al*., 2006, Fodor *et al*., 1994, Lamb & Krug, 2001, Li *et al*., 1998b). Subsequent interaction of the 3' end of the vRNA template with the viral polymerase triggers cleavage of the host-cell mRNA by PB1, thus retaining the 5'-methylated host cap structure together with 9-15 cellular mRNA nucleotides (Cianci *et al*., 1995, Hagen *et al*., 1994, Li *et al*., 2001, Plotch *et al*., 1981). Interestingly, viral capped mRNAs are protected from cleavage when they interact with the viral polymerase (Shih & Krug, 1996b). The cleaved host cap structure acts as a primer for viral transcription initiation by PB1 (Elton *et al*., 2006, Lamb & Krug, 2001, Shaw & Lamb, 1984), which is enhanced by the physical interaction of PB2 with the cap structure (Penn & Mahy, 1984). Addition of ribonucleotides (elongation) occurs according to the viral template (vRNA), and at some point PB2 dissociates from the cap (Elton *et al*., 2006, Portela & Digard, 2002).

Synthesis of viral mRNA terminates at a stretch of uridine (U) residues ~15-22 nucleotides from the 5' end of the vRNA, where steric hindrance of the viral polymerase probably causes it to reiteratively copy the poly(U) tract ("stutter") resulting in the generation of a poly(A) tail (Elton *et al*., 2006, Fodor *et al*., 1994, Lamb & Krug, 2001, Portela & Digard, 2002) (**Fig. 1.4B**). It is unclear what role, if any, is played by the PA polymerase subunit in viral mRNA synthesis, although it may contribute to the nuclear localisation of PB1, as well as efficient endonuclease activity and/or elongation (Fodor *et al*., 2002, Fodor *et al*., 2003, Fodor & Smith, 2004).

Although pre-mRNAs of most eukaryotic cells are fully spliced (i.e. introns removed) in order to generate functionally mature mRNAs, only two influenza A virus mRNAs require splicing: NS1 and M1 (Inglis *et al*., 1979, Inglis & Brown, 1981, Lamb &

Krug, 2001). Additionally, unlike complete cellular mRNA splicing, the splicing of viral NS1 and M1 mRNAs (to produce NEP and M2 mRNAs, respectively) appears to be regulated such that spliced mRNA is only ~10% that of unspliced mRNA (Lamb *et al*., 1980, Lamb & Lai, 1981). M1 mRNA splicing is controlled by both the viral polymerase and cellular splicing factors, such as the serine/arginine-rich splicing factor SF2/ASF (Shih & Krug, 1996a, Shih *et al*., 1995). Splicing of NS1 mRNA to produce NEP mRNA can be regulated by the NS1 protein itself (Garaigorta & Ortin, 2007). It is not exactly clear how viral mRNAs (both spliced and un-spliced) can specifically leave the nucleus to be translated into protein, particularly as NS1 has been reported to block the nucleo-cytoplasmic transport of cellular mRNAs (Fortes *et al*., 1994, Qiu & Krug, 1994, Satterly *et al*., 2007).

### *1.1.7 Replication cycle – genome replication.*

The replication of genomic virion RNA (vRNA) occurs in two stages: the initial synthesis of "template" RNA that is complementary to full-length vRNA (i.e. a positive-sense strand: cRNA), and the subsequent copying of cRNA into new vRNAs [reviewed in (Elton *et al*., 2006, Lamb & Krug, 2001, Portela & Digard, 2002)] (**Fig. 1.4C**). Although viral mRNAs and cRNAs are both positive-sense, mRNA cannot act as a replicative intermediate in the formation of new vRNAs as it has a 5' host-derived cap and is truncated relative to the full-length genomic segments. Thus, the viral polymerase initiates cRNA synthesis in a cap-independent manner, and must "read-through" the polyadenylation signal at the 5' end of vRNA (Elton *et al*., 2006). Additionally, cRNA (but not mRNA) is encapsidated by NP. Unlike mRNA transcription, the incoming viral RNP alone is insufficient to stimulate cRNA synthesis (Plotch & Krug, 1977, Skorko *et al*., 1991), and evidence suggests that an initial round of mRNA and protein synthesis is necessary prior to initiation of cRNA synthesis

(Beaton & Krug, 1984, del Rio *et al*., 1985, Hay *et al*., 1977, Nagata *et al*., 1989, Shapiro & Krug, 1988, Takeuchi *et al*., 1987).

It is not clear what viral components constitute the replication polymerase complex. Soluble viral NP appears to be a major factor regulating replication, and its involvement would correlate with the requirement for *de novo* protein synthesis. Soluble NP may simply act in a concentration-dependent manner (i.e. stabilising cRNA transcripts when high enough levels of NP protein are available) (Beaton & Krug, 1984, Shapiro & Krug, 1988), by modifying the transcription template (Fodor *et al*., 1994, Hsu *et al*., 1987), or by directly modifying polymerase function (i.e. "switching" protein:protein interactions between NP and PB1/PB2 in order to favour a replication polymerase over a transcription polymerase) (Elton *et al*., 2006, Medcalf *et al*., 1999, Poole *et al*., 2004). Additionally, all three polymerase subunits (PA, PB1, PB2) are required for efficient cRNA synthesis (Nakagawa *et al*., 1996, Perales & Ortin, 1997, Perales *et al*., 2000), and it is possible (although uncertain) that PA acts in a proteolytic manner to clip components of the transcription polymerase, and thus convert it into a replication polymerase (Naffakh *et al*., 2001, Perales *et al*., 2000). Possible roles of numerous host-cell proteins (including hCLE, HSP90 and UAP56/BAT1; a DEAD-box ATP-dependent RNA helicase) in viral RNA synthesis have not been fully clarified (Huarte *et al*., 2001, Mayer *et al*., 2007, Momose *et al*., 2001, Momose *et al*., 2002).

Both PA and soluble NP are important for the synthesis of vRNA from cRNA (Elton *et al*., 2006, Shapiro & Krug, 1988). NP is probably required for encapsidation of the nascent RNA (Shapiro & Krug, 1988). NS1 protein has also been suggested to be an additional cofactor that directly interacts with the viral replication polymerase complex

and affects accumulation of vRNA, but not cRNA (Falcon *et al*., 2004, Marion *et al*., 1997b). Intriguingly, vRNA only appears to be synthesised at sites of insoluble "nuclear matrix" within the nucleus, whilst both mRNA and cRNA synthesis is detectable in both soluble and insoluble nuclear fractions (Lopez-Turiso *et al*., 1990).

### *1.1.8 Replication cycle –virion assembly and budding.*

Nuclear export of newly synthesised negative-sense vRNPs to the cytoplasm is an active and regulated process [reviewed in (Whittaker & Digard, 2006)]. Early in infection these RNPs act as templates for mRNA transcription, therefore their premature exit from the nucleus is likely to be detrimental. NEP (nuclear export protein (O'Neill *et al*., 1998); formerly designated non-structural protein 2 (NS2) (Lamb *et al*., 1978)) is a ~14 kDa protein found in both virions and virus-infected cells, and is essential for vRNP nuclear export (Boulo *et al*., 2007, Neumann *et al*., 2000, Richardson & Akkina, 1991, Yasuda *et al*., 1993).

NEP binds directly to the viral M1 protein (Ward *et al*., 1995, Yasuda *et al*., 1993), and can also form complexes with members of the cellular nucleoporin family (i.e. Rab/hRIP1, yRIP1, yNup100 and yNup116 (O'Neill *et al*., 1998)), as well as hCRM1 and Ran-GTP (Akarsu *et al*., 2003, Neumann *et al*., 2000, Paragas *et al*., 2001). At late times in the infection cycle, a nuclear fraction of M1 binds and inhibits the transcriptional activity of vRNPs (Perez & Donis, 1998, Zvonarjev & Ghendon, 1980). The interaction between M1 and NEP then links vRNPs to the cellular nucleo-cytoplasmic transport machinery, which contributes to the nuclear export of vRNPs prior to virion formation at the plasma membrane (Akarsu *et al*., 2003, Neumann *et al*., 2000, O'Neill *et al*., 1998). However, NP [which can also bind hCRM1 (Akarsu *et al*., 2003, Elton *et al*., 2001)] appears to have a direct (and possibly NEP-independent) role

in regulating vRNP nuclear export (Bui *et al*., 2000, Elton *et al*., 2001), suggesting that additional (and possibly overlapping) vRNP nuclear export mechanisms exist. Furthermore, mitogen-activated protein kinase and caspase cascades may play some role in mediating the exit signal (Pleschka *et al*., 2001, Wurzer *et al*., 2003). Once in the cytoplasm, newly synthesised RNPs are directed to the cell surface, either via interaction with the M1 protein (which associates with membranes), or directly via cytoskeletal elements (Digard *et al*., 1999, Simpson-Holley *et al*., 2002, Whittaker & Digard, 2006).

In infected epithelial cells, virion assembly is polarised and only occurs at the apical side of the cell (Boulan & Sabatini, 1978). Such polarity is directed by the independent targeting of the viral HA, NA, and M2 proteins to the apical plasma membrane (Hughey *et al*., 1992, Kundu *et al*., 1996, Roth *et al*., 1983, Whittaker & Digard, 2006). NA, M2, and the precursor HA ($HA_0$) are synthesised on membrane-bound ribosomes and translocated across the membrane into the lumen of the endoplasmic reticulum (ER). In the ER, these proteins are all individually and correctly folded (Lamb & Krug, 2001), and (in some cell-types) $HA_0$ molecules with a "multi-basic" cleavage site are cleaved by the intra-cellular protease furin to produce the disulphide-linked, fusion-ready, $HA_1$ and $HA_2$ (Stieneke-Grober *et al*., 1992). In this case, activity of the viral M2 ion channel is required in the ER/trans-Golgi network to regulate the intra-compartmental pH, thus preventing the conformational change in HA that is associated with fusion (Grambas & Hay, 1992, Sakaguchi *et al*., 1996, Takeuchi & Lamb, 1994, Takeuchi *et al*., 1994). However, for $HA_0$ molecules with only a single arginine residue at this cleavage site (most influenza A virus strains), $HA_0$ cleavage can only occur in response to extra-cellular proteases, thus ensuring that full

maturation of the virion (and infectivity) is achieved subsequent to viral exit from the cell [reviewed in (Lamb & Krug, 2001)].

Evidence indicates that the HA and NA glycoproteins are specifically sorted to sphingolipid- and cholesterol- enriched lipid raft domains within the apical plasma membrane (Nayak & Barman, 2002, Scheiffele *et al*., 1997). Such domains are dynamic platforms where signal transduction events can be initiated, and from where a number of enveloped viruses bud [reviewed in (Suomalainen, 2002)]. Indeed, accumulation of viral HA within these surfaces appears to trigger a protein kinase Cα (PKCα)-dependent Raf/MEK/ERK signalling cascade that initiates nuclear export of vRNPs ready for virus assembly (Marjuki *et al*., 2006). Budding from these rafts also means that influenza A virus particles contain high concentrations of HA and NA surface glycoproteins, and subsequently have increased infectivity (Scheiffele *et al*., 1999, Takeda *et al*., 2003, Zhang *et al*., 2000b).

The mechanics of influenza A virus budding from the infected cell likely requires host-cell "pinching machinery", as well as interactions between the cytoplasmic tails of the viral integral membrane proteins (HA, NA, and M2) and the internal proteins/vRNPs (Chen *et al*., 2007, Iwatsuki-Horimoto *et al*., 2006, Jin *et al*., 1997, Lamb & Krug, 2001, McCown & Pekosz, 2005, Zhang *et al*., 2000a, Zhang *et al*., 2000b). M1 can associate with the sites of budding (lipid rafts) via the cytoplasmic tails of HA and/or NA (Barman *et al*., 2001, Zhang *et al*., 2000b), and this could aid in the localisation of vRNPs into the assembly sites [reviewed in (Lamb & Krug, 2001)]. The vRNP segments are selectively (rather than randomly) incorporated into each budding virus particle, as efficient infectious virion formation appears to require all 8 segments (Duhaut & McCauley, 1996, Fujii *et al*., 2003, Muramoto *et al*., 2006, Noda *et al*.,

2006, Odagiri & Tashiro, 1997). The mechanism of vRNP selection is unclear, but may be hierarchical and involve unique segment-specific packaging sequences/structures, similar to those already determined in the coding and non-coding regions of the HA, NA, PB1, PB2, PA, and NS segments (Fujii *et al*., 2003, Fujii *et al*., 2005, Gog *et al*., 2007, Marsh *et al*., 2007, Muramoto *et al*., 2006). The functional role of neuraminidase (NA) activity during viral budding is to catalyse the removal of sialic acid from the surface of infected cells (and from HA and NA molecules), thus allowing efficient virus egress, and preventing self-aggregation of progeny virions (Lamb & Krug, 2001, Palese *et al*., 1974). Additionally, NA activity may permit transport of virions through the mucin layer of the respiratory tract (Lamb & Krug, 2001).

### *1.1.9 "Accessory" proteins of influenza A virus.*

During infection, two viral proteins are expressed that are not packaged into budding virions: NS1 and PB1-F2. NS1 is described in detail in the following section (**1.2**). PB1-F2 is a 87 amino-acid protein encoded by an alternate (+1) reading frame within the PB1 gene (segment 2) (Chen *et al*., 2001). It is likely that both PB1 and PB1-F2 are translated from the same bicistronic mRNA by ribosomal scanning (Chen *et al*., 2001). PB1-F2 is maximally expressed at ~5h post-infection, and localizes to mitochondrial membranes (Chen *et al*., 2001, Gibbs *et al*., 2003, Yamada *et al*., 2004).

PB1-F2 expression alone can induce the formation of non-specific pores within membranes (Chanturiya *et al*., 2004), and PB1-F2 interacts with the mitochondrial apoptotic mediators ANT3 and VDAC1 (Zamarin *et al*., 2005), thus sensitising some cell-types (mainly monocyte-derived) to apoptotic cell-death (Chanturiya *et al*., 2004, Chen *et al*., 2001, Gibbs *et al*., 2003, Lamb & Takeda, 2001, Zamarin *et al*., 2005). However, the PB1-F2 ORF is not conserved in all influenza A virus genomes (Lamb &

Takeda, 2001, Obenauer *et al*., 2006, Zell *et al*., 2007), and can be "knocked-out" without seriously attenuating virus replication (or virus-induced cell-death) in a number of tissue-culture cell-lines (e.g. MDCK, MBCK, A549, HeLa) (Chen *et al*., 2001, Zamarin *et al*., 2006), and in an *in vivo* mouse model (Zamarin *et al*., 2006). Despite this, given the pro-apoptotic effect of PB1-F2 is most pronounced in "immune" monocyte/macrophage cells (Chen *et al*., 2001), and data from reassortment viruses indicate that PB1-F2 might contribute towards viral pathogenesis (Zamarin *et al*., 2006), it has been suggested that PB1-F2 acts to limit efficient immune-cell mediated virus clearance *in vivo* (Zamarin *et al*., 2006).

## 1.2 The non-structural (NS1) protein.

NS1 protein is widely regarded as a multifunctional virulence factor, and is expressed at high levels in virus-infected cells [reviewed in (Garcia-Sastre, 2005, Kochs *et al*., 2007b, Li *et al*., 2006b, Noah & Krug, 2005)]. It is not a structural component of the virion (Krug & Etkind, 1973, Lazarowitz *et al*., 1971). During infection, NS1 performs a plethora of activities that each contribute to efficient virus replication: temporal regulation of viral RNA synthesis (Min *et al*., 2007), enhancement of viral mRNA translation (Burgui *et al*., 2003), regulation of virus particle morphogenesis (Garaigorta *et al*., 2005), and suppression of the host immune/apoptotic responses (Garcia-Sastre, 2005, Lowy, 2003, Nemeroff *et al*., 1998, Satterly *et al*., 2007). The protein:protein and protein:RNA interactions that mediate these functions are detailed below.

### *1.2.1 Synthesis of the NS1 protein.*

The smallest genomic segment of influenza A virus (segment 8) is transcribed into two separate mRNAs, which encode the non-structural (NS1) and nuclear export (NEP) viral polypeptides (Inglis *et al*., 1979, Lamb & Choppin, 1979, Lamb & Krug, 2001). A co-linear mRNA transcript directly codes for NS1, whilst NEP is translated from spliced mRNA (Lamb & Lai, 1980). Both mRNA species share a 56-nucleotide viral leader sequence, which contains the AUG initiation codon and encodes 9 N-terminal amino acid residues common to NS1 and NEP (Lamb & Lai, 1980). Removal of the 473-nucleotide intron in order to form NEP mRNA means that after the 9 shared residues, translation of NEP continues in the +1 ORF relative to NS1 (Lamb & Lai, 1980). Thus, after the splicing site, the NS1 and NEP ORFs overlap by the equivalent of ~70 residues (Lamb & Lai, 1980). In infected cells, the steady-state

amount of spliced NEP mRNA is only ~10% that of unspliced NS1 mRNA (Lamb *et al*., 1980). Regulation of this splicing is thought to be controlled, in part, by the viral NS1 protein itself (Garaigorta & Ortin, 2007).

### *1.2.2 Biochemistry of NS1 protein.*

NS1 is a ~26 kDa protein, and has a strain specific length of 230-237 amino acids (Lamb & Krug, 2001). A proportion of the NS1 protein population appears to be phosphorylated within infected cells (Petri & Dimmock, 1981, Petri *et al*., 1982, Privalsky & Penhoet, 1978, Privalsky & Penhoet, 1981). Comparison of NS1 proteins derived from a range of human and animal influenza A viruses has revealed considerable differences in charge and phosphorylation between strains (Petri *et al*., 1982). NS1 proteins derived from "avian" influenza A viruses are predominantly acidic, whereas "human" NS1 proteins are generally basic (Petri *et al*., 1982). This is due primarily to sequence differences, but is intriguing given the high degree of reported charge homogeneity among other viral proteins (PA, PB1, PB2, NP, M1, HA, NA) (Petri *et al*., 1982).

Phosphorylation of NS1 is also highly virus-specific, and some NS1 proteins (almost exclusively the acidic "avian" ones) were not found to be phosphorylated in tissue culture studies (Petri *et al*., 1982). For NS1 proteins that can be phosphorylated, there are at least two distinct sites of modification (Privalsky & Penhoet, 1981), and these have recently been speculated to be serine-195 and threonine-197 (Bornholdt & Prasad, 2006). Mono- or di- phosphorylation of NS1 sub-populations occurs rapidly after translation, and appears to occur in the cell nucleus (Privalsky & Penhoet, 1981). This is compatible with evidence that indicates NS1 can be phosphorylated *in vitro* by a kinase activity associated with purified virion RNPs, which would normally be

located within the nucleus of infected cells (Skorko *et al*., 1991). It is still largely unknown if any physiological role for NS1 phosphorylation exists.

NS1 contains two distinct structural/functional domains: an N-terminal RNA-binding domain (residues 1-73), which binds with low affinity to double-stranded RNA (dsRNA) in a sequence independent manner (Chien *et al*., 2004, Qian *et al*., 1995); and a C-terminal "effector" domain (residues 74-237), which mediates interactions with a number of host-cell proteins (Krug *et al*., 2003, Qian *et al*., 1994) (see below). *In vitro* and *in vivo* studies show that NS1 exists as a homodimer, and both the RNA-binding domain and effector domain contribute to functional multimerisation (Nemeroff *et al*., 1995, Wang *et al*., 2002). Evidence to support this also comes from individual x-ray crystallographic and nuclear magnetic resonance structures of both NS1 domains (Bornholdt & Prasad, 2006b, Chien *et al*., 1997, Liu *et al*., 1997).

The RNA-binding domain alone is a symmetrical homodimer, and each monomer consists of three α-helices (Chien *et al*., 1997, Liu *et al*., 1997) (**Fig. 1.5**). Dimerisation is essential for binding dsRNA (Wang *et al*., 1999), and the stoichiometry of dimer:dsRNA is 1:1 (Chien *et al*., 2004). Further studies have also shown that the structure and position of all six α-helices in the dimer do not alter upon dsRNA binding (Chien *et al*., 2004, Yin *et al*., 2007). Two identical helices from each NS1 monomer contribute significantly to dsRNA binding (termed α1/α1' and α2/α2') (Yin *et al*., 2007) (**Fig. 1.5**). The α2/α2' helices form antiparallel "tracks" on either side of a deep cleft that is bounded at its base by the α1/α1' helices (Liu *et al*., 1997, Yin *et al*., 2007). The "tracks" are made up of highly conserved basic and hydrophilic residues that likely form complementary contacts with the polyphosphate backbone of dsRNA (Yin *et al*., 2007). Residues in NS1 that mediate this interaction

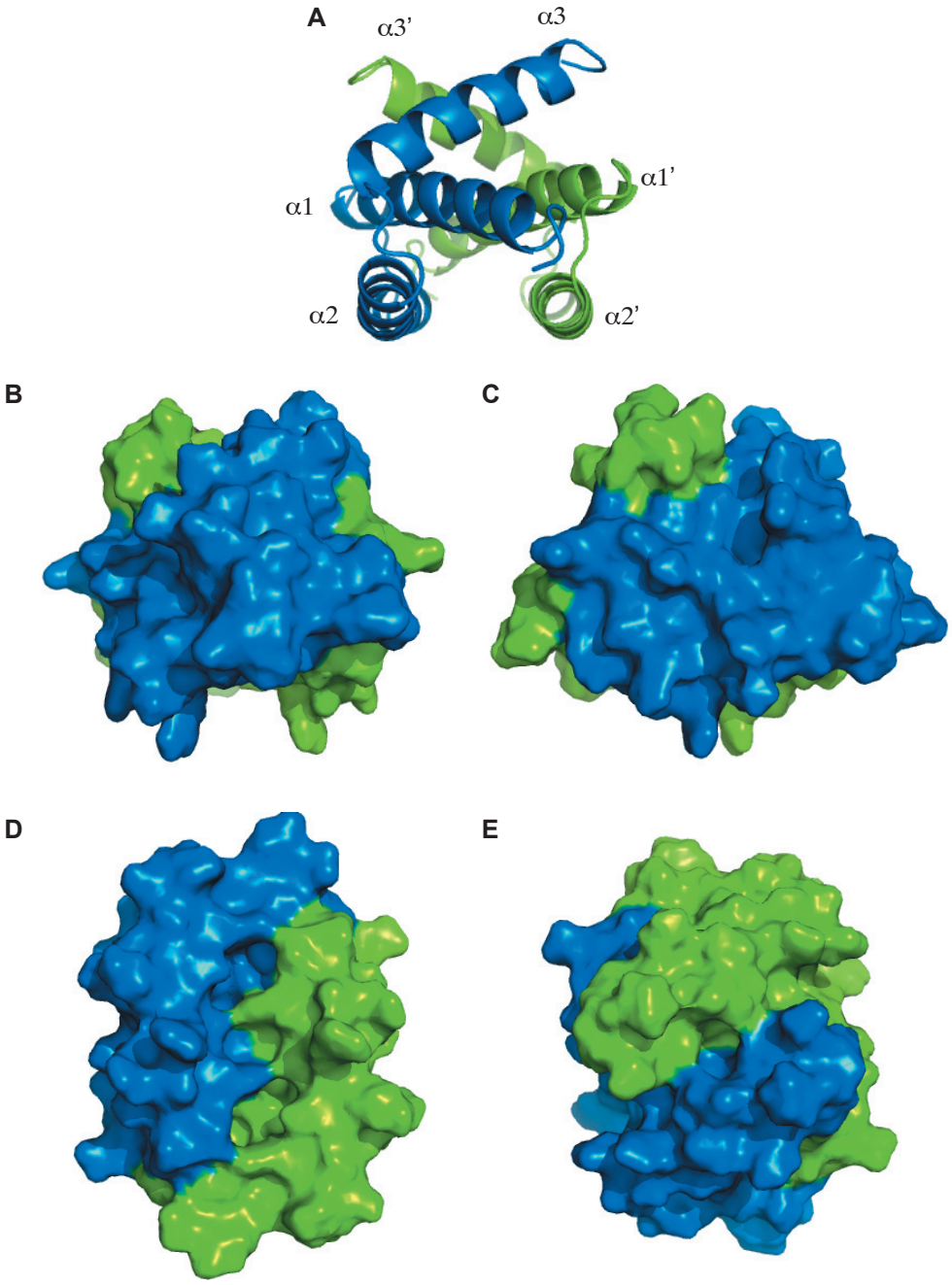



**Fig. 1.5. Structure of the influenza A virus NS1 protein (RNA-binding domain).** The N-terminal 73 amino-acids of NS1 form the functional RNA-binding domain. Cartoon (**A**), and surface (**B-E**) representations of the RNA-binding domain homodimer. The two monomers are coloured green and blue. The 3 helices of each monomer are labelled a1, a2, and a3 (or a1`, a2`, and a3`). Views are arbitrarily designated front (**A & B**), side (**C**), bottom (**D**), and top (**E**). *Images were prepared using MacPyMol (Protein Data Bank file: 1NS1).*

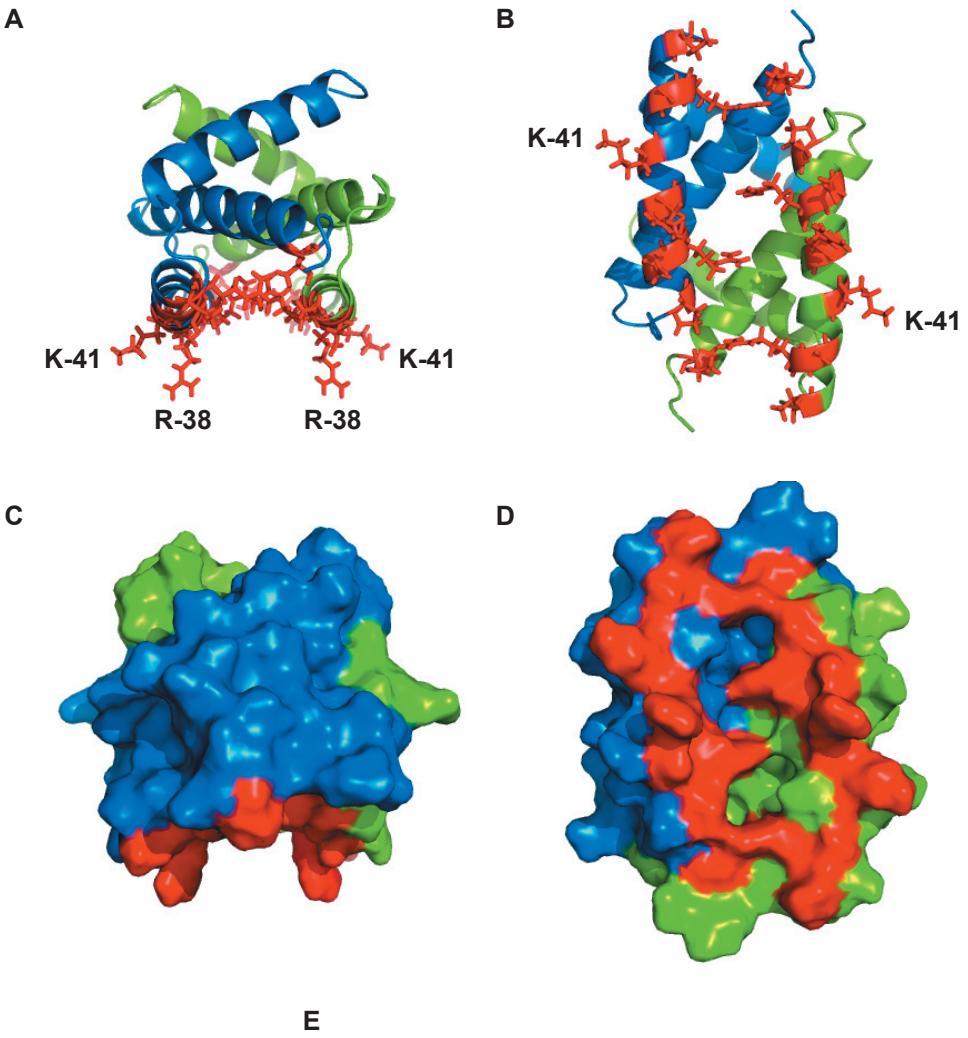
(either directly, or via improving complex stability), include: threonine-5, proline-31, aspartic acid-34, arginine-35, arginine-38, lysine-41, glycine-45, arginine-46, and threonine-49 (Wang *et al*., 1999, Yin *et al*., 2007) (**Fig. 1.6**).

The C-terminal effector domain of NS1 is also a homodimer, and each monomer consists of seven β-strands and three α-helices (Bornholdt & Prasad, 2006b) (**Fig. 1.7**). The structure/function relationship of this domain with cellular proteins is discussed further in the following sections (see also **Fig. 1.8**).

### *1.2.3 Sub-cellular localisation of NS1 protein.*

The sub-cellular localisation of NS1 protein appears to be dependent on several factors, including: virus strain, expression level of NS1, cell-type used, state of cell-polarity, and time post-infection (Newby *et al*., 2007). Experimental detection of NS1 within cells is also determined by the fixation procedure used, as some fixatives (such as acetone) have been suggested to preferentially extract small proteins like NS1 from the cytoplasm (Li *et al*., 1998c). Nevertheless, in infected tissue-culture cells, most NS1 protein localises predominantly to the nucleus, but a significant proportion can also be found in the cytoplasm (Greenspan *et al*., 1988, Krug *et al*., 2003, Newby *et al*., 2007). Indeed, early work identified the NS1 protein in crystalline cytoplasmic inclusions that could be isolated from cells late in infection (Morrongiello & Dales, 1977, Shaw & Compans, 1978).

NS1 proteins contain one or two nuclear localisation sequences (NLSs; NLS1 and NLS2) (Greenspan *et al*., 1988), which mediate their active nuclear import via binding to cellular importin-α molecules (Melen *et al*., 2007). As such, translocation of NS1 from cytoplasmic ribosomes to the nucleus is thought to be extremely rapid (Privalsky & Penhoet, 1981). NLS1 in NS1 is monopartite, and involves arginine-35,




**Original image can be found at the reference given below.**

**Alternatively, e-mail bgh1@st-andrews.ac.uk for a full copy of this thesis, including all figures.**

**Fig. 1.6. Residues of the NS1 protein important for RNA-binding.** Cartoon/stick (**A & B**), and surface (**C & D**) representations of the RNA-binding domain homodimer. The two monomers are coloured green and blue. All residues implicated in RNA-binding are coloured red. The two most important residues (arginine-38 (R-38) and lysine-41 (K-41)) are labelled (**A & B**). Views are arbitrarily designated front (**A & C**), and bottom (**B & D**). *Images were prepared using MacPyMol (Protein Data Bank file: 1NS1).* (**E**) Model of the NS1 RNA-binding domain homodimer (bottom view) in complex with 16-bp dsRNA (green). Both monomers are coloured silver, and the basic residues of NS1 involved in contacting dsRNA are coloured blue. *Image modified from Yin et al., 2007.*

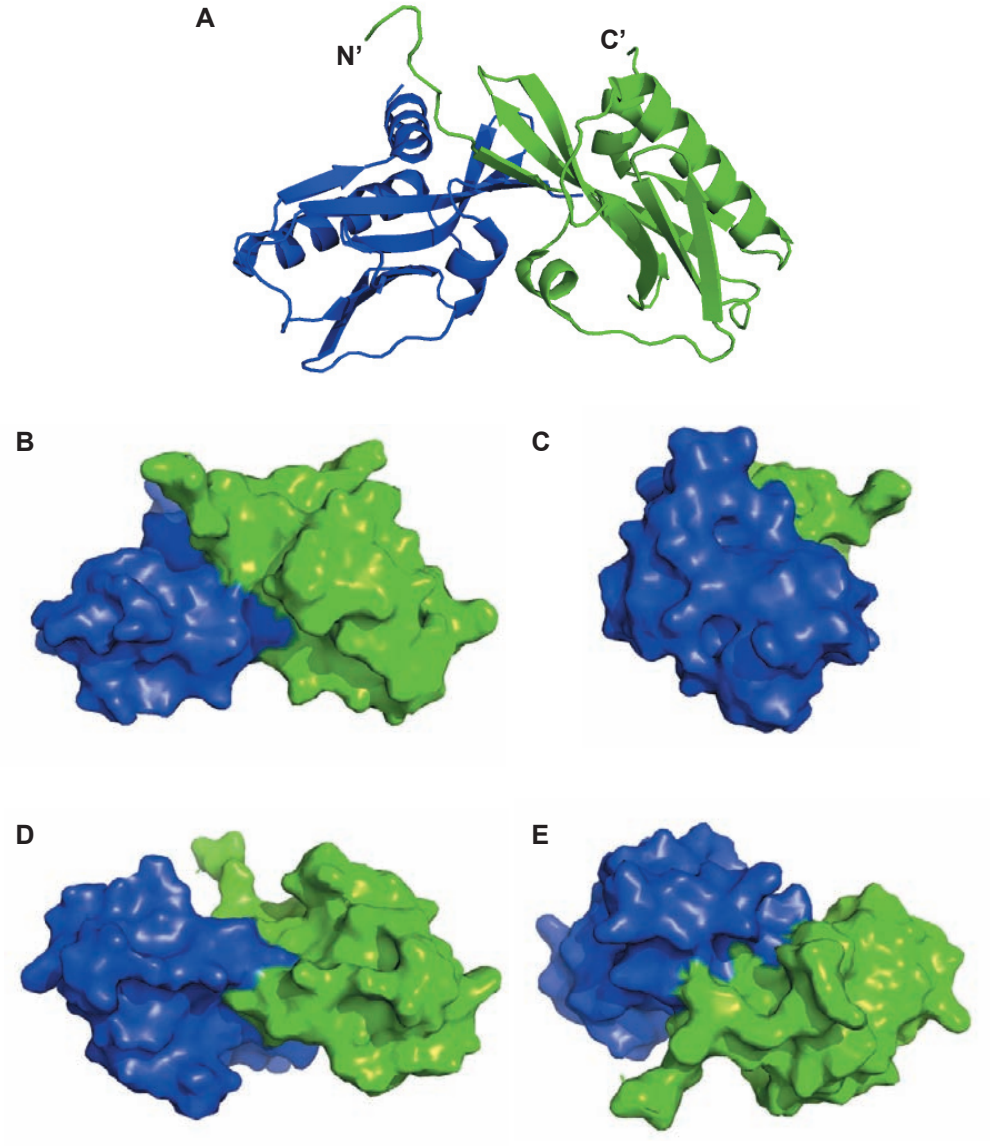



**Fig. 1.7. Structure of the influenza A virus NS1 protein (C-terminal effector domain).** Cartoon (**A**), and surface (**B-E**) representations of the C-terminal effector domain homodimer. The two monomers are coloured green and blue. The N-terminal and C-terminal ends of one monomer are labelled (**A**). Views are arbitrarily designated front (**A & B**), side (**C**), bottom (**D**), and top (**E**). *Images were prepared using MacPyMol (Protein Data Bank file: 2GX9).*

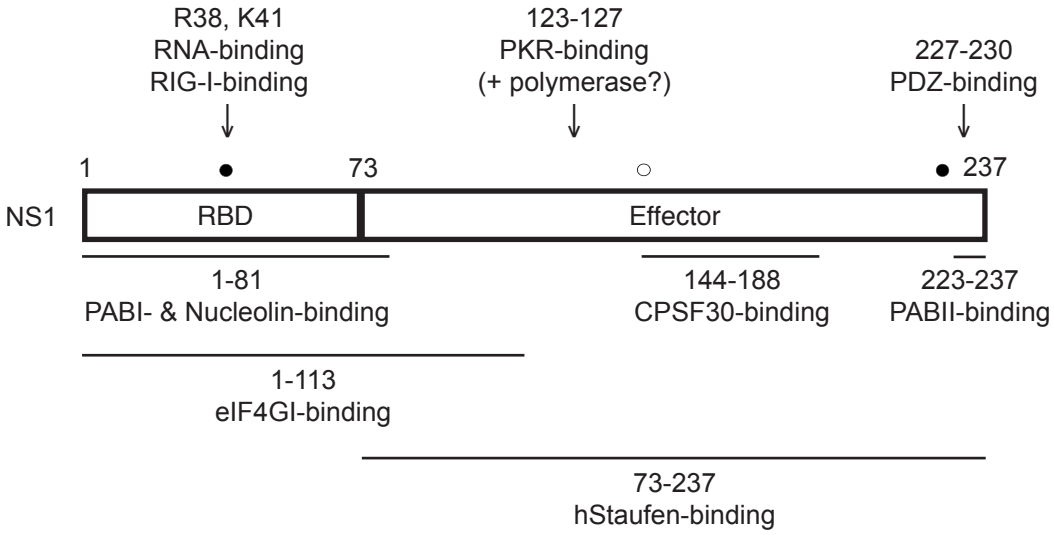



**Fig. 1.8. Schematic representation of the influenza A virus NS1 protein, together with its known interactors.** The influenza A virus NS1 protein is 230-237 amino-acids long depending upon the strain. The N-terminal 73 amino-acids form a functional RNA-binding domain (RBD), whilst the C-terminal effector domain mediates interactions with host-cell proteins. NS1 contains two nuclear localisation sequences (black circles), and a nuclear export sequence (white circle). Residues involved in RNA-binding (arginine-38 [R38] and lysine-41 [K41]) are also implicated in the inhibition of cellular 2`-5`-oligo (A) synthetase/RNase L, the inhibition of Jun N-terminal kinase, and the interaction with/inhibition of RIG-I. Additionally, NS1 contains binding sites for: poly (A)-binding protein I (PABI), nucleolin, eIF4GI, hStaufen, protein kinase R (PKR), 30 kDa subunit of cleavage and polyadenylation specificity factor (CPSF30), poly (A)-binding protein II (PABII), PDZ domain-containing proteins, and the viral polymerase. For diagrammatic clarity, the following interactors have not been included: NS1-BP, NS1-I, and components of the mRNA nuclear export machinery. *See text for references.*

arginine-38, and lysine-41, residues also involved in dsRNA-binding (Melen *et al*., 2007) (**Fig. 1.6**). This NLS is well-conserved among all known influenza A virus NS1 proteins (Melen *et al*., 2007). In contrast, the bipartite NLS2 comprises specific amino-acids at the C-terminus of only some NS1 proteins (lysine-219, arginine-220, arginine-231, and arginine-232) (Melen *et al*., 2007).

Concurrent with NLS2 is a functional nucleolar localisation signal (NoLS), which requires additional basic residues (usually arginine and lysine, respectively) at positions 224 and 229 (Melen *et al*., 2007). Thus, although the NS1 proteins of most strains appear predominantly nuclear during virus infection, only a subset that contain a functional NoLS localise into nucleoli (Melen *et al*., 2007). The nucleolar function of NS1 is unknown, however a mutant influenza A virus (A/Udorn/72 strain) expressing a truncated NS1 protein unable to localise into nucleoli was not attenuated for replication under tissue-culture conditions (Melen *et al*., 2007). Therefore, it has been suggested that any nucleolar function of NS1 may only be necessary for the *in vivo* pathogenesis of some viruses (Melen *et al*., 2007). As NLS2 is absent from the majority of pathogenic and non-pathogenic influenza A virus strains, it is difficult to ascribe a critical function to this sequence with regards viral replication/pathogenesis.

In infected cells, the cytoplasmic localisation of a sub-population of NS1 is probably regulated by two mechanisms. Some newly synthesised NS1 may be sequestered by a cellular/viral binding partner, thus masking the NLSs and retaining NS1 in the cytoplasm. Alternatively, a latent nuclear export signal (NES) in NS1 may cause the nucleo-cytoplasmic transport of nuclear NS1 back into the cytoplasm (Li *et al*., 1998c). The NS1 NES lies within residues 138-147, and requires leucines at positions 144 and 146 (Li *et al*., 1998c) (**Figs. 1.9A & 1.9B**). In the full-length molecule, the

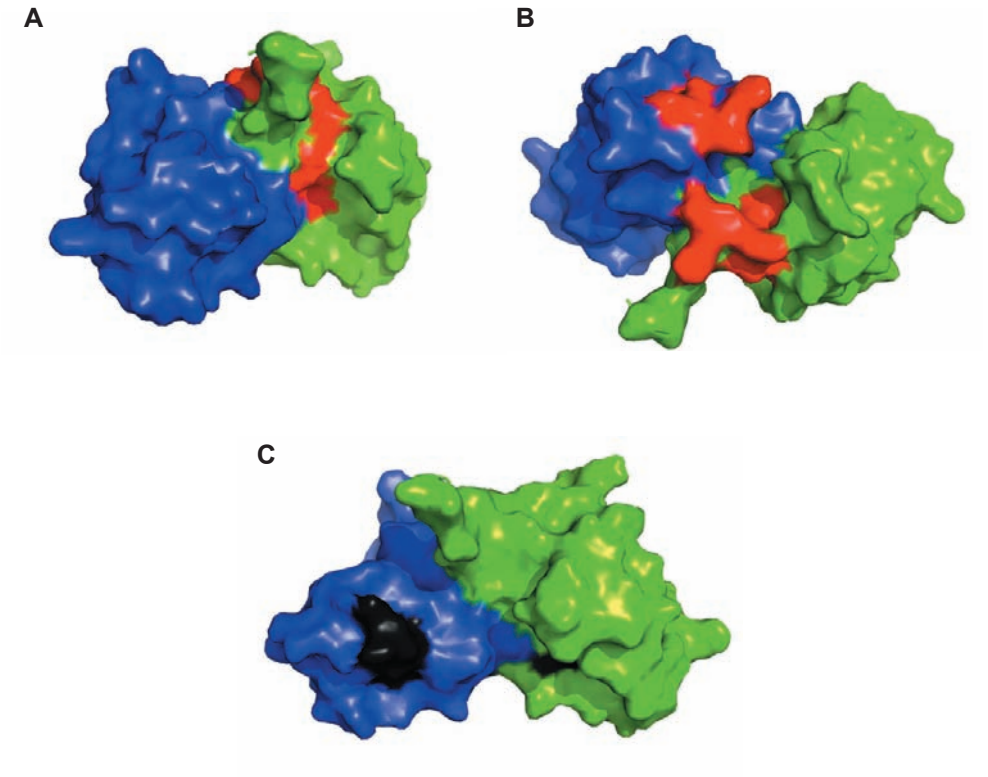



**Fig. 1.9. The nuclear export signal (NES) of NS1.** Surface representations of the C-terminal effector domain homodimer (monomers coloured green and blue) (**A-C**). Residues comprising the NES are highlighted in red (**A & B**). Residues comprising the NES "mask" are highlighted in black (**C**). Views are arbitrarily designated as for **Fig. 1.7**: angled front (**A**), top (**B**), and front (**C**). *Images were prepared using MacPyMol (Protein Data Bank file: 2GX9).*

NES is functionally "masked" by residues spatially adjacent to it: 148-161 (particularly arginine-148, glutamic acid-152, and glutamic aid-153) (Li *et al*., 1998c) (**Fig. 1.9C**). Thus, for NS1 to be re-localised back into the cytoplasm of infected cells, it has been postulated that the NES must be "unmasked", possibly by interaction of residues 148-161 in NS1 with another virus protein (Li *et al*., 1998c). This would potentially allow spatial control of NS1 to be regulated temporally by the relative expression levels of other viral proteins. However, host-cell factors also clearly play an important role in determining the sub-cellular localisation of NS1. For example, in infected murine tracheal epithelial cell cultures, the NS1 protein of A/WSN/33 localises mainly to the cytoplasm, a striking contrast to the nuclear distribution of the same protein observed in MDCK cells (Newby *et al*., 2007).

Thus, many regulatory signals are responsible for the varied distribution of NS1 during infection (i.e. nuclear, nucleolar, or cytoplasmic). Such varied localisations presumably contribute to the reported ability of NS1 to perform a number of different functions, as detailed below.

### *1.2.4 NS1 and viral RNA synthesis.*

Gene expression in influenza A virus-infected cells can be divided into "early" and "late" phases [reviewed in (Elton *et al*., 2006, Lamb & Krug, 2001)]. Immediately after nuclear import, all the viral segments appear to be equally transcribed into mRNA (Barrett *et al*., 1979, Hay *et al*., 1977). However, the NS and NP vRNA segments become preferentially synthesised, and there is a consequent increase in NS1 and NP mRNA transcription leading to high levels of NS1 and NP proteins (Barrett *et al*., 1979, Inglis *et al*., 1979, Lamb & Krug, 2001, Shapiro *et al*., 1987, Smith & Hay, 1982). In contrast, at "late" phases of infection all the viral segments

are efficiently replicated, and there is a high rate of transcription/translation for all the viral genes, particularly HA and M1 (Hay *et al*., 1977, Inglis *et al*., 1979, Shapiro *et al*., 1987, Skehel, 1973).

The NS1 protein has been reported to control the temporal synthesis of both viral mRNAs and vRNAs (Falcon *et al*., 2004, Min *et al*., 2007, Wolstenholme *et al*., 1980). The highly conserved (>95%) isoleucine-123 and methionine-124 residues in the C-terminal effector domain of NS1 have been implicated in this activity (**Fig. 1.10**), as the substitution of these two residues for alanine resulted in the increased synthesis of all viral mRNAs and vRNAs early in infection (Min *et al*., 2007). In particular, both HA and M1 (usually only synthesised in the "late" phase of infection) were synthesised "early" in the context of a mutant virus encoding NS1 with amino-acid substitutions at isoleucine-123 and methionine-124 (Min *et al*., 2007). The mechanism by which NS1 might regulate switches in viral vRNA, mRNA, and protein synthesis is unclear, but is not thought to involve PKR, a cellular protein bound by NS1 that can regulate translation (see below) (Min *et al*., 2007). One possibility is that NS1 functionally interacts with the viral polymerase complex to affect these processes (Marion *et al*., 1997b).

### *1.2.5 NS1 and viral mRNA translation.*

As an RNA-binding protein, NS1 has been shown to interact *in vitro* with a variety of RNA species: dsRNA (Hatada & Fukuda, 1992, Wang *et al*., 1999), viral genomic RNA (Hatada *et al*., 1997), viral mRNA (Marion *et al*., 1997a, Park & Katze, 1995), poly(A)-containing RNAs (Qiu & Krug, 1994), and snRNAs (small nuclear RNAs) derived from the U6 promoter (Qiu *et al*., 1995). As described below, the interactions of NS1 with putative viral dsRNA, viral genomic RNA, and cellular poly(A)-

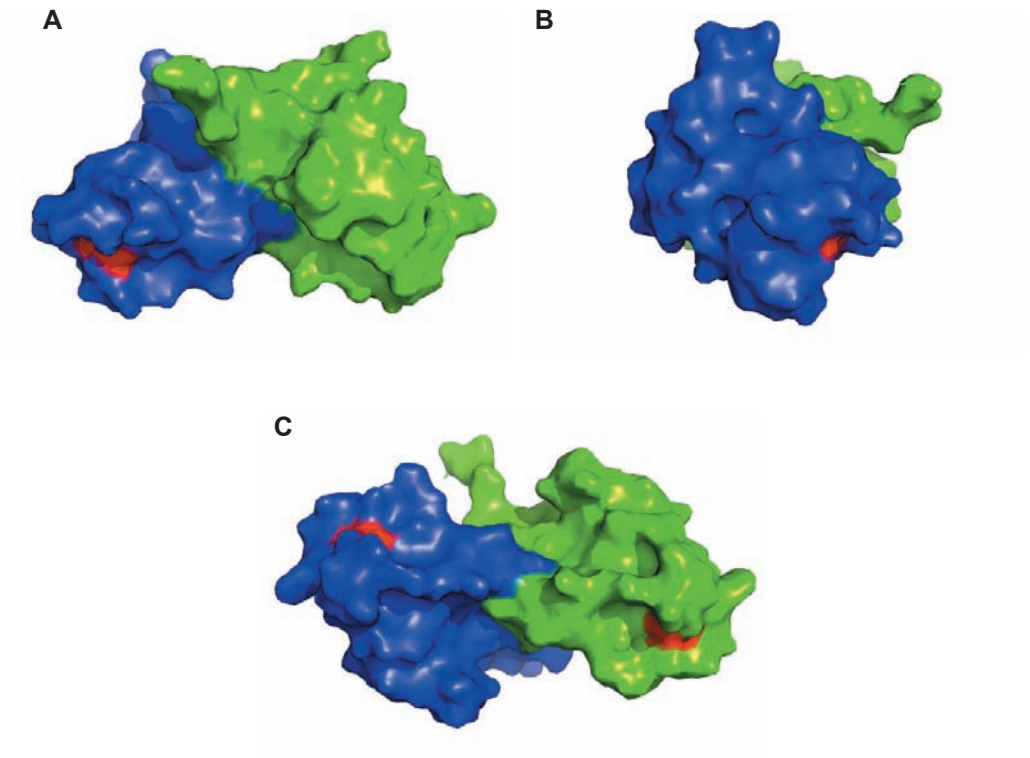



**Fig. 1.10. Residues of NS1 involved in controlling RNA synthesis.** Surface representations of the C-terminal effector domain homodimer (monomers coloured green and blue) (**A-C**). Residues of NS1 that contribute to the regulation of viral RNA synthesis (isoleucine-123 and methionine-124) are highlighted in red. Views are arbitrarily designated as for **Fig. 1.7**: front (**A**), side (**B**), and bottom (**C**). *Images were prepared using MacPyMol (Protein Data Bank file: 2GX9).*

containing RNAs (as well as the cellular proteins that may bind these ligands) likely play predominant roles in countering the host antiviral response (Kochs *et al*., 2007a, Min & Krug, 2006, Nemeroff *et al*., 1998, Pichlmair *et al*., 2006). Moreover, the possible interaction of NS1 with the 5'UTR (untranslated region) of viral mRNAs (Garfinkel & Katze, 1993, Park & Katze, 1995) has been linked to the ability of NS1 to directly enhance the translation initiation of viral mRNAs (de la Luna *et al*., 1995, Enami *et al*., 1994, Marion *et al*., 1997b).

Other than possibly binding the 5'UTR of viral mRNAs, NS1-mediated enhancement of viral protein synthesis may also require its interaction with host-cell proteins: NS1 can form an RNA-protein complex consisting of the eukaryotic translation initiation factor eIF4GI, poly(A)-binding protein I (PABI), and viral mRNAs (Aragon *et al*., 2000, Burgui *et al*., 2003). The RNA-independent binding of NS1 to eIF4GI requires the N-terminal 113 residues of NS1 (Aragon *et al*., 2000), whilst the RNA-independent interaction with PABI requires residues in the N-terminal 81 amino acids of NS1 (Burgui *et al*., 2003) (**Fig. 1.8**). Thus, mutant NS1 proteins unable to bind eIF4GI are also defective in enhancing viral mRNA translation (Marion *et al*., 1997a).

In addition, NS1 interacts with hStaufen, a dsRNA- and tubulin- binding protein that is related to PKR (Falcon *et al*., 1999). The interaction is independent of RNA, and appears to be mediated by the C-terminal effector domain of NS1 and the dsRNA-binding domains of hStaufen (Falcon *et al*., 1999) (**Fig. 1.8**). hStaufen is normally a cytoplasmic protein, but a sub-population can be relocalised to the nucleus upon co-expression with NS1 (Falcon *et al*., 1999). As hStaufen contributes to the microtubular transport of cellular mRNAs to sites of enhanced translation (such as cytoplasmic polysomes – collections of ribosomes translating the same mRNA), it has

been speculated that the interaction between NS1 and hStaufen may also promote efficient protein synthesis from viral mRNAs (Falcon *et al*., 1999). Indeed, a proportion of both NS1 and hStaufen has been found to co-fractionate with cytoplasmic polysomes in influenza A virus-infected cells (Compans, 1973, Falcon *et al*., 1999, Krug & Etkind, 1973, Marion *et al*., 1999). Thus, in order to promote efficient viral protein synthesis, NS1 may interact with cellular hStaufen, eIF4GI and PABI to specifically recruit viral mRNAs to multi-protein translation-initiation complexes. The additional contribution of NS1 to viral protein synthesis by inhibiting PKR is discussed below.

### *1.2.6 NS1 and the host immune response.*

The innate interferon (IFN) response of the host-cell is a potent antiviral mechanism that can limit the replication and spread of viruses [reviewed in (Goodbourn *et al*., 2000, Haller *et al*., 2006)]. IFNs (such as IFNα or IFNβ) are soluble cytokines synthesised by cells in response to viral infection [reviewed in (Goodbourn *et al*., 2000)]. Secreted IFNs act in both an autocrine and paracrine fashion to upregulate >300 genes (Der *et al*., 1998), and thereby induce an "antiviral state" within target host cells [reviewed in (Goodbourn *et al*., 2000)].

Studies using modified influenza A viruses (strain A/Puerto Rico/8/34; PR8) that are either unable to express NS1 (termed PR8delNS1), or express truncated NS1 proteins, have revealed that a major role for NS1 during infection is to counter the innate immune response (Egorov *et al*., 1998, Fernandez-Sesma *et al*., 2006, Garcia-Sastre *et al*., 1998, Kochs *et al*., 2007b). PR8delNS1 replicates to titres ~1000-fold lower than wild-type (WT) PR8 in "IFN-competent" MDCK cells (Garcia-Sastre *et al*., 1998). However, in Vero cells (which have the genes encoding both IFNα and IFNβ deleted

(Diaz *et al*., 1988, Mosca & Pitha, 1986)), PR8delNS1 replicates to titres only ~10-fold lower than WT PR8 (Garcia-Sastre *et al*., 1998). Additionally, PR8delNS1 virus is only pathogenic in mice with a homozygous deletion in STAT1 (an essential molecule that mediates the cellular response to exogenous IFN), and PR8delNS1 (but not WT PR8) causes high transactivation of an IFN-stimulated reporter gene (Garcia-Sastre *et al*., 1998). Together, these data indicate that NS1 normally functions to limit the host innate IFN-mediated antiviral response. However, given that viruses completely lacking NS1 still replicate to titres ~10-100-fold lower than WT in Vero cells (Garcia-Sastre *et al*., 1998, Kochs *et al*., 2007b), it is clear that NS1 has functions that are independent of its IFN-antagonistic abilities (see above sections).

A number of studies have demonstrated, at the molecular level, how NS1 functions to limit both the production and downstream effects of IFN. As described below, it is becoming increasingly apparent that the mechanisms by which NS1 achieves this are likely to be strain and/or cell-type specific (Geiss *et al*., 2002, Hayman *et al*., 2006, Kochs *et al*., 2007a).

#### *1.2.6.1 Limitation of IFN production by NS1.*

The H1N1 influenza virus A/Puerto Rico/8/34 (PR8) is a commonly used laboratory strain that has been extensively passaged in tissue culture and eggs. Unusually, it is highly mouse-adapted, and is attenuated in humans. Studies on the PR8/NS1 protein show that it can specifically prevent dsRNA- and virus- mediated activation of the transcription factors interferon regulatory factor-3 (IRF-3), NFκB, and c-Jun/ATF-2, and thus limit induction of IFNβ (Ludwig *et al*., 2002, Talon *et al*., 2000a, Wang *et al*., 2000). This inhibition appears to occur pre-transcriptionally, and has been attributed to two residues in the dimerised RNA-binding domain of PR8/NS1:

arginine-38 and lysine-41 – both of which mediate efficient RNA-binding (Talon *et al*., 2000a, Wang *et al*., 1999, Wang *et al*., 2002) (**Fig. 1.6**).

Although it was postulated that PR8/NS1 might sequester aberrant dsRNA produced during virus infection (and thus limit the activation of transcription factors necessary for IFNβ gene transcription), dsRNA has not been detected in influenza A virus infected cells (Pichlmair *et al*., 2006, Weber *et al*., 2006), and recent work indicates that the mechanism of IFN inhibition may actually involve PR8/NS1 forming a complex with retinoic acid-inducible gene product I (RIG-I) (Guo *et al*., 2007, Mibayashi *et al*., 2007, Opitz *et al*., 2006, Pichlmair *et al*., 2006).

RIG-I is a cytoplasmic RNA helicase that acts as a cellular sensor of RNA virus infection, and can lead to the induction of IFNβ synthesis (Yoneyama *et al*., 2004). The co-precipitation of RIG-I with PR8/NS1 is largely dependent upon arginine-38 and lysine-41 in PR8/NS1 (Pichlmair *et al*., 2006), suggesting that these two residues are involved in the protein:protein interaction, or that RNA acts as an intermediary component (**Fig. 1.6**). Indeed, studies to date have failed to confirm direct binding of PR8/NS1 to RIG-I (Mibayashi *et al*., 2007), and the presence of 5'-phosphorylated ssRNA clearly enhances the stability of a PR8/NS1:RIG-I complex (Pichlmair *et al*., 2006). However, it is intriguing to note that PR8/NS1 has also been shown to block the function of a constitutively active RIG-I construct lacking its RNA-binding helicase domain (Mibayashi *et al*., 2007), indicating that inhibition of RIG-I mediated IFNβ induction is complex, and could involve multiple mechanisms.

Studies using the human H3N2 virus A/Udorn/72 (Ud) have revealed that Ud/NS1 can limit IFNβ induction by preventing the global post-transcriptional processing of cellular pre-mRNAs (Lu *et al*., 1994). For this, the C-terminal effector domain of

Ud/NS1 binds directly to two zinc-finger regions in the 30 kDa subunit of cleavage and polyadenylation specificity factor (CPSF30), and also binds poly(A)-binding protein II (PABII) (Chen *et al*., 1999, Nemeroff *et al*., 1998, Noah *et al*., 2003, Twu *et al*., 2006). NS1 binding to CPSF30 prevents the cellular protein from binding pre-mRNAs, thus inhibiting the correct cleavage and polyadenylation of host mRNAs (Nemeroff *et al*., 1998). NS1 binding to PABII is thought to block the nuclear export of mRNAs that might partially escape from the inhibition of 3'-end formation (Chen *et al*., 1999). Retention of cellular pre-mRNAs within the nucleus of infected cells usually leads to their rapid degradation (Katze & Krug, 1984).

The CPSF30-binding site on Ud/NS1 includes residues 184-188 (glycine-leucine-glutamic acid-tryptophan-asparagine) (Noah *et al*., 2003), as well as leucine-144 (Twu *et al*., 2006). Binding is also stabilised by either phenylalanine-103 and methionine-106, or by an NS1-mediated interaction with the cognate viral polymerase complex (Kochs *et al*., 2007a, Twu *et al*., 2007) (**Fig. 1.11**). The PABII interaction site requires Ud/NS1 residues 223-237 (Noah *et al*., 2003) (**Fig. 1.8**). Recombinant Ud viruses expressing NS1 with a mutated CPSF30-binding site are highly attenuated under tissue culture conditions, and induce the synthesis of large amounts of IFNβ mRNA, as well as other antiviral mRNAs (such as ISG15, p56, and 2'-5'-oligoadenylate synthetase mRNAs) (Noah *et al*., 2003).

Despite the discrepancy in proposed "modes of action" for the IFN-antagonistic properties of NS1, recent comparative studies suggest that influenza A viruses may have evolved two strategies to circumvent the host immune response, and these strategies might normally synergise to mediate efficient evasion of the IFN system (Hayman *et al*., 2006, Kochs *et al*., 2007a). For example, PR8/NS1 cannot bind

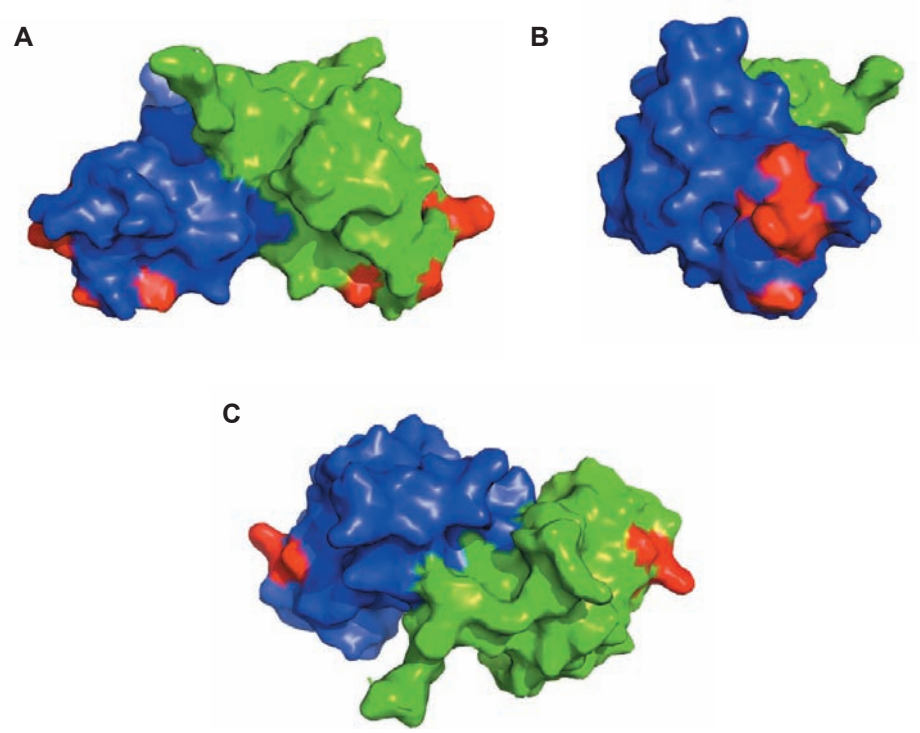



**Fig. 1.11. Residues of NS1 that mediate the interaction with CPSF30.** Surface representations of the C-terminal effector domain homodimer. The two monomers are coloured green and blue. Residues involved in mediating the stable interaction between NS1 and CPSF30 are shown in red (amino-acids 103, 106, 144, and 184-188). Views are arbitrarily designated front (**A**), side (**B**), and top (**C**). *Images were prepared using MacPyMol (Protein Data Bank file: 2GX9).*

directly to CPSF30, and is therefore unable to block the activation of gene reporter constructs (Hayman *et al*., 2006, Kochs *et al*., 2007a). For comparison, the NS1 proteins of other influenza A virus strains, including Ud, A/Brevig Mission/1/18 (BM), A/Texas/36/91 (Tx) and A/Victoria/3/75 (Vic), can efficiently limit host-cell gene transcription (and specifically RNA polymerase II transcripts (Kochs *et al*., 2007a)), a property shown for Tx/NS1 to be dependent upon its binding to CPSF30 (Hayman *et al*., 2006, Kochs *et al*., 2007a). However, during infection PR8/NS1 efficiently limits IFNβ synthesis by a pre-transcriptional block on IRF-3 activation, which probably involves its interaction with RIG-I (Kochs *et al*., 2007a). In contrast, Tx/NS1 (which interacts poorly with RIG-I) only partially limits IRF-3 activation in response to virus infection, yet completely blocks IFNβ mRNA synthesis (Kochs *et al*., 2007a). Furthermore, during virus infection Vic/NS1 limits both IRF-3 translocation to the nucleus and host-cell gene transcription (Hayman *et al*., 2006), suggesting that this NS1 protein might functionally interact with both RIG-I and CPSF30. It is of interest to note that PR8/NS1 (unlike other NS1 proteins) has been reported to enhance the translation of cellular mRNAs (Salvatore *et al*., 2002), which may be due in part to its inability to suppress the 3'-end processing of cellular pre-mRNAs prior to translation.

Thus, evidence indicates that different NS1 proteins may limit the synthesis of IFNβ by both pre-transcriptional and/or post-transcriptional processes. The existence and evolution of two mechanisms may increase the capacity of influenza A viruses to adapt to new environments and hosts (Kochs *et al*., 2007a). However, it is also possible that the laboratory adaptation of some strains of influenza A virus has lead to the loss of one or other of these mechanisms, resulting in viruses that inhibit IFNβ

pathways by distinct means. Either way, there is clearly a potential problem in interpreting data from studies using only a single virus strain.

### *1.2.6.2 Inhibition of host-cell mRNA processing by NS1.*

Given that most NS1 proteins cause the bulk nuclear retention of host mRNAs, but not viral mRNAs (Fortes *et al*., 1994, Qiu & Krug, 1994), it should be noted that NS1 can also form a complex with components of the mRNA nuclear export machinery: NXF1, p15, Rae1, E1B-AP5, and to some extent Nup98 (Satterly *et al*., 2007). Interestingly, within influenza A virus infected cells, Nup98 protein appears to be actively degraded (Satterly *et al*., 2007).

Complex formation between NS1 and NXF1, Rae1, and E1B-AP5 requires residues in both the RNA-binding and C-terminal effector domains of NS1, whilst the interaction with p15 appears to be mediated solely by the C-terminal effector domain (Satterly *et al*., 2007) (**Fig. 1.8**). Although the RNA-binding domain of NS1 is implicated in the interaction with NXF1, Rae1, and E1B-AP5, treatment with RNase A only affects the co-precipitation of E1B-AP5 with NS1 (Satterly *et al*., 2007). Overexpression of these mRNA nuclear export factors is able to overcome the NS1-mediated block on mRNA nucleo-cytoplasmic transport, suggesting that the inhibitory effect of NS1 can be titrated out by functional NXF1, Rae1, p15, and E1B-AP5 (Satterly *et al*., 2007). Thus, in addition to inhibiting the 3'-end processing of host-cell pre-mRNAs (and thereby mRNA export) by binding CPSF30 and PABII, NS1 can act directly at the level of nuclear mRNA export transporters in order to prevent host protein synthesis.

Yeast two-hybrid studies also identified a novel ~70 kDa NS1-binding protein (subsequently termed NS1-BP) (Wolff *et al*., 1998). NS1-BP predominantly co-localises with the spliceosome assembly factor SC35 into nuclear “speckle” domains,

suggesting that NS1-BP is involved in the cellular mRNA splicing machinery (Wolff *et al*., 1998). During influenza A virus infections, the cytoplasmic fraction of NS1-BP redistributes into the nucleus, and thus colocalises with NS1 (Wolff *et al*., 1998). In addition, NS1 also induces a change in the nuclear localisation of other cellular splicing factors (Fortes *et al*., 1995), and the "speckle domains" appear to change dramatically in size, shape and composition (Fortes *et al*., 1995, Wolff *et al*., 1998). It has been proposed that the interaction between NS1 and NS1-BP may contribute towards the inhibition of host-cell mRNA processing, although a role for the interaction in regulating viral mRNA splicing cannot be ruled out (Garaigorta & Ortin, 2007, Wolff *et al*., 1998).

It should be noted that, in contrast to the effect of NS1 on host-cell mRNAs, NS1 does not inhibit the nuclear export of most viral mRNAs, with the apparent exception of its own NS1 mRNA (Garaigorta & Ortin, 2007, Satterly *et al*., 2007). The mechanism by which NS1 achieves this specificity is unknown, but suggests that viral mRNAs leave the nucleus by a means independent of nuclear pore complexes containing NXF1, Rae1, p15, and E1B-AP5.

#### *1.2.6.3 Inhibition of IFN-inducible enzymes by NS1.*

The ability of some NS1 proteins to efficiently limit IFN and tumour necrosis factor α (TNFα)-stimulated gene expression (thereby conferring resistance to their antiviral effects), has a beneficial effect on virus replication and pathogenicity (Geiss *et al*., 2002, Hayman *et al*., 2006, Kobasa *et al*., 2007, Kochs *et al*., 2007a, Seo *et al*., 2002). However, NS1 also specifically blocks the activation of two cytoplasmic IFN-inducible anti-viral proteins: 2'-5'-oligoadenylate synthetase (2'-5'-OAS), and the dsRNA-dependent serine/threonine protein kinase R (PKR).

dsRNA (a putative by-product of RNA virus replication) acts as co-factor to activate 2'-5'-OAS, which is then able to polymerize adenosine triphosphate (ATP) into 2'-5' oligoadenylate (2'-5' oligo(A)) chains [reviewed in (Sen, 2000)]. These 2'-5' oligo(A) chains in turn cause dimerisation and activation of the latent ribonuclease, RNaseL that inhibits viral replication (translation) by degrading single-stranded RNAs, such as mRNAs and ribosomal RNAs [reviewed in (Sen, 2000)].

dsRNA also activates PKR - it binds to specific motifs within the N-terminal domain of PKR, and thereby releases its conformationally-induced autoinhibition (Nanduri *et al*., 2000). Activated PKR phosphorylates the α-subunit of eIF2 (eukaryotic translation initiation factor 2), a process which causes the inhibition of both cellular and viral protein synthesis [reviewed in (Samuel, 1993)]. PKR also has roles in upregulating gene expression (IFNβ) from IRF-3 and NFκB responsive promoters [reviewed in (Goodbourn *et al*., 2000, Sen, 2000)], and is involved in initiating the apoptotic response (Balachandran *et al*., 1998). Thus, both 2'-5'-OAS and PKR play important roles in limiting virus replication, and their effects are dramatically increased by a functional IFN system.

A study using a recombinant influenza A virus (strain A/Udorn/72) that expresses an RNA-binding deficient NS1 (single amino-acid substitution at arginine-38) revealed that RNA-binding by NS1 plays a major role in circumventing the host IFN-induced antiviral state (Min & Krug, 2006). Pretreatment of A549 cells with IFNβ resulted in ~1000-fold decrease in the yield of this virus (rUd NS1-R38A) as compared with WT rUd (Min & Krug, 2006). This effect was mediated mostly (but not exclusively) by RNaseL, as short-interfering RNA (siRNA) targeting this latent ribonuclease restored the yield of rUd NS1-R38A by ~100-200-fold (Min & Krug, 2006). Given that

RNaseL is activated solely by 2'-5'-OAS, and NS1 likely has a higher binding affinity for dsRNA than 2'-5'-OAS, it has been postulated that the primary function of the NS1 RNA-binding domain is to out-compete 2'-5'-OAS for binding dsRNA, thus preventing the activation of this antiviral enzyme (Min & Krug, 2006). The cytokine sensitivity of influenza A viruses expressing NS1 proteins with a single amino-acid substitution at arginine-38 has also been confirmed in an *in vivo* (mouse) model, and in primary murine tracheal epithelial cells (Newby *et al*., 2007). In addition, it is interesting to note that *in vivo* such a mutant virus induces large amounts of TNFα, interleukin-6 (IL-6), and IFNβ (Newby *et al*., 2007). [As RNaseL has recently been shown to augment the actual induction of IFNβ in a 2'-5' oligo(A)-dependent manner (Malathi *et al*., 2007), it is possible that inhibition of 2'-5'-OAS activity by NS1 may also contribute to limiting IFNβ induction.]

Influenza A viruses have developed two direct mechanisms by which to counteract the powerful antiviral activity of PKR (Katze *et al*., 1988). Firstly, influenza A virus upregulates the activity of the normal cellular inhibitor of PKR, $p58^{IPK}$ (Lee *et al*., 1990). The mechanism by which this upregulation occurs is not entirely clear, although there is no increase in the total amount of $p58^{IPK}$ protein (Lee *et al*., 1992). Rather, evidence suggests that influenza A virus infection may cause the dissociation of $p58^{IPK}$ from its own cellular inhibitor, HSP40 (Melville *et al*., 1997, Melville *et al*., 1999). The influenza A virus NS1 protein can also directly negatively regulate PKR activity (Bergmann *et al*., 2000). Although *in vitro* data indicate that NS1 effectively blocks PKR activation by competing with PKR for dsRNA (Hatada *et al*., 1999, Lu *et al*., 1995), the relatively low affinity of NS1 for dsRNA (Chien *et al*., 2004) means it is unlikely to successfully achieve this block during an *in vivo* infection (Li *et al*.,

2006a). It has since been shown that NS1 can bind directly to PKR (Tan & Katze, 1998) in a dsRNA-independent manner (Li *et al*., 2006a, Min *et al*., 2007).

The binding of NS1 to PKR requires amino acids 123-127 in the C-terminal effector domain of NS1, and these amino acids are essential for the NS1-mediated inhibition of PKR during virus infection (Min *et al*., 2007). Thus, NS1 has been proposed to interact with a linker region in the PKR molecule and mechanically prevent the autoinhibitory relief conferred upon PKR when dsRNA binds to its N-terminal domain (Li *et al*., 2006a). NS1-mediated inhibition of PKR therefore allows efficient viral protein synthesis to occur during infection (Salvatore *et al*., 2002). [Given that PKR may also contribute to IFN induction [reviewed in (Goodbourn *et al*., 2000, Sen, 2000)], its inhibition by NS1 could play an additional role in the antagonism of IFN production by influenza A viruses.]

#### *1.2.6.4 NS1 and host RNAi pathways.*

In eukaryotic organisms, RNA silencing (RNA interference; RNAi) is a unique RNA-guided mechanism for suppressing the expression of specific genes [reviewed in (Denli & Hannon, 2003)]. The host endoribonuclease, Dicer, produces small ~22 nucleotide RNAs from dsRNA, which act individually in a homology-dependent manner to form a ribonucleoprotein complex – the RNA-induced silencing complex (RISC) [reviewed in (Cullen, 2006)]. RISC acts on the homologous target mRNA in two ways: translational inhibition requires only partial homology between the "guide" RNA and the target mRNA, whilst degradation of the mRNA requires extensive homology between the two RNA species [reviewed in (Bartel, 2004)]. The possible role of endogenous host RNAi pathways in the anti-viral response to RNA viruses in

mammalian cells is controversial and unclear [reviewed in (Cullen, 2006, Gitlin & Andino, 2003)].

In the case of influenza A virus, artificially introduced RNAi responses can protect mammalian cells from infection, both in tissue-culture cells and in *in vivo* mouse models (Ge *et al*., 2004a, Ge *et al*., 2004b, Ge *et al*., 2003, Hui *et al*., 2004, Tompkins *et al*., 2004, Zhou *et al*., 2004). Despite this, only recent data have suggested that mammalian cells may potentially mount an innate RNAi-associated anti-influenza virus response (Matskevich & Moelling, 2007). Studies on Vero cells showed that siRNA-mediated knock-down of Dicer increased the replication of influenza A virus, and enhanced virus-induced apoptosis (Matskevich & Moelling, 2007). However, as knock-down of Dicer also causes the modest upregulation of several hundred host-cell gene transcripts (Schmitter *et al*., 2006), it is possible that this effect is unrelated to RNAi-degradation of viral RNAs. Nevertheless, Dicer mRNA and protein appear to be preferentially downregulated during normal influenza A virus infections (Matskevich & Moelling, 2007), suggesting that this host-cell protein may be specifically targeted by the virus to suppress an anti-viral function.

Although involvement of RNA silencing in the anti-viral response of mammalian cells to viruses is yet to be fully resolved, the influenza A virus NS1 protein has already been proposed to antagonize such a host defence (Li *et al*., 2004). In this regard, overexpression of NS1 can demonstrably inhibit the induction of RNAi pathways in heterologous *Drosophila* (insect) and plant cell systems (Bucher *et al*., 2004, Delgadillo *et al*., 2004, Li *et al*., 2004). However, this may simply be due to the non-specific sequestration of dsRNA, as arginine-38 and lysine-41 in NS1 significantly contribute to this suppression (Cullen, 2006, Li *et al*., 2004) (**Fig. 1.6**).

Evidence indicates that NS1 is unable to exert the same inhibitory effect in mammalian cells (Kok & Jin, 2006), thus the role of mammalian RNAi pathways during virus infection (and their antagonism by viral factors), still needs to be fully determined.

***1.2.6.5 NS1 and the adaptive immune response.***

During viral/bacterial infections *in vivo*, dendritic cells (DCs) act as sentinels to survey infected tissues (Mellman & Steinman, 2001). Upon contact with a pathogen, DCs mature, leading to the upregulation of major histocompatibility complex (MHC) class II molecules and the release of pro-inflammatory cytokines/chemokines (including IFNα/IFNβ) (Fernandez-Sesma *et al*., 2006). Mature DCs migrate to lymph nodes and present pathogen-specific antigens on their surface to both cytotoxic and helper T-cells, thus stimulating initiation of adaptive immune responses specific for the infecting pathogen (Mellman & Steinman, 2001). Activated cytotoxic ($CD8^+$) T-cells are able to directly kill (virus)-infected cells, whilst helper ($CD4^+$) T-cells produce cytokines such as IFNγ and TNFβ, which further activate the killing capacity of macrophages and $CD8^+$ T-cells (Mellman & Steinman, 2001).

Studies on human-derived primary DCs have revealed that the influenza A virus NS1 protein is able to prevent the correct maturation of DCs in response to virus infection, and thereby limits host T-cell activation (Fernandez-Sesma *et al*., 2006). Specifically, infection of DCs with PR8delNS1 virus (lacking the ability to express full-length NS1) lead to the secretion of higher amounts of IFNα, IFNβ, TNFα, and IL-6, as compared to the WT virus (Fernandez-Sesma *et al*., 2006). This is in agreement with *in vivo* mouse studies done using a mutant influenza A virus (WSN strain) expressing NS1 with a single amino-acid substitution at arginine-38, and which is thus unable to

bind RNA (Newby *et al*., 2007). Expression of WT NS1 also specifically limited the virus-induced expression of several genes involved in DC maturation and migration, including: MHC, IL-8, IL-12 p35, IL-23 p19, IRF-7, RANTES, TRIF, MIP-1β, and CCR7 (Fernandez-Sesma *et al*., 2006). Thus, DCs infected with influenza A viruses expressing WT NS1 are unable to mature, and thereby fail to stimulate the secretion of IFNγ from T-cells (Fernandez-Sesma *et al*., 2006). This effect of NS1 would clearly have a profound inhibitory effect on the adaptive immune response to infection.

### *1.2.7 Effect of NS1 on other cell signalling pathways.*

A number of studies have reported and attempted to characterise novel cellular binding partners for NS1 [most recently nucleolin (Murayama *et al*., 2007)]. The NS1 proteins from five different human and avian influenza A virus strains can associate with NS1-I, a human protein highly homologous to the porcine 17β-estradiol dehydrogenase precursor protein, which may mediate steroid hormone levels (Wolff *et al*., 1996). Although the function of this interaction is not well understood, overexpression of NS1-I in 293 cells specifically reduces the expression of influenza A virus proteins, suggesting that NS1-I has antiviral activity that may be inhibited by NS1 (Wolff *et al*., 1996).

Large-scale sequence analyses of avian influenza A viruses have also revealed two other putative protein-binding motifs within NS1: a Src-homology 3 (SH3) binding motif (residues 212-216) (Finkelstein *et al*., 2007), and a PDZ (postsynaptic density, PSD-95; discs large, Dlg; zonula occludens-1, ZO-1) domain ligand (final four residues at the C-terminus) (Obenauer *et al*., 2006) (**Fig. 1.8**). Cellular proteins

containing SH3 or PDZ domains often coordinate the assembly of a diverse array of localized signalling complexes.

No host-cell proteins have, to date, been identified as interacting with the putative SH3 binding motif in NS1 (though Crk and CrkL are strong possibilities (Heikkinen *et al*., 2007)). However, *in vitro* studies have shown that the PDZ domain ligand of highly pathogenic avian influenza virus NS1s (including those from H5N1 and 1918 strains) can bind (and therefore potentially disrupt the activity of) over 30 different human PDZ-proteins (Obenauer *et al*., 2006).

### *1.2.8 NS1 and the host apoptotic response.*

During infection, influenza A viruses induce cell death by both apoptosis (Fesq *et al*., 1994, Hinshaw *et al*., 1994, Lowy, 2003, Takizawa *et al*., 1993), and (in certain cell types) necrosis (Arndt *et al*., 2002, Seo *et al*., 2004). Most infected cells undergo apoptosis by intrinsic mechanisms, however influenza A virus can induce apoptosis in neighbouring, uninfected cells by triggering the release of extracellular cytokines that act in a paracrine fashion (Zhirnov & Klenk, 2007).

The role of apoptosis during infection is somewhat controversial. For example, cellular pro-apoptotic factors, such as caspase-3 or the NFκB/FasL/TRAIL (TNF-related apoptosis-inducing ligand) pathway, have been reported to promote the efficient propagation of influenza A viruses (Wurzer *et al*., 2004, Wurzer *et al*., 2003). Alternatively, apoptosis can be regarded as a host antiviral defence mechanism that is activated in response to infection and limits virus replication (Barber, 2001, Kurokawa *et al*., 1999). As such, cell-death induction is probably dependent upon the temporal balance between pro- and anti-apoptotic functions of several viral proteins, as well as the rate of production of other viral apoptosis “inducers”, such as dsRNA

(Morris *et al*., 2005). The kinetics of apoptosis induction throughout each stage of infection are likely to be important for virus propagation. "Early" apoptosis may be severely detrimental to virus replication, packaging, and spread, whilst "late" apoptosis may benefit the virus by clearing infected cells that might otherwise stimulate cell-mediated cytotoxic responses and/or the adaptive immune system.

Several influenza A virus proteins have been shown to directly modulate apoptosis: NS1 can have both pro- and anti- apoptotic functions (Morris *et al*., 2002, Schultz-Cherry *et al*., 2001, Zhirnov *et al*., 2002a); NA activates transforming growth factor β (TGFβ), a known inducer of apoptosis in epithelial cells (Morris *et al*., 1999, Schultz-Cherry & Hinshaw, 1996); PB1-F2 promotes the formation of ion channels in the inner mitochondrial membrane (see **section 1.1**), either by itself or through interactions with ANT3 and VDAC (Chanturiya *et al*., 2004, Chen *et al*., 2001, Gibbs *et al*., 2003, Zamarin *et al*., 2005); and M1 may enhance apoptosis by interacting with caspase-8 (Zhirnov *et al*., 2002b).

The exact function of NS1 with regard to virus-induced apoptosis/cell-death is unclear, and may be dependent upon specific host-cell factors or virus strain – for example, Ud/NS1 on its own appears to be extremely toxic to MDCK cells, but not HeLa cells (Schultz-Cherry *et al*., 2001); and Ud/NS1 (but not PR8/NS1) inhibits the processing of cellular pre-mRNAs, a process likely to be toxic to the cell (Kochs *et al*., 2007a, Noah *et al*., 2003). Indeed, experiments using transiently expressed Ud/NS1 have demonstrated that residues in the RNA-binding domain of NS1 can augment NS1-induced apoptosis in MDCK cells independently of other virus-derived factors (Schultz-Cherry *et al*., 2001). However, in the context of virus infection, NS1 appears to have anti-apoptotic functions, which have been linked largely to its ability

to limit the production and/or downstream effects of IFN (Zhirnov *et al*., 2002a). As such, catalytically active PKR is reported to play an important role in influenza A virus-mediated induction of apoptosis (Takizawa *et al*., 1996), thus the direct binding and inhibition of PKR by NS1 may contribute to cell-death suppression (Li *et al*., 2006a). The same may be true for the pro-apoptotic functions of 2'-5' OAS/RNaseL (Diaz-Guerra *et al*., 1997, Min & Krug, 2006, Zhou *et al*., 1997). In addition, by limiting the production of IFN and other cytokines, NS1 might reduce paracrine-mediated (extrinsic) apoptosis induction in neighbouring uninfected cells (Zhirnov & Klenk, 2007, Zhirnov *et al*., 2002a). Overall, there may be a temporal role for NS1 in contributing to both "early" (active) suppression of apoptosis, and "late" (toxic) induction cell-death during infection.

As part of this thesis, a functional interaction between NS1 and the phosphoinositide 3-kinase (PI3K) signalling pathway was identified (Hale *et al*., 2006). This was subsequently confirmed by several other groups (Shin *et al*., 2007a, Ehrhardt *et al*., 2007a, Zhirnov & Klenk, 2007), and a possible role for the interaction in delaying virus-induced apoptosis was inferred (Ehrhardt *et al*., 2007a, Zhirnov & Klenk, 2007). The remainder of this introduction is therefore concerned with detailing the molecular details of PI3K signalling (**section 1.3**), and reviewing how different viruses hijack this pathway in order to benefit their replication (**section 1.4**).

## 1.3 Phosphoinositide 3-kinase (PI3K) signalling.

### *1.3.1 Membrane lipids as signalling molecules.*

Phosphatidylinositol (PtdIns) is a component of eukaryotic cell membranes [reviewed in (Fruman *et al*., 1998)]. Despite its relatively low abundance, PtdIns plays a crucial role in many cell signalling events as a metabolic precursor of several second messenger molecules [reviewed in (Cantley, 2002, Cantrell, 2001, Fruman *et al*., 1998, Hawkins *et al*., 2006, Vanhaesebroeck *et al*., 2005)]. As a phospholipid, PtdIns is unique in that its inositol head group can become phosphorylated at multiple free hydroxyl positions [reviewed in (Fruman *et al*., 1998, Hawkins *et al*., 2006)]. Thus, within cells seven different phosphorylated PtdIns species (phosphoinositides) have been detected, each presumably with unique signalling functions [reviewed in (Hawkins *et al*., 2006)] (**Fig. 1.12**). The generation and regulation of phosphoinositides is controlled by a variety of lipid kinases and phosphatases that act directly on PtdIns or its phosphoinositide derivatives (see below). An alternative mechanism involves phospholipase C, which hydrolyses phosphatidylinositol (4,5) biphosphate (PtdIns(4,5)$P_2$) to produce soluble inositol (1,4,5) triphosphate (Ins(1,4,5)$P_3$), and membrane-associated diacylglycerol (DAG). Ins(1,4,5)$P_3$ acts via receptors in the endoplasmic reticulum to increase intracellular $Ca^{2+}$ levels, whilst DAG can activate pathways downstream of the serine/threonine protein kinase C family [reviewed in (Cantrell, 2001, Vanhaesebroeck *et al*., 1997a)]. However, the events concerning this PtdIns metabolism are not considered further here.

Phosphoinositide kinases are enzymes that catalyse the transfer of phosphate to a particular position on the inositol ring of PtdIns or one of its phosphorylated forms [reviewed in (Fruman *et al*., 1998)]. They are highly conserved (at the amino acid

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**Fig. 1.12. Structure of phosphatidylinositol and its seven phosphorylated derivatives.** Phosphatidylinositol (PtdIns; top left) found in mammalian cells comprises fatty acid chains (which are inserted into the plasma membrane), and a cytosolic head-group (inositol). The inositol head-group can become phosphorylated at multiple hydroxyl (-OH) positions to form various PtdIns derivatives (as shown). Each derivative is named for the position of phosphorylation - consensus numbering is indicated on the PtdIns (top left) diagram. PtdIns(3,4,5)$P_3$ (the product of class I PI3K) is shown top right. The regulation of PtdIns phosphorylation is controlled by lipid kinases and phosphatases (see text). *Schematic modified from Hawkins et al., 2006.*

level) amongst eukaryotic organisms (including yeasts) [reviewed in (Vanhaesebroeck *et al*., 1997a)], and are classified with respect to the position on the inositol ring that they phosphorylate (note: although PtdIns has free hydroxyls at positions 2-6, only positions 3-5 are known to be phosphorylated *in vivo* [reviewed in (Hawkins *et al*., 2006)]): phosphoinositide 3-kinases (PI3Ks) phosphorylate the 3'-OH position of PtdIns; phosphoinositide 4-kinases (PI4Ks) phosphorylate the 4'-OH position of PtdIns; and phosphoinositide (P) 5-kinases (PIP5Ks) phosphorylate the 5'-OH position of PtdInsP [reviewed in (Fruman *et al*., 1998)]. PI3Ks have been the most intensively studied phosphoinositide kinases, and are the subjects of the following sections.

### *1.3.2 PI3K enzymes, substrates, and products.*

Lipid second messengers generated by PI3Ks regulate an array of protein kinase signalling cascades which, in turn, control diverse cellular processes such as cell survival, metabolism, proliferation, and inflammation/immunity [reviewed in (Cantley, 2002, Hawkins *et al*., 2006, Vanhaesebroeck *et al*., 2005)]. Several PI3K enzymes (which specifically catalyse the reactions shown in **Fig. 1.13**), have been characterised, and are grouped into four classes (IA, IB, II, and III) based upon their structural and functional homologies [reviewed in (Fruman *et al*., 1998, Hawkins *et al*., 2006)] (**Fig. 1.14A**). Class IA PI3Ks (the subject of this thesis) and class IB PI3Ks preferentially generate PtdIns(3,4,5)$P_3$ from PtdIns(4,5)$P_2$, whilst class II and class III PI3Ks are primarily responsible for the synthesis of PtdIns3P (how PtdIns(3,4)$P_2$ is made from PtdIns4P is not yet clear) (Hawkins *et al*., 2006). Class I PI3K-generated PtdIns(3,4,5)$P_3$ is part of a major signal transduction pathway downstream of cell-surface receptors [reviewed in (Cantley, 2002, Hawkins *et al*.,

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**Fig. 1.13. Phosphorylation of PtdIns derivatives by various PI3K classes.** Each PI3K activity transfers phosphate from ATP to the 3`-OH position of the inositol head-group of a specific PtdIns derivative. Class II and class III PI3Ks phosphorylate PtdIns to produce PtdIns(3)P, whilst class I PI3K phosphorylates PtdIns(4,5)$P_2$ to produce PtdIns(3,4,5)$P_3$. The kinase responsible for producing PtdIns(3,4)$P_2$ from PtdIns(4)P is unknown. For clarity, only the inositol head-groups are shown, although it should be noted that the fatty-acid chains would normally extend from the 1` position. *Schematic modified from Hawkins et al., 2006.*

2006)], and class III PI3K-generated PtdIns3P regulates endocytosis, vesicle transport and lysosome formation [reviewed in (Cantrell, 2001, Hawkins *et al*., 2006)].

### *1.3.3 Regulation of class IA PI3K activity.*

Class IA PI3Ks are dimeric enzymes consisting of a p110 catalytic subunit (p110α, p110β, or p110δ) tethered to a smaller non-catalytic ("p85") regulatory subunit (typically p85α, p85β, p55α, p50α, or p55γ) (**Fig. 1.14A**). p85α, p85β, and p55γ are all encoded by separate genes, but p55α and p50α are splice variants from the p85α gene (and thus lack the SH3 and BH domain sequences of full-length p85α.) [reviewed in (Vanhaesebroeck *et al*., 2005)]. All regulatory subunits (with the exception of p55γ) exhibit a wide tissue distribution (Vanhaesebroeck *et al*., 2005). The interaction of "p85" with p110 functions to both stabilize heat-labile p110, and suppress its enzymatic activity (Yu *et al*., 1998). Thus, within cells, "p85" and p110 exist as obligate heterodimers (Geering *et al*., 2007, Yu *et al*., 1998), and subsequent activation of PI3K must occur via inter- or intra- molecular allosteric changes. "p85s" are also essential for recruiting p110 subunits to the plasma membrane, where the necessary lipid substrates for PI3K reside (Vanhaesebroeck *et al*., 2005).

The p85 regulatory subunits contain an N-terminal SH3 (Src homology 3) domain, a BH (B-cell receptor homology) domain flanked by proline-rich sequences, and two SH2 (Src homology 2) domains, which are on either side of the p110-binding inter-SH2 (iSH2) domain (**Fig. 1.14B**) (Dhand *et al*., 1994, Vanhaesebroeck *et al*., 2005). All the domains of p85 contribute to the regulation of p110, and stimulatory signals (such as growth factors, hormones, neurotransmitters, and many inflammatory stimuli including IFNs) act through multiple mechanisms in order to modulate the basal inhibition of PI3K (Hawkins *et al*., 2006, Kaur *et al*., 2005). For example, tyrosine

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**Fig. 1.14. Classification and organisation of the four PI3K classes.** (**A**) Schematic domain structures for the regulatory and catalytic subunits comprising each class of PI3K. After each structure is the protein and gene name. Classes are determined by the homology between the relevant catalytic subunits. Class IA PI3Ks are heterodimers of a p85/p55/p50 regulatory subunit complexed to a p110 subunit. Class IB PI3Ks are heterodimers of a p84/p101 regulatory subunit complexed to a specific p110 subunit. Class II PI3Ks consist of only a catalytic subunit. Class III PI3K is a heterodimer of a p150 protein kinase in complex with the human homologue of VPS34. The kinase specificity of each PI3K class is detailed further in **Fig. 1.13**. (**B**) Larger schematic representation of the p85 regulatory subunit of class IA PI3K (724 amino-acids long). p85 consists of an N-terminal SH3 (Src homology 3) domain, a BH (B-cell receptor homology) domain, and two SH2 (Src homology 2) domains (N-terminal: nSH2, and C-terminal: cSH2), which flank the inter-SH2 (iSH2) domain. The iSH2 domain binds the p110 catalytic subunit of PI3K. Asterisks denote two proline-rich regions within the p85 protein. *Schematic modified from Hawkins et al., 2006.*

phosphorylation of consensus Y*XX*M motifs in activated growth factor receptors (or their specific adaptor substrates, including the insulin receptor substrates: IRS1-IRS-4), provides docking sites for the two p85 SH2 domains and relieves the effect of p85 on p110 (Carpenter *et al*., 1993a, Rordorf-Nikolic *et al*., 1995). Binding to the BH domain of p85 by GTPases such as Cdc42 or Rac have also been shown to increase the activity of the p85:p110 heterodimer (Tolias *et al*., 1995, Zheng *et al*., 1994). In contrast, a novel adapter protein, Ruk, can interact with the N-terminal SH3 domain of p85 and negatively regulate PI3K (Gout *et al*., 2000). Additional direct phosphorylations of p85 also determine p110 activity: Src kinase-mediated phosphorylation of tyrosine-688 alleviates p110 inhibition (Cuevas *et al*., 2001), whilst autophosphorylation of serine-608 restores inhibition (Foukas *et al*., 2004). Thus, docking of adapter proteins to particular domains, or phosphorylation of key residues, probably induces conformational changes in p85 that are transmitted to p110 and affect its catalytic activity (**Fig. 1.15**). It should be noted that p110 itself can also be directly regulated by other host-cell proteins, such as activated Ras (Rodriguez-Viciana *et al*., 1994, Rodriguez-Viciana *et al*., 1996).

The generation of PtdIns(3,4,5)$P_3$ is countered by the lipid phosphatases PTEN (phosphatase and tensin homologue deleted on chromosome ten), and SHIP1/SHIP2 (SH2 domain-containing inositol 5'-phosphatases). PTEN acts to convert PtdIns(3,4,5)$P_3$ to PtdIns(4,5)$P_2$, whilst SHIP1/SHIP2 dephosphorylate the 5'-OH of PtdIns(3,4,5)$P_3$ to produce PtdIns(3,4)$P_2$ [reviewed in (Cantley, 2002, Vanhaesebroeck *et al*., 2005)]. PTEN is a tumour suppressor protein that is often mutated or deleted in a variety of different human cancers (Cantrell, 2001). Cells lacking functional PTEN have increased levels of PtdIns(3,4,5)$P_3$ and PtdIns(3,4)$P_2$, which leads to the constitutive activation of signalling pathways downstream of class

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**Fig. 1.15. Model for class IA p85-p110 lipid kinase (PI3K) regulation.** The interaction of p85 with p110 functions to stabilise the heat-labile p110 subunit, as well as inhibit its catalytic activity. p85 interacts with the N-terminus of p110 via the coiled-coil inter-SH2 domain of p85, but another interaction (via a putative leucine zipper region) has been postulated. The PtdIns inositol ring is represented by a hexagon that contacts the C-terminus of p110 and possibly the inter-SH2 domain of p85. As p85 and p110 are obligate heterodimers, release of p85-mediated inhibition of p110 occurs via inter- and intra- molecular steric changes: binding of the two SH2 domains of p85 to phospho-tyrosines (pY) in activated cell surface receptors, Src kinase-mediated phosphorylation of tyrosine-688 in p85, or binding of GTPases to the p85 BH domain. Ras can also directly activate p110 lipid kinase activity. PI3K catalytic activity is negatively regulated by Ruk binding to the SH3 domain of p85, as well as the autophosphorylations of serine-608 in p85, and/or serine-1039 in p110. Legend as for **Fig. 1.14**. *Diagram reproduced from Vanhaesebroeck et al., 1999.*

I PI3K (see below). Members of the SHIP phosphatase family mediate an important inhibitory role in lymphocytes, and loss of SHIP1/SHIP2 can lead to unbalanced immune responses and/or autoimmune problems [reviewed in (Cantrell, 2001, Vanhaesebroeck *et al*., 2005)].

### *1.3.4 Signalling downstream of PI3K.*

Membrane-associated PtdIns(3,4,5)$P_3$ (generated by active PI3Ks) causes the specific recruitment of proteins containing pleckstrin homology (PH) domains [reviewed in (Hawkins *et al*., 2006)] (**Fig. 1.16**). Thus, PI3K activity is "interpreted" by multiple primary effectors, which will have a large influence on a variety of signalling cascades. Indeed, upwards of ~20 different PtdIns(3,4,5)$P_3$-binding proteins (with PH domains) have been identified from a single cell-type (Krugmann *et al*., 2002). The serine/threonine protein kinase Akt (also known as protein kinase B; PKB) is perhaps one of the best understood (and arguably most important) PH-domain containing PI3K effectors (**Fig. 1.16**), and is ubiquitously expressed in all cell-types [reviewed in (Manning & Cantley, 2007)]. Although the remainder of this section (and thesis) will focus on the activation and downstream effects of Akt, it should be kept in mind that other effectors exist. Indeed, there are at least three different Akt isoforms (Manning & Cantley, 2007). Thus, the functional consequences of PI3K activation are likely to extend well beyond Akt signalling, and may display cell-type, temporal, or spatial specificity. As such, the differential metabolism of phosphoinositides may act as a "regulatable scaffold", which influences the protein composition (and subsequent signalling) in a particular membrane location (Hawkins *et al*., 2006).

Akt is activated by phosphorylation at two sites: Thr308 and Ser473. PtdIns(3,4,5)$P_3$/PH domain-dependent membrane recruitment of PDK1

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**Fig. 1.16. Downstream of PI3K: Akt.** Model for PI3K-dependent Akt activation. The serine/threonine protein kinase Akt (also known as PKB; protein kinase B), is a major downstream effector of class IA PI3K signalling. In response to increased PtdIns(3,4,5)$P_3$, both PDK1 (phosphoinositide-dependent kinase-1) and Akt are recruited to the plasma membrane via PtdIns(3,4,5)$P_3$-binding PH (pleckstrin homology) domains. The constitutively active PDK1 is therefore brought into close proximity with Akt, and phosphorylates Akt at Thr308 (T308) in its activation loop. Ser473 (S473) is then further phosphorylated by the mTORC2 protein complex in a PI3K-dependent, PDK1-independent, manner. Phosphorylation of Ser473 may act to regulate the degree of Akt activation once Thr308 has been phosphorylated by PDK1, although both phosphorylations are necessary for the full activation of Akt. *Diagram modified from Hawkins et al., 2006.*

(phosphoinositide-dependent kinase-1) brings this kinase into close proximity with Akt, which is also recruited to the plasma membrane in a PtdIns(3,4,5)$P_3$/PH domain-dependent fashion [reviewed in (Hawkins *et al*., 2006, Manning & Cantley, 2007)]. The increase in "effective concentration" of these two enzymes allows the constitutively active PDK1 to phosphorylate Akt at Thr308 in its activation loop (**Fig. 1.16**). PI3K also stimulates the function of the multi-protein mTORC2 complex, which contains mTOR, Rictor, SIN1, and mLST8 (Bayascas & Alessi, 2005, Brazil *et al*., 2004, Manning & Cantley, 2007). The exact upstream mechanism of this regulation is unclear, but mTORC2 is responsible for phosphorylating Akt at Ser473 in its hydrophobic motif, thus causing full PI3K-dependent activation of Akt (Manning & Cantley, 2007). There are over 100 reported protein substrates immediately regulated by Akt phosphorylation, and some Akt substrates control more than one cellular function, which often varies in a cell- and signal- dependent manner [reviewed in (Manning & Cantley, 2007)]. It is impossible to describe all of these targets/activities, however a number of common (and sometimes overlapping) themes relating to Akt activity can be inferred, particularly with respect to cell survival, growth, and proliferation (**Fig. 1.17**).

### *1.3.4.1 PI3K-Akt and anti-apoptosis.*

A major and critical role for Akt is to promote cell survival downstream of growth factors, oncogenes, and cell stress. The Bcl-2 homology domain 3 (BH3)-only family of proteins normally exert a pro-apoptotic effect by binding to and inactivating the pro-survival Bcl-2 proteins at the mitochondrial membrane (Manning & Cantley, 2007). Active Akt blocks the function of these BH3-only proteins at several levels. Firstly, Akt can directly phosphorylate some BH3-only proteins (i.e. BAD), causing their cytosolic sequestration and inactivation by 14-3-3 proteins (Cooray, 2004, Datta

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**Fig. 1.17. Akt/PKB substrates and physiological functions**. Activated Akt/PKB can phosphorylate >100 different proteins (10 examples shown), which regulates various cellular processes (e.g. survival, growth, proliferation, glucose uptake, metabolism, and angiogenesis). Akt/PKB-mediated phosphorylation of its substrates leads to their activation (arrows) or inactivation (blocked arrows). *Diagram reproduced from Manning & Cantley, 2007.*

*et al*., 1997, Datta *et al*., 2000, del Peso *et al*., 1997). The 14-3-3 cytosolic regulatory proteins generally act to cause conformational changes in proteins they bind to, and thus mask/reveal functional motifs that control the localisation, activity, phosphorylation status and/or stability of the target protein [reviewed in (Dougherty & Morrison, 2004)]. Akt can also directly limit the expression of several BH3-only proteins by phosphorylating and inactivating FOXO (also known as Forkhead; FKHR) transcription factors, which may additionally be sequestered and inactivated by 14-3-3 proteins (Tran *et al*., 2003) [it should be noted that phosphorylation of FOXO/FKHR transcription factors also reduces the expression of the pro-apoptotic cytokine, Fas ligand (FasL) (Brunet *et al*., 1999)]. Further, the expression of some pro-apoptotic BH3-only proteins (specifically Puma and Noxa) is reduced by Akt-mediated phosphorylation of the E3 ubiquitin ligase, MDM2 (also known as HDM2 in humans), which is translocated into the nucleus and negatively regulates the transcriptional activity of p53 (Manning & Cantley, 2007, Mayo & Donner, 2001, Zhou *et al*., 2001b).

Active Akt can also phosphorylate and inactivate the serine/threonine protein kinase, glycogen synthase kinase-3 (both GSK-3α and GSK-3β isoforms) [reviewed in (Manning & Cantley, 2007)]. Inhibition of GSK-3β function leads to increased activity of the pro-survival Bcl-2 protein, MCL-1 (Maurer *et al*., 2006), which together with other Bcl-2 proteins (see above) acts to limit the release of cytochrome *c* from mitochondria [reviewed in (Manning & Cantley, 2007)]. This prevents the proteolytic cleavage of procaspase-9, thus blocking the "initiator" effect of mature caspase-9 on the pro-apoptotic caspase cascade. In addition, Akt has been reported to directly phosphorylate procaspase-9, thus rendering it less proteolytically active (Cardone *et al*., 1998).

### *1.3.4.2 PI3K-Akt and cell growth.*

Akt-mediated activation of a Raptor-containing mTOR complex (mTORC1: consisting mTOR, mLST8, and Raptor) plays an essential function in increasing cell growth (protein and membrane synthesis) in response to nutrients and growth factor signalling [reviewed in (Manning & Cantley, 2007)]. Akt directly phosphorylates and inactivates the tuberous sclerosis complex 2 (TSC2), and thereby indirectly activates mTORC1 (Manning *et al*., 2002, Potter *et al*., 2002). Additionally, phosphorylation of the mTORC1 inhibitor, PRAS40, by Akt causes it to dissociate from mTORC1 and be sequestered by 14-3-3 proteins, thus releasing the PRAS40-mediated inhibition of mTORC1 (Kovacina *et al*., 2003, Sancak *et al*., 2007). mTORC1 enhances protein synthesis by modulating the activities of both p70S6K and 4E-BP1 (eukaryotic initiation factor 4E (eIF4E)-binding protein 1), which act to regulate ribosome biogenesis and translation initiation (Manning & Cantley, 2007). Inhibition of 4E-BP1 by mTORC1 phosphorylation leads to the activation of eIF4E, and thus promotes the cap-dependent translation of numerous target mRNAs (Mamane *et al*., 2004). In addition, lipid membrane synthesis (an essential requirement for cell growth) may be enhanced by the Akt-mediated phosphorylation and activation of ATP-citrate lyase (Berwick *et al*., 2002).

### *1.3.4.3 PI3K-Akt and cell proliferation.*

Akt-dependent phosphorylation of the cyclin-dependent kinase inhibitors $p21^{Cip1/WAF1}$ and $p27^{Kip1}$ causes their cytosolic sequestration by 14-3-3 proteins, and leads to cell-cycle progression (Liang *et al*., 2002, Sekimoto *et al*., 2004, Shin *et al*., 2002, Viglietto *et al*., 2002, Zhou *et al*., 2001a, Zhou *et al*., 2001b). Akt also regulates $p21^{Cip1/WAF1}$ and $p27^{Kip1}$ protein levels by decreasing the activity of p53 and FKHR/FOXO transcription factors (see above) (Collado *et al*., 2000, Dijkers *et al*.,

2000, Mayo & Donner, 2001, Medema *et al*., 2000, Zhou *et al*., 2001b). GSK-3β may also contribute to driving cell proliferation. Akt-mediated phosphorylation (and thus inactivation) of GSK-3β prevents GSK-3β from targeting β-catenin for proteasome-mediated degradation. This leads to the accumulation of β-catenin protein, and an increase in the transcription of genes such as cyclin D1 and *c-myc*, the encoded proteins of which are involved in cell-cycle entry or phase transition (Manning & Cantley, 2007). The Akt-mediated phosphorylation/inactivation of GSK-3β also directly stabilises the protein levels of cyclin D, cyclin E, *c-jun*, and *c-myc* by preventing their proteasome-mediated degradation (Diehl *et al*., 1998, Manning & Cantley, 2007, Welcker *et al*., 2003) . In addition, it is likely that translational effects downstream of mTORC1/4E-BP1 (see above) contribute significantly to cell proliferation [reviewed in (Manning & Cantley, 2007)].

***1.3.4.4 PI3K-Akt and cytokine production/signalling.***

Recent work has uncovered an important role for the PI3K-Akt signalling pathway in regulating the host innate immune response downstream of cell-surface Toll-like receptors (TLRs). TLRs recognise a number of microbial products (often termed pathogen-associated molecular patterns (PAMPs)), and can trigger the production of various immunomodulatory cytokines, including IFNα/IFNβ, TNFα, and interleukin-12 (IL-12) (Fukao & Koyasu, 2003). IL-12 is a key regulator of host innate and adaptive immune responses during infection: it enhances the cytolytic activity of natural killer (NK) cells and $CD8^+$ T-cells, and contributes to the optimal production of IFN-γ (Trinchieri, 1995). In dendritic cells (DCs), the synthesis of IL-12 is negatively regulated by active PI3K-Akt signalling (Fukao *et al*., 2002). Interestingly, the PI3K pathway is also implicated in limiting TNFα induction (Guha & Mackman, 2002).

The role of PI3K in IFNβ production is somewhat controversial and confusing, and may be cell-type specific. Studies done in 293 cells have shown PI3K and Akt to be essential for TLR3-mediated IFNβ induction (Sarkar *et al*., 2004). Such PI3K-mediated activation is not required for the nuclear localisation or dimerisation of the IRF-3 transcription factor, but appears to regulate the full activation of IRF-3 by hyperphosphorylation (Sarkar *et al*., 2004). However, similar to the situation with IL-12 synthesis, recent work using DCs has proposed a role for active PI3K in the negative regulation of IFNβ production in response to extracellular TLR (TLR3/TLR4) ligands (such as the dsRNA analogue, poly I:C), a mechanism likely to involve a limitation of NFκB transcriptional activity (Aksoy *et al*., 2005). It is not known if PI3K is also involved in the regulation of IFNα/IFNβ production downstream of the intracellular viral PAMP receptors, RIG-I and mda-5. However, tissue-culture studies on two types of viruses that can induce IFN via RIG-I (namely, influenza A viruses and flaviviruses) have shown PI3K inhibitors and/or dominant negative constructs dramatically prevent virus-induced IFNβ promoter activity (Chang *et al*., 2005, Ehrhardt *et al*., 2006).

The PI3K pathway is also implicated in IFN-mediated signalling, independent of classical JaK-STATs (Janus kinase; signal transducer and activator of transcription) [reviewed in (Kaur *et al*., 2005)]. Receptor binding of IFNα/IFNβ induces the tyrosine phosphorylation of IRS1/IRS2 adapter proteins, and the IFN-dependent recruitment and activation of p85:p110 heterodimers (Platanias *et al*., 1996, Uddin *et al*., 1995). IFNα/β-induced, IRS-mediated, and STAT-independent PI3K stimulation increases both the lipid kinase and serine kinase activities of PI3K, although the functional consequences of these events are unclear (Platanias, 2005, Platanias *et al*., 1996). As STAT-dependent anti-viral effects of IFNs can be partly attributed to the

PKR-mediated inhibition of viral mRNA translation (see **section 1.2**), one hypothesis is that IFN-induced PI3K activation may contribute to the selective regulation of cellular mRNA translation initiation, thus ensuring the production of other IFN-induced anti-viral proteins (Platanias, 2005). To this end, IFN has been shown to activate a PI3K-dependent, STAT-independent, mTOR-p70S6K/4E-BP1 pathway (Lekmine *et al*., 2003), although how cellular versus viral mRNA translation might possibly be selected for is not known.

Specific PI3K activation by IFNγ has an important role in IFNγ-induced transcriptional regulation [reviewed in (Platanias, 2005)]. PI3K causes the IFNγ-dependent phosphorylation of STAT1 at Ser727, which although not essential for the dimerisation or nuclear translocation of STAT1, contributes significantly to STAT1-driven transcription (Nguyen *et al*., 2001). Mechanistically, this involves the PI3K-dependent activation of PKCδ (protein kinase C, δ isoform) that directly phosphorylates STAT1 at Ser727 (Deb *et al*., 2003), and leads to the induction of pro-apoptotic genes (DeVries *et al*., 2004). Although IFNs are widely implicated in contributing to apoptosis, it seems surprising that PI3K is involved given its well-documented anti-apoptotic roles. However, in response to IFNs, the PI3K signalling pathway can mediate either pro- or anti- apoptotic signals, which probably depends upon the cellular context and the simultaneous activation of other cytokine-induced signalling pathways (e.g. by TNFα) (Platanias, 2005, Yang *et al*., 2001).

## 1.4 PI3K signalling and virus infections.

Given the diverse array of cellular processes regulated by signalling downstream of PI3K, it is not surprising that viruses hijack this pathway in order to support their own replication. In particular, activating PI3K is a popular means of delaying virus-induced cell-death and/or apoptosis. During acute virus infections, increased short-term cell viability is likely to increase efficient replication, whilst for more chronic infections PI3K signalling can modify the cell cycle, and may contribute towards latency or transformation. However, viral activation of this pathway is clearly important for functions independent of cell survival, including virus entry, RNA/protein synthesis, persistence, and immunomodulation. Additionally, some DNA and RNA viruses actually down-regulate PI3K signalling during infection. Thus, it is possible that the requirement and/or consequences of PI3K signalling for virus replication may depend upon a range of factors, including the host species, cell-type, or stage of infection.

It should be stressed that although many viruses have been reported to affect the PI3K signalling pathway, it is often unclear whether this kinase is actively stimulated by the pathogen, or if activation is simply a cellular response to infection (for examples see section **1.3**). Furthermore, it has yet to be determined if PI3K activation globally stimulates all substrates downstream of it, or if additional (spatial/temporal) regulation at distinct levels of the pathway ensure the mediation of particular signals. Thus, although many investigations report upon specific virus-modulated PI3K-dependent pathways, it may be that virus-activated PI3K does not discriminate with regards its normal cellular targets. With this in mind, viral strategies to modulate components of the PI3K pathway are detailed (as they have been reported) in the text

below. DNA virus families are dealt with first, followed by RNA virus families (indicated within parentheses).

### *1.4.1 PI3K and the Adenoviridae family (DNA).*

Binding of the adenovirus (AdV) capsid penton to cell-surface integrins not only initiates the PI3K-dependent uptake (endocytosis) of virions (Li *et al*., 1998a), but has been shown to stimulate the PI3K-dependent induction of TNFα and the maturation of dendritic cells (Philpott *et al*., 2004). Additionally, the E4-ORF1 oncoproteins of some AdV subtypes bind directly to PDZ domain-containing proteins and directly stimulate PI3K activity, thereby inducing the phosphorylations of Akt and p70S6K (Frese *et al*., 2003). This also causes GSK-3β phosphorylation, stabilizes β-catenin levels, and decreases the activity of the FKHR transcription factor (Frese *et al*., 2003, Zhang *et al*., 2004). These signalling events have been associated with AdV-mediated mammary tumourigenesis (Frese *et al*., 2003), selective endothelial cell survival (Rajala *et al*., 2005, Zhang *et al*., 2004), gap junction expression, and vascular angiogenesis (Zhang *et al*., 2004, Zhang *et al*., 2005). In contrast to the stimulation of PI3K by virus binding or E4-ORF1 activity, the AdV E1A protein can down-regulate the basal level of Akt phosphorylation in some human cell-types (probably by acting upstream of PI3K), and can thus potentiate apoptosis and tumour suppression (Viniegra *et al*., 2002). This function of E1A is remarkable, given that E1A was originally described as a transforming oncoprotein in rodent cells [reviewed in (Frisch & Mymryk, 2002)].

### *1.4.2 PI3K and the Hepadnoviridae family (DNA).*

Hepatitis B virus (HBV; *Hepadnoviridae*) causes acute liver disease, although ~10% of infections become chronic and may subsequently result in the development of

hepatocellular carcinoma (Cooray, 2004). The HBV X protein is multifunctional, and regulates a variety of host-cell processes which may contribute to HBV-induced transformation [reviewed in (Murakami, 1999)]. In particular, HBV X activates a Src (and possibly Ras)-mediated, PI3K-dependent signalling cascade involving the phosphorylation and activation of Akt, the phosphorylation of BAD, and the down-regulation of caspase-3 (Klein & Schneider, 1997, Lee *et al*., 2001, Shih *et al*., 2003). These processes have been linked to increased cell viability during HBV infections (Lee *et al*., 2001), and it has been noted that the X protein can specifically prevent apoptosis induced by transforming growth factor β (TGFβ) (Shih *et al*., 2000). In addition, the PI3K activating function of HBV X leads to the overexpression of matrix metalloproteinase-9, the secretion of which causes breakdown of the extra-cellular matrix, and may be responsible for the increased likelihood of HBV-infected tumour cells spreading (Chung *et al*., 2004).

### *1.4.3 PI3K and the Herpesviridae family (DNA).*

Epstein-Barr virus (EBV; *Herpesviridae*) is associated with the development of both lymphoid (B-cell) and epithelial tumours [reviewed in (Rickinson & Kieff, 2001). Transformation of B-cells is linked to the expression of a limited set of latent viral genes, including the integral membrane proteins LMP1 and LMP2A [reviewed in (Rickinson & Kieff, 2001)]. LMP1 is the major transforming protein of EBV (Dawson *et al*., 2003), and can act as a constitutively activate viral homologue of the cellular TNF receptor (TNFR) superfamily (Gires *et al*., 1997). The cytoplasmic tail of LMP1 recruits a number of signalling adapter proteins, and upregulates many host-cell pathways [reviewed in (Rickinson & Kieff, 2001)]. Oligomerised LMP1 can also interact with the PI3K p85 subunit, potentially bringing it into contact with other activating signals at the plasma membrane, and causing constitutive PI3K-Akt activity

(Dawson *et al*., 2003). This not only provides a cell survival signal, but also induces actin remodelling associated with cell migration, both factors which could contribute to B-cell transformation and/or EBV persistence (Dawson *et al*., 2003).

The other latency-associated EBV integral membrane protein, LMP2A, also causes the PI3K-dependent phosphorylations of Akt, GSK-3β, and FKHR (Morrison *et al*., 2003, Swart *et al*., 2000). However, as LMP2A is not associated with B-cell survival (Speck *et al*., 1999), this activity may regulate another function in lymphoid tissues, such as cell-cycle progression (Swart *et al*., 2000). In epithelial cells (often the first cell-type infected by EBV), LMP2A-mediated PI3K activation likely contributes to anchorage-independent cell growth and survival (Scholle *et al*., 2000). Thus, LMP2A may play an important role in early virus spread, and ultimately in epithelial cell tumour development. In addition to the contribution of LMP1- and LMP2A- mediated PI3K activation in EBV-induced lymphoid and epithelial cell survival, PI3K-Akt signalling is also necessary for EBV reactivation from latency (Darr *et al*., 2001). Indeed, the viral BRLF1 protein activates PI3K and disrupts EBV latency, thereby inducing the lytic form of EBV (Darr *et al*., 2001). As EBV expresses several proteins that modulate PI3K signalling at different stages of its latent/lytic replication cycle, it is clear that the overall regulation of this pathway during infection is complex.

A number of other herpesviruses modulate the activity of PI3K pathway components by various means, and for various reasons: Kaposi's sarcoma-associated herpesvirus (KSHV) latency-associated nuclear antigen (LANA), and the herpesvirus saimiri homologue ORF73, both bind to GSK-3β and sequester it in the nucleus, thus stabilising β-catenin levels and stimulating β-catenin-dependent transcription (Fujimuro & Hayward, 2003, Fujimuro *et al*., 2003); Varicella-Zoster virus (VZV)

activates a PI3K-Akt-GSK-3β pathway to enhance virus replication, a process dependent upon expression of two VZV kinases: ORF47 and ORF66 (Rahaus *et al*., 2007); Herpes simplex virus type-1 (HSV-1) infection induces the early (anti-apoptotic) PI3K-dependent phosphorylation of Akt at Ser473, which is then down-regulated by the late expression of the HSV-1 U(S)3 serine/threonine protein kinase via an unknown mechanism (Benetti & Roizman, 2006); and human cytomegalovirus (hCMV) activates a PI3K-Akt-p70S6K-NFκB response in order to upregulate cellular gene expression and promote the transcription/translation of hCMV immediate-early genes, which may promote hCMV DNA replication (Johnson *et al*., 2001).

### *1.4.4 PI3K and the Papillomaviridae family (DNA).*

Human papillomavirus (HPV) usually causes benign epithelial warts, but some high-risk sub-types (such as HPV-16) are associated with severe cervical and urogenital cancers [reviewed in (zur Hausen, 2002)]. HPV-16 E5 protein associates with the cellular epidermal growth factor (EGF) receptor, and can augment the EGF-induced stimulation of PI3K-Akt-BAD, resulting in reduced sensitivity of cells to apoptosis (Zhang *et al*., 2002). Intriguingly, the HPV-16 E7 protein (a major oncoprotein), also contributes to this phenotype (Pim *et al*., 2005). E7 binds to the two subunits of protein phosphatase 2A (PP2A), and prevents PP2A from dephosphorylating Akt, thus maintaining activation of cell survival signalling (Pim *et al*., 2005).

### *1.4.5 PI3K and the Polyomaviridae family (DNA).*

The polyomavirus "tumour" (or "T") antigens are produced early during infection, and stimulate cellular DNA replication leading to the re-entry of resting cells into the cell cycle (Cooray, 2004). The middle "T" antigen (MT) of murine polyomavirus can be tyrosine-phosphorylated by cellular Src-kinases, and the resulting phospho-motifs

in MT provide consensus docking sites for the SH2 domains of p85 subunits (Courtneidge & Heber, 1987, Kaplan *et al*., 1987, Talmage *et al*., 1989, Yoakim *et al*., 1992). In this way, phosphorylated MT is associated with increased anti-apoptotic PI3K-Akt activity (Dahl *et al*., 1998, Whitman *et al*., 1985), a process likely to contribute to MT-induced transformation (Dahl *et al*., 1998, Whitman *et al*., 1985). In addition, the large "T" antigen (LT) of some human neurotropic polyomaviruses can bind directly to β-catenin, inducing its nuclear accumulation, and increasing transcription of β-catenin-dependent genes (Gan & Khalili, 2004). Thus, polyomaviruses can act via different mechanisms (and at different levels) in order to activate unique, virus-specific components of the PI3K pathway.

### *1.4.6 PI3K and the Coronaviridae family (RNA).*

The severe acute respiratory syndrome (SARS)-associated coronavirus (SARS-CoV; *Coronaviridae*) was identified as an emerging and important infectious disease agent in 2003 (Ksiazek *et al*., 2003, Rota *et al*., 2003). SARS-CoV infection of Vero-E6 cells has been demonstrated to cause the transient phosphorylation (Ser473) of Akt (Mizutani *et al*., 2004), which may delay virus-induced apoptosis and contribute towards the establishment of persistent tissue-culture infections (Mizutani *et al*., 2004, Mizutani *et al*., 2005). The mechanism of Akt phosphorylation during SARS-CoV infection is controversial, but appears to require active replication and PI3K function (Mizutani *et al*., 2004). Additionally, one study has demonstrated that vaccinia virus-mediated expression of the SARS-CoV N (nucleocapsid) protein (but not Green Fluorescent Protein; GFP) is sufficient to cause Akt phosphorylation (Ser473) in Vero-E6 cells (Mizutani *et al*., 2006). However, work in COS-1 cells previously showed that N expression actually induces a host apoptotic response, and no striking differences in Akt phosphorylation (Thr308) were observed (Surjit *et al*., 2004). The

situation is also complicated by possible roles for the SARS-CoV S (spike) and M (membrane) proteins in de-regulating Akt signalling (Chan *et al*., 2007, Liu *et al*., 2007). Indeed, expression of M protein in *Drosophila* cells has been shown to cause the de-phosphorylation of Akt, and is pro-apoptotic (Chan *et al*., 2007). Clearly, more work is needed to clarify the temporal activity and functions of PI3K-Akt signalling during SARS-CoV infections.

### *1.4.7 PI3K and the Flaviviridae family (RNA).*

Viruses of the *Flaviviridae* family are enveloped, with a single-stranded positive sense RNA genome. They are perhaps within one of the most characterised RNA virus families with regards viral interaction with host-cell PI3K. Hepatitis C virus (HCV; *Flaviviridae*; hepacivirus) is a major public health concern, and commonly establishes long-term infections culminating in chronic inflammation, cirrhosis, and hepatocellular carcinoma [reviewed in (Cooray, 2004, Street *et al*., 2005)]. The HCV NS5A protein interacts with the p85:p110 heterodimer by binding directly to the SH3 domain of p85 (He *et al*., 2002b, Street *et al*., 2004). Although NS5A clearly increases the lipid kinase activity of PI3K (Street *et al*., 2004), the mechanism of activation is unclear. For example, the interaction between NS5A and p85 appears to be enhanced *in vivo* by EGF stimulation, and NS5A has been suggested to form a heterotrimeric complex consisting NS5A, p85, and Gab1 (an EGF receptor docking protein) (He *et al*., 2002b). Thus, NS5A may promote the interaction of p85:p110 heterodimers with active Gab1:EGFRs, thereby increasing EGF-induced PI3K activity (Cooray, 2004, He *et al*., 2002b). This would be consistent with the observation that expression of NS5A in mammalian cells only significantly increases p85 tyrosine phosphorylation (a marker of active PI3K) in response to EGF stimulation (He *et al*., 2002b). However, *in vitro* experiments have shown that

baculovirus- (but not *E. coli*-) expressed and purified NS5A can directly increase the lipid kinase activity associated with p85 immunoprecipitates (M. Harris, *personal communication*), suggesting that membrane and/or receptor recruitment of p85:p110 is not essential for NS5A-mediated PI3K activation (Street *et al*., 2004), and that eukaryotic post-translational modification of NS5A (such as phosphorylation [reviewed in (Macdonald & Harris, 2004)]) may be necessary.

The effect of NS5A on PI3K causes an increase in the Ser473 phosphorylation (and kinase activity) of Akt (He *et al*., 2002b, Street *et al*., 2004, Street *et al*., 2005). Downstream of Akt, BAD and GSK-3β have been identified as phosphorylation substrates of NS5A-activated PI3K (He *et al*., 2002b, Street *et al*., 2004, Street *et al*., 2005). As consequences of these phosphorylation events, NS5A has been reported to stimulate β-catenin dependent transcription, as well as reduce the transcriptional activity of FOXO/FKHR (Street *et al*., 2005). Additionally, HCV can stimulate mTOR activity downstream of Akt, and thus induces the activation of p70S6K and the inactivation of 4E-BP1, thus leading to overall translational enhancement (Mannova & Beretta, 2005).

The biological consequences of NS5A activating the PI3K signalling pathway are not entirely known, although anti-apoptotic and some translational effects have clearly been demonstrated (Mannova & Beretta, 2005, Street *et al*., 2004). Thus, by limiting cell-death, NS5A-activated PI3K may augment the persistence of HCV infections, a factor which could contribute to the development of infection-associated chronic liver disease and carcinoma (He *et al*., 2002b, Street *et al*., 2004, Street *et al*., 2005).

Two flavivirus members of the *Flaviviridae* family (namely, DEN and JEV) have also been reported to activate PI3K-Akt signalling in order to counter virus-induced

apoptosis (Lee *et al*., 2005). Intriguingly, activation occurs at an early stage of infection (possibly virus binding), and appears to require intact lipid-rafts (Lee *et al*., 2005). DEN- and JEV- induced Akt phosphorylation peaks during the first few hours of infection and then subsides, although the mechanism behind this negative-regulation is unknown (Lee *et al*., 2005). Despite the anti-apoptotic function of this pathway during infection, PI3K inhibitors have little effect on DEN and JEV RNA synthesis or protein levels, suggesting that these viruses [unlike HCV (Mannova & Beretta, 2005)] do not absolutely require host PI3K-mediated translational functions (Lee *et al*., 2005). However, the absolute role of PI3K during flavivirus infections is complicated by the report implicating PI3K as an essential mediator of JEV- and DEN- induced IFNβ production (Chang *et al*., 2005). Similarly, although some biotypes of Bovine Viral Diarrhoea Virus (BVDV; *Flaviviridae*; pestivirus) can induce Akt phosphorylation during infection (Bendfeldt *et al*., 2007), it is unclear whether this is functionally virus-mediated, or if it is part of the cellular anti-viral response.

### *1.4.8 PI3K and the Paramyxoviridae family (RNA).*

Human respiratory syncytial virus (hRSV; *Paramyxoviridae*; pneumovirus) is a globally important cause of lower respiratory tract disease in young children [reviewed in (Collins *et al*., 2001)]. hRSV infection of tissue-culture cells induces the lipid kinase activity of PI3K, and the subsequent PI3K-dependent phosphorylations of Akt and GSK-3β (Thomas *et al*., 2002). PI3K activation during hRSV infection appears to be transient – phosphorylation of Akt is first detectable ~30 mins after infection, and is maintained until at least 6h post-infection (Thomas *et al*., 2002). Interestingly, phospho-Akt/phospho-GSK-3β is undetectable at ~24h post-infection, although total levels of Akt/GSK-3β are unaffected (Thomas *et al*., 2002). hRSV-

induced PI3K-dependent phosphorylation of Akt causes the activation of the E3-ubiquitin ligase, MDM2, which targets the pro-apoptotic tumour suppressor, p53, for proteasome-mediated degradation (Groskreutz *et al*., 2007). Although, the mechanism of hRSV-induced PI3K activation is unclear, specific siRNA-mediated knock-down of either NS1 or NS2 mRNAs during infection has been shown to completely abrogate the phosphorylation of Akt (Bitko *et al*., 2007). Similarly, viruses unable to express either NS1, NS2 (or both NS1 and NS2) are unable to cause Akt phosphorylation (Bitko *et al*., 2007). Thus, studies using such NS1/NS2-deleted viruses (as well as work done with inhibitors of PI3K) have lead to the proposition that PI3K activation during hRSV infection induces the Akt-MDM2 pathway to reduce levels of p53, and thereby limit virus-induced apoptosis (Bitko *et al*., 2007, Groskreutz *et al*., 2007, Thomas *et al*., 2002).

Suppression of immune functions is a major contributory factor associated with infant deaths during acute measles virus (MV; *Paramyxoviridae*; paramyxovirus) infections (Avota *et al*., 2001). This immunosuppression is thought to be caused by the co-interaction of both MV fusion (F) and haemagglutinin (H) glycoproteins with the lipid-raft surfaces of T-cells (Avota *et al*., 2004), which has been shown to reduce T-cell proliferation *in vitro* (Avota *et al*., 2001, Schlender *et al*., 1996). This cell-surface contact event appears to specifically prevent IL-2-induced phosphorylation of Akt (Ser473), as (surprisingly) it does not affect BAD phosphorylation (Avota *et al*., 2001, Avota *et al*., 2004). Thus, MV modulates T-cell immune function without sensitizing the cell to apoptosis (Avota *et al*., 2001). Mechanistically, contact of MV with T-cell lipid rafts induces the nuclear translocation of splicing proteins, which specifically upregulate expression of the lipid phosphatase, SIP110 (Avota *et al*., 2006). SIP110 can dephosphorylate PtdIns(3,4,5)$P_3$, and thus depletes the cellular

reservoir of this second messenger that would otherwise cause sustained Akt phosphorylation (Avota *et al*., 2006). Additionally, contact of MV glycoproteins with T-cell lipid rafts may stabilize the levels of Cbl-b, a cellular protein that can chaperone the PI3K p85 subunit away from lipid raft signalling hubs (Avota *et al*., 2004), thereby attenuating PtdIns(3,4,5)$P_3$ generation.

### *1.4.9 PI3K and the Picornaviridae family (RNA).*

Studies on human rhinovirus type-2 (hRV2; *Picornaviridae*) and enterovirus-71 (EV71; *Picornaviridae*) have demonstrated a role for PI3K in the transport of internalized virion particles from early vesicles to late (low pH) endosomes (Brabec *et al*., 2006, Wong *et al*., 2005). Indeed, an early receptor (integrin) binding event mediated by the VP-1 capsid protein has been shown to induce Akt phosphorylation during Foot-and-Mouth disease virus (FMDV) infections (Peng *et al*., 2004).

In addition to viral entry into the cytoplasm, PI3K may also be important at later times during the picornavirus replication cycle. Coxsackievirus B3 (CVB3) infection stimulates the PI3K-dependent phosphorylations of Akt (both Ser473 and Thr308) and GSK-3β at ~6h post-infection (Esfandiarei *et al*., 2004). Such stimulation is dependent upon CVB3 genome replication, and appears to delay apoptosis, augment viral RNA synthesis, increase protein expression, and (ultimately) increase progeny virus production (Esfandiarei *et al*., 2004, Zhang *et al*., 2003). Intriguingly, the delay in apoptosis is not responsible for increasing viral protein expression (Esfandiarei *et al*., 2004), suggesting that CVB3-mediated activation of PI3K-Akt regulates viral protein expression directly and independently.

The mechanism of PI3K activation during CVB3 infection is unclear, but it is interesting to note that RasGAP (a cellular negative-regulator of the PI3K-activating

protein, Ras) is cleaved in CVB3-infected cells (Huber *et al*., 1999). Thus, as the kinetics of this cleavage event correlate well with the observed phosphorylations of Akt (Huber *et al*., 1999), it has been speculated that this may contribute to PI3K activation (Esfandiarei *et al*., 2004). Such a mechanism may be common to many picornaviruses, as echovirus-11 and echovirus-12 also induce the cleavage of RasGAP during infection (Huber *et al*., 1999). Additionally, IFNγ inducible GTPase [IGTPase; the expression of which is up-regulated during CVB3 infections (Yang *et al*., 1999)], can also stimulate PI3K activity, and this protein may therefore also play a role in CVB3-mediated PI3K-Akt activation (Zhang *et al*., 2003). However, overexpression of IGTPase actually causes a reduction in CVB3 replication (Zhang *et al*., 2003), indicating a potential anti-viral function for this IFN-inducible enzyme. Thus (as stated previously), it is often difficult to distinguish between possible virus- and/or cell- instigated PI3K activities, which are likely to direct opposing effects.

### *1.4.10 PI3K and the Retroviridae family (RNA).*

Many retroviruses activate the PI3K pathway during infection. Indeed, Akt was originally identified as a cellular homologue of an oncoprotein encoded by the acutely transforming retrovirus, AKT-8 (Bellacosa *et al*., 1991, Staal, 1987). The human T-cell leukaemia virus type-1 (HTLV-1) Tax protein stimulates the PI3K-Akt signalling pathway to increase the accumulation and transcriptional activity of β-catenin and HIF-1α transcription factors (Liu *et al*., 2001, Tomita *et al*., 2006, Tomita *et al*., 2007). Such activity may contribute towards the survival of infected T-cells, and likely plays a crucial role in transformation (Liu *et al*., 2001, Tomita *et al*., 2006).

For human immunodeficiency virus type-1 (HIV-1), binding of the virion gp120 glycoprotein to the CD4 surface molecule of T-cells results in the G-protein and Lck-

mediated activation of class IB PI3K (Briand *et al*., 1997, Francois & Klotman, 2003). gp120 binding to CD4 stimulates phosphorylation of both Akt and the downstream p70S6K, and appears to be required for a function prior to HIV-1 genome integration (Francois & Klotman, 2003). In addition to gp120, soluble (and secreted) HIV-1 Tat protein also causes the PI3K-dependent phosphorylation and activation of Akt (Borgatti *et al*., 1997, Deregibus *et al*., 2002, Zauli *et al*., 2001). Such Tat-mediated PI3K activation is biphasic, with initial stimulation probably occurring by the binding of soluble Tat to the cell-surface receptor VEGFR-2, and the later stimulation requiring internalised Tat-mediated synthesis of IGF-1 and IL-3 (Deregibus *et al*., 2002). Thus, a suggested role for the multifunctional Tat protein with regards PI3K may be to regulate cell proliferation or apoptosis (Borgatti *et al*., 1997, Deregibus *et al*., 2002, Zauli *et al*., 2001).

HIV-1 proteins can also directly stimulate components of the PI3K pathway. For example, the HIV-1 Vpu protein blocks the ubiquitination and proteasomal degradation of β-catenin by binding and inactivating the βTrCP E3-ligase (Besnard-Guerin *et al*., 2004). In addition, expression of HIV-1 Nef protein can increase PI3K activity (Schibeci *et al*., 2000), and Nef has been reported to bind directly to the C-terminal half of p85 (Linnemann *et al*., 2002). Such an interaction between Nef and PI3K has been proposed to contribute towards the Nef-mediated stimulation of IL-2 production (Schibeci *et al*., 2000), the downregulation of surface MHC class I molecules (Blagoveshchenskaya *et al*., 2002, Larsen *et al*., 2004, Swann *et al*., 2001), and the anti-apoptotic phosphorylation of BAD (Wolf *et al*., 2001).

Chapter 2

# Materials and Methods

## 2.1 Mammalian cells and tissue culture.

### *2.1.1 Cell types used in this study.*

293T: human embryonic kidney cells.

HEp2: human larynx carcinoma epithelial cell-line.

1321N1: human brain astrocytoma cell-line (from Prof. C. Peter Downes).

MDCK: derived from the kidney of an adult cocker spaniel.

Vero: fibroblast-like cells originating from the kidney of an African Green monkey (from ICN Pharmaceuticals Ltd., U.K.).

In addition to the basic cell-lines mentioned above, the following permanent cell-lines (generated by others) were also used:

HEp2-PR8/NS1: HEp2 cells stably expressing the influenza A virus NS1 protein (PR8; produced by Dr. Yun-Hsiang Chen, Hale *et al*., 2006).

HEp2-SeV/V: HEp2 cells stably expressing the V protein of Sendai virus (produced by Dr. Yun-Hsiang Chen, Hale *et al*., 2006).

HEp2-SeV/C: HEp2 cells stably expressing the C protein of Sendai virus (produced by Dr. Yun-Hsiang Chen, *manuscript in prep*).

MDCKΔPTEN: MDCK cells stably expressing GFP and shRNA targeting PTEN (provided by Dr. Nick Leslie; Lackey *et al*., 2007).

MDCK-PIV5/V: MDCK cells stably expressing the V protein of PIV5 (Precious *et al*., 2005).

A549-NFκBLuc: A549 cell-line (human lung epithelial) stably expressing the Firefly luciferase gene under the control of the NFκB promoter (provided by Prof. Ron Hay, University of Dundee, U.K.).

The following stable cell-lines were also generated and used as part of this study:

1321N1-NS1: 1321N1 cells stably expressing the wild-type influenza A virus NS1 protein (PR8; Hale *et al*., 2006).

1321N1-NS1(Y89F): 1321N1 cells stably expressing the PR8/NS1 protein with a Y89F amino-acid substitution (Hale *et al*., 2006).

MDCK-iSH2: MDCK cell-line constitutively expressing the C-terminally V5-tagged iSH2 domain of bovine p85β.

MDCK-NLS-iSH2: MDCK cell-line constitutively expressing the C-terminally V5-tagged iSH2 domain of bovine p85β with an N-terminal NLS derived from the SV40 T antigen (MPKKKRKV).

### *2.1.2 Cell maintenance.*

Cell monolayers were generally maintained in 25cm$^2$ or 75cm$^2$ tissue culture flasks (Greiner, U.K.) in DMEM (Dulbecco's modified Eagle's medium; Invitrogen, U.K.) supplemented with 10% (v/v) heat-inactivated FBS (foetal bovine serum; Biowest) and incubated at 37ºC/5% $CO_2$. Cells were routinely trypsinised (Trypsin, EDTA; Becton Dickinson Ltd., U.K.), and diluted every 4-7 days as appropriate.

### *2.1.3 Cell stock freezing and resuscitation.*

Adherent cells were trypsinised, resuspended in the relevant growth medium, and pelleted at ~500x*g* for 5mins. Cells were then resuspended in "freezing medium" (DMEM supplemented with 20% FBS and 10% DMSO), aliquoted into cryovials, and (slowly) frozen at -70ºC before long-term storage in liquid $N_2$. For resuscitation of cells, cryovials were rapidly thawed at 37ºC before centrifugation at ~500x*g*. Cells were then resuspended and grown in normal growth medium (tissue culture flasks) at

37ºC/5% $CO_2$ overnight. Medium was replaced after 24h in order to remove traces of DMSO.

### *2.1.4 Treatment of cells.*

As required, cells were treated with media supplemented with recombinant human IFNαA/D (PBL Biomedical Labs) or recombinant human TNFα (Dr. Rachel Evans, University of Dundee, U.K.). For stimulation of IFN-responsive promoters, cells were incubated overnight with $10^3$ units/ml IFNα. For stimulation of NFκB-responsive promoters, cells were incubated with TNFα at a concentration of 50ng/ml for ~6h. LY294002 (Calbiochem, U.S.A), LY303511 (Alexis, U.S.A.), and wortmannin (Sigma-Aldrich, U.K.) were dissolved in DMSO and used as directed by the respective manufacturer. Transfection of cells by plasmids (and synthetic dsRNA; poly I:C) was carried out using the FuGENE 6 transfection reagent (Roche Diagnostics, U.K.), according to the manufacturer's instructions. As required, cells were metabolically labelled for 1h with [$^{35}$S]methionine (25 μCi/25cm$^2$ flask; Amersham International Ltd., U.K.) in methionine-free tissue-culture medium.

## 2.2 Mammalian viruses and infection of cells.

### *2.2.1 Main replicating viruses used in this study.*

**PIV5 (W3):** wild-type laboratory strain of PIV5.

**CPI-:** canine isolate of PIV5 unable to block IFN signalling. Is also reported to induce the synthesis of IFN (Poole *et al*., 2002).

**PR8:** influenza virus strain A/Puerto Rico/8/34 (stock provided by Dr. A. Douglas, National Institute for Medical Research, U.K.).

**rUd WT:** recombinant wild-type influenza A virus (A/Udorn/72) generated and provided by Dr. Dave Jackson, Northwestern University, U.S.A. (Hale *et al*., 2006).

**rUd NS1-Y89F:** recombinant influenza A virus (A/Udorn/72) expressing NS1 with the Y89F amino-acid substitution. Generated and provided by Dr. Dave Jackson, Northwestern University, U.S.A. (Hale *et al*., 2006).

**rWSN WT:** recombinant wild-type influenza A virus (A/WSN/33) generated and provided by Dr. Dave Jackson, Northwestern University, U.S.A. (Hale *et al*., 2006).

**rWSN NS1-Y89F:** recombinant influenza A virus (A/WSN/33) expressing NS1 with the Y89F amino-acid substitution. Generated and provided by Dr. Dave Jackson, Northwestern University, U.S.A. (Hale *et al*., 2006).

As required, UV-inactivation of viruses was performed as described by others (Hayman *et al*., 2006). Efficiency of UV-inactivation was assessed using virus titration by plaque assay (see below).

### 2.2.2 Infection of cells.

For infections where determination of infectious virus was not required, no trypsin was added to the medium. Monolayers of cells were infected with virus diluted in serum-free DMEM at an appropriate multiplicity of infection (MOI). Monolayers were washed prior to infection in PBS to remove all traces of serum. After an adsorption period of 1-2h on a rocking platform at 37°C, the virus inoculum (or DMEM only for mock infections) was removed and replaced with serum-free DMEM. Cells were incubated at 37°C/5% $CO_2$ until harvested.

### 2.2.3 Virus yield assays.

To determine infectious titres over multiple replication cycles, 90% confluent MDCK cell monolayers were infected at a MOI of 0.001 PFU/cell in serum-free DMEM for 1h on a rocking platform at 37°C. Monolayers were washed twice in sterile PBS prior to infection in order to remove all traces of serum. After the adsorption period, the virus inoculum was removed by washing twice in PBS, and the cultures were subsequently incubated in serum-free DMEM (supplemented with 0.2% BSA and 2μg/ml TPCK- [L-1-tosylamido-2-phenylethylchloromethylketone] treated trypsin) at 37°C/5% $CO_2$. As required, chemical compounds under test were also included in this medium. Samples of the culture supernatants were removed at 24h, 48h, 72h, and 96h p.i., and virus titres were determined by plaque assay on Vero cells (PIV5), or MDCK cells (influenza A virus). as described below.

### 2.2.4 Preparation of virus stocks.

Stocks of the paramyxoviruses, PIV5(W3) and CPI-, were kindly maintained and provided as required by Mr. Dan Young (University of St. Andrews, U.K.). For stocks of the influenza A viruses, 90% confluent MDCK cells (2x75$cm^2$ flasks) were washed

twice in sterile PBS in order to remove traces of serum, and then infected in serum-free DMEM at an MOI of ~ 0.001 PFU/cell. After ~2h rocking at 37°C, the inoculum was removed and monolayers were washed with ~5mls serum-free DMEM. 12mls (per 75cm$^2$ flask) of serum-free DMEM supplemented with 2μg/ml TPCK-treated trypsin (Sigma-Aldrich, U.K.) was added to the monolayers. Cells were incubated at 37°C/5% $CO_2$ for ~36-48h, and supernatants were harvested when 50-70% CPE (cytopathic effect) was observed. Debris was removed by centrifugation (~3000x*g* for 10mins), and aliquots were frozen at -70°C. Titres were determined by plaque assay.

### *2.2.5 Titration of viruses.*

For titrations of PIV5, monolayers of Vero cells were grown in 6-well plates (Greiner, U.K.) until 80-90% confluent. Virus preparations were serially diluted 10-fold in DMEM supplemented with 2% FBS, and cells were inoculated with 1ml of each virus dilution per well. After adsorption for ~2h on a rocking platform at 37°C/5% $CO_2$, the virus inoculum was removed, and ~6mls of overlay (DMEM supplemented with 2% FBS and 0.5% carboxy methyl cellulose [Methocel MC; Sigma-Aldrich, U.K.]) was added to each well. Cells were incubated at 37°C/5% $CO_2$ for 8-10 days (without agitation) until distinct plaques had formed. The overlay was then removed, and monolayers were fixed for 1h with 5% formaldehyde in PBS. Areas of cell lysis (plaques) were visualised by staining with crystal violet (0.1% crystal violet, 3.6% formaldehyde, 1% methanol, 20% ethanol in $H_2O$) for 20mins, followed by rinsing in $H_2O$. Plaques were counted, and titres estimated as plaque-forming-units per ml of virus preparation; PFU/ml), taking into account the original dilutions made.

For titrations of influenza A viruses, monolayers of MDCK cells were grown in 6-well plates (Greiner, U.K.) until 80-90% confluent, and washed twice in serum-free

DMEM in order to remove all traces of serum. Virus preparations were serially diluted 10-fold in serum-free DMEM, and cells were inoculated with 1ml of each virus dilution per well. After adsorption for ~2h on a rocking platform at 37°C/5% $CO_2$, the virus inoculum was removed, and ~6mls of molten overlay (serum-free DMEM supplemented with 0.2% BSA, 2µg/ml TPCK-trypsin, and 0.9% agarose [NuSeive GTG; Cambrex, U.K.]) was added to each well. After the overlay had set, monolayers were incubated at 37°C/5% $CO_2$ for 3-4 days (without agitation) until distinct plaques had formed. 5% formaldehyde in PBS was then added on top of the agarose plugs, and the monolayers were left to fix overnight. Plaques were visualised by immunostaining as described below. Titres were estimated as plaque-forming-units per ml of virus preparation; PFU/ml), taking into account the original dilutions made.

### *2.2.6 Visualisation of virus plaques by immunostaining.*

To visualise influenza A virus plaques, an immunostaining technique was used. Fixed MDCK monolayers (see above) were permeablised (5% NP-40, 10% sucrose in PBS) for 15 min, and then washed in PBS supplemented with 1% FBS. Areas of virus infection (plaques) were visualised by detecting viral structural proteins. Monolayers were incubated for 1h at room-temperature with 500µl/well of appropriately diluted primary antisera (rabbit antisera raised against whole X31 [H3N2] or PR8 [H1N1]; diluted in PBS/1% FBS). Cells were washed with PBS/1% FBS, and monolayers were incubated for 1h at room-temperature with 500µl/well of appropriately diluted secondary anti-rabbit IgG alkaline phosphatase (AP)-conjugated antibody. Monolayers were subsequently washed with PBS/1% FBS, and incubated for ~30mins with 500µl/well of alkaline phosphatase substrate (as per manufacturer's instructions; Sigma-Aldrich. U.K.), or until sites of virus infection were easily visualised. The reaction was stopped by rinsing with water.

## 2.3 Molecular biology.

### *2.3.1 Polymerase chain reaction (PCR).*

Polymerase chain reaction (PCR) was used to amplify DNA fragments required for cloning. Products were obtained by PCR amplification using either Taq or *Pfu*I polymerases (Promega Ltd., U.K.) according to the manufacturer's instructions. The 50µl reaction mixture (made up in sterile water) consisted of enzyme buffer (at an appropriate dilution), 0.2mM of each dNTP, ~100-200ng of DNA template, ~0.1µM of each specific primer (forward and reverse), and ~3 units of DNA polymerase. PCR was generally performed using a GeneAmp PCR System 2400 (Applied Biosystems, U.K.) as follows: melting at 94°C for 30 secs, annealing at 55°C for 45 secs, and strand extension at 72°C (for Taq) or 68°C (for *Pfu*I) for 45 secs. The PCR set (typically 30 cycles) was preceded by an initial step of melting at 94°C for 5 min followed by a final step of strand extension at 72°C (for Taq) or 68°C (for *Pfu*I) for 7 mins.

### *2.3.2 Agarose gel electrophoresis.*

DNA was analysed by gel electrophoresis in horizontal mini-gels of 1% (w/v) agarose (Sigma-Aldrich, U.K.) in TBE buffer (45mM Tris-borate, 1mM EDTA). DNA samples were mixed with the appropriate volume of DNA loading buffer (Promega Ltd., U.K.), prior to electrophoresis. Samples were run at 90V in TBE buffer (containing 1 $\mu$g/ml ethidium bromide), until bands were clearly resolved. Along with the samples, known DNA size markers were also run (1kb ladder; Promega Ltd., U.K.). Resolved DNA bands of interest were excised under UV light, and DNA was recovered using a QIAquick™ Gel Extraction Kit (following manufacturer's instructions; QIAGEN® Ltd., U.K.).

### *2.3.3 Restriction enzyme digestion of DNA.*

Backbone vector DNA and purified PCR products were individually mixed with 2$\mu$l 10X enzyme buffer, 0.5$\mu$l (5U) each of the desired restriction enzymes (New England Biolabs, U.K.), and 0.2$\mu$l 10mg/ml acetylated BSA, in a reaction mixture made up to 20$\mu$l with de-ionised $H_2O$. Reactions were incubated at 37ºC (for the majority of enzymes) for 2-4h, or overnight as appropriate.

### *2.3.4 Ligation of DNA fragments (vector/insert).*

Insert and vector DNA were prepared by enzyme digestion/gel purification as described above. 10$\mu$l insert was mixed with 1$\mu$l vector, 2$\mu$l 10X ligase buffer, 1$\mu$l T4 DNA ligase and made up to 20$\mu$l with de-ionised $H_2O$. Reactions were incubated overnight at 16ºC and stored at –20ºC until ready for use. Ligation reaction products were used for heat-shock transformation of competent *E. coli* DH5α cells as described below.

### *2.3.5 Preparation of (heat-shock) competent bacterial cells.*

*E. coli* strain DH5α was used to propagate plasmids. *E. coli* strain BL21(DE3) was used exclusively for protein expression. Generally, bacteria were grown in liquid Luria-Bertani (LB) medium (10g/l bacto-tryptone, 5g/l yeast extract, 10mM NaCl, pH 7.5), or plated on solid LB medium supplemented with 1.5% (w/v) agar and 10mM $MgSO_4$. As appropriate, media were supplemented with ampicillin (100 μg/ml), or kanamycin (10 μg/ml).

For competent cells, a fresh bacterial mini-culture (10mls LB broth without antibiotic) was diluted 1/100 into 100mls of the same medium and incubated in a 37ºC shaking incubator until $OD_{600nm}$ reached ~0.6 (approx. 2h). The culture was then chilled on ice (15mins), before being pelleted by centrifugation at ~4000x*g* for 15mins. After

removal of the supernatant, cells were resuspended in 20mls filter sterilised TFB1 buffer (30mM potassium acetate, 10mM $CaCl_2$, 50mM $MnCl_2$, 100mM RbCl, 15% (v/v) glycerol, pH 5.8 – with 1M acetic acid). After 30mins on ice, cells were re-pelleted at ~4000xg for 15mins and resuspended in 2mls filter sterilised TFB2 (10mM MOPS (pH 6.5), 75mM $CaCl_2$, 10mM RbCl, 15% glycerol (v/v), pH 6.5 – with 1M KOH). Aliquots (100$\mu$l) were snap-frozen in liquid $N_2$ and stored at –70ºC until required.

### *2.3.6 Heat-shock transformation of competent cells.*

10$\mu$l of ligated reaction mix (or ~1$\mu$g pre-purified plasmid) was added directly to 100$\mu$l of thawed, competent cells. After incubation on ice for 1h, cells were heat-shocked in a 42ºC water bath for 2mins before being immediately transferred back to ice for a further 2mins. Cells were resuspended in 1ml LB broth and incubated at 37ºC for 1h (in order to assist cell recovery). The suspension was then plated out onto solid LB-agar plates supplemented with an appropriate antibiotic (90 mm-diameter Petri dishes; Scientific Laboratory Supplies Ltd., U.K.). Plates were inverted and incubated at 37ºC overnight. Mini-cultures were prepared from selected colonies.

### *2.3.7 Preparation of plasmid DNA.*

For small-scale preparations, bacterial cell cultures of 2-3 ml (in LB broth containing appropriate antibiotic) were grown overnight at 37°C in a shaking incubator. DNA was extracted from cells and purified on silica gel membrane columns using the QIAGEN® DNA mini-prep kit (QIAGEN® Ltd., U.K.), according to the manufacturer's instructions. For large-scale preparations, bacterial cultures of 200-250 ml were grown overnight at 37°C in a shaking incubator, and DNA was extracted from cells using the QIAfilter™ Plasmid Maxi Kit (following manufacturer's

instructions; QIAGEN® Ltd., U.K.). The extraction of DNA using these QIAGEN® kits is based on the alkaline lysis of bacterial cells, followed by adsorption of DNA onto silica in the presence of high salt.

### *2.3.8 Colony screening.*

Following heat-shock transformation of ligation reactions into competent cells, amplification of growing colonies, and mini-prep purification of DNA (see above), DNA samples were screened to find positive clones. 7.5µl of miniprep DNA was incubated with ~2U of the appropriate restriction enzyme(s), and 10X buffer in a 10µl reaction mix, and incubated at the appropriate temperature (37°C for the majority of enzymes) for 2-4h. DNA samples were analysed by agarose gel electrophoresis as described above.

### *2.3.9 UV spectrophotometry.*

Appropriately diluted plasmid DNA was quantified by measuring $Abs_{260}$ using a UVIKON 923 UV spectrophotometer (Bio-Tek Kontron Instruments Ltd., U.K.). An $Abs_{260}$ of 1 was taken to correspond to a dsDNA concentration of 50 $\mu$g/ml. The purity of DNA preparation (i.e. protein contamination) was estimated by calculating the $Abs_{260}/Abs_{280}$ ratio (ratios greater then 1.8 were considered acceptable).

## 2.4 Plasmid DNAs.

### *2.4.1 Plasmids used in this study.*

Several plasmids were used in this study, either directly, as backbone vectors for cloning, or to provide DNA sequences for the construction of new plasmids:

*pdl'MCS(C'V5)*: empty lentivirus vector for expressing proteins with a C-terminal V5-tag. Expression is controlled by the constitutive SFFV (spleen focus forming virus) promoter, which produces a single bicistronic mRNA that encodes for the protein of interest together with a puromycin resistance product for mammalian cell selection (modified from the self-inactivating lentivirus vector pHR-SIN-CSGW by Dr. Yun-Hsiang Chen; see **appendix A.1**).

*pdl'PR8/NS1(C'V5)*: lentivirus vector expressing C-terminally V5-tagged PR8/NS1; provided by Dr. Yun-Hsiang Chen (Hale *et al*., 2006).

*pdl'PR8/NS1(N'V5)*: lentivirus vector expressing N-terminally V5-tagged PR8/NS1; provided by Ms. Marian Killip (Hale *et al*., 2006).

*pdl'Vic/NS1(C'V5)*: lentivirus vector expressing C-terminally V5-tagged Vic/NS1; provided by Dr. Yun-Hsiang Chen (Hale *et al*., 2006).

*pdl'SeV/V(C'V5)*: lentivirus vector expressing C-terminally V5-tagged SeV/V; provided by Dr. Yun-Hsiang Chen (Hale *et al*., 2006).

*pCMVR8.91*: plasmid expressing the *gag/pol*, *tat* and *rev* genes of HIV-1 (used in lentivirus production, provided by Dr. Yun-Hsiang Chen).

*pMD-G*: plasmid expressing the vesicular stomatitis virus glycoprotein (VSV-G) gene (used in lentivirus production, provided by Dr. Yun-Hsiang Chen).

*pGEX-4T3*: used for bacterial expression of GST, and to generate vectors encoding GST-TEV-fusion proteins (Amersham Biosciences Ltd., U.K.).

*pHH21-Ud/NS*: vector containing the entire NS segment of Ud virus (provided by Prof. Robert A. Lamb, Northwestern University, U.S.A.). Used to amplify Ud/NS1 cDNA for other vectors, as well as to substitute nucleotides encoding the Y89F mutation prior to recombinant Ud virus rescue.

*pHH21-WSN/NS*: vector containing the entire NS segment of WSN virus (provided by Prof. Robert A. Lamb, Northwestern University, U.S.A.). Used to amplify WSN/NS1 cDNA for other vectors, as well as to substitute nucleotides encoding the Y89F mutation prior to recombinant WSN virus rescue.

*pEF.mda-5'c-myc*: mammalian expression vector encoding myc-tagged mda-5 (Andrejeva *et al*., 2004).

*pGFP.RIG-I(Heli)*: mammalian expression vector encoding the GFP-tagged helicase domain of RIG-I (provided by Dr. Friedemann Weber, University of Freiburg, Germany).

*pGFP.RIG-I(CARD)*: mammalian expression vector encoding the GFP-tagged CARD domain of RIG-I (provided by Dr. Friedemann Weber, University of Freiburg, Germany).

*pEF'tag*: mammalian expression vector derived from pEF-plink2 (provided by Dr. Jelena Andrejeva, University of St. Andrews, U.K.), and used to

express domains of bovine p85β with an N-terminal myc-tag.

*pMT2-bov.p85β*: used as template to amplify fragments of bovine p85β (provided by Prof. Bart Vanhaesebroeck, Queen Mary, University of London, U.K.).

*pEHISTEV*: used to generate T7-driven 6His-tagged constructs where the 6His-tag can be removed from the recombinant protein by digestion with TEV protease (provided by Dr. Huanting Lui, University of St. Andrews, U.K.).

*pGAD.p110α.ABD*: T7-driven expression of the adapter-binding domain of human p110α (provided by Dr. Marie DeFrances, University of Pittsburgh, U.S.A.; Zu *et al*., 2007).

### *2.4.2 Plasmids generated in this study.*

Several novel plasmids were generated for transfections, the isolation of stable cell-lines, or bacterial expression. The integrity of all new constructs was confirmed by sequencing prior to use (DNA Sequencing Service, Dundee, U.K.):

*pdl'WSN/NS1(C'V5)*: cDNA encoding for the NS1 protein of WSN was amplified by PCR from pHH21-WSN/NS using gene-specific primers. Silent splice acceptor mutations and a silent *Sma*I site were made by overlap PCR (i.e. pHH21-WSN/NS nucleotides 525-530 were changed from CCAGGA to CCCGGG without altering any amino acids). This prevents the production of NEP mRNA. The resulting PCR product was ligated between the *Spe*I and *Nde*I sites of pdl'MCS(C'V5).

*pGEX'TEV'PR8/NS1*: cDNA encoding for the PR8/NS1 protein was amplified from pdl'PR8/NS1(C'V5) by PCR using gene-specific primers. The forward primer contained a coding region (GAAAACCTGTATTTTCAGGGCGCC) for the cleavage

sequence of tobacco etch virus (TEV) protease. The digested PCR product was ligated between the *Eco*RI and *Not*I sites of pGEX-4T3.

*pGEX'TEV'Ud/NS1*, *pGEX'TEV'Vic/NS1, pGEX'TEV'PR8/NS1Δ72*: constructed as for pGEX'TEV'PR8/NS1 using specific templates. The template for Ud/NS1 was pHH21-Ud/NS, therefore the same splice acceptor mutation was generated as for pdl'WSN'NS1(C'V5). The template for Vic/NS1 was pdl'Vic'NS1(C'V5). Primers for pGEX'TEV'PR8/NS1Δ72 were designed to amplify nucleotides encoding amino acid residues 73-230 of PR8/NS1. All forward primers used to construct these plasmids contained nucleotides encoding for the TEV protease cleavage sequence (see above).

*pdl'PR8/NS1Δ73(C'V5):* nucleotides encoding amino acid residues 74-230 were amplified by PCR from pdl'PR8/NS1(C'V5) and ligated between the *Spe*I and *Nde*I sites of pdl'MCS(C'V5).

*pdl'β-iSH2(C'V5)*: nucleotides encoding amino acids 433-610 of bovine p85β were amplified by PCR from pMT2-bov.p85β and ligated between the *Spe*I and *Nde*I sites of pdl'MCS(C'V5).

*pdl'NLS'β-iSH2(C'V5)*: nucleotides encoding amino acids 433-610 of bovine p85β were amplified by PCR from pMT2-bov.p85β. The forward primer contained nucleotides encoding for the NLS of SV40 T antigen (namely, MPKKKRKV). The digested fragment was ligated between the *Spe*I and *Nde*I sites of pdl'MCS(C'V5).

*pHH21-Ud/NS (NS1-Y89F)* and *pHH21-WSN/NS (NS1-Y89F)*: A ~900bp fragment of the respective NS segment was amplified by PCR, and digested at the "internal" restriction sites: *Nco*I and *Apa*I. The gene-specific forward primer consisted of nucleotides encoding for the Y89F amino acid substitution, and *Nco*I/*Apa*I were

unique within the vector. Thus, after excision of the ~900bp *Nco*I/*Apa*I fragment from each wild-type vector, the "mutated" fragment was ligated in. Ligated constructs were screened by sequencing. The plasmids were sent to Prof. Robert Lamb (Northwestern University, U.S.A.) in order to rescue recombinant influenza A viruses.

*pEF-p85β'myc* and *pEHISTEV-p85β* plasmids: For mammalian expression vectors encoding N-terminally myc-tagged domains of bovine p85β, cDNA fragments corresponding to each domain [SH3 (aa 1-100), nSH2 (aa 313-433), iSH2 (aa 433-610), and cSH2 (aa 611-724)] were amplified by PCR from pMT2-bov.p85β and ligated between the *Nco*I and *Spe*I sites of pEF'tag. For T7-driven 6His-tagged β-iSH2 (aa 433-610), nSH2-iSH2 (aa 313-610), β-iSH2'565 (aa 433-564), and nSH2-iSH2'565 (aa 313-564), the relevant cDNAs were PCR amplified from pMT2-bov.p85β and ligated between the *Nco*I and *Not*I sites of pEHISTEV.

*pGEX'TEV'β-iSH2(565-610)*: A cDNA fragment encoding the C-terminal 45 amino acids of the p85β iSH2 domain was amplified by PCR using gene-specific primers and ligated between the *Nco*I and *Not*I sites of digested pGEX'TEV'PR8/NS1. The resulting plasmid encodes β-iSH2(565-610) with a GST-tag fused to its N-terminus. The GST-tag can be removed from the fusion protein by digestion with TEV protease.

*pEHISTEV'PR8/NS1*: 6His-tagged full-length PR8/NS1 was generated by partially digesting pGEX'TEV'PR8/NS1 with *Nco*I/*Not*I and ligating the ~700 bp fragment into the corresponding sites of linearised pEHISTEV.

For all plasmids, 4-primer overlap PCR was used to introduce specific site-directed point mutations into a variety of constructs as required (e.g. NS1-Y89F, NS1-M93A, NS1-D92E, NS1-Y89E, NS1-R38AK41A).

## 2.5 Lentivirus-mediated generation of stable cell-lines.

### *2.5.1 Lentivirus production.*

To generate recombinant lentiviral particles, 293T cells (70% confluent; 75 $cm^2$ tissue culture flask) were co-transfected with three plasmids: 3$\mu$g pCMVR8.91, 3$\mu$g pMD-G, and 5$\mu$g of the pdl vector containing the construct of interest. Supernatants were harvested at 48h and 72h post-transfection, and pooled. Cell debris was removed from supernatants by centrifugation at 3,000x*g* for 10mins, and filtering through 0.45$\mu$m Tuffryn membrane filters (Invitrogen, U.K.). Virus aliquots were frozen at -70°C until required.

### *2.5.2 Transduction of target cells.*

30% confluent target cell monolayers (25$cm^2$ flask) were transduced with the harvested lentivirus supernatant (estimated MOI of ~1PFU/cell) in the presence of Polybrene (Sigma-Aldrich, U.K.; 8µg/ml). 48h post-infection, pools of transformed cells were selected by resistance to puromycin (HEp2/1321N1 cells, 2µg/ml; MDCK cells, 5µg/ml). Puromycin-containing medium was replaced every four days until control naïve cells were dead. Stocks of stable cell-lines were frozen away, and working cell-lines were regularly subjected to re-selection with puromycin. Expression of the required protein was further confirmed by immunoblot/immunofluorescence analysis of cells using an anti-V5 antibody.

## 2.6 Immunoprecipitations.

### *2.6.1 General immunoprecipitations.*

For most applications, cells were washed twice in ice-cold PBS and lysed for 10mins on ice with 500µl (per $4.5x10^6$ cells) immunoprecipitation buffer (20 mM Tris-HCl (pH 7.8), 5 mM EDTA, 0.5% (v/v) NP-40, and 650mM NaCl, freshly supplemented with a protease inhibitor cocktail; Roche Diagnostics, Germany). Concentrations of NaCl were adjusted as required for each specific experiment. Lysates were further disrupted by sonication with an ultrasonic probe. Soluble antigen extract was prepared by centrifuging the cell lysate at 12,000x*g* for 50mins to pellet particulate matter. Large volume extracts were additionally clarified by filtration (pore size 0.1-0.2 µm).

Immune complexes were formed by incubating 1ml samples of the supernatant (soluble antigen extract) with ~1-5µg of the desired antibody for 2h at 4°C. The immune complexes were isolated by incubation with protein G-Sepharose 4B Fast Flow beads (Sigma-Aldrich Ltd., U.K.) for 1h at 4°C. Alternatively, immune complexes were formed by incubating the soluble antigen extracts with an excess of cross-linked anti-V5 mAb protein-G beads (see below). For some applications siliconised tubes (Sigma-Aldrich, U.K.) were used to minimize any non-specific protein binding. After extensive washing in immunoprecipitation buffer, proteins in the immune complexes were dissociated by heating in SDS-PAGE loading buffer at 100°C for 5min. Proteins were analysed by SDS-PAGE as described below.

### *2.6.2 Production of cross-linked anti-V5 mAb beads.*

The anti-V5 mAb is a high affinity antibody raised against a linear 14 amino-acid epitope (GKPIPNPLLGLDST) found on the paramyxovirus PIV5 P/V common N-

terminal domain (produced from a mouse hybridoma cell-line as required by Mr. Dan Young).

Protein G-Sepharose beads (binding capacity for IgG >20mg/mL) were pre-washed several times with ice-cold PBS. An appropriate amount of isolated beads was mixed with anti-V5 mAb tissue culture fluid (approx. 4mg of mAb per mL of beads), resuspended in binding buffer A (20mM Tris-HCl (pH 7.8); 650mM NaCl), and incubated at 4°C for 4h ("end over end" tumbler).

Antibody-bead complexes were rescued and isolated after four repeated wash cycles of: low-speed centrifugation to pellet beads, removal of supernatant, resuspension of beads in binding buffer A. Antibody-bead complexes were then washed twice in buffer B (3M NaCl; 1.5M glycine [buffered to pH 8.9 with 3M NaOH]), and twice with buffer C (3M NaCl; 50mM Borate [buffered to pH 8.9 with 3M NaOH]).

The antibody-bead complexes were then incubated with freshly prepared buffer D (40mM di-methyl-pimelimidate; 3M NaCl; 200mM Borate [buffered to pH 8.9 with 3M NaOH]) for 1h at 4°C ("end over end" tumbler). The beads were pelleted by low-speed centrifugation and incubated on ice (10mins) with buffer E (200mM ethanolamine [buffered to pH 8.0 with HCl]). Complexes were washed twice more with buffer E and then re-incubated for a further 2h at room temperature with buffer E ("end over end" tumbler).

Finally, the cross-linked anti-V5 mAb beads were subjected to four wash cycles with buffer A. The efficiency of cross-linking was determined by analysing bead samples (SDS-PAGE) at various stages throughout the procedure. Cross-linked beads were produced as required and stored as a 50% (v/v) slurry in buffer A at 4°C.

## 2.7 Protein expression/purification from *E. coli* and insect cells.

### *2.7.1 Protein purification from E. coli.*

GST- or 6His- tagged fusion proteins were expressed and purified from *E. coli* BL21(DE3) cells. Briefly, 10ml overnight mini-cultures (37°C) were diluted 1:100 into 3 litres fresh LB liquid medium (containing antibiotic), and grown at 30°C in a shaking incubator. When $OD_{600}$ reached 0.5-0.6 units, protein expression was induced by addition of 1mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 4h. After centrifugation, the bacterial cells were resuspended in buffer [50mM Tris-HCl (pH 7·8), 200mM NaCl, together with appropriate protease-inhibitor tablets (Roche, Germany)], and lysed by sonication. Lysates were clarified by centrifugation at 30,000 rpm for 30mins in a Beckman 42.1 rotor, and supernatants were filtered through a 0.45μm Tuffryn membrane filter (Invitrogen, U.K.). GST fusion proteins were purified by binding to glutathione-agarose beads (Sigma-Aldrich, U.K.), and (if required) eluted in buffer containing 10mM glutathione. 6His-tagged proteins were purified on Ni-NTA resin (GE Healthcare, Sweden), and eluted in buffer containing 100mM imidazole. As necessary, cleavage by TEV protease (1:50 dilution; Invitrogen, U.K.) was carried out overnight at room temperature with accompanying dialysis in 50mM Tris-HCl (pH 7.8), 200mM NaCl, 1mM DTT.

### *2.7.2 p85α and p85β expression in Sf9 (insect) cells.*

The *Spodoptera frugiperda* (*Sf9*) insect cell-line was maintained at 28°C as a suspension culture in TC-100 medium (Invitrogen, U.K.) supplemented with 7% FBS. Cells were routinely passaged and diluted every 3-5 days.

Titrated recombinant baculoviruses expressing untagged bovine p85α and p85β were kindly provided by Prof. Bart Vanhaesebroeck (Queen Mary University of London,

U.K.) (Gout *et al*., 1992). For p85α or p85β expression, monolayers of approximately $1x10^8$ *Sf9* cells in $300cm^2$ flasks (Helena Biosciences Ltd., U.K.) were infected with recombinant baculoviruses at an MOI of ~3 for 1h at 28°C with frequent rocking. After removal of inoculum, cells were incubated at 28°C for 72h in fresh medium. To harvest, the medium was removed and cells were scraped into 10mls PBS, centrifuged at 500x*g* for 5mins, and the cell pellet was resuspended in 5mls of a lysis buffer containing 50mM HEPES (pH 7.6), 10mM KCl, 1.5mM $MgCl_2$, 1% NP40, 5mM DTT, and 10% glycerol [supplemented with protease inhibitors (Roche, U.K.)]. Samples were centrifuged at 35,000x*g* for 30min at 4°C in a Beckman SW50.1 rotor, and supernatants were frozen at –70°C until required.

## 2.8 Miscellaneous assays.

### *2.8.1 EMCV-inhibition assays.*

The amount of IFN secreted by HEp2 cells was estimated by using a biological EMCV (Encephalomyocarditis virus)-inhibition assay in Vero cells. 50% confluent Vero cells in 96-well plates were pre-treated with UV-inactivated 2-fold serial dilutions of culture media containing IFN for 24h prior to infection with EMCV [which is sensitive to IFN]. The development of CPE was monitored 36h p.i. by crystal violet staining (see above). Units of IFN were estimated as the dilution of media required to reduced the CPE by ~50%.

### *2.8.2 Luciferase assays.*

Following virus infection and TNFα treatment, A549-NFκBLuc cell monolayers (in 6-well plates) were washed once in PBS and lysed for 2mins in 300µl (per well) luciferase buffer A (25mM Tris-phosphate (pH 7.8), 8mM $MgCl_2$, 1mM DTT, 1mM EDTA, 1% TritonX-100). Following lysis, 300µl (per well) of luciferase buffer B (30% glycerol, 0.8mM ATP, 2% BSA in luciferase buffer A) was added to each sample. A portion of the soluble cell extract (300µl) was subsequently transferred to a luciferase cuvette into which 100µl of luciferase substrate (1.5mM luciferin in TE buffer) was injected. Substrate injection, as well as measurement of the light emitted over the first 10s following substrate addition (measured in relative light units; RLU), was performed using a Lumat LB9501 luminometer (Bertold, Germany).

### *2.8.3 In vitro PI3K assays.*

Monolayers from 6-well plates were fixed and lysed by addition of ice-cold lysis buffer (1% (v/v) TritonX-100, 120mM NaCl, 50mM NaF, 1mM $MgCl_2$, 1mM EGTA,

1mM EDTA, 5mM β-glycerophosphate, 25mM HEPES, pH 7.6 at 4°C, supplemented with 0.1 mM PMSF, 0.1mM benzamidine, 10μM leupeptin, 1mM $Na_3VO_4$, and 1mM DTT). Cell debris was centrifuged for 5min at 20,000x*g* (4°C), and lysate supernatants were frozen in liquid $N_2$ before storage at -80°C. Analysis of NS1-associated PI3K activity was performed by Dr. Ian Batty (University of Dundee. U.K.). Briefly, cell lysates were mixed for ~2h at 4°C with 10μg of anti-V5 antibody pre-coupled to protein G-Sepharose beads. Immunoprecipitates were collected by brief centrifugation, washed, and analysed for PI3K activity as described previously (Herbert *et al*., 2000, Tang & Downes, 1997).

### *2.8.4 In vitro synthesis of [$^{35}$S]methionine-labelled proteins.*

[$^{35}$S]methionine-labelled proteins were synthesised individually from plasmid DNA via T7-dependent *in vitro* translations carried out using the TnT® Quick Coupled Transcription/Translation system (Promega, U.S.A.) according to the manufacturer's instructions. For protein interaction experiments, lysates expressing different proteins were mixed on ice for 30mins prior to addition of antibody (1h). Mixtures were then diluted up to 0.5ml with immunoprecipitation buffer (see above), and incubated with protein G-Sepharose beads for 1h to precipitate immune complexes. Samples were washed and analysed as described in **sections 2.6.1** and **2.9.1**.

## 2.9 Protein analysis.

### *2.9.1 SDS polyacrylamide gel electrophoresis (SDS-PAGE).*

Protein samples were prepared in SDS-PAGE disruption buffer (6M Urea, 2M β-mercaptoethanol, 4% (w/v) SDS, with bromophenol blue colouring) and heated at 100°C for 2 mins prior to analysis. Polypeptides were separated through 4-12% NuPAGE polyacrylamide gradient gels (Invitrogen, U.K.) by electrophoresis at 180-200V until maximum resolution of polypeptide bands was noted. MOPS [3-(*N*-morpholino)propanesulphonic acid] or MES [2-(*N*-morpholino)ethanesulphonic acid] buffer was used as appropriate (Invitrogen, U.K.). Gels were stained (0.5% (w/v) R250 Coomassie Blue, 40% (v/v) methanol, 10% (v/v) acetic acid) and de-stained (20% (v/v) methanol, 10% (v/v) acetic acid) as necessary. If appropriate, gels were dried and exposed to an imaging plate (Fuji) in order to visualise [$^{35}$S]methionine-labelled polypeptides (Fuji FLA-5000 phosphorimager).

### *2.9.2 Antibodies.*

Antibodies were used for immunoprecipitations and immunostaining (see above), immunoblotting, immunofluorescence, and ELISAs. ***Primary antibodies.*** All anti-V5 antibodies (as well as the anti-p85α and anti-p85β antibodies) were purchased from Serotec, U.K. The β-actin and GFP antibodies were from Sigma-Aldrich Ltd., U.K. Antibodies specific for Akt, phospho-Akt (Ser473), phospho-Akt (Thr308), and phospho-eIF2α (Ser51) were from Cell Signaling Technology, U.S.A. The HA-tag mAb was from BAbCo, U.S.A. PKR, STAT1, myc-tag, and phospho-tyrosine antibodies were purchased from Santa-Cruz Biotechnology, U.S.A. The polyclonal rabbit anti-PR8/NS1 antibody was kindly provided by Dr. Paul Digard (University of Cambridge, U.K.), and Dr. Alan Douglas (NIMR, U.K.) provided rabbit antisera to

the influenza A virus strains X31 (H3N2) and PR8 (H1N1). ***Secondary antibodies.*** Anti-mouse IgG Texas Red and FITC conjugated antibodies were from Oxford Biotechnology Ltd., U.K. Anti-mouse IgG and anti-rabbit IgG HRP conjugated antibodies were from Amersham Bioscience, U.K. Goat anti-rabbit IgG AP (alkaline phosphatase)-conjugated antibody was from Santa Cruz Biotechnology, U.S.A.

### *2.9.3 Immunoblotting.*

Polypeptides were separated by SDS-PAGE as described in **2.9.1**, and transferred to polyvinylidene difluoride (PVDF) membrane using the XCell II Blot Module according to the manufacturer's instructions (Invitrogen, U.K.). Following transfer, membranes were blocked for 30 mins in 5% (w/v) skimmed milk powder, 0.1% (v/v) Tween 20 in PBS (blocking buffer), and incubated for 1h with the appropriate primary antibodies diluted (in blocking buffer) as directed by the supplier. After extensive washing with 0.1% (v/v) Tween 20 in PBS, the protein:antibody interactions were detected by incubation for 1h with horseradish peroxidase (HRP) conjugated anti-mouse or anti-rabbit IgG (Amersham Bioscience, U.K.) diluted appropriately in blocking buffer. Following final washing in 0.1% (v/v) Tween 20 in PBS, specific polypeptide bands were visualised by enhanced chemiluminescence (Amersham Biosciences Ltd., U.K.) according to the manufacturer's protocol.

### *2.9.4 Immunofluorescence.*

For immunofluorescence analysis, cells grown on 10mm coverslips were fixed for 10mins in 5% formaldehyde, 2% sucrose (in PBS), before permeabilisation with 0.5% NP-40, 10% sucrose (in PBS). After repeated washing with 1% FBS (in PBS), monolayers were incubated with appropriately diluted primary antibody for 1h. Following further washes, cells were incubated for 1h with a cocktail solution

containing Texas Red or FITC conjugated secondary antibody (Oxford Biotechnology Ltd., U.K.), together with the DNA-binding fluorochrome 4', 6-diamidino-2-phenylindole (DAPI; 0.5 μg/ml; Sigma-Aldrich Ltd., U.K.). All reactions were performed at room temperature in a humidified chamber. Coverslips were washed in PBS and mounted on slides using Citifluor AF-1 mounting solution (Citifluor Ltd., U.K.). Immunofluorescence was visualised using a Nikon Microphot-FXA microscope.

### *2.9.5 Enzyme-linked immunosorbant assay (ELISA).*

Wells of a flat-bottomed 96-well plate were coated overnight at 4°C with 100μl of the target protein solution (10μg/ml in PBS). Wells were subsequently blocked for 1h at room temperature with 400μl skimmed milk solution (3% (w/v) skimmed milk powder in PBS). Lysates containing the ligand protein were serially diluted in skimmed milk solution and added to the protein-coated wells for 1h. Wells were washed 3 times with 0.1% (v/v) Tween-20 in PBS, and bound ligand proteins were detected with a specific primary antibody and an HRP-conjugated secondary antibody as in **2.3.3**. All antibodies were diluted in 3% (w/v) skimmed milk powder in PBS. HRP activity was detected using 100μl/well of an equal volume mixture of TMB peroxidase substrate (0.4g/l 3,3',5,5'-tetramethylbenzidine in an organic base) and peroxidase solution B (0.02% (w/v) $H_2O_2$ in citric buffer). After sufficient colour had developed, the reaction was stopped by adding 100μl/well 1M HCl, and the OD was measured at 450nm.

Chapter 3

# Results

## 3.1 Host-cell proteins that interact with viral IFN-antagonists.

The initial aim of this study was to develop a methodology for identifying host-cell proteins that are bound by virus-encoded proteins. As part of a previous study within the group concerning viral evasion of host innate immunity (Galiano, Chen and Randall, *manuscript in preparation*), a panel of HEp2 cell-lines had already been isolated that constitutively express C-terminal V5-tagged forms of well-characterised virus-encoded IFN-antagonists: Sendai virus V protein (strain Fushimi; SeV/V), Sendai virus C protein (strain Fushimi; SeV/C), and influenza A virus NS1 protein (strain A/PuertoRico/8/34; PR8/NS1) (Andrejeva *et al*., 2004, Childs *et al*., 2007, Garcia-Sastre, 2001, Garcin *et al*., 1999, Komatsu *et al*., 2004, Krug *et al*., 2003). As host-cell binding partners for these viral proteins have already been identified (see **Introduction**, and below), it was speculated that work on such cell-lines might prove useful for determining if protein:protein interactions could be resolved by simple anti-V5 immunoprecipitations.

### *3.1.1 Characterisation of cells expressing IFN-antagonists.*

Characterisation of the HEp2 cell-lines was first performed in order to confirm some of the previously documented IFN-antagonistic activities associated with the stably expressed viral proteins. Western blot analysis of total-cell lysates using the anti-V5 mAb determined the expression and predicted molecular weight of all three viral proteins in their respective HEp2 cell-lines (**Fig. 3.1A**). Indirect immunofluorescence also indicated that >95% (estimated) of cells were expressing each protein (**Fig. 3.1B**). As expected, expression of these proteins significantly reduced the ability of HEp2 cells to produce IFN in response to infection with CPI-, a virus known to normally induce high amounts of IFN (Poole *et al*., 2002) (**Fig. 3.2A**). Note that the

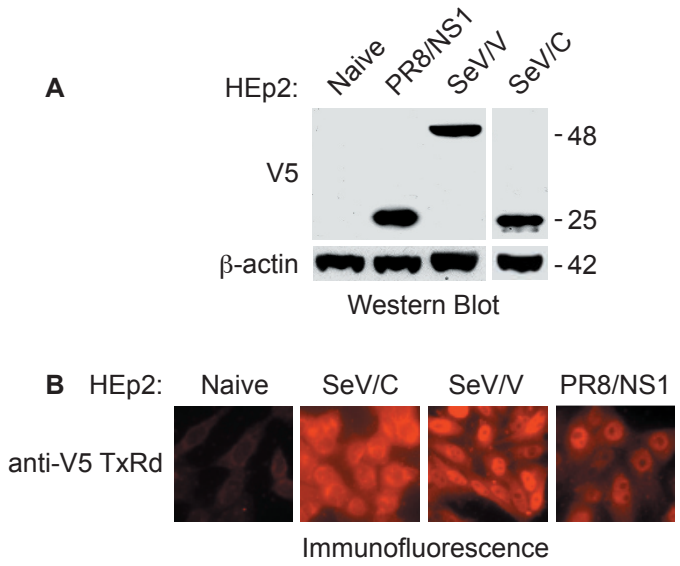


**Fig. 3.1. Expression of different viral IFN-antagonists in HEp2 cells.** (**A**) Protein lysates were prepared from monolayers of HEp2 cells constitutively expressing V5-tagged PR8/NS1, SeV/V, or SeV/C. Naive HEp2 cells acted as a negative control. Lysates were separated by SDS-PAGE followed by transfer to PVDF membrane. V5-tagged proteins were detected using an anti-V5 mAb. Detection of β-actin acted as a loading control. Molecular weight markers (kDa) are indicated on the right. (**B**) Expression of V5-tagged proteins in HEp2 cells as determined by indirect-immunofluorescence using an anti-V5 Texas Red (TxRd)-conjugated mAb. Naive HEp2 cells acted as a negative control.

variation of IFN-antagonism demonstrated by these viral proteins may be due to differences in relative expression levels (**Fig. 3.1**). Alternatively, the efficiency of antagonism may be affected by the type of "inducer" used, particularly as each viral protein limits IFN production by a distinct mechanism: SeV/V binds mda-5 (Andrejeva *et al*., 2004, Childs *et al*., 2007); PR8/NS1 interacts with RIG-I and/or dsRNA (Garcia-Sastre, 2001, Guo *et al*., 2007, Mibayashi *et al*., 2007, Opitz *et al*., 2006, Pichlmair *et al*., 2006); and SeV/C binds STAT1 (Takeuchi *et al*., 2001). Thus, SeV/V and PR8/NS1 probably have a direct effect on IFN induction, whilst the antagonistic role of SeV/C is likely due to limitation of the IFN induced positive-feedback loop.

Confirmation that SeV/C (but not PR8/NS1) is able to block a specific step in the IFN signalling cascade (Garcin *et al*., 1999) was determined by examining levels of the IFN-inducible STAT1 protein after treatment of cells with exogenous IFNα (**Fig. 3.2B**). Further characterisation of these HEp2 cell-lines also verified that PR8/NS1 limits the activity of the dsRNA-activated protein kinase, PKR (Hatada *et al*., 1999, Li *et al*., 2006a). Thus, expression of PR8/NS1 prevented the Ser51 phosphorylation of eIF2α (the antiviral effector of PKR) in response to both virus infection (CPI-) and synthetic dsRNA (poly I:C) (**Fig. 3.2C**). As the binding of NS1 to PKR may be responsible for PKR inactivation (Li *et al*., 2006a), the co-precipitation of PR8/NS1 with endogenous PKR was also assessed (**Fig. 3.2D**). PR8/NS1 was clearly precipitated with anti-PKR antibody from PR8/NS1-expressing HEp2 cell extracts, and the amount precipitated was proportional to the amount of PKR bound by the antibody (protein levels of PKR were increased by prior treatment of cells with exogenous IFNα, which did not affect the protein level of PR8/NS1) (**Fig. 3.2D**).

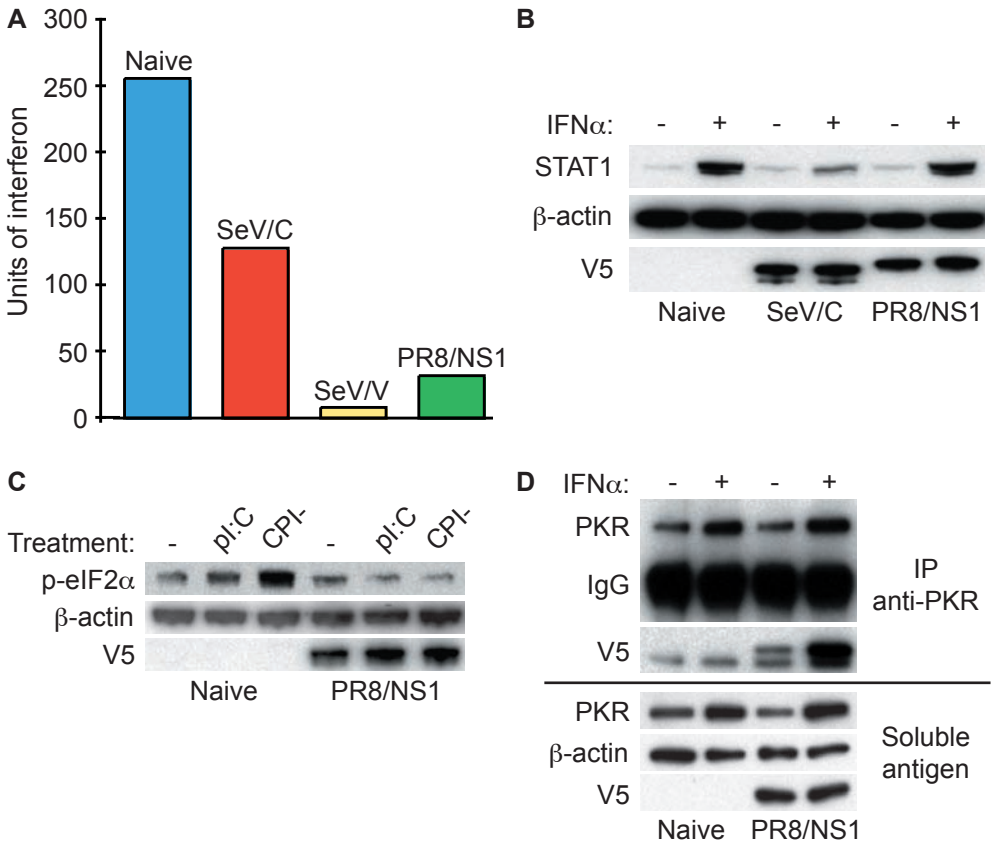


**Fig. 3.2. Functional characterisation of different viral IFN-antagonists in HEp2 cells.** (**A**) **IFN production.** Monolayers of HEp2 cell-lines were infected with CPI- at an MOI of 5 PFU/cell, and culture supernatants were harvested at 48h post-infection. After UV inactivation, IFN concentration was estimated by EMCV-inhibition assay on Vero cells. Bars represent relative units of protection, which has previously been shown to correlate well with the total amount of IFN in the supernatant (*M.C. Galiano, unpublished observations*). (**B**) **IFN signalling.** Monolayers of HEp2 cell-lines constitutively expressing SeV/C or PR8/NS1 were treated for 20h with recombinant human IFNα (or mock treated). Naive HEp2 cells acted as a positive control. Protein lysates were separated by SDS-PAGE followed by transfer to PVDF membrane. Total STAT1 and β-actin were detected using specific antibodies. Expression of V5-tagged proteins was confirmed using an anti-V5 mAb. (**C**) **PKR activation.** PKR activation in naive or PR8/NS1-expressing HEp2 cells was assessed by the induction of phospho-eIF2α (Ser51) in response to mock-transfection (-), transfection of poly I:C (pI:C), or CPI- infection (MOI of 5 PFU/cell). 20h post-treatment monolayers were harvested, and protein lysates separated by SDS-PAGE followed by transfer to PVDF membrane. Phospho-eIF2α (Ser51) and β-actin were detected using specific antibodies. NS1 expression was confirmed using an anti-V5 mAb. (**D**) **PKR co-precipitation.** PR8/NS1-expressing HEp2 cells (or naive HEp2 cells) were treated as for (**B**). Soluble antigen extracts were immunoprecipitated with anti-PKR antibody and separated by SDS-PAGE followed by transfer to PVDF membrane. PKR and PR8/NS1 were detected in the precipitates using a specific anti-PKR pAb or an anti-V5 mAb, respectively. Levels of PKR, β-actin, and PR8/NS1 were also determined in the input soluble antigen.

### *3.1.2 Identification of IFN-antagonist binding partners.*

Immunoprecipitations were carried out on each HEp2 cell-line in order to identify host-cell proteins that specifically interact with the viral IFN-antagonists. Antibody to the V5-tag was used to immunoprecipitate each recombinant protein from [$^{35}$S]-methionine labelled cell lysates (~5x10$^5$ cells per experiment), and precipitated protein complexes were separated by SDS-PAGE and visualised by phosphorimager analysis. Similarly labelled naïve HEp2 cell extract acted as a negative control (**Fig. 3.3A**). A number of radiolabelled protein bands specifically co-precipitating with each IFN-antagonist were observed. Bands corresponding to V5-tagged viral proteins are marked with an arrow.

In order to identify polypeptides by mass spectrometry, immunoprecipitations were performed using extracts from a larger number of cells (~5x10$^7$), such that protein bands would be visible by Coomassie blue staining. In order to reduce the amount of immunoglobulin heavy and light chain on the SDS-polyacrylamide gel, anti-V5 mAb was chemically cross-linked onto protein-G beads (see **Methods and Materials**). Coomassie blue staining of co-precipitated proteins revealed a similar (but not identical) pattern to that observed using [$^{35}$S]-methionine labelled extracts (**Fig. 3.3B**). Specifically, the high molecular weight protein band (>200 kDa) co-precipitated with SeV/V was only observed in the radiolabelled experiment, as was an apparent doublet band of ~40 kDa in the PR8/NS1 immunoprecipitation (**Fig. 3.3A**, both bands marked with an asterix). The thick protein bands of ~20 kDa clearly visible by Coomassie blue staining (**Fig. 3.3B** – not present in the radiolabelled experiment), probably correspond to modified anti-V5 immunoglobulin light chain that can dissociate from the protein-G bead.

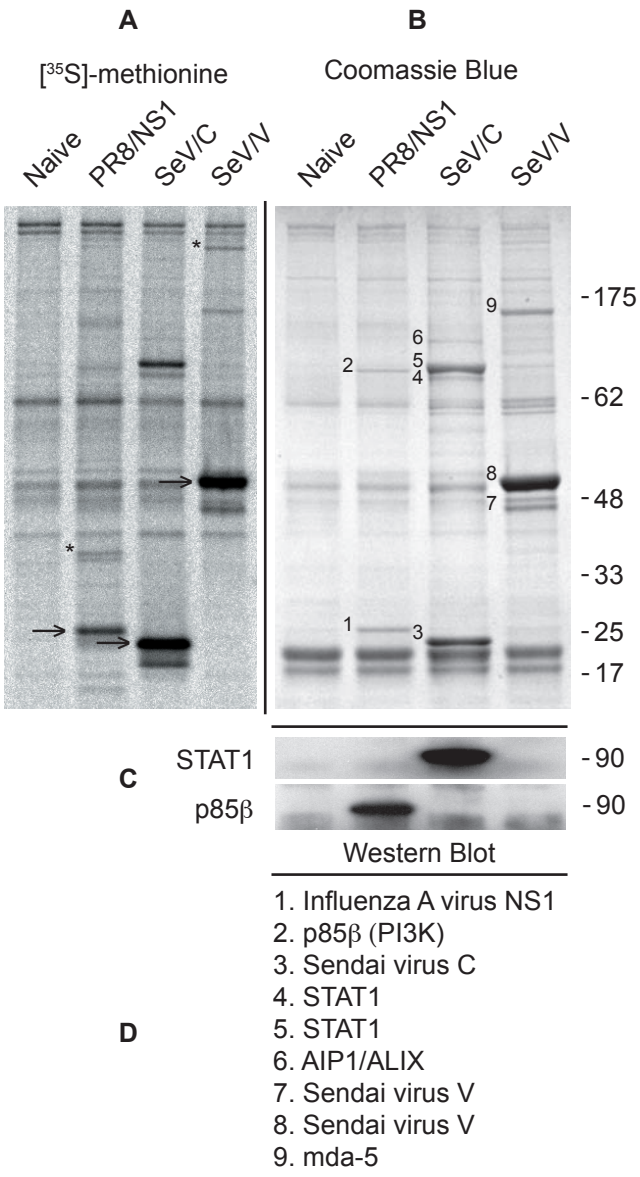


**Fig. 3.3. Identification of host-cell proteins that interact with different viral IFN-antagonists in HEp2 cells.** (**A**) **[$^{35}$S]-methionine labelling.** Monolayers of HEp2 cell-lines constitutively expressing different viral IFN-antagonists (~5x10$^5$ cells per experiment) were metabolically labelled for 1h with [$^{35}$S]-methionine. Soluble antigen extracts were immunoprecipitated using anti-V5 mAb. Precipitated protein complexes were separated by SDS-PAGE and visualised by phosphorimager analysis. Bands presumed to correspond to the immunoprecipitated V5-tagged viral proteins are marked with arrows. Bands not identified by Coomassie Blue staining (**B**) are marked with asterices. (**B**) **Coomassie Blue staining and protein identification.** Soluble antigen extracts from ~2x10$^7$ cells were immunoprecipitated with anti-V5 mAb cross-linked to protein G Sepharose. Precipitated proteins were separated by electrophoresis through 4-12% polyacrylamide gradient gels and visualised by Coomassie Blue staining. Polypeptide bands denoted by numbers 1-9 were excised and identified by the University of St. Andrews mass spectrometry service (**D**). Molecular weight markers (kDa) are indicated on the right. (**C**) **Immunoblot confirmation.** Immunoprecipitates were prepared as for (**B**), and separated by electrophoresis followed by transfer to PVDF membrane. STAT1 and p85β were detected using specific antibodies.

Mass spectrometry (performed by the University of St. Andrews Mass Spectrometry and Proteomics Facility) showed that, as expected, the ~150 kDa protein associated with SeV/V is mda-5, and the two ~90 kDa polypeptides associated with SeV/C are both STAT1. This is in agreement with previously described interactions for SeV/V and SeV/C that have been shown to play a role in their IFN-antagonistic properties (Andrejeva *et al*., 2004, Childs *et al*., 2007, Takeuchi *et al*., 2001). The specific interaction of STAT1 with SeV/C protein was confirmed by Western blot analysis of similar immuno-precipitates (**Fig. 3.3C**). Additionally, the ~100 kDa polypeptide species precipitated with SeV/C (**Fig. 3.3B**) was identified as AIP1/Alix, a protein previously reported to bind SeV/C (Sakaguchi *et al*., 2005), and which may play a role in either virus maturation/budding (Sakaguchi *et al*., 2005), or in viral escape from the host immune/apoptotic response (Gosselin-Grenet *et al*., 2007).

Analysis of cellular proteins co-precipitated with the PR8/NS1 protein identified only a single polypeptide species of ~90 kDa (**Fig. 3.3B**). This was surprising given the number of host-cell proteins that have been reported to bind NS1 (see **Introduction**). The mass spectrometry service identified this ~90 kDa protein as p85β, a regulatory subunit of phosphoinositide 3-kinase (PI3K; see **Introduction**). Interestingly, p85α (a related PI3K adapter subunit that is ~55% identical to p85β at the amino-acid level) was not identified in the band. Specificity of the PR8/NS1:p85β interaction was confirmed by Western blot analysis of similar immunoprecipitates (**Fig. 3.3C**).

Given that an interaction between the influenza A virus NS1 protein and host-cell p85β had not been reported previously, it was decided that this observation should be characterised further. Thus, the remainder of this work focuses on determining a role for this interaction in the influenza A virus replication cycle.

## 3.2 Influenza A virus NS1 proteins bind p85β, but not p85α.

### *3.2.1 Co-precipitation of p85β with various NS1 proteins.*

To determine if the interaction of p85β with PR8/NS1 is a feature common to other influenza A virus strains, the co-precipitation of p85β with the NS1 proteins of A/WSN/33 (H1N1; WSN), and A/Victoria/3/75 (H3N2; Vic) was examined. A HEp2 cell-line stably expressing Vic/NS1 could not be isolated, possibly due to toxicity caused by this protein inhibiting cellular pre-mRNA processing (Nemeroff *et al*., 1998, Qiu & Krug, 1994); a function not shared by PR8/NS1 (Hayman *et al*., 2006). Therefore, human 293T cells were transfected with plasmids expressing either the C-terminal V5-tagged NS1 protein of each strain, N-terminal V5-tagged PR8/NS1, or empty vector (negative control). Analysis of proteins immunoprecipitated with the V5 antibody showed that endogenous p85β interacted with the NS1 proteins of all three influenza A virus strains (**Fig. 3.4A**). The position of the V5-tag (N-terminal or C-terminal) had no effect on the interaction.

### *3.2.2 Direct binding of NS1 proteins to p85β, but not p85α.*

To confirm the specific and direct binding of NS1 to p85β *in vitro*, a GST-NS1 fusion protein pull-down assay was used. Recombinant GST or GST-NS1 (NS1 of PR8, Vic, or Ud) was expressed and purified from *E. coli* and immobilised onto glutathione-agarose beads. Equal amounts of these beads were then used as bait to affinity isolate an excess of baculovirus-expressed p85α or p85β (the expression of these proteins in infected *Sf*9 cells was confirmed prior to the assay by Western blot [**Fig. 3.4B**]). Strikingly, a single protein band of ~90 kDa was specifically isolated from the p85β expressing lysate by each GST-NS1 (PR8, Vic and Ud), but not by GST alone (**Fig. 3.4C**). The identity of the ~90 kDa polypeptide was confirmed as p85β by mass

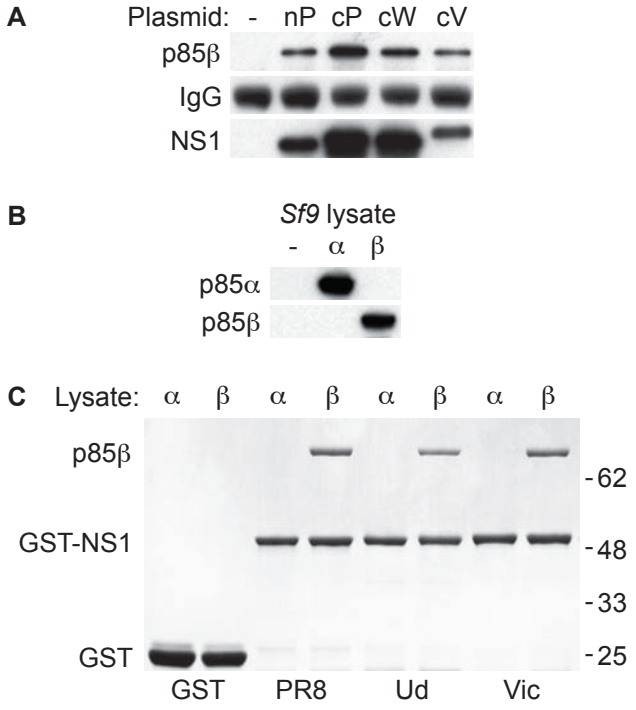


**Fig. 3.4. The NS1 proteins of several influenza A virus strains bind efficiently to p85β, but not p85α.** (**A**) 293T cells were transfected for 48h with either empty vector (-), a plasmid expressing the N-terminal V5-tagged NS1 protein of PR8 (nP), or plasmids expressing the C-terminal V5-tagged NS1 proteins of PR8 (cP), WSN (cW), or Vic (cV). Soluble antigen extracts were immunoprecipitated with anti-V5 antibody and precipitates separated by SDS-PAGE followed by transfer to PVDF membrane. Endogenous p85β was detected using a specific mAb. Anti-V5 mAb was used to detect V5-tagged NS1 proteins. (**B**) *Sf9* cells were infected (or mock [-]) with recombinant baculoviruses expressing either p85α or p85β. Equal amounts of soluble lysate were separated by electrophoresis, transferred to PVDF membrane, and probed for p85α or p85β expression using specific mAbs. (**C**) Equal amounts of soluble *Sf9* cell lysate from (**B**) were mixed with GST or GST-NS1 (PR8, Ud or Vic) immobilized onto glutathione-agarose beads. After washing, protein complexes were dissociated from the beads and separated by SDS-PAGE through 4-12% polyacrylamide gradient gels. Polypeptides were stained with Coomassie Blue, and protein identification was confirmed by mass spectrometry. Molecular weight markers (kDa) are indicated on the right.

spectrometry. As GST-NS1 and p85β were the only protein bands visible in this pull-down experiment, the interaction between NS1 and p85β is likely to be direct. Interestingly, no proteins were precipitated by GST-NS1 from the p85α expressing lysate (**Fig. 3.4C**).

## 3.3 NS1 induces the PI3K-dependent phosphorylation of Akt.

### *3.3.1 Influenza A virus infection induces phospho-Akt.*

As described in the **Introduction**, Akt is a serine/threonine protein kinase that serves as a key downstream mediator of PI3K signalling [reviewed in (Brazil *et al*., 2004, Cantley, 2002)]. In response to active PI3K, Akt is recruited to membranes and phosphorylated at residues Thr308 and Ser473 [by PDK1 and the mTORC2 complex, respectively] (Bayascas & Alessi, 2005, Brazil *et al*., 2004). As NS1 was found to bind the p85β regulatory subunit of PI3K (**Fig. 3.4**), the phosphorylation of Akt following influenza A virus infection was investigated as a marker for PI3K activity. Serum-starved monolayers of human 1321N1 astrocytoma cells (used widely in PI3K activation studies) were infected with influenza A virus (PR8) at a MOI of ~5 PFU/cell, and the phosphorylation state of Akt (Thr308 and Ser473) was analysed in total cell lysates at various times post-infection (p.i.) (**Fig. 3.5A**). Increasing levels of phosphorylated Akt at both sites were evident during the time-course of infection, and phospho-Akt levels appeared concomitant with the expression of NS1 protein (an increase in phospho-Akt was first detectable ~4-6h p.i., and was maintained up to 24h p.i.). Induction of phospho-Akt (Ser473) was dependent on influenza A virus replication, as prior UV-inactivation of the virus prevented phosphorylation of Akt (**Fig. 3.5B**).

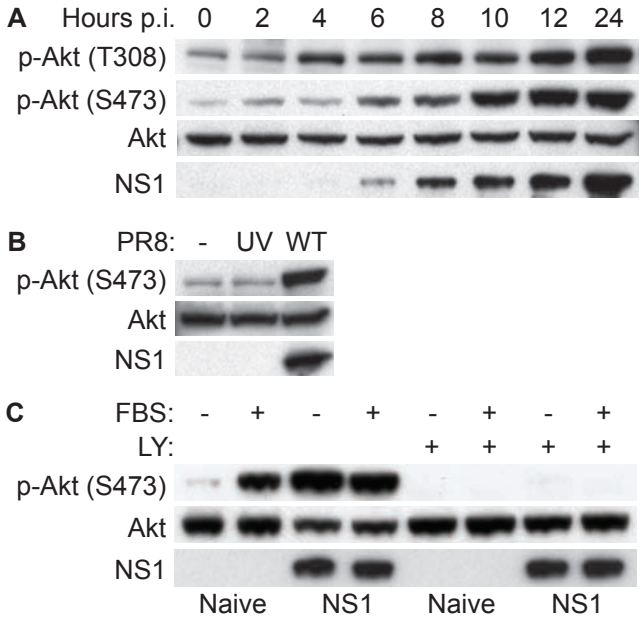


**Fig. 3.5. NS1 induces the Ser473 and Thr308 phosphorylations of Akt in a PI3K-dependent manner.** (**A**) **Time-course of Akt phosphorylation at Ser-473 and Thr-308 following influenza A virus (PR8) infection.** Confluent serum-starved monolayers of 1321N1 cells were infected (or mock [-]) with PR8 at an MOI of 5 PFU/cell, and total cell lysates were harvested at various times p.i.. Lysates were separated by SDS-PAGE followed by transfer to PVDF membrane. Phospho-Akt (Ser473 and Thr308) was detected using specific mAbs. Total Akt was detected using a pAb and acted as a loading control. NS1 expression was detected using a specific pAb. (**B**) **UV-inactivated influenza A virus (PR8) does not induce Akt phosphorylation.** Serum-starved 1321N1 monolayers were infected (or mock [-]) as for (**A**), with either untreated (WT) or UV-inactivated (UV) virus. Lysates were prepared 20h p.i. and analysed as for (**A**). (**C**) Serum-starved naive or PR8/NS1-expressing 1321N1 monolayers were treated (or mock) with 5% FBS for 1h. In a duplicate experiment, cells were also treated with 25$\mu$M LY294002 (a specific PI3K inhibitor; LY). Cell lysates were analysed as for (**A**). PR8/NS1 was detected using an anti-V5 mAb.

### *3.3.2 NS1 induces PI3K-dependent phospho-Akt.*

To determine if NS1 is responsible for influenza A virus-mediated activation of PI3K, a 1321N1 cell-line was isolated that constitutively expressed V5-tagged PR8/NS1, and the phosphorylation state of Akt within these cells was investigated. In naïve 1321N1 cells, Akt phosphorylation (Ser473) decreased rapidly in response to serum-withdrawal (**Fig. 3.5C**, lanes 1-2). In contrast, there was no apparent reduction in phospho-Akt when NS1-expressing cells were similarly serum-starved (**Fig. 3.5C**, lanes 3-4). This indicates that NS1 expression alone can induce the phosphorylation of Akt without the absolute requirement for additional virus-derived factors. Furthermore, it was found that the phosphorylation of Akt induced by NS1 is dependent on PI3K activation, as treatment of cells with a well-characterised inhibitor of PI3K, LY294002 (Vlahos *et al*., 1994), was able to negate the effect of NS1 expression (**Fig. 3.5C**, lanes 5-8).

### *3.3.3 Kinetics of Akt phosphorylation during infection.*

The observation that influenza A virus infection causes a sustained increase in the level of Akt phosphorylation has recently been challenged by a report noting only transient Akt phosphorylation at ~6-8 h p.i., which was not evident at later timepoints (Zhirnov & Klenk, 2007). The apparent discrepancy between these two results may be explained by the cell-type used, as the experiments presented here were done only in 1321N1 cells, whilst those of Zhirnov and Klenk were performed in MDCK, CV-1, and Vero cells (Zhirnov & Klenk, 2007). Indeed, a comparative study of phospho-Akt induction in response to influenza A virus (PR8) infection did reveal kinetic differences between 1321N1 cells and MDCK cells (**Fig. 3.6**). As previously noted, in 1321N1 cells, Ser473 phosphorylation of Akt is first detectable ~4h p.i., and is maintained throughout infection (**Fig. 3.6A**). By contrast, in MDCK cells, Akt

phosphorylation peaks at ~8h p.i. before falling significantly – at 24h p.i. phospho-Akt (Ser473) is not even detectable (**Fig. 3.6B**).

Interestingly, 1321N1 cells (unlike MDCK cells), appear to lack PTEN (Lackey *et al*., 2007, Orchiston *et al*., 2004), the lipid phosphatase that negatively regulates PI3K signalling by dephosphorylating the 3'-OH position of PtdIns(3,4,5)$P_3$ to produce PtdIns(4,5)$P_2$ (see **Introduction**). MDCK cells with a stable shRNA-mediated knock-down of PTEN have recently been generated by others (Lackey *et al*., 2007). Thus, the kinetics of Akt phosphorylation (Ser473) in response to influenza A virus infection were investigated in these MDCKΔPTEN cells, and the results compared with the kinetics observed for naïve MDCK cells. As shown in **Fig. 3.6C**, during the course of infection no differences in the phosphorylation state of Akt (Ser473) were observed between the two cell-lines. Interestingly, MDCKΔPTEN cells appear to undergo less cell death during virus infection than naïve cells (**Fig. 3.6D**), however it is clear from **Fig. 3.6C** that this is not due to sustained (and potentially anti-apoptotic) phosphorylation of Akt. As the expression of some shRNA vectors has been implicated in the overproduction of dsRNA, and thus the overstimulation of an IFN response (Bridge *et al*., 2003), this survival effect may be due solely to an enhanced anti-viral state within the cells. Although not observed in previous experiments, it is also worth noting a band possibly corresponding to cleaved Akt [a known consequence of apoptosis induction (Bachelder *et al*., 2001, Rokudai *et al*., 2000)], is readily detectable in virus-infected MDCK cells at 24h p.i. (**Fig. 3.6C**).

Overall, the data suggest that PTEN activity does not play a critical role in the kinetics of Akt phosphorylation during influenza A virus infection. Clearly some other host-cell factor must act directly or indirectly to determine these kinetics.

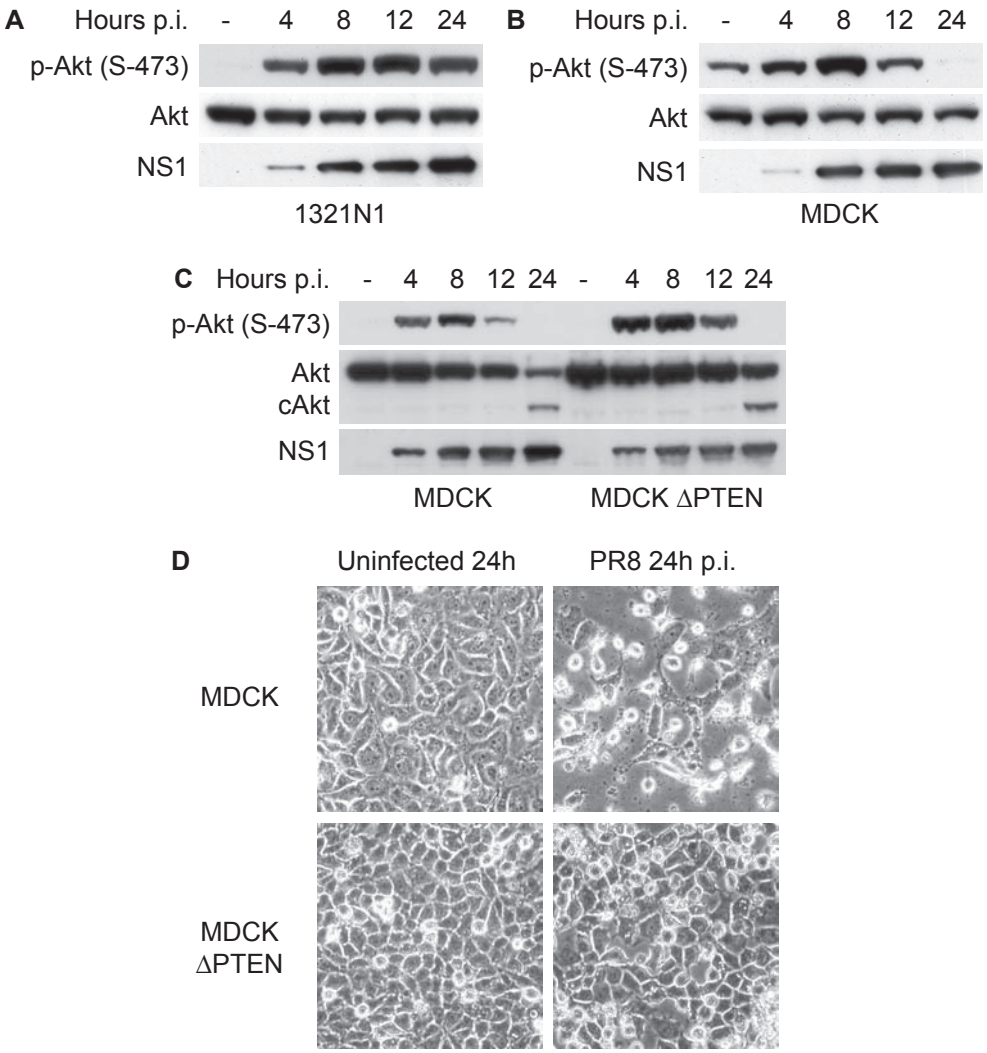


**Fig. 3.6. Kinetics of Akt phosphorylation during influenza A virus infections is cell-type specific.** (**A & B**) **Time-course of Akt phosphorylation at Ser473 following influenza A virus (PR8) infection of 1321N1 or MDCK cells.** Confluent serum-starved monolayers of 1321N1 or MDCK cells were infected (or mock [-]) with PR8 at an MOI of 5 PFU/cell, and total cell lysates were harvested at various times p.i.. Lysates were separated by SDS-PAGE followed by transfer to PVDF membrane. Phospho-Akt (Ser473) was detected using a specific mAb. Total Akt was detected using a pAb and acted as a loading control. NS1 expression was detected using a specific pAb. (**C**) **PTEN expression does not alter the kinetics of influenza A virus induced Akt phosphorylation in MDCK cells.** Serum-starved MDCK or MDCKΔPTEN monolayers were infected (or mock [-]) as for (**A**), and total cell lysates were harvested at various times p.i.. Lysates were also analysed as for (**A**). The band labelled cAkt is presumed to be an apoptosis-induced cleavage product of full-length Akt. (**D**) Phase-contrast pictures of PR8-infected monolayers from (**C**) at 24h p.i..

## 3.4 Binding of PI3K requires Tyr89 and Met93 of NS1.

### *3.4.1 Assessment of a potential YXXM-like motif in NS1.*

In order to define the NS1-specific role of PI3K signalling during influenza A virus infection, it was of interest to identify single-amino acid substitutions in NS1 that prevented its binding and activation of PI3K. Tyrosine-phosphorylated Y*XX*M motifs are considered consensus docking sites and activators of the SH2 domains of PI3K p85 subunits (Songyang *et al*., 1993). The amino-acid sequence of PR8/NS1 contains a single tyrosine residue (Tyr89), which is four residues upstream of a methionine (Met93) (**Fig. 3.7A**). Alignment of 1546 NS1 protein sequences available in the public domain indicated that Tyr89 is absolutely conserved amongst all influenza A virus strains sequenced to date. Given the similarity in sequence between NS1 residues 89-93 and classical Y*XX*M motifs, the importance of this sequence for binding p85β was investigated. As the substitution of aspartic acid for glutamic acid at position 92 has been previously identified as a potential pathogenicity factor in some H5N1 isolates (Seo *et al*., 2002), it was also included in a panel of NS1 mutants that were assessed for their ability to interact with endogenous p85β. V5-tagged NS1 constructs were expressed transiently in human 293T cells and immunoprecipitated with anti-V5 antibody. Co-precipitating p85β was detected by Western blot analysis. As shown in **Fig. 3.7B**, both wild-type PR8/NS1 and PR8/NS1-D92E precipitated p85β efficiently. In contrast, PR8/NS1 constructs containing Y89F or M93A substitutions were unable to precipitate p85β. The requirement for Tyr89 in binding p85β was further investigated by expressing and purifying a GST-PR8/NS1-Y89F fusion protein, and testing its *in vitro* interaction with baculovirus-expressed p85β. As expected, the PR8/NS1-Y89F mutant did not bind to either p85α or p85β (**Fig. 3.7C**).

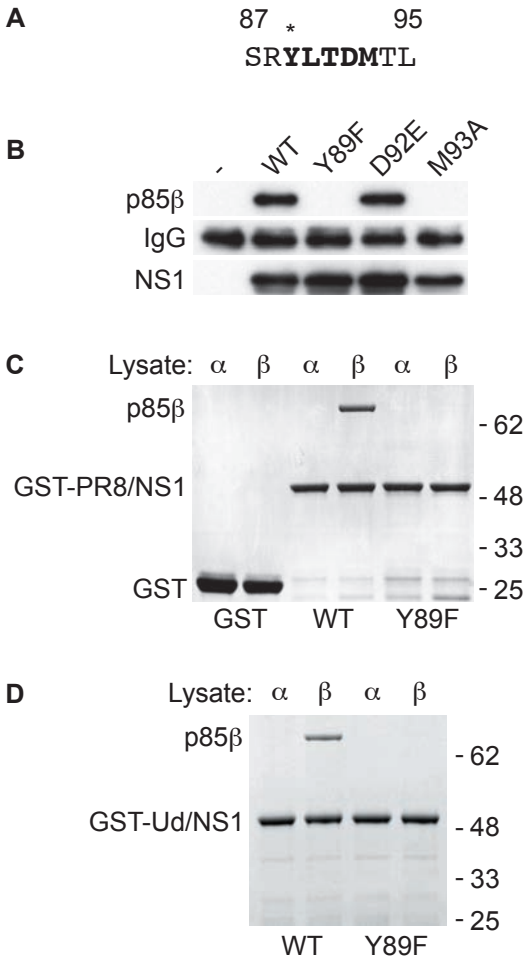


**Fig. 3.7. Binding of p85β requires residues from a YXXM-like motif in NS1.** (**A**) **Amino-acid sequence of PR8/NS1 residues 87-95.** The putative YXXM-like motif is shown in bold. Tyr-89 (denoted by *) is totally conserved among all influenza A virus NS1 proteins sequenced to date. (**B**) **Binding of NS1 to p85β requires Tyr-89 and Met-93.** 293T cells were transfected for 48h with either empty vector (-), a plasmid expressing WT V5-tagged PR8/NS1, or plasmids expressing V5-tagged PR8/NS1 proteins with single amino-acid substitutions (Y89F, D92E or M93A). Soluble antigen extracts were immunoprecipitated with anti-V5 antibody and precipitates separated by SDS-PAGE followed by transfer to PVDF membrane. p85β was detected using a specific mAb. Anti-V5 mAb was used to detect the V5-tagged NS1 mutants. (**C**) WT GST-PR8/NS1 and GST-PR8/NS1 with the Y89F mutation were used as bait to precipitate p85α or p85β from baculovirus-infected *Sf9* cell lysates. GST acted as a negative control. Protein complexes were analysed as for **Fig. 3.4C**. (**D**) As for (**C**), but using WT GST-Ud/NS1 and GST-Ud/NS1-Y89F. Molecular weight markers (kDa) are indicated on the right.

These results were confirmed in the context of *E. coli* expressed GST-Ud/NS1 and GST-Ud/NS1-Y89F (**Fig. 3.7D**).

### *3.4.2 Mutation of Tyr89 prevents induction of phospho-Akt.*

To investigate if NS1-Y89F was able to activate PI3K signalling, a 1321N1 cell-line that constitutively expresses the PR8/NS1-Y89F mutant protein was isolated. The intra-cellular localisation of PR8/NS1-Y89F in these cells was, like that of WT PR8/NS1 (**Fig. 3.1B**), predominantly nuclear (but not nucleolar; **Fig. 3.8A**). Serum-starvation of both naïve and NS1-Y89F expressing cells for 1h caused a marked reduction in phospho-Akt (Ser473) levels (**Fig. 3.8B**, lanes 1-2, 5-6). In contrast, there was no apparent reduction in Akt phosphorylation when cells expressing WT NS1 were similarly serum-starved (**Fig. 3.8B**, lanes 3-4). These data indicate that the binding of p85β (and activation of PI3K) absolutely requires the highly conserved tyrosine residue at position 89 of NS1.

## 3.5 Effect of NS1-Tyr89 mutation on other interactions.

Amino-acid substitution of Tyr89 for phenylalanine in NS1 could potentially disrupt the three-dimensional structure of NS1, or inhibit homodimerisation, thus preventing other functions of NS1. Although the cellular localisation of PR8/NS1-Y89F is similar to that of WT PR8/NS1 (compare **Fig. 3.8A** with **Fig. 3.1B**), it was necessary to determine if the Y89F mutation would prevent NS1 from interacting with host-cell proteins other than p85β.

### *3.5.1 Interaction of PR8/NS1 with RIG-I, but not mda-5.*

A recently identified cellular partner for PR8/NS1 is RIG-I, a cytoplasmic RNA helicase involved in sensing RNA virus infection and initiating the innate antiviral

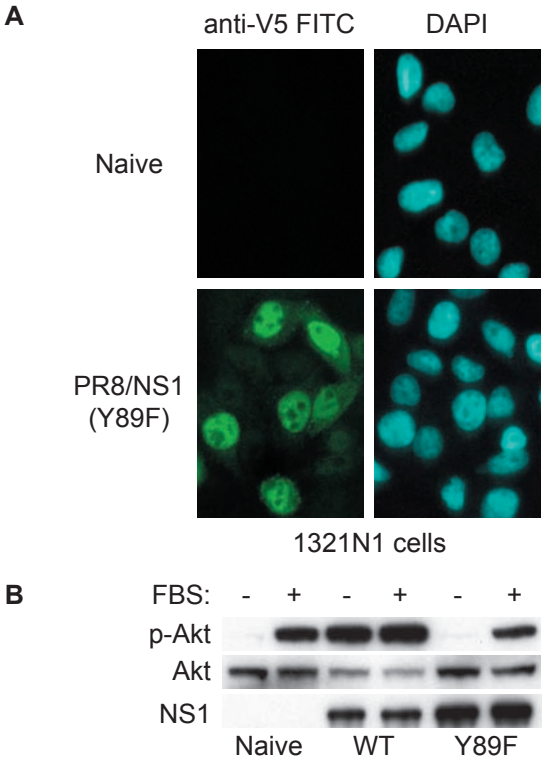


**Fig. 3.8. Effect of Y89F amino-acid substitution on PR8/NS1.** (**A**) **Expression of PR8/NS1 with the single amino-acid substitution Y89F does not affect its subcellular localisation.** Localisation of V5-tagged PR8/NS1-Y89F in 1321N1 cells was determined by indirect-immunofluorescence using an anti-V5 FITC-conjugated mAb. Naive 1321N1 cells acted as a negative control. Cell nuclei were stained with DAPI. (**B**) **Expression of PR8/NS1 with the single amino-acid substitution Y89F does not induce the phosphorylation of Akt at Ser473.** Serum-starved naive, PR8/NS1-expressing (WT), or mutant PR8/NS1-expressing (Y89F) 1321N1 monolayers were treated (or mock) with 5% FBS for 1h. Cell lysates were separated by SDS-PAGE followed by transfer to PVDF membrane. Phospho-Akt (Ser473) was detected using a specific mAb. Total Akt was detected using a pAb and acted as a loading control. PR8/NS1 (WT and Y89F) was detected using an anti-V5 mAb.

response (Guo *et al*., 2007, Mibayashi *et al*., 2007, Opitz *et al*., 2006, Pichlmair *et al*., 2006) [see **Introduction**]. Thus, the ability of PR8/NS1-Y89F to interact with RIG-I was assessed. Initially, V5-tagged PR8/NS1 constructs (full-length or lacking the RNA-binding domain: PR8/NS1Δ73) were transiently co-expressed in 293T cells with either the GFP-tagged CARD domain or Helicase domain of RIG-I. Interestingly, immunoprecipitations using anti-V5 antibody revealed that both full-length PR8/NS1 and PR8/NS1Δ73 could precipitate the CARD domain of RIG-I (**Fig. 3.9A**). In contrast, only full-length PR8/NS1 (and not PR8/NS1Δ73) could efficiently precipitate the Helicase domain (**Fig. 3.9A**), an observation consistent with data indicating that the interaction between PR8/NS1 and RIG-I is enhanced by residues in the RNA-binding domain of NS1 (Pichlmair *et al*., 2006). However, the data presented here also indicate that PR8/NS1 may form a complex with the CARD domain of RIG-I independently of RNA-binding activity. It should be noted that (unlike for paramyxovirus V proteins), PR8/NS1 was not found to interact with mda-5, a CARD/Helicase domain-containing protein functionally related to RIG-I (**Fig. 3.3B**, **Fig. 3.9B**, (Andrejeva *et al*., 2004, Childs *et al*., 2007, Pichlmair *et al*., 2006)).

### *3.5.2 Interaction of PR8/NS1-Y89F with RIG-I.*

Human 293T cells were co-transfected with plasmids expressing WT V5-tagged PR8/NS1, V5-tagged PR8/NS1-Y89F, or empty vector (negative control), together with either GFP-tagged RIG-I CARD or GFP-tagged RIG-I Helicase. Western blot analysis of proteins immunoprecipitated with the anti-V5 antibody showed that both RIG-I domains were co-precipitated with WT PR8/NS1 and PR8/NS1-Y89F (**Fig. 3.9C**). Thus, substitution of Tyr89 for phenylalanine specifically prevents PR8/NS1 from binding p85β, and probably does not disrupt the overall three-dimensional structure of NS1.

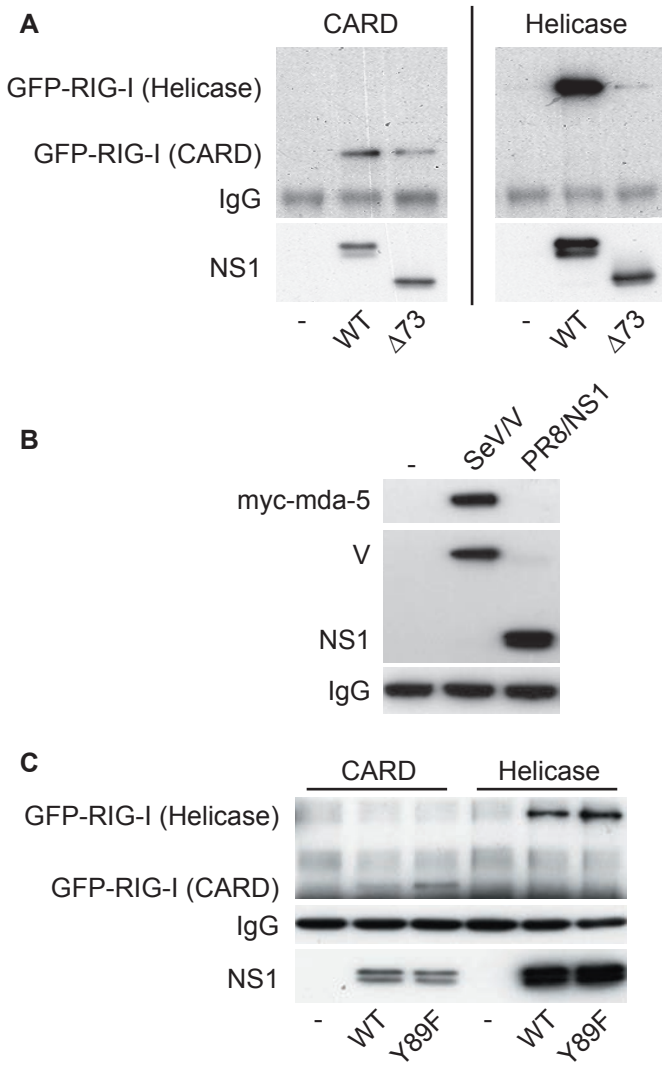


**Fig. 3.9. Effect of Y89F amino-acid substitution on the ability of PR8/NS1 to interact with RIG-I.** (**A**) **PR8/NS1 interacts with RIG-I.** 293T cells were co-transfected for 48h with a plasmid expressing the GFP-tagged CARD domain of RIG-I, or the GFP-tagged Helicase domain of RIG-I, together with either empty vector (-), a plasmid expressing V5-tagged full-length PR8/NS1 (WT), or a plasmid expressing the V5-tagged effector domain of PR8/NS1 (Δ73). Soluble antigen extracts were immunoprecipitated with anti-V5 antibody and precipitates separated by SDS-PAGE followed by transfer to PVDF membrane. Co-precipitated GFP-tagged proteins were detected using a specific mAb. Anti-V5 mAb was used to detect V5-tagged NS1 proteins. (**B**) **PR8/NS1 does not interact with mda-5.** 293T cells were co-transfected for 48h with a plasmid expressing myc-tagged mda-5, together with either empty vector (-), a plasmid expressing V5-tagged SeV/V, or a plasmid expressing V5-tagged PR8/NS1. Soluble antigen extracts were immunoprecipitated with anti-V5 antibody and precipitates separated by SDS-PAGE followed by transfer to PVDF membrane. Co-precipitated myc-mda-5 was detected using an anti-myc mAb. Anti-V5 mAb was used to detect V5-tagged proteins. (**C**) **Y89F amino-acid substitution does not affect the interaction of PR8/NS1 with RIG-I.** 293T cells were co-transfected for 48h with a plasmid expressing the GFP-tagged CARD domain of RIG-I, or the GFP-tagged Helicase domain of RIG-I, together with either empty vector (-), a plasmid expressing V5-tagged PR8/NS1 (WT), or a plasmid expressing V5-tagged PR8/NS1-Y89F (Y89F). Soluble antigen extracts were immunoprecipitated and analysed as for (**A**).

## 3.6 Requirement for PI3K during influenza A virus replication.

### *3.6.1 PI3K inhibitors specifically reduce influenza A virus replication.*

Given that NS1 was found to bind and activate PI3K, the requirement of PI3K activation for efficient influenza A virus replication was determined. Initially, low multiplicity multi-step growth analyses were performed using commercially available PI3K inhibitors (such as the well-characterised LY294002 and wortmannin (Powis *et al*., 1994)). In MDCK cells, neither compound had a detrimental effect on the paramyxovirus, PIV5 (**Fig. 3.10A**), whilst a significant decrease in the yield of PR8 was noted (**Fig. 3.10B**). The observed "recovery" of PR8 virus titres 72h and 96h p.i. may be due to degradation of the compounds, as only a fixed concentration was added to the cells at the time of inoculation. LY294002 and wortmannin are known to bind/inhibit a multitude of non-PI3K lipid and protein kinases (and thus may affect unrelated signalling pathways (Gharbi *et al*., 2007)), therefore LY303511 – a compound structurally related to LY294002 but which does not inhibit PI3K (Ding *et al*., 1995) – acted as a specific negative control. The results presented here are in agreement with those recently published by several other groups (Ehrhardt *et al*., 2006, Shin *et al*., 2007b). However, studies have also shown that inhibitors of PI3K may actually block influenza A virus entry into cells (Ehrhardt *et al*., 2006), and LY294002 appears to prevent the production of IFN in HEp2 cells challenged by CPI- (*data not shown*; LY294002 also inhibits dsRNA:TLR3-mediated IRF-3 phosphorylation/activation (Sarkar *et al*., 2004)). Thus, interpretation of studies using these compounds (particularly with specific regards to NS1-mediated PI3K activation) is likely to be confused by off-target effects, or by the inhibition of unrelated PI3K pathways.

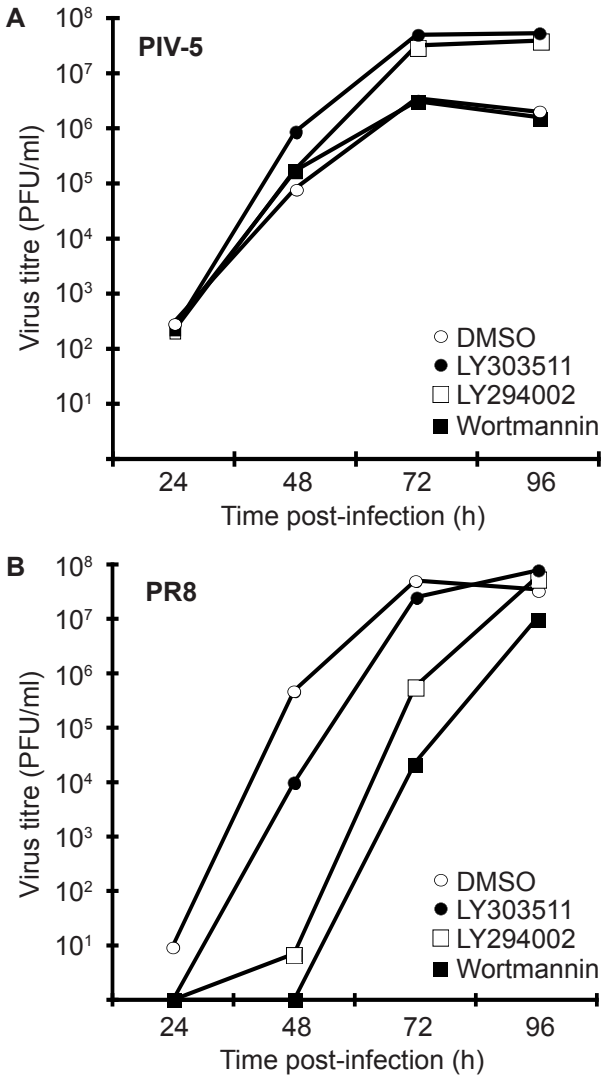


**Fig. 3.10. Specific inhibition of influenza A virus replication by the PI3K inhibitors, LY294002 and wortmannin.** Confluent MDCK monolayers were infected for 1h with parainfluenza virus-5 (PIV-5; A) or influenza A virus (PR8; B) at an MOI of 0.001 PFU/cell. Following virus adsorption, monolayers were treated with DMSO (open circles) [solvent control], 25$\mu$M LY303511 (closed circles) [LY294002 negative control], 25$\mu$M LY294002 (open squares) [PI3K inhibitor], or 1$\mu$M wortmannin (closed squares) [potent PI3K inhibitor]. Virus containing supernatants were harvested at regular times p.i. and titres determined by plaque assay on Vero cells (PIV-5), or MDCK cells (PR8).

### *3.6.2 Tissue culture replication of NS1-Y89F viruses.*

Given the potential difficulties in interpreting results from studies using LY294002 and wortmannin, together with the identification of NS1-Y89F as a construct specifically unable to bind or activate PI3K, the particular importance of NS1-mediated PI3K activation during influenza A virus replication was studied using recombinant mutant viruses. Nucleotides encoding the Y89F amino-acid substitution in NS1 were engineered into pHH21 vectors containing the Ud or WSN NS segments (such a mutation does not affect the amino-acid sequence of NEP). These plasmids were then used in the laboratory of Prof. Robert Lamb (Northwestern University, U.S.A.) to rescue recombinant rWSN and rUd viruses that expressed their strain-specific NS1 proteins with Y89F amino-acid substitutions. Subsequent characterisation of the viruses by Dr. David Jackson (Northwestern University, U.S.A.) showed that the rUd NS1-Y89F mutant virus was clearly attenuated: it formed small plaques in MDCK cells (**Fig. 3.11A**), and grew to infectious titres ~10-fold lower than WT rUd virus during single-step growth analysis (**Fig. 3.11B**). Haemagglutination assays showed a similar reduction in rUd NS1-Y89F titres, indicating that the attenuation was not due to an increase in the number of defective virus particles (*data not shown*). Western blot analysis confirmed that PI3K activation (i.e. phosphorylation of Akt at Ser473) did not occur in cells infected with the rUd NS1-Y89F virus (**Fig. 3.11C**). Interestingly, the rWSN mutant virus did not appear to exhibit an attenuated phenotype under tissue culture conditions. In MDCK cells, the plaque phenotype of rWSN NS1-Y89F was indistinguishable from that of WT rWSN NS1-Y89F (**Fig. 3.12A**), and no replication defects were observed during single-step growth analysis (**Figs. 3.12B**). Despite this, rWSN NS1-Y89F was unable to induce the phosphorylation of Akt (Ser473) during infection (**Fig. 3.12C**).

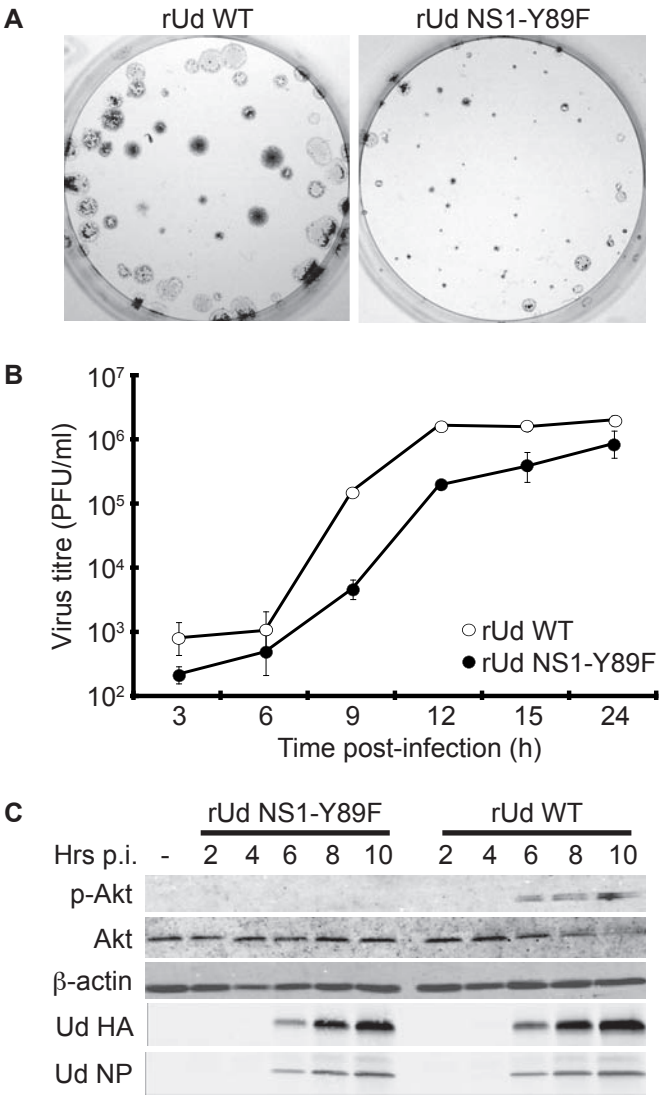


**Fig. 3.11. Characterisation of a recombinant Ud virus (rUd) expressing NS1 with the Y89F amino-acid substitution.** (**A**) **rUd NS1-Y89F forms smaller plaques than WT rUd virus.** Confluent MDCK monolayers were infected at equal MOI with either WT rUd or rUd NS1-Y89F. Plaques were fixed 4 days p.i. and immuno-stained with goat anti-Ud serum. (**B**) **Single-step growth analysis of WT rUd (open circles) and rUd NS1-Y89F (closed circles).** Recombinant Ud viruses were used to infect MDCK cell monolayers (in the absence of TPCK-trypsin) at an MOI of 3 PFU/cell. Virus-containing supernatants were harvested at various times p.i. and titrated by plaque assay. The mean titres from 3 independent experiments are shown - error bars represent standard deviation. (**C**) **Phosphorylation of Akt in response to either WT rUd or rUd NS1-Y89F viruses.** Confluent serum-starved monolayers of 1321N1 cells were infected at an MOI of 5 PFU/cell, and total cell lysates were harvested at various times p.i.. Lysates were separated by SDS-PAGE followed by transfer to PVDF membrane. Phospho-Akt (Ser473) was detected using a specific mAb. Total Akt was detected using a pAb, and β-actin acted as a loading control. Ud HA and NP were detected using goat anti-Ud serum. *Data courtesy of Dr. Dave Jackson, Northwestern University, U.S.A.*

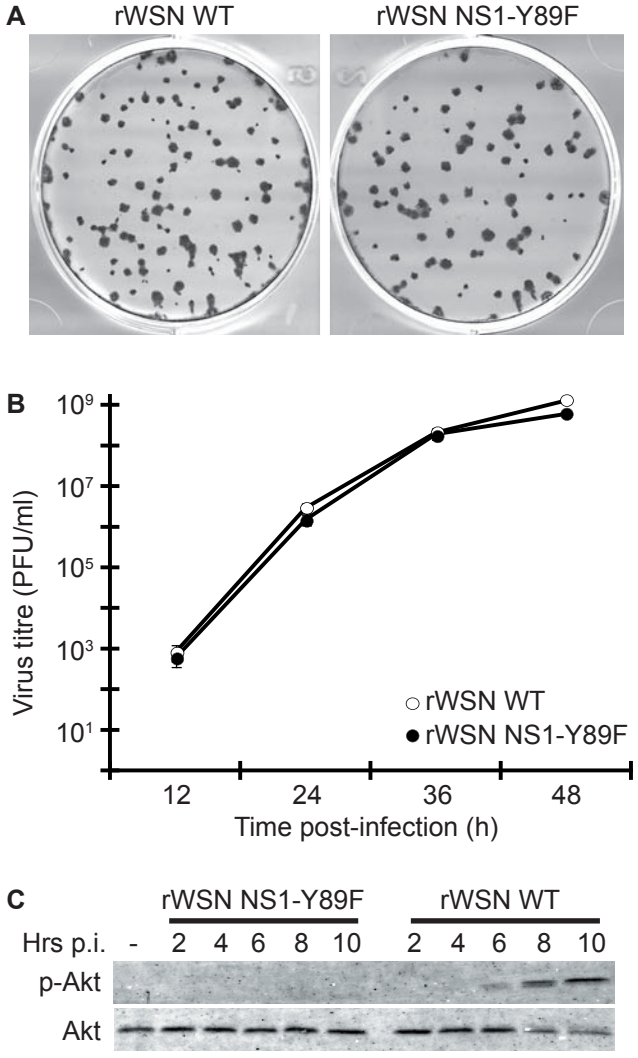


**Fig. 3.12. Characterisation of a recombinant WSN virus (rWSN) expressing NS1 with the Y89F amino-acid substitution.** (**A**) **rWSN NS1-Y89F has a plaque phenotype indistingushable from WT rWSN.** Confluent MDCK monolayers were infected at equal MOI with either WT rWSN or rWSN NS1-Y89F. Plaques were fixed 4 days p.i. and immuno-stained with rabbit anti-PR8 serum. (**B**) **Single-step growth analysis of WT rWSN (open circles) and rWSN NS1-Y89F (closed circles).** Recombinant WSN viruses were used to infect MDCK cell monolayers (in the absence of TPCK-trypsin) at an MOI of 3 PFU/cell. Virus-containing supernatants were harvested at various times p.i. and titrated by plaque assay. Mean titres from 3 independent experiments are shown. (**D**) **Phosphorylation of Akt in response to either WT rWSN or rWSN NS1-Y89F viruses.** Confluent serum-starved monolayers of 1321N1 cells were infected at an MOI of 5 PFU/cell, and total cell lysates were harvested at various times p.i.. Lysates were separated by SDS-PAGE followed by transfer to PVDF membrane. Phospho-Akt (Ser473) was detected using a specific mAb. Total Akt was detected using a pAb, and acted as a loading control. *Data for figs. B & C courtesy of Dr. Dave Jackson, Northwestern University, U.S.A.*

### *3.6.3 In vivo replication of NS1-Y89F viruses.*

It is not clear why the Y89F mutation in Ud/NS1 should attenuate this virus strain, but appears to have no effect on the WSN strain. Ud virus is considered to be more closely related to human influenza A viruses than WSN, which is a highly neurovirulent and mouse-adapted strain of the virus (Castrucci & Kawaoka, 1993). Thus, it is possible that the indistinguishable phenotype between WT rWSN and rWSN NS1-Y89F observed in MDCK cells is due to host species differences. Therefore, it was determined if these recombinant WSN viruses differed in their pathogenicity and/or replication abilities in an *in vivo* mouse model (collaboration with Dr. Daniel Perez, University of Maryland, U.S.A.).

Results indicated that approximately 10-fold less rWSN NS1-Y89F infectious virus (compared with WT rWSN) could be detected in the lungs of infected mice 2 days post-inoculation (**Fig. 3.13A**). However, at 4 and 6 days post-inoculation the amount of recovered rWSN WT and rWSN NS1-Y89F virus was the same (**Fig. 3.13A**). The body weight of infected mice over the timecourse of infection was also monitored. With an input inoculum of $10^5$ PFU/mouse, WT rWSN induced a faster loss of body weight than rWSN NS1-Y89F, and the mice died (or had to be sacrificed) approximately 1 day earlier (**Fig. 3.13B**). With a lower infectious dose ($10^4$ PFU/mouse), the differences were even more striking: WT rWSN caused a relatively rapid loss of body weight (~30% over 6-10 days) and mice died (or had to be sacrificed) at 7, 9 and 11 days (**Fig. 3.13C**). In contrast, the average body weight loss induced by infection with rWSN NS1-Y89F was only ~10%, and all the mice appeared to recover (**Fig 3.13C**). One exception was a single mouse infected with rWSN NS1-Y89F which lost body weight at a rate comparable to WT rWSN (**dotted red line, Fig. 3.13C**). The possibility that the virus in this host may have reverted

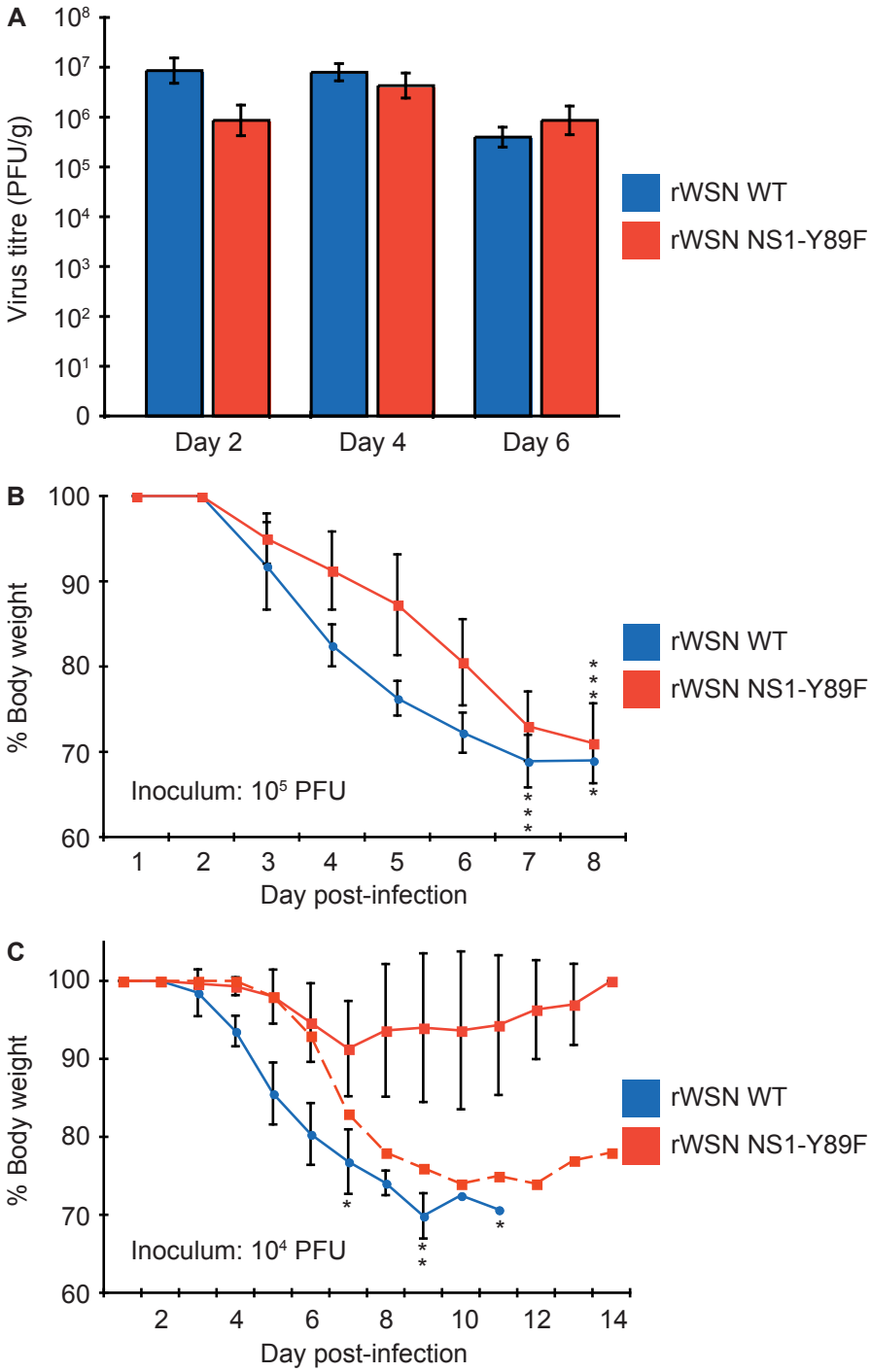


**Fig. 3.13. Effect of Y89F amino-acid substitution on the pathogenicity of WSN in mice.** (**A**) Mice were inoculated intra-nasally with either rWSN WT or rWSN NS1-Y89F. At 2, 4, or 6 days post-inoculation mice were sacrificed, and virus titres within lung homogenates were determined by plaque assay. Data show mean titres (PFU/g) from 3 mice at each time-point. Error bars represent standard deviation. (**B & C**) Mice were inoculated intra-nasally with either rWSN WT or rWSN NS1-Y89F ($10^5$ PFU, **B**; $10^6$ PFU, **C**). Body weight for each mouse was determined daily. Data show mean body weight (%) for 4 mice per virus. Error bars represent standard deviation. Asterisks indicate sacrifice/death of a mouse. For (**C**), 1 mouse infected with rWSN NS1-Y89F lost body weight with kinetics similar to mice infected with WT rWSN (dotted red line), and was therefore excluded from analysis (*see text*). *Raw data courtesy of Dr. Daniel Perez, University of Maryland, U.S.A.*

back to wild-type is currently under investigation. In summary, these *in vivo* data indicate that Tyr89 in NS1 (and thus inferred PI3K activation) may play an important role in the pathogenicity and replication of WSN in mice.

## 3.7 Studies on the biological role of PI3K activation by NS1.

The results presented above demonstrate that NS1-mediated PI3K activation is important for the efficient replication of influenza A viruses, both in MDCK cells (for Ud) and in an *in vivo* mouse model (for WSN). In an attempt to define the biological reasons for PI3K stimulation during infection, WT rUd and rUd NS1-Y89F viruses were used as a comparative model system to investigate the biological role of NS1-activated PI3K in tissue-culture cells.

### *3.7.1 Phenotype of rUd NS1-Y89F is not IFN-dependent.*

Given that NS1 is a well-documented IFN-antagonist (Garcia-Sastre, 2005, Garcia-Sastre *et al*., 1998, Krug *et al*., 2003), and that PI3K has been linked to the ability of cells to both produce and respond to IFNs (see **Introduction**), it was of interest to determine if the attenuation of rUd NS1-Y89F in MDCK cells was due to the inability of NS1-Y89F to counter the host innate immune response.

MDCK cells constitutively expressing the V protein of PIV5 have previously been described (Precious *et al*., 2005). PIV5/V limits IFNβ production by binding directly to mda-5 (Andrejeva *et al*., 2004, He *et al*., 2002a, Poole *et al*., 2002), and completely blocks the IFN signalling response by targeting STAT1 for proteasome-mediated degradation (Didcock *et al*., 1999). Thus, cells constitutively expressing PIV5/V can support the replication of viruses lacking a fully-functional IFN-antagonist (Young *et al*., 2003). Plaque reduction assays clearly showed that rUd NS1-Y89F has a small

plaque phenotype in both naïve MDCK cells and MDCK cells constitutively expressing PIV5/V (**Fig. 3.14A**; the ability of PIV5/V to degrade canine STAT1 in MDCK cells is confirmed in **Fig. 3.14B**). These data indicate that the ability of NS1 to activate PI3K is unlikely to play a major part in the IFN-antagonistic function of NS1, as attenuation of rUd NS1-Y89F appears independent of host IFN-mediated innate immune responses.

### *3.7.2 PI3K does not limit host-cell gene expression.*

A number of influenza A virus NS1 proteins can counter cellular antiviral defences by inhibiting the processing and nuclear export of host-cell mRNAs (Nemeroff *et al.*, 1998, Noah *et al.*, 2003, Satterly *et al.*, 2007) [see also **Introduction**]. To assess if the Y89F mutation of NS1 in rUd NS1-Y89F and rWSN NS1-Y89F lead to their inability to block pre-mRNA processing, a TNFα-responsive luciferase reporter assay was used. A549 cells stably expressing the Firefly luciferase gene under the control of an NFκB promoter were infected overnight with either WT rUd, rUd NS1-Y89F, WT rWSN, or rWSN NS1-Y89F. Infected monolayers were treated (or not) with recombinant human TNFα for 6h in order to stimulate NFκB activity, and lysates were subsequently assayed for luciferase activity. As shown in **Fig. 3.15**, TNFα treatment of uninfected cells caused an approximate 8-fold increase in luciferase gene expression. In contrast, TNFα treatment of cells pre-infected with either WT rUd or rUd NS1-Y89F did not lead to a significant increase in luciferase gene expression (**Fig. 3.15**). Identical results were obtained for WT rWSN and rWSN NS1-Y89F (**Fig. 3.15**). Thus, the Y89F mutation in NS1 does not prevent either Ud or WSN viruses from inhibiting the expression of host-cell genes, suggesting (as might be expected) that this function of NS1 is wholly independent of PI3K activity.

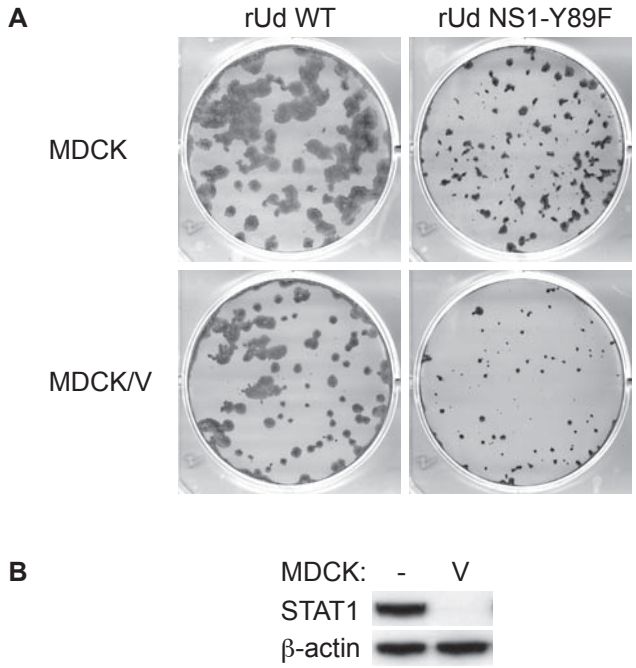


**Fig. 3.14. Plaque phenotype of rUd NS1-Y89F is not rescued in cells deficient in IFN-signalling.** (**A**) Confluent MDCK or MDCK/V monolayers were infected at equal MOI with either WT rUd or rUd NS1-Y89F. Plaques were fixed 4 days p.i. and immuno-stained with rabbit anti-X31 serum. (**B**) Protein lysates from confluent uninfected MDCK or MDCK/V monolayers were separated by SDS-PAGE followed by transfer to PVDF membrane. STAT1 and β-actin were detected using specific antibodies.

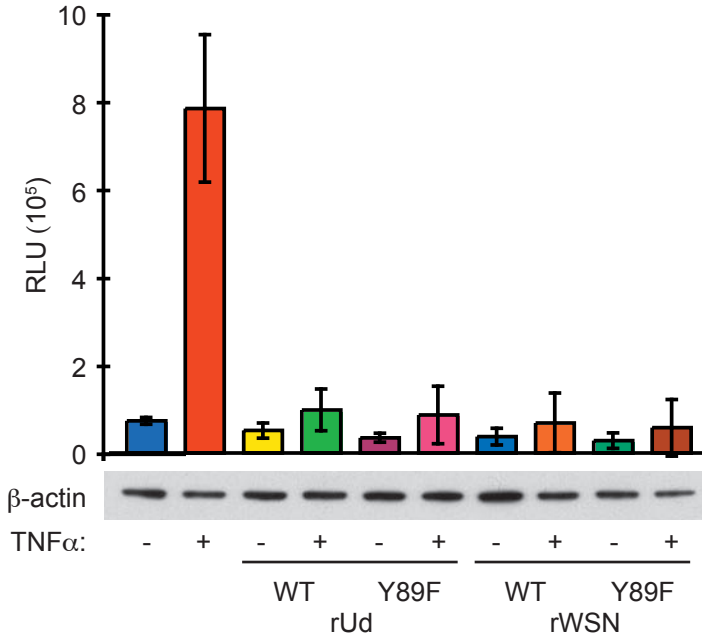


**Fig. 3.15. Inhibition of host-cell gene expression is not affected by the Y89F amino-acid substitution in NS1.** A549 cells constitutively expressing the luciferase gene under the control of an NFkB-dependent promoter were infected (or mock) for 16h with rUd WT, rUd NS1-Y89F, rWSN WT, or rWSN NS1-Y89F (MOI 5 PFU/cell). Monolayers were then treated (or not) with recombinant human TNFα for 6h (in order to stimulate NFkB), and lysates were subsequently assayed for luciferase activity. Data show mean values from triplicate assays (RLU; relative light units), and are representative of two independent experiments. Error bars indicate standard deviation. Samples were also immunoblotted for β-actin (representative blot shown).

### *3.7.3 PI3K delays virus-induced PARP cleavage.*

Influenza A virus-infected cells often show significant signs of apoptosis (Fesq *et al*., 1994, Hinshaw *et al*., 1994, Lowy, 2003, Takizawa *et al*., 1993), a defined form of cell death that is widely regarded as contributing to host antiviral defence. However, NS1 is reported to play a role in limiting the induction of apoptosis during infection (Li *et al*., 2006a, Stasakova *et al*., 2005, Zhirnov *et al*., 2002a) [see also **Introduction**]. Given that the PI3K/Akt signalling pathway is often exploited by viruses in order to prevent early cell death [see **Introduction**], a possible function of NS1-activated PI3K in delaying influenza A virus-induced apoptosis was investigated. A concurrent project within the group (carried out by *Ms. Marian Killip*) was focused on determining viral anti-apoptotic mechanisms, and the data shown below were generated as part of that study.

PARP (poly-(ADP-ribose) polymerase) is a nuclear enzyme involved in DNA repair, and its direct cleavage by caspase-3 is commonly used as a marker for (influenza A) virus-induced apoptosis (Lin *et al*., 2002, Wurzer *et al*., 2003). Thus, HEp2 cells were infected (or mock) with either WT rUd or rUd NS1-Y89F (~50 PFU/cell), and cell lysates were immunoblotted for PARP cleavage at various times post-infection. As shown in **Fig. 3.16**, only the uncleaved (full-length) 116 kDa form of PARP can be detected in uninfected cells. In contrast, the 85 kDa proteolytic fragment indicative of apoptosis is readily visible in lysates from both WT rUd and rUd NS1-Y89F infected cells (**Fig. 3.16**). For WT rUd, the 85 kDa fragment is first observed at ~20h p.i., whilst for rUd NS1-Y89F this fragment is detectable at ~16h p.i. (**Fig. 3.16**). As rUd NS1-Y89F is unable to stimulate PI3K signalling (**Fig. 3.11D**), yet has no apparent defect in countering the innate immune response (**Figs. 3.9C, 3.14A and 3.15**), it is likely that NS1-mediated PI3K activation may delay virus-induced cell death. This

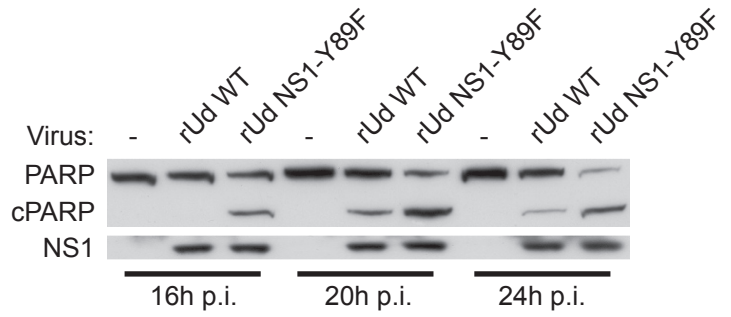


**Fig. 3.16. rUd NS1-Y89F induces PARP cleavage faster than rUd WT in HEp2 cells.** HEp2 cells were infected (or mock, [-]) with rUd WT or rUd NS1-Y89F (MOI 50 PFU/cell), and monolayers were harvested at 16h, 20h, and 24h p.i.. Lysates were separated by SDS-PAGE followed by transfer to PVDF membrane. Full-length (PARP) and cleaved PARP (cPARP) were detected using a specific pAb. NS1 was detected using a rabbit pAb. *Data courtesy of Ms. Marian Killip, University of St. Andrews.*

hypothesis has recently been supported by indirect evidence from a number of groups (Ehrhardt *et al*., 2007a, Zhirnov & Klenk, 2007), and is currently being investigated further by another member of the laboratory (Ms. Marian Killip). Preliminary data also suggest that (in HEp2 cells), there is little difference in PARP cleavage (apoptosis induction) between the WT rWSN virus and the rWSN NS1-Y89F mutant (*data not shown*), which may explain why NS1-mediated PI3K activation appears to play no role in the replication cycle of WSN in MDCK cells (**Fig. 3.12**). Potential differences between Ud and WSN with regards apoptosis induction are also currently under investigation.

## 3.8 Investigating the mechanism of PI3K activation by NS1.

### *3.8.1 NS1 RNA binding is not essential for p85β binding.*

The ability of influenza A virus NS1 to bind RNA has been mapped to a number of basic residues in the first 73 amino-acids of the protein (Qian *et al*., 1995, Wang *et al*., 1999). In particular, arginine-38 probably interacts directly with the RNA target, whilst lysine-41 contributes significantly towards binding affinity (Wang *et al*., 1999). As these residues have been implicated in the NS1-mediated regulation of numerous host-cell processes (Donelan *et al*., 2003, Min & Krug, 2006, Pichlmair *et al*., 2006, Talon *et al*., 2000a, Wang *et al*., 2000), their importance for the interaction of NS1 with p85β was investigated. 293T cells were transfected with plasmids expressing either WT V5-tagged PR8/NS1, or a V5-tagged PR8/NS1 construct that lacks RNA-binding activity (R38A and K41A amino-acid substitutions). Immunoblot analysis of proteins co-precipitated with the anti-V5 antibody revealed that both WT PR8/NS1 and PR8/NS1-R38AK41A efficiently precipitated endogenous p85β (**Fig. 3.17A**). As described previously (**section 3.5.1**), a PR8/NS1 construct containing the single Y89F substitution is unable to precipitate p85β (**Fig. 3.17A**).

To confirm that the entire RNA-binding domain of NS1 is not essential for binding p85β, the *in vitro* interaction of p85β with a recombinant *E. coli* expressed GST-PR8/NS1 protein lacking the first 72 amino-acids of NS1 (termed GST-PR8/NS1Δ72) was tested. GST-PR8/NS1 fusion proteins (WT, Y89F, or Δ72) were immobilised onto glutathione-agarose beads and used to affinity isolate an excess of baculovirus-expressed p85β. SDS-PAGE and Coomassie blue staining revealed that a single 90 kDa protein (p85β) could be specifically isolated by both WT GST-PR8/NS1 and GST-PR8/NS1Δ72, but not by the GST-PR8/NS1-Y89F mutant (**Fig. 3.17B**). These

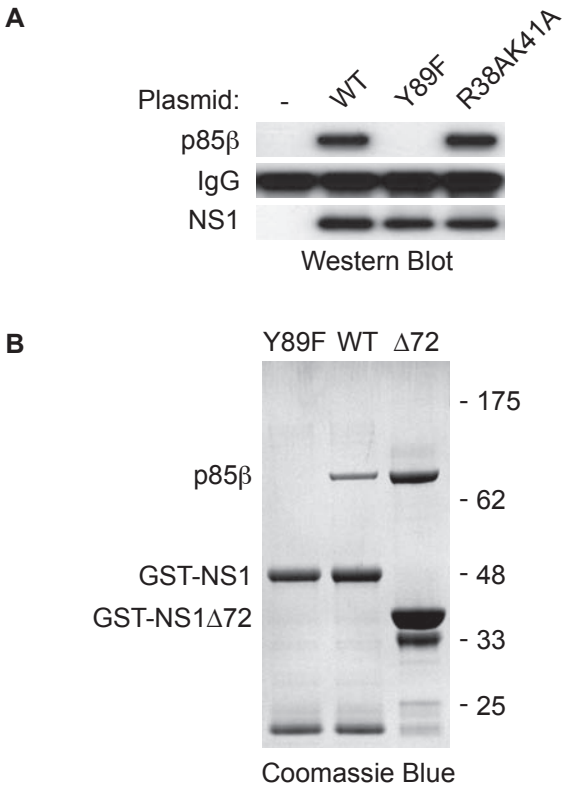


**Fig. 3.17. The RNA-binding domain of PR8/NS1 is not required for the interaction with p85β.** (**A**) 293T cells were transfected for 48h with empty vector (-), a plasmid expressing WT V5-tagged PR8/NS1, or plasmids expressing V5-tagged PR8/NS1 proteins with the amino-acid substitutions Y89F or R38AK41A. Soluble antigen extracts were immunoprecipitated with anti-V5 antibody and precipitates separated by SDS-PAGE followed by transfer to PVDF membrane. Endogenous p85β and V5-tagged NS1 proteins were detected using specific mAbs. (**B**) Equal amounts of soluble *Sf9* cell lysate (expressing p85β) were mixed with recombinant GST-NS1/Y89F, GST-NS1/WT, or GST-NS1Δ72 protein immobilized onto glutathione-agarose beads. After washing, protein complexes were dissociated from the beads and separated by SDS-PAGE through 4-12% polyacrylamide gradient gels. Polypeptides were stained with Coomassie Blue, and protein identification was confirmed by mass spectrometry. Molecular weight markers (kDa) are indicated to the right.

data indicate that the C-terminal effector domain of NS1 alone is sufficient to form a stable complex with p85β.

### *3.8.2 NS1 binds the inter-SH2 domain of p85β.*

In order to determine the domain of p85β that NS1 targets, N-terminal myc-tagged constructs corresponding to four domains of p85β were generated: SH3 (aa 1-100), nSH2 (aa 313-433), iSH2 (aa 433-610), and cSH2 (aa 611-724) (**Fig. 1.14B**). Transient expression of these constructs in 293T cells was followed by affinity isolation using *E. coli* expressed WT GST-PR8/NS1. Immunoblot analysis using an anti-myc antibody revealed that only the inter-SH2 domain of p85β (herein referred to as β-iSH2) co-precipitated with GST-PR8/NS1 (**Fig. 3.18A**).

To verify the specific interaction between PR8/NS1 and β-iSH2 in a wholly mammalian expression system, [$^{35}$S]methionine-labelled 6His-tagged β-iSH2 and unlabelled 6His-tagged PR8/NS1 were synthesised in reticulocyte lysates, mixed, and then immunoprecipitated with anti-NS1 antibody [unlabelled PR8/NS1 was used as it has a molecular weight similar to that of β-iSH2]. SDS-PAGE followed by phosphorimager analysis showed that β-iSH2 was efficiently precipitated from lysate mixtures containing both β-iSH2 and NS1, but not from lysates containing β-iSH2 only (**Fig. 3.18B**). Similar experiments using an unrelated "prey" protein revealed that the observed interaction was not due to both β-iSH2 and PR8/NS1 constructs encoding 6His tags (data not shown, see also **Fig. 3.21A**). In addition, a recombinant 6His-tagged form of β-iSH2 was expressed and purified from *E. coli* (see construct in **Fig. 3.19A**), and was assessed for its ability to compete with full-length p85β in the binding of WT GST-PR8/NS1. As shown in **Fig. 3.18C**, the binding of increasing amounts of β-iSH2 to *E. coli* expressed GST-PR8/NS1 prevented the subsequent

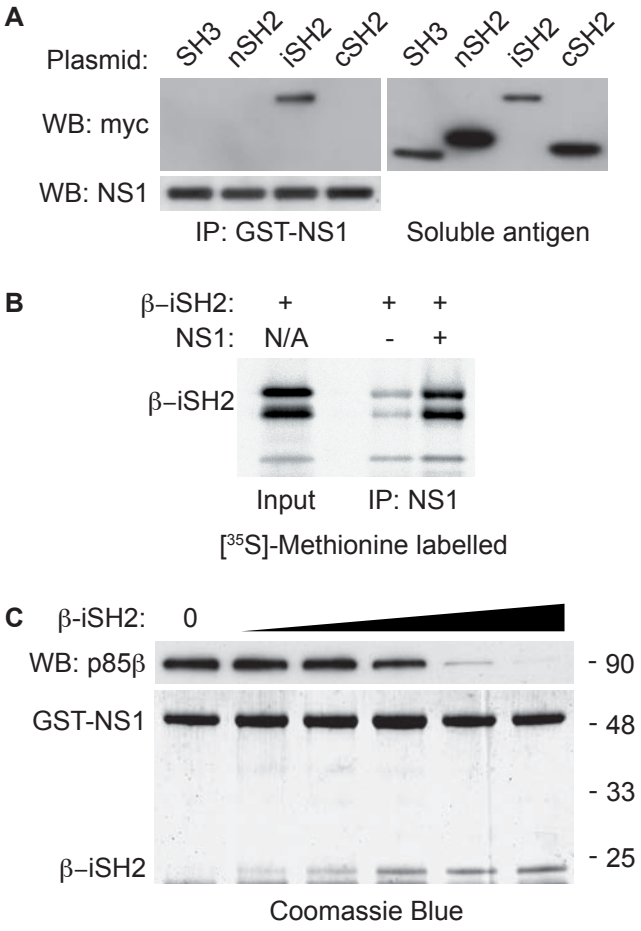


**Fig. 3.18. PR8/NS1 binds the inter-SH2 domain of p85β.** (**A**) 293T cells were transfected for 48h with plasmids expressing myc-tagged domain constructs of p85β: SH3, nSH2, iSH2 or cSH2. Soluble antigen extracts were mixed with GST-NS1 glutathione-agarose beads and precipitates were analysed by SDS-PAGE followed by immunoblotting using an anti-myc mAb. Input GST-NS1 amount was confirmed by detecting NS1 with a specific pAb. The original soluble antigen extracts were also probed with an anti-myc mAb in order to assess relative expression of the myc-tagged constructs. (**B**) *In vitro* synthesised [$^{35}$S]-methionine-labelled β-iSH2 was mixed with unlabelled PR8/NS1 and immunoprecipitated using anti-NS1 pAb. Immunoprecipitation of β-iSH2 alone acted as a negative control. Protein complexes were separated by SDS-PAGE through 4-12% polyacrylamide gradient gels and subjected to phosphorimager analysis. (**C**) **The inter-SH2 domain of p85β is the primary site of interaction for NS1.** GST-NS1 immobilized onto glutathione-agarose beads was mixed with 2-fold increasing amounts of purified 6His-tagged β-iSH2 (*E. coli* expressed). After washing, a fixed amount of soluble p85β-expressing *Sf9* cell lysate was added. Subsequent protein complexes were separated by SDS-PAGE, and GST-NS1 and β-iSH2 were visualised by Coomassie Blue staining. Levels of precipitated full-length p85β were determined by immunoblot analysis. Molecular weight markers (kDa) are indicated to the right.

interaction of GST-PR8/NS1 with baculovirus-expressed p85β. Overall, these data reveal that β-iSH2 is the primary site of interaction between NS1 and p85β.

### *3.8.3 Purification of NS1 in complex with β-iSH2.*

To further confirm direct binding between the C-terminal effector domain of NS1 and β-iSH2, an un-tagged complex of these proteins was purified. Constructs encoding GST-PR8/NS1Δ72 and 6His-tagged β-iSH2 (**Fig. 3.19A**) were co-expressed in *E. coli*, and glutathione-agarose beads were used to affinity isolate GST-PR8/NS1Δ72 together with any associated proteins. It should be noted that both constructs have a cleavage sequence for the Tobacco Etch virus (TEV) protease between the protein of interest and the affinity tag. Glutathione eluted the bound proteins (**Fig. 3.19B** lane (i)), and this eluate was further purified on Ni-NTA resin. After imidazole elution (**Fig. 3.19B**, lane (ii)), the GST and 6His tags were cleaved from the recombinant proteins by TEV protease (**Fig. 3.19B**, lane (iii)). A second glutathione-agarose column removed free GST and uncleaved GST-PR8/NS1Δ72 (**Fig. 3.19B**, lane (iv)), whilst a second Ni-NTA column removed TEV (also 6His-tagged), uncleaved 6His-β-iSH2, and the small ~2kDa cleavage product from 6His-β-iSH2. The flow-through from this column contained only two polypeptide species [as revealed by SDS-PAGE and Coomassie blue staining – **Fig. 3.19B**, lane (v)], which were confirmed as untagged PR8/NS1Δ72 and untagged β-iSH2 by mass spectrometry. In collaboration with Dr. Rupert Russell (University of St. Andrews, U.K.), protein complexes from similar purifications are currently in crystallisation trials.

### *3.8.4 Does NS1:p85β interaction require phospho-Tyr89?*

The observation that an NS1:β-iSH2 complex can be formed *in vitro* from *E. coli* expressed recombinant proteins argues against a phosphorylation-dependent

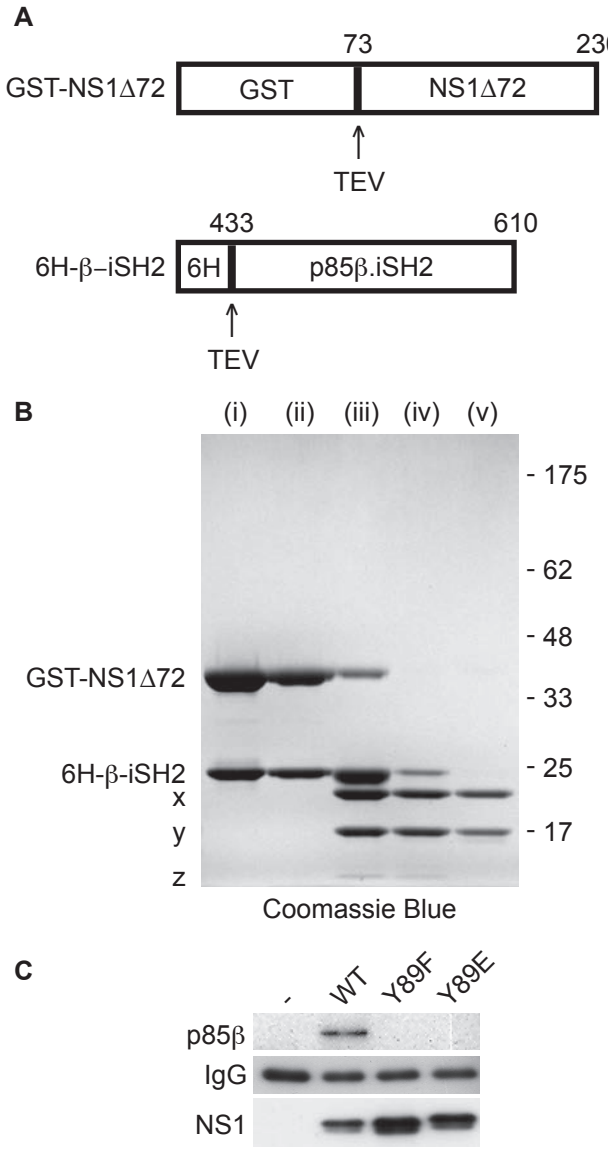


**Fig. 3.19. Purification of an untagged NS1Δ72:β-iSH2 complex.** (**A**) **Schematic representation of the GST-NS1Δ72 and 6His-tagged β-iSH2 constructs.** Numbers refer to residues from full-length PR8/NS1 and p85β. Sites of TEV protease cleavage are indicated. (**B**) **SDS-PAGE analysis of the purification process.** Co-expressed GST-NS1Δ72 and 6His-β-iSH2 from *E. coli* were purified on glutathione-agarose beads and eluted using 10mM glutathione (i). The eluate was further purified on Ni-NTA resin and eluted in 100mM imidazole (ii). After TEV protease cleavage (iii), a second glutathione-agarose column was used to remove free GST (~25 kDa) and uncleaved GST-NS1Δ72 (iv). A second Ni-NTA column was used to remove 6His-TEV (~25 kDa), uncleaved 6His-β-iSH2 (~25 kDa), and the small ~2 kDa cleavage product from 6His-β-iSH2 (band z). The flow-through (v) contained only two polypeptide species as determined by Coomassie Blue staining – untagged NS1Δ72 (band y) and untagged β-iSH2 (band x). Molecular weight markers (kDa) are indicated to the right. (**C**) **The interaction is independent of NS1 Tyr-89 phosphorylation**. 293T cells were transfected for 48h with empty vector (-), a plasmid expressing WT V5-tagged PR8/NS1, or plasmids expressing V5-tagged PR8/NS1 proteins with the amino-acid substitutions Y89F or Y89E. Soluble antigen extracts were immunoprecipitated with anti-V5 antibody and precipitates separated by SDS-PAGE followed by transfer to PVDF membrane. Endogenous p85β and V5-tagged NS1 proteins were detected using specific mAbs.

interaction. However, abrogation of NS1 binding to p85β by a single Y89F mutation in NS1 (**Fig. 3.7**) is highly suggestive of a role for tyrosine-89 phosphorylation. In order to investigate the possibility that tyrosine-89 must first be phosphorylated in order to allow NS1 to bind p85β, a construct encoding V5-tagged PR8/NS1 with a Y89E amino-acid substitution (a mimic for the negative charge of phospho-tyrosine) was generated. Thus, plasmids encoding V5-tagged PR8/NS1-Y89E, V5-tagged PR8/NS1-Y89F, or V5-tagged WT PR8/NS1 were transiently expressed in human 293T cells and subsequently immunoprecipitated with anti-V5 antibody. Western blot analysis of the immunoprecipitates revealed that only WT PR8/NS1 (and not the Y89F or Y89E mutants) was able to precipitate endogenous p85β (**Fig. 3.19C**). Together with the direct *in vitro* binding results from **Fig. 3.19B**, the data imply that phosphorylation of tyrosine-89 is not a determinant of the NS1:p85β interaction.

### *3.8.5 Binding of NS1 to β-iSH2 does not displace p110.*

In the original identification of p85β as a direct binding partner for NS1, no other polypeptide band corresponding to any p110 isoform could be detected in NS1 immunoprecipitates from HEp2 cells (**Fig. 3.3B**). This was surprising given that class IA PI3Ks are reported to be obligate p85:p110 heterodimers (Geering *et al*., 2007, Yu *et al*., 1998). Thus, an equimolar amount of p110 and p85β might have been expected to be co-precipitated with NS1. One explanation for this discrepancy might come from the observation that HEp2 cells express relatively low levels of p110α protein as compared with other commonly used laboratory cell-lines (Vanhaesebroeck *et al*., 1997b). However, given that both p85 and p110 are unstable in the absence of their corresponding PI3K binding partner (Brachmann *et al*., 2005, Yu *et al*., 1998, Zhao *et al*, 2006), it is supposed that the level of p85β in HEp2 cells would mirror the reduced

level of p110α. An alternative explanation might therefore be that the immunoprecipitation conditions used in the initial study caused disruption of p85:p110, or that the C-terminal V5-tag of NS1 in complex with antibody caused the dissociation of p85 from p110. A combination of these factors may be possible, as analysis of NS1 immunoprecipitates from HEp2 cell extracts at low NaCl concentrations (150mM) did reveal a protein that was later identified as p110α by mass spectrometry (*data not shown*).

Despite this, given that NS1 interacts directly with the p110-binding domain of p85β (**section 3.8.2**), it was necessary to investigate the possibility that NS1 might displace p110 subunits from p85β. Thus, in collaboration with Prof. Peter Downes and Dr. Ian Batty (University of Dundee, U.K.), a kinase activity assay was used to determine if NS1 could associate with a p110-containing complex. V5-tagged WT PR8/NS1 or V5-tagged PR8/NS1-Y89F were immunoprecipitated from human 1321N1 cells stably expressing the appropriate construct, and these immunoprecipitates were subjected to *in vitro* PI3K activity assays (immunoprecipitates from naïve 1321N1 cells acted as a negative control). It should be noted that these immunoprecipitations were performed using NaCl concentrations of ~150mM. As shown in **Fig. 3.20A**, significant PI3K activity was readily detectable in V5 immunoprecipitates from lysates expressing WT PR8/NS1, but not from naïve lysates, or from lysates expressing PR8/NS1-Y89F (which is unable to bind p85β).

The ability of WT PR8/NS1 (but not PR8/NS1-Y89F) to precipitate PI3K activity is most likely due to the indirect co-precipitation of a p110 catalytic subunit via p85β, although attempts to physically detect co-precipitating p110α by immunoblot failed. Therefore, to truly establish if the interaction was possible, the formation of a

heterotrimeric complex comprising NS1, β-iSH2, and the p85-binding domain of p110α (also known as the p110α adaptor binding domain; p110α-ABD) was investigated. Initially, [$^{35}$S]methionine-labelled β-iSH2 and HA-tagged p110α-ABD were individually or co-synthesised in reticulocyte lysates and immunoprecipitated using anti-HA antibody. SDS-PAGE and phosphorimager analysis showed that β-iSH2 could be specifically co-precipitated together with p110α-ABD (**Fig. 3.20B**), indicating that the β-iSH2 construct was able to interact with p110α-ABD. Individually synthesised [$^{35}$S]methionine-labelled β-iSH2 or p110α-ABD, as well as unlabelled PR8/NS1, were mixed in various combinations and immunoprecipitated with anti-NS1 antibody. As expected, only β-iSH2 (and not p110α-ABD) could be co-precipitated directly with PR8/NS1 (**Fig. 3.20C**, lanes 4-7). However, p110α-ABD was clearly evident in immunoprecipitates of PR8/NS1 that contained β-iSH2 (**Fig. 3.20C**, lane 8). This confirms that the binding of NS1 to β-iSH2 does not prevent interactions between β-iSH2 and p110α-ABD, and suggests that a heterotrimeric complex consisting of NS1, p85β, and p110α could potentially form.

### *3.8.6 Binding of NS1 requires the C-terminus of β-iSH2.*

As the interaction between NS1 and β-iSH2 is independent of the p110-binding site (**Fig. 3.20C**), it is likely that NS1 must bind elsewhere on the molecule. Previously, it has been shown that truncation of p85α at residue 571 causes constitutive p110 catalytic activity (Jimenez *et al*., 1998). Thus, given that residues downstream of 571 in p85α must therefore play an inhibitory role with regards to PI3K activation, it was investigated whether this same regulatory region in p85β was targeted (and thus potentially masked) by NS1. *In vitro* synthesised [$^{35}$S]methionine-labelled β-iSH2, or a β-iSH2 construct lacking residues downstream of amino-acid 564 (equivalent to

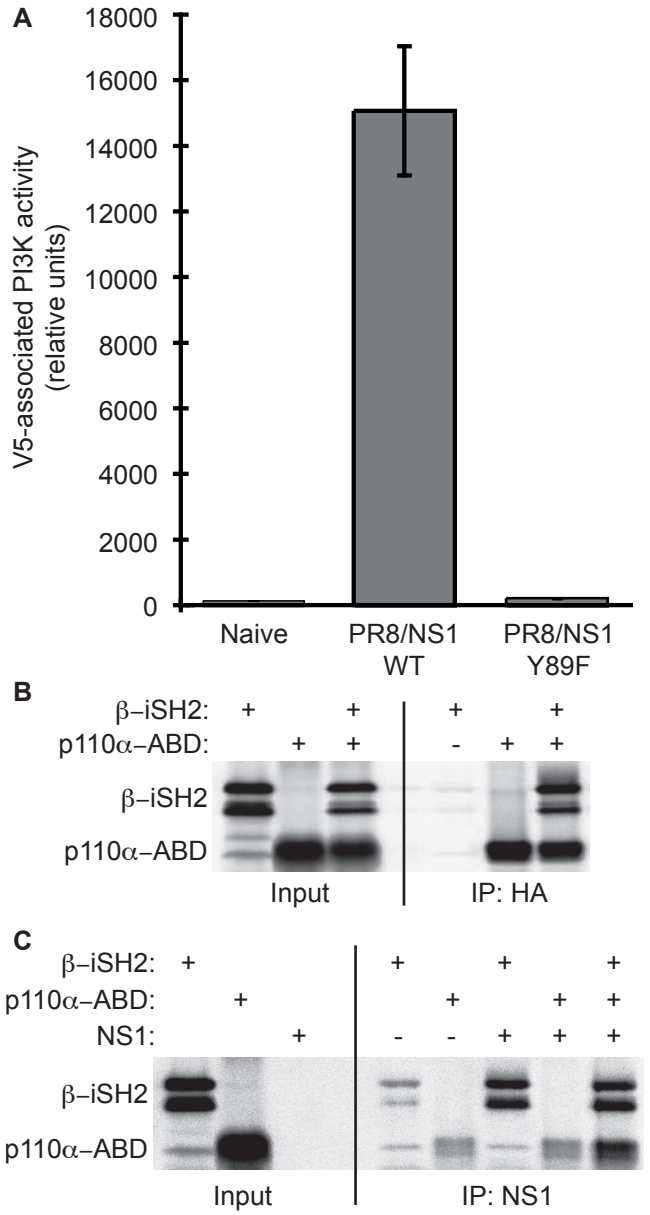


**Fig. 3.20. NS1 can form a heterotrimeric complex containing β-iSH2 and p110.** (**A**) V5-tagged WT PR8/NS1 or V5-tagged PR8/NS1-Y89F were immunoprecipitated from equal amounts of stably expressing 1321N1 cell lysates and assayed for associated PI3K activity. Naïve 1321N1 cell lysate acted as a negative control. Results show the mean and standard deviation of triplicate values obtained in a single experiment, and are representative of two similar experiments. (**B**) Individually synthesised [$^{35}$S]-methionine-labelled β-iSH2 and HA-tagged p110α-ABD (or co-synthesised β-iSH2 and HA-p110α-ABD) were immunoprecipitated using anti-HA mAb. Labelled complexes were separated by SDS-PAGE through 4-12% polyacrylamide gradient gels and subjected to phosphorimager analysis. (**C**) [$^{35}$S]-methionine-labelled β-iSH2 and HA-p110α-ABD were mixed individually (or together) with unlabelled PR8/NS1, and immunoprecipitated using anti-NS1 pAb. Labelled proteins co-precipitating with PR8/NS1 were visualised as for (**B**). *The immunoprecipitations and in vitro PI3K activity assays for (**A**) were performed by Dr. Ian Batty, University of Dundee.*

truncation at residue 571 in p85α; termed β-iSH2'565), were mixed with unlabelled PR8/NS1 and then immunoprecipitated with anti-NS1 antibody. SDS-PAGE and phosphorimager analysis clearly showed that β-iSH2'565, unlike β-iSH2, was not precipitated by PR8/NS1 (**Fig. 3.21A**, lanes 1-6). These data were replicated using longer β-iSH2 constructs that also contained the adjacent nSH2 domain of p85β (**Fig. 3.21A**, lanes 7-12). The inability of PR8/NS1 to bind β-iSH2'565 is unlikely to be caused by gross non-specific disruption of the coiled-coil structure, as the nSH2-iSH2'565 construct could still interact with p110α-ABD (**Fig. 3.21B**). These data indicate that residues at the C-terminal end of β-iSH2 are required for NS1 binding. To confirm this, the C-terminal 45 residues of β-iSH2 (i.e. amino acids 565-610 of full-length p85β) were expressed and purified as a GST fusion protein, and subsequently used to affinity isolate *in vitro* synthesised [$^{35}$S]methionine-labelled PR8/NS1. As shown in **Fig. 3.21C**, the GST-tagged form of β-iSH2 (565-610) was able to precipitate more PR8/NS1 than the GST control alone.

### *3.8.7 Other proteins and NS1-mediated PI3K activation?*

As NS1 expression alone causes the constitutive Ser473 phosphorylation of Akt (**Fig. 3.5**), it is apparent that no other influenza A virus-derived factors are required to activate PI3K signalling. However, it is unclear exactly how NS1 can increase p110 catalytic activity simply by binding to a C-terminal site of β-iSH2 (the means by which this region normally negatively regulates p110 function is currently unknown). Thus, it may be that NS1 recruits a host-cell "activating" factor to the p85β:p110 complex rather than masking an inhibitory domain. In this regard, it is of interest to note that the NS1:p85β complex can precipitate a host-cell protein of ~180 kDa (**Fig. 3.22A**). This interaction was identified as part of the initial effort to determine if NS1

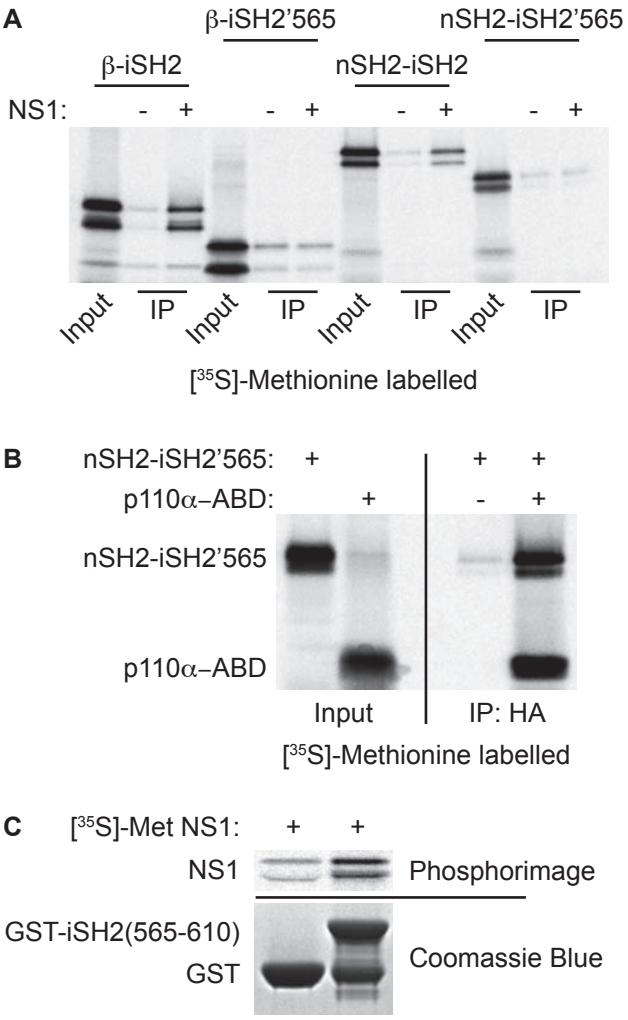


**Fig. 3.21. Interaction between NS1 and β-iSH2 requires residues at the C-terminus of β-iSH2.** (**A**) [$^{35}$S]-methionine-labelled β-iSH2 and nSH2-iSH2 (or similar constructs containing a stop codon at residue 565: β-iSH2'565 and nSH2-iSH2'565) were mixed individually with unlabelled PR8/NS1, and immunoprecipitated using anti-NS1 pAb. An identical procedure in the absence of PR8/NS1 (-) acted as a negative control. Precipitated labelled proteins were visualised as for **Fig. 3.19B**. (**B**) **Truncation at residue 565 of the nSH2-iSH2 construct does not affect the p110-binding site.** [$^{35}$S]-methionine-labelled nSH2-iSH2'565 and HA-tagged p110α-ABD were individually synthesised, mixed, and immunoprecipitated with anti-HA mAb. Immunoprecipitation of nSH2-iSH2'565 alone acted as a negative control. Detection of labelled proteins was as for (**A**). (**C**) Equal amounts of [$^{35}$S]-methionine-labelled PR8/NS1 were mixed with recombinant GST or GST-iSH2(565-610) immobilized onto glutathione-agarose beads. After washing, protein complexes were dissociated from the beads and separated by SDS-PAGE through 4-12% polyacrylamide gradient gels. Polypeptides were visualised by either phosphormager analysis or Coomassie Blue staining.

was tyrosine phosphorylated. V5-tagged PR8/NS1 immunoprecipitates from transiently transfected 293T cells were immunoblotted using an anti-phosphotyrosine antibody. Although, a phospho-tyrosine band corresponding to NS1 could not be detected due to the experimental procedure used (it was obscured by immunoglobulin light chain), a single tyrosine-phosphorylated host-cell polypeptide of ~180 kDa was observed specifically co-precipitating with WT PR8/NS1, but not with PR8/NS1-Y89F (**Fig. 3.22A**). As PR8/NS1-Y89F cannot bind p85β, this indicated that the tyrosine-phosphorylated protein was either competing with p85β for the same site on NS1, or was co-precipitated indirectly via p85β. To address this (and to identify the protein), GST-PR8/NS1 alone, or GST-PR8/NS1 complexed with baculovirus-expressed p85β, was used to affinity isolate proteins from a 293T cell extract (~$1x10^7$ cells). SDS-PAGE and Coomassie Blue staining revealed a single protein (~180 kDa) that specifically precipitated with the GST-PR8/NS1:p85β complex, but not with GST-PR8/NS1 alone (**Fig. 3.22B**), indicating that p85β probably acted as a bridge between NS1 and the host-cell protein. Mass spectrometry identified the ~180 kDa protein as insulin receptor substrate 4 (IRS-4), a highly tyrosine-phosphorylated protein abundantly expressed in 293 cells (Fantin *et al*., 1998, Kuhne *et al*., 1995, Lavan *et al*., 1997). IRS-4 normally binds to the SH2 domains of p85 molecules, and thus acts as an activating adapter between insulin/growth factor receptors and the PI3K heterodimer (Fantin *et al*., 1998). As IRS-4 is estimated to be the major IRS in 293 cells (Fantin *et al*., 1998), it is possible that the observed co-precipitation of IRS-4 with GST-PR8/NS1:p85β is an artifact of excess soluble IRS-4 being "mopped-up" by the free SH2 domains of p85β. Thus, work in other cell-lines is currently underway to determine if IRS-4 (or any other IRS) actually plays a specific role in NS1-mediated PI3K activation.

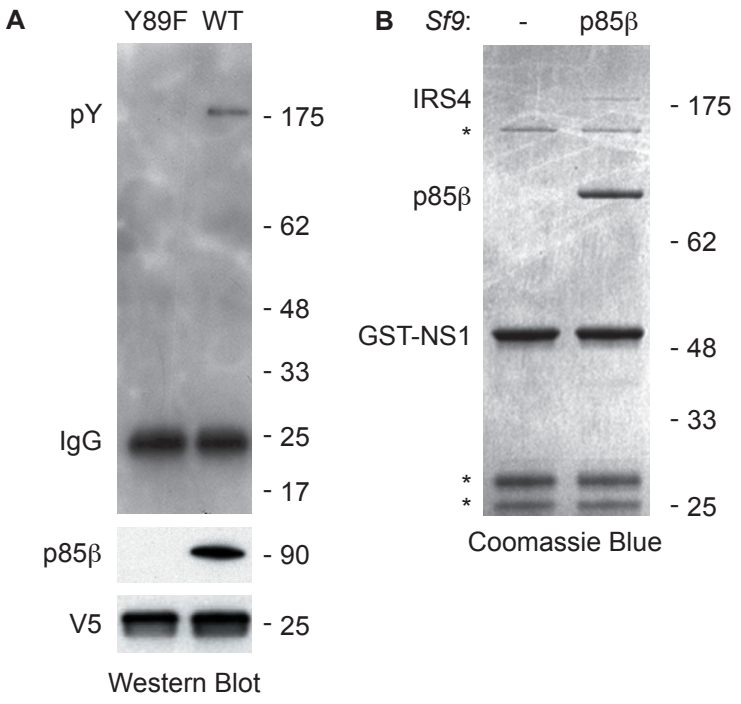


**Fig. 3.22. Identification of IRS-4 as a host-cell protein that co-precipitates with the PR8/NS1:p85β complex in 293T cells.** (**A**) 293T cells were transfected for 48h with either a plasmid expressing the WT V5-tagged PR8/NS1 protein, or the V5-tagged PR8/NS1-Y89F mutant. Soluble antigen extracts were immunoprecipitated with anti-V5 antibody (rabbit) and precipitates separated by SDS-PAGE followed by transfer to PVDF membrane. Tyrosine phosphorylated (pY) proteins were detected using a specific mouse mAb. Endogenous co-precipitating p85β was detected using a specific mAb. Anti-V5 mAb was used to detect V5-tagged NS1 proteins. Molecular weight markers (kDa) are indicated to the right. (**B**) Equal amounts of soluble *Sf9* cell lysate (expressing p85β, or mock [-]) were mixed with *E. coli* expressed recombinant GST-PR8/NS1 (WT) immobilized onto glutathione-agarose beads and subsequently mixed with 293T cell extract equivalent to ~5x$10^6$ cells. After washing, protein complexes were dissociated from the beads and separated by SDS-PAGE through 4-12% polyacrylamide gradient gels. Polypeptides were stained with Coomassie Blue, and protein identification was performed by the University of St. Andrews mass spectrometry facility. Breakdown products of GST-NS1 are indicated by asterisks. Molecular weight markers (kDa) are indicated to the right.

## 3.9 PI3K signalling as a potential anti-influenza drug target.

### *3.9.1 Engineering of cell-lines that limit virus replication.*

Blocking the ability of NS1 protein to bind one of its cellular targets has recently been described as a "proof-of-principle" approach to limit influenza A virus replication (Twu *et al*., 2006). Twu *et al*. generated an MDCK cell-line stably expressing a ~60 amino acid fragment of CPSF30 that interacts with NS1, and demonstrated that expression of this fragment could prevent Ud virus from inhibiting the post-transcriptional processing of cellular pre-mRNAs (Twu *et al*., 2006). This lead to attenuation of Ud virus replication, presumably via a mechanism involving an increased innate immune response (Twu *et al*., 2006).

In this study, a similar approach was undertaken to determine if blocking the NS1:p85β interaction could attenuate influenza A virus replication. Lentivirus technology was used to generate MDCK cell-lines that stably express either C-terminal V5-tagged β-iSH2, or identically tagged β-iSH2 with an N-terminal nuclear localisation sequence (NLS; MPKKKRKV, derived from the SV40 T antigen). β-iSH2 is able to compete with full-length p85β for binding to NS1 (**Fig. 3.18C**), therefore the rationale was to express a polypeptide that would prevent NS1 from forming a complex with, and activating, PI3K. The V5-tag facilitated detection of the recombinant proteins, whilst the NLS was added in order to redirect the protein to the nucleus (where NS1 is usually predominantly localised in virus-infected cells). Immunoblot analysis of the cell lysates using anti-V5 antibody verified the stable expression of each construct (**Fig. 3.23A**). As expected, treatment of starved, naïve MDCK cells with either 10% FBS or PR8 infection (MOI ~5 PFU/cell) resulted in the phosphorylation of Akt at Ser473 (**Fig. 3.23A**). Similar treatment of β-iSH2

expressing MDCK cells yielded identical results, indicating that this construct had no effect on the ability of either FBS or influenza A virus to activate PI3K (**Fig. 3.23A**). Surprisingly, no Ser473 phosphorylation of Akt could be detected in cells expressing the N-terminally NLS-tagged β-iSH2 construct (**Fig. 3.23A**). Basal levels of phospho-Akt were also undetectable in these cells (**Fig. 3.23A**), suggesting that this construct inhibited general PI3K activity independently of the stimulus (i.e. FBS or influenza A virus infection). The reasons why such a nuclear localised construct might block PI3K are unknown, but one possibility may be that NLS-β-iSH2 sequesters p110 into the nucleus away from its plasma membrane substrates, or that the NLS from SV40 T antigen has some effect. Nevertheless, plaque reduction assays revealed that WT Ud virus was attenuated in the MDCK cell-line expressing NLS-β-iSH2, as compared with the naïve cell-line and the cell-line expressing β-iSH2 only (**Fig. 3.23B**).

Given the previous observation that rWSN NS1-Y89F showed no attenuation in naïve MDCK cells despite its inability to activate PI3K (**Fig. 3.12**), it was perhaps not surprising that WT WSN did not display any attenuation (as determined by plaque reduction assays) in the cells expressing NLS-β-iSH2 (**Fig. 3.23B**). Together, these data confirm previous results which indicate that PI3K activation is important for the tissue-culture replication of Ud (but not WSN) influenza A viruses. However, it should be noted that the ability to activate PI3K still appears to be important for the pathogenicity of WSN *in vivo* (**Fig. 3.13**).

### *3.9.2 Assay development to screen for NS1:p85β interaction inhibitors.*

Small molecules are not generally considered suitable for disrupting the large and hydrophobic interfaces that often mediate protein:protein interactions (Cochran, 2000, Cochran, 2001, Toogood, 2002). Nevertheless, several studies have shown that

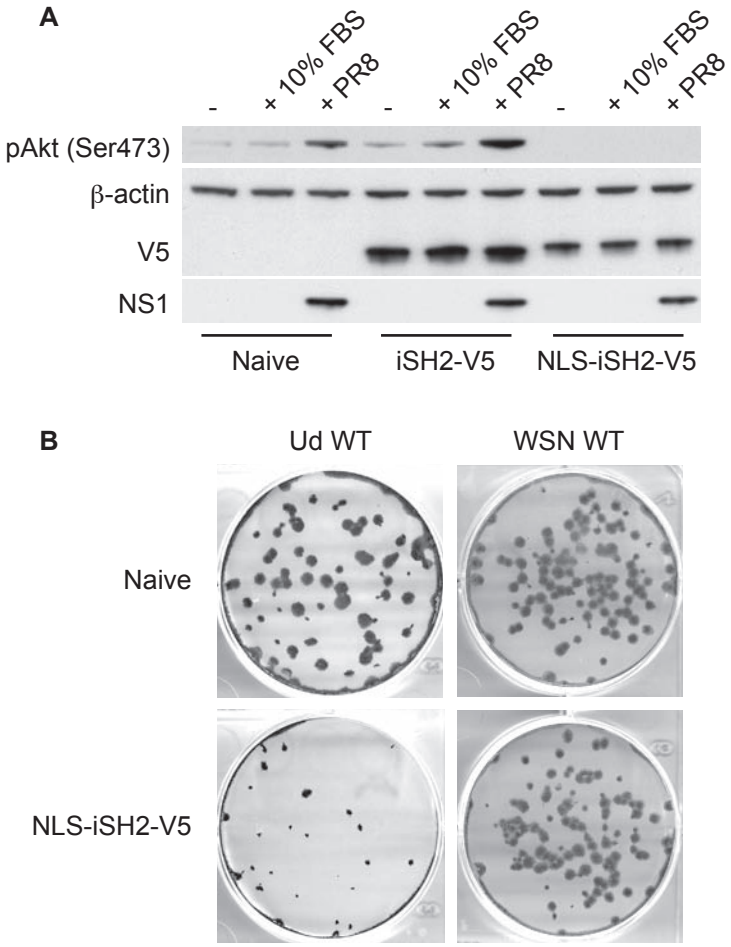


**Fig. 3.23. Characterisation of MDCK cell-lines constitutively expressing β-iSH2 constructs.** (**A**) **Phosphorylation of Akt in response to either serum or PR8.** Confluent serum-starved monolayers of naive MDCK cells, or MDCK cells constitutively expressing either V5-tagged β-iSH2 (iSH2-V5) or V5-tagged β-iSH2 with an N-terminal NLS (NLS-iSH2-V5) were left untreated, treated with 10% FBS, or infected with PR8 at an MOI of 5 PFU/cell, and total cell lysates were harvested ~8h p.i.. Lysates were separated by SDS-PAGE followed by transfer to PVDF membrane. Phospho-Akt (Ser473) was detected using a specific mAb. Detection of β-actin acted as a loading control. Anti-V5 mAb was used to detect V5-tagged β-iSH2 constructs, and a specific pAb was used to detect NS1. (**B**) **Ud virus (but not WSN virus) forms smaller plaques in MDCK cells expressing NLS-iSH2-V5 than in naive MDCK cells.** Confluent MDCK cell-line monolayers were infected at equal MOI with either WT rUd or WT rWSN. Plaques were fixed 4 days p.i. and immuno-stained with either rabbit anti-X31 or rabbit anti-PR8 serum.

protein associations can be inhibited by small molecules (Berg *et al*., 2002, Degterev *et al*., 2001, Duncan *et al*., 2001, Lepourcelet *et al*., 2004, Stoll *et al*., 2001), leading to the possibility that aberrant protein:protein interactions (including virus:host interactions) could be novel sites for drug intervention. Protein binding events commonly rely on a few important amino-acid contacts that would be ideal targets. Although the x-ray crystal structure of the NS1:p85β complex interface would provide detailed information on these essential residues, single point mutation analysis has already shown that Tyr89 of NS1 is a critical determinant (**Fig. 3.7**).

In an identical strategy to the successful ELISA-based screening technique utilised by others (Lepourcelet *et al*., 2004), an assay was developed that might be used to identify small molecular weight compounds that specifically inhibit the interaction between NS1 and p85β. Purified GST-NS1 was coated onto microtitre plates and incubated with *Sf9* cell lysate expressing p85β. GST alone, or naive *Sf9* cell lysate acted as the relevant negative control. Anti-p85β antibody was used to detect bound p85β, which was visualised using an alkaline phosphatase conjugated secondary antibody, together with the appropriate substrate. As shown in **Fig. 3.24A**, a positive result (binding) was clearly obtained when p85β-expressing lysate was incubated with GST-NS1. No alkaline phosphatase activity was detectable when using GST alone as "bait", or when using naïve *Sf9* cell lysate as "prey", indicating the specificity of the ELISA. The results are quantified and plotted in **Fig. 3.24B**. This strategy has been used in preliminary assays to screen ~200 compounds for potential inhibitory effects on the NS1:p85β interaction (collaboration with Dr. Nick Westwood, University of St. Andrews, U.K.). As might be expected from such a small-scale screen, no potential "hits" were identified. The methodology is currently under refinement in order to make it more amenable to high-throughput screening.

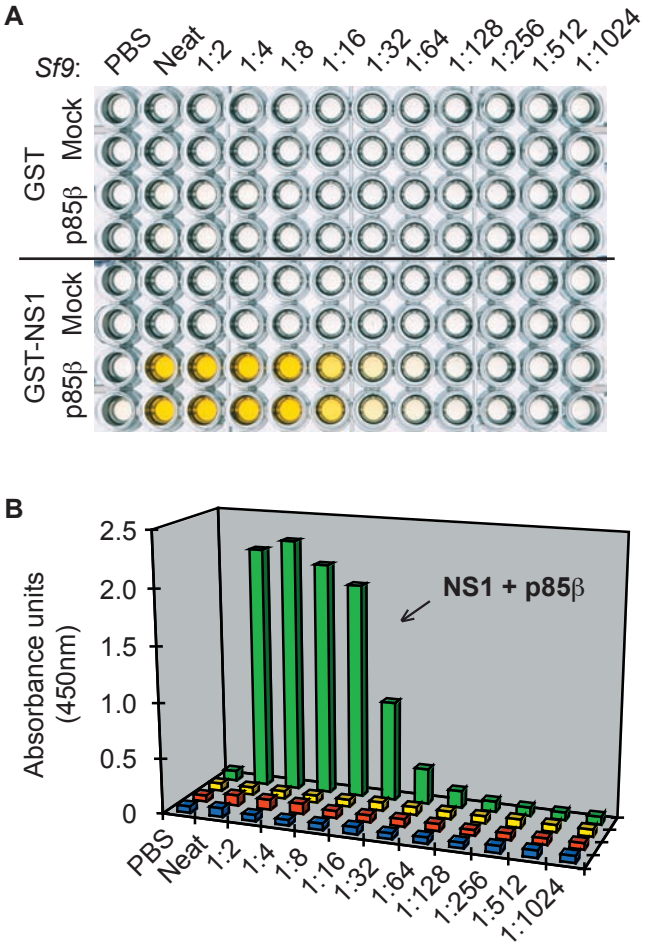


**Fig. 3.24. Development of an ELISA-based assay to screen for inhibitors of the NS1: p85β interaction.** (**A**) 1$\mu$g of recombinant, purified, *E. coli* expressed GST or GST-NS1 was adsorbed to wells of a flat-bottomed 96-well ELISA plate, and doubling dilutions of *Sf9* cell lysate expressing (or not) p85β were applied in duplicate. After washing, bound p85β was detected using a specific mAb in combination with an HRP-conjugated secondary antibody. TMB peroxidase substrate was used to visualise wells containing p85β (yellow). (**B**) Plot of the relative absorbance units (450nm) per well determined for the plate shown in (**A**). Data represent the mean values for each duplicate. Legend: blue, GST + mock; red, GST + p85β; yellow, GST-NS1 + mock; green, GST-NS1 + p85β.

Chapter 4

# Discussion

## 4.1 Influenza A virus NS1 protein binds and activates PI3K.

In this thesis, the interaction of several influenza A virus NS1 proteins with the p85β regulatory subunit of PI3K is reported. PI3K activity could be co-precipitated with NS1 from cell-lysates, and expression of NS1 alone in cells was sufficient to cause the PI3K-dependent phosphorylation of Akt, a major downstream effector of PI3K. During the course of this study, these results were confirmed and published by several other groups (Ehrhardt *et al*., 2007a, Shin *et al*., 2007a, Shin *et al*., 2007b, Shin *et al*., *in press*, Zhirnov & Klenk, 2007).

### *4.1.1 NS1 proteins bind p85β, but not p85α.*

The two PI3K p85 isoforms (p85α and p85β) are encoded by separate genes, yet exhibit ~55% protein sequence identity and high structural homology. Both are expressed in a wide range of tissues, but p85α is generally thought to be involved in the major response pathway for most PI3K-mediated stimuli. The physiological role of p85β *in vivo* is largely unknown. Given that p85β expression is reported to be ~40% that of p85α (Ueki *et al*., 2003), it was remarkable to find that influenza A virus NS1 proteins directly bind to p85β (and activate PI3K), whilst no evidence for NS1 binding p85α could be established. This intriguing observation has recently been confirmed by Shin and colleagues, who used different experimental conditions to those described here (Shin *et al*., *in press*).

Subsequent to this study, Ehrhardt and colleagues reported the co-precipitation of NS1 with both p85α and p85β from influenza A virus-infected human cells (Ehrhardt *et al*., 2007a). However, the co-precipitation of NS1 with p85α was weak, and could not be detected when both NS1 and p85α were transiently expressed from plasmids (Ehrhardt *et al*., 2007a). Additionally, no significant interaction between NS1 and

p85α was observed in yeast two-hybrid assays (Ehrhardt *et al*., 2007a). Together, these data suggest that the observed NS1:p85α interaction is indirect, and may require an important (and possibly virus-encoded) co-factor. Alternatively, NS1 may have a significantly higher binding affinity for p85β than for p85α.

A number of studies have attempted to establish distinct roles for p85α and p85β in the regulation of different PI3K-dependent or -independent pathways (Deane *et al*., 2004, Hartley *et al*., 1995, Ueki *et al*., 2003, Ueki *et al*., 2002, Vanhaesebroeck *et al*., 2005), but other than a possible function in limiting T-cell expansion (Deane *et al*., 2004), p85β has yet to be specifically associated with a role directly relevant to virus infection. As detailed below, further work is clearly required to determine the (as yet unknown) consequences of NS1 primarily targeting p85β, both for the virus and for the host. Excitingly, such studies may yield valuable information regarding the normal cellular function of p85β in specific PI3K signalling pathways. Additionally, there is the possibility that NS1 also discriminates between the various differentially expressed p110 isoforms, which may contribute to cell-type dependent NS1-mediated PI3K responses.

### *4.1.2 Mechanism of PI3K activation by NS1.*

A major part of this study was to map the interaction between NS1 and p85β in an attempt to determine the mechanism of NS1-mediated PI3K activation. Although it is clear that NS1 proteins from different strain origins may possess distinct biological properties (as determined by their ability to differentially interact with some host-cell proteins [Kochs *et al*. 2007a]), there is no reason at present to believe that NS1 proteins bind and activate PI3K by separate means. Thus, PR8/NS1 was used as the sole model to investigate NS1-mediated binding to p85β.

***4.1.2.1 The binding site on NS1.***

Here it is demonstrated that the direct binding of NS1 to p85β is independent of NS1 RNA-binding activity, and is primarily mediated via the C-terminal effector domain of NS1. Additionally, tyrosine-89 and methionine-93 (both in the C-terminal effector domain of NS1) were identified as essential residues for binding p85β. These two residues were initially of interest as they formed part of a putative motif with similarity to the well-documented PI3K SH2-binding motif, phospho-Y*XX*M (Songyang *et al*., 1993).

Results from this and other studies indicate that the activation of PI3K by NS1 probably does not involve tyrosine-phosphorylated NS1 occupying a p85β SH2 domain. Firstly, an interaction could not be detected between NS1 and either of the two p85β SH2 domains. Secondly, NS1 with glutamic acid substituted as a mimic of phospho-tyrosine at residue 89 was unable to bind full-length p85β, and the interaction domains of NS1 and p85β could be expressed and purified as a protein complex entirely from *E. coli*. Thirdly, previous Y*XX*M phosphopeptide binding studies have shown relatively conserved pockets in both p85 SH2 domains, which are strongly selective for methionine only at the Tyr+3 position (Songyang *et al*., 1993). In NS1, the relevant methionine is at the Tyr+4 position and is thereby unlikely to be presented in the appropriate orientation. Finally, recent structural analysis of the PR8/NS1 effector domain indicates that whilst tyrosine-89 appears exposed within a cleft formed at the interface between the two PR8/NS1 monomers, methionine-93 is mostly buried within the PR8/NS1 homodimer (Bornholdt & Prasad, 2006) (**Fig. 4.1**). Thus, whilst the location of tyrosine-89 on the border of an extended chain region/helix means it is well positioned for a role in binding p85β, it is not possible for methionine-93 to be directly involved in the interaction with p85β without a major

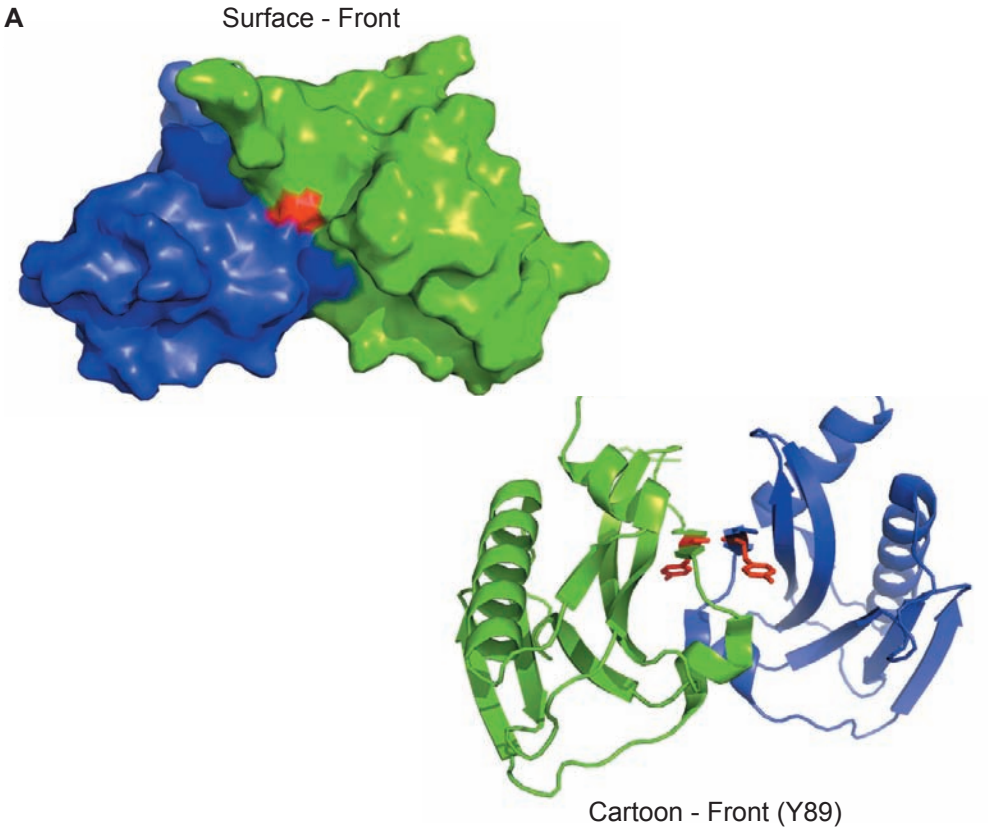


**Fig. 4.1. Position of tyrosine-89 and methionine-93 in the C-terminal effector domain of NS1.** (**A**) Front-surface and front-cartoon representations of the C-terminal effector domain homodimer with tyrosine-89 highlighted in red. The two monomers are coloured green and blue. (**B**) Front-cartoon representation of the C-terminal effector domain with methionine-93 highlighted in red. A surface representation cannot be shown as methionine-93 is buried within the homodimer. *Images were prepared using MacPyMol (Protein Data Bank file: 2GX9).*

conformational change in NS1. The observation that mutation of methionine-93 abrogates p85β binding may therefore be due to destabilisation of the NS1 homodimer, as this residue could be important for maintaining the functional integrity of the NS1 structure.

Of course, it is entirely possible that substitution of phenylalanine for tyrosine-89 also destabilises NS1. However, the observation that this mutant (PR8) protein, like WT, predominantly localises to the nucleus of cells (and can co-precipitate RIG-I), strongly argues against structural disruption of the protein. Moreover, the rUd NS1-Y89F virus was only attenuated ~10-fold in MDCK cells compared to WT rUd, whilst influenza A viruses completely unable to express NS1 are reported to be attenuated ~1000-fold in MDCK cells (Garcia-Sastre *et al*., 1998). Indeed, a substitution of phenylalanine for tyrosine is relatively conservative (as compared with alanine for methionine), and it is difficult to envisage how this change could seriously affect protein structure (**Fig. 4.2**).

#### *4.1.2.2 The binding site on p85β.*

Competition assays from this work clearly establish that the primary direct binding site for NS1 in p85β is the iSH2 domain. Structurally, iSH2 is predicted to be a rigid 100-110Å coiled-coil of two anti-parallel α-helices (Dhand *et al*., 1994, Fu *et al*., 2003). The helices are ~70 amino-acids long (helix-1; residues 434-505, and helix-2; residues 511-581), and are connected by a loop of 5 residues (506-510) (Dhand *et al*., 1994). At the C-terminal end of helix-2 is a ~30 residue "tail" that links the coiled-coil to the cSH2 domain. The acidic nature of this stretch of amino-acids suggests that it could fold back and pack against exposed basic residues at the C-terminus of helix-2 (Dhand *et al*., 1994). Biochemical studies to determine the p110-binding site on

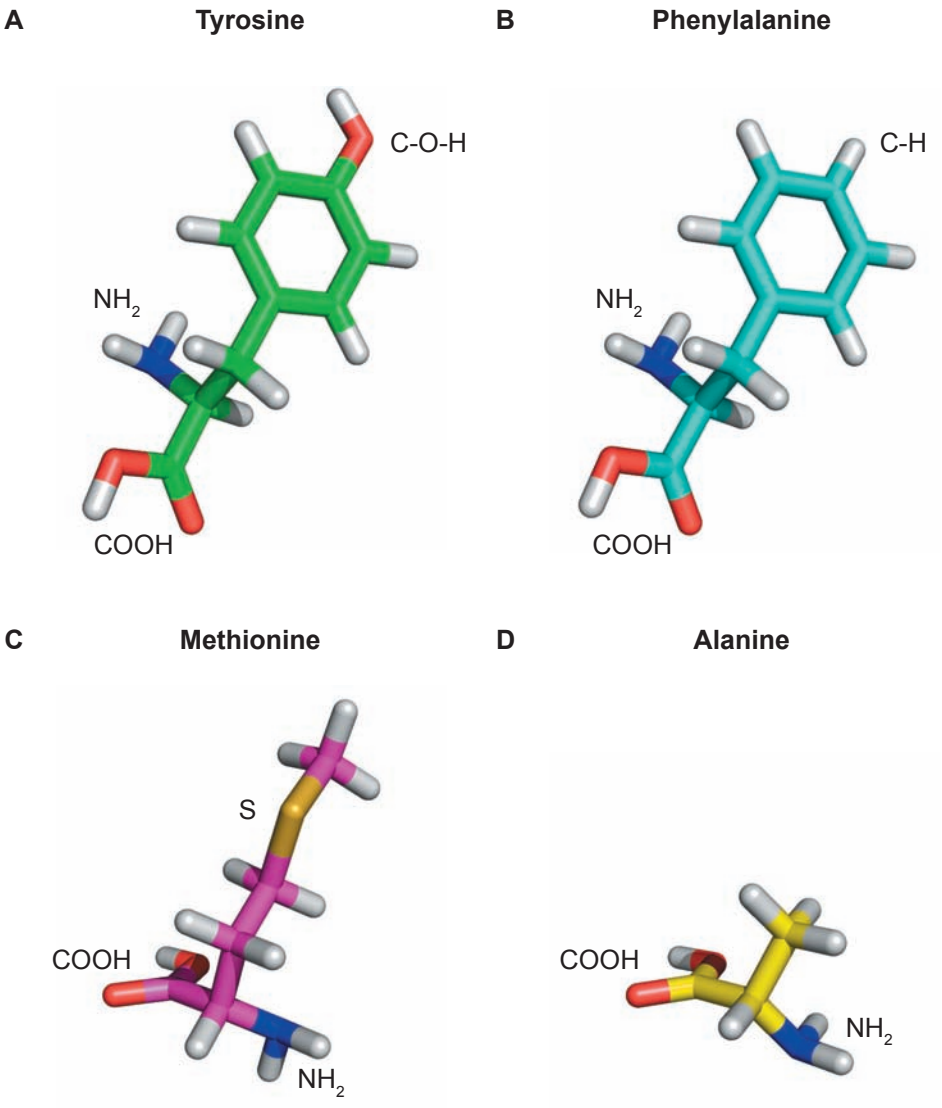


**Fig. 4.2. Amino-acid changes made in NS1.** (**A**) Tyrosine to (**B**) phenylalanine. (**C**) Methionine to (**D**) alanine. Amino- and carboxyl- ends of the backbone are shown . The major colour of each amino-acid represents carbon. Hydrogen is represented in silver. *Images were prepared in MacPyMol using PDB files from http://www.chemistry.gsu.edu/glactone*.

p85β have indicated essential residues in helix-1 as 445-485 (and in particular 475-477) (Dhand *et al*., 1994, Fu *et al*., 2004). Important residues for p110-binding in helix-2 (adjacent to the binding site on helix-1) are 525-534 (Fu *et al*., 2004).

Truncation of p85α at residue 571 removes part of helix-2 from the iSH2 domain and the whole of cSH2, but leaves the p110-binding site intact. Consequently, this mutant leads to constitutive activation of the resulting p85α:p110α heterodimer (Jimenez *et al*., 1998). It is not absolutely clear why this mutant (unlike full-length p85α) is unable to repress PI3K function. However, one possibility is that deletion of the auto-inhibitory phosphorylation site at Ser608 (in the "tail" of iSH2) leads to the de-regulated phenotype (Carpenter *et al*., 1993b, Foukas *et al*., 2004). Alternatively, specific inter- or intra- subunit interactions that repress p110 activity might be lost (Chan *et al*., 2002, Shekar *et al*., 2005). For example, evidence suggests that the nSH2 domain of p85α is normally in close contact with residues 581-593 of iSH2, a conformation which may present nSH2 in such a way as to inhibit p110 activity (Shekar *et al*., 2005). Indeed, whilst this thesis was in preparation, the x-ray crystallographic structure of the p85α-iSH2:p110α-ABD complex was solved (Miled *et al*., 2007). The authors of this paper (Miled *et al*., 2007) were able to propose a model whereby kinase activity is negatively regulated by a charge-charge interaction between the p85 nSH2 domain and the p110 helical domain, an interaction which would be disrupted upon competitive phosphopeptide binding to nSH2, thus leading to increased PI3K activity (Miled *et al*., 2007) (**Fig. 4.3A**).

#### *4.1.2.3 Possible model for PI3K activation by NS1.*

It is intriguing to find that the binding of influenza A virus NS1 protein to the p85β iSH2 domain requires residues equivalent to those lost in the constitutively active

**Original image can be found at the reference given below.**

**Alternatively, e-mail bgh1@st-andrews.ac.uk for a full copy of this thesis, including all figures.**

**Fig. 4.3.** (**A**) **Model of p110 lipid kinase regulation by p85.** p110 binds the inter-SH2 (coiled-coil) domain of p85. Charge-charge contacts between the p85-nSH2 domain and the p110 helical domain maintain p110 in an inactive state. Activation of PI3K results from a phospho-Y*XX*M motif in an activated receptor out-competing p110 for binding the p85-nSH2 domain, thus relieving p85-mediated inhibition of p110. (**B**) **Proposed model of PI3K activation by NS1.** NS1 binds the C-terminal region of the p85β inter-SH2 domain, but does not displace p110. The binding of NS1 to this region may disrupt the regulatory charge-charge contact between p85β–nSH2 and p110, resulting in a receptor-independent increase in PI3K activity. *Images modified from Miled et al., 2007.*

p85α mutant (i.e. part of helix-2 and the acidic "tail") (**Fig. 4.4**). Thus, these data are suggestive of a novel mode-of-action whereby NS1 interacts with and activates heterodimeric PI3K by potentially masking the p110-inhibitory function of the p85β iSH2 domain. Currently the mechanistic details can only be speculated upon: NS1 may modify inter- and intra- molecular contacts within the PI3K heterodimer, displace an unknown repressive element, or recruit additional cellular co-stimulatory factors to the complex (such as IRS-4).

Given the model recently proposed by Miled *et al*. (Miled *et al*., 2007), it is tempting to think that NS1 simply displaces nSH2 from the p110 helical domain (**Fig. 4.3B**). Such a mode-of-action would directly target mechanical aspects of PI3K regulation, and would be an efficient way to activate the kinase, as the multiple inputs that normally regulate p85:p110 function would be short-circuited (Carpenter *et al*., 1993a, Cuevas *et al*., 2001, Foukas *et al*., 2004, Gout *et al*., 2000, Rordorf-Nikolic *et al*., 1995, Tolias *et al*., 1995, Zheng *et al*., 1994). For example, stimulation of PI3K by NS1 during virus infection would be independent of phosphopeptide binding, thus ensuring that signalling was not linked to host activity and could occur even if the infected cells were quiescent (when normal receptor signalling might be low). Additionally, NS1 might also mask the inhibitory Ser608 autophosphorylation site, and in this way circumvent any automatic negative feedback by p110 (Foukas *et al*., 2004). It is noteworthy that analogous host-cell or viral proteins that regulate PI3K activity by binding to the iSH2 domain of p85β (or even p85α) have yet to be identified.

The *in vitro* binding studies presented here are unable to account for the possibility that other regions of either full-length NS1 or full-length p85β may play some minor

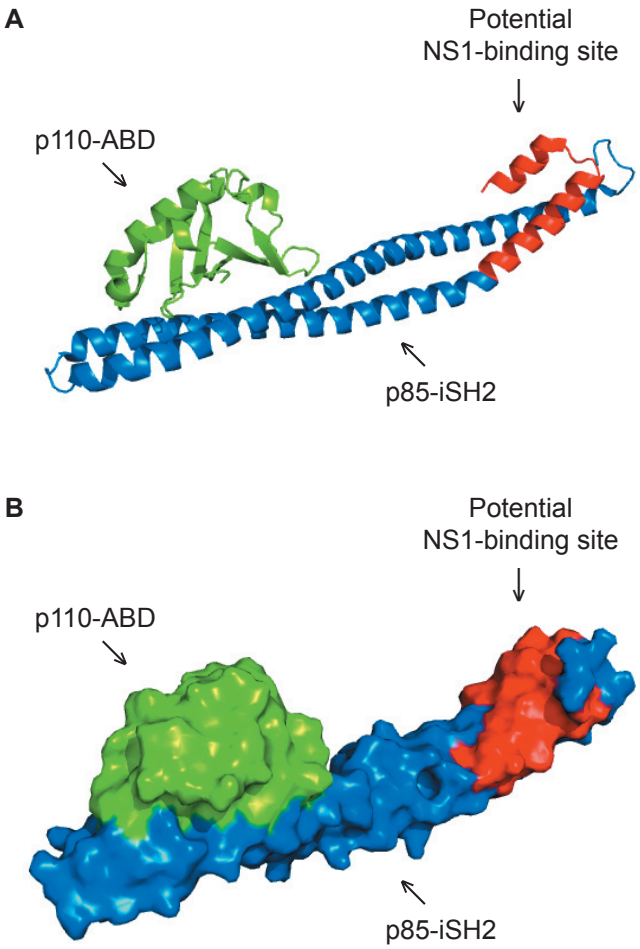


**Fig. 4.4. Structure of the p85-iSH2 domain in complex with the p110-ABD.** Cartoon (**A**), and surface (**B**) representations of the p85-iSH2 domain (coiled-coil; blue) bound to p110-ABD (green). Full-length p110 is predicted to extend over the whole length of iSH2. Residues that are absent in the truncated p85-iSH2 construct are highlighted in red. These make up the putative NS1-binding site. It should be noted that both the nSH2 and cSH2 domains are located at this end of iSH2, and thus may form secondary contacts with NS1. Charge-charge contacts between nSH2 and the p110 helical domain are thought to normally negatively-regulate PI3K activity. *Images were prepared using MacPyMol (Protein Data Bank file: 2V1Y).*

role in the functional interaction *in vivo*. For example, the RNA-binding domain of NS1 is not absolutely essential for complex formation, but residues within this domain could contribute to NS1:p85β stability and/or the activation of PI3K. Indeed, Ehrhardt *et al*. noted that transient expression of a WSN/NS1 construct containing amino-acid substitutions at arginine-38 and lysine-41 (which abrogate the binding of NS1 to RNA) induced less Akt phosphorylation than the wild-type construct (Ehrhardt *et al*., 2007a). However, interpretation of such data is complicated by a report that mutation of arginine-38 may cause mislocalisation of WSN/NS1 in infected cells (Min & Krug, 2006). Additional work is therefore required to investigate any potential role for the NS1 RNA-binding domain with respect to PI3K stimulation.

The data presented here also appear to contradict a recent publication in which the co-precipitation of NS1 with the SH3 and cSH2 domains of p85 [isoform not reported] is noted (Shin *et al*., 2007a). Although such interactions were not identified under the experimental conditions described here, it is possible that the binding of NS1 to p85β is relatively complex, and dynamic interplay between NS1 and multiple domains of p85β occurs *in vivo*. Indeed, the proposed positioning of NS1 with respect to the iSH2 domain (**Figs. 4.3B & 4.4**) would potentially bring it into close contact with the SH3 and cSH2 domains of p85β, which are likely to be distal from the p110-binding site on iSH2 (**Fig. 4.4**). Thus, the observed co-precipitation of NS1 with these two domains by Shin *et al.* (Shin *et al.*, 2007a) may yet be compatible with this model. Indeed, it is clear that mutation of a single polyproline motif (residues 164-167 [P*XX*P]; putative p85 SH3-binding motif) in NS1 completely abrogates the ability of NS1 to interact with p85β and activate PI3K (Shin *et al.*, *in press*). However,

mutation of proline residues can severely disrupt protein structure, and the specificity of these amino-acid substitutions for NS1 function has yet to be demonstrated.

The model proposed here also does not yet take into account the recent unpublished finding that NS1 proteins of some avian influenza A virus strains display enhanced PI3K-activating abilities, which may be due to their direct interaction with the SH3 domains of cellular Crk/CrkL proteins (Heikkinen *et al*., 2007). Work to refine this model and define the possible role of Crk/CrkL is currently underway.

## 4.2 Consequences of PI3K activation for virus replication.

The biological consequences of NS1 binding and activating PI3K were not fully tackled as part of this thesis, and still remain to be fully elucidated. However, from the preliminary work done it is clear that PI3K activity is important for multiple stages of efficient influenza A virus replication.

### *4.2.1 PI3K and influenza A virus entry into cells.*

Chemical inhibition of PI3K (using wortmannin or LY294002) specifically reduced the infectious titres of PR8 in MDCK cells (**Fig. 3.10**). This is in agreement with a previous study performed by Ehrhardt and colleagues, who also observed that inhibition of PI3K activity lead to a decrease in the growth of influenza A virus (Ehrhardt *et al*., 2006). Interestingly, a decrease was only noted if PI3K was inhibited prior to 4h p.i., after which PI3K inhibition had no effect on virus yield in single-step growth analyses (Ehrhardt *et al*., 2006). As determined by immunofluorescence studies, the block on virus replication appears to be at the level of early virus uptake into cells (Ehrhardt *et al*., 2006). Such a role for PI3K is likely to be independent of Akt activity, as transient expression of a kinase-dead, dominant-negative form of Akt

was unable to suppress the amount of infectious influenza A virus generated (Ehrhardt *et al*., 2006).

It is unlikely that NS1-activated PI3K is responsible for promoting virus uptake, as the NS1 protein is non-structural and is thus only produced subsequent to RNP delivery into the nucleus. Indeed, Ehrhardt and colleagues noted that PI3K activation (as determined by Akt phosphorylation) occurs in a bivalent manner during infection, with an initial weak "peak" at 30mins-1h p.i., followed by a much greater and sustained "peak" beginning ~4h p.i. (Ehrhardt *et al*., 2006, Ehrhardt *et al*., 2007b). The early activation of PI3K could be due to virus adsorption, and might be responsible for the noted role of PI3K in viral entry processes.

Interestingly, this specific early requirement for PI3K activation may be conserved among other members of the *Orthomyxoviridae* family, as weak transient Akt phosphorylation at 30mins-1h p.i. has also been observed during influenza B virus infection of tissue-culture cells (Ehrhardt *et al*., 2007b). In a situation similar to influenza A virus, PI3K inhibitors also reduce the infectious titres of influenza B virus, but only when treatment is applied up to ~4h p.i. (Ehrhardt *et al*., 2007b).

### *4.2.2 PI3K and influenza A virus replication.*

The induction of Akt phosphorylation beginning at ~4h p.i. during influenza A virus infections is likely due to activation of PI3K by the NS1 protein. In this study, to dissect the direct contribution of NS1-activated PI3K with regards virus replication, recombinant influenza A viruses expressing NS1 proteins specifically unable to activate PI3K (NS1-Y89F) were generated. These viruses are convenient tools, given that they can be used to circumvent the problem of LY294002 and wortmannin blocking influenza A virus entry into cells, as well as other "off-target" effects of

these compounds. Surprisingly, characterisation of these viruses in MDCK cells revealed a strain-specific requirement for NS1-mediated PI3K activation. Whereas rUd NS1-Y89F exhibited a small plaque phenotype and grew to titres lower than WT rUd virus, rWSN NS1-Y89F did not exhibit an altered phenotype as compared with WT rWSN. The reasons for this remain unclear, but given that Tyr89 of NS1 is totally conserved (and if its sole function is to promote the interaction of NS1 with p85β), it seems probable that the activation of PI3K is necessary for all natural non-laboratory adapted strains of influenza A virus. Indeed, the need to activate PI3K may be a requirement primarily for efficient virus replication and spread *in vivo*, as such a function appears to enhance the pathogenicity of WSN in mice.

Recent unpublished work by Dr. Dave Jackson (Northwestern University, U.S.A.) suggests that NS1-mediated PI3K activation may be required to counter another function encoded by the influenza A virus NS segment. Recombinant Ud viruses were generated that contained either the WT WSN NS segment, or the WSN NS segment that expresses NS1-Y89F. Initial characterisation of these rUd/WSN viruses in MDCK cells revealed that both viruses replicate to the same titres in single-cycle and multi-cycle growth analyses (Dr. Dave Jackson, *unpublished observations*). Thus, the inability of WSN NS1-Y89F to activate PI3K does not attenuate virus replication in a Ud background. This observation may indicate that PI3K activation is required by Ud virus in order to counter a putative effect encoded by its virus-specific NS segment. In contrast, the ability to carry out such a "putative function" in MDCK cells may have been lost by the WSN NS segment, possibly as a result of its adaptation to the mouse host. Despite this, it would appear that activation of PI3K by the WSN/NS1 protein has been retained, which may still be required for WSN to oppose the "putative function" in murine cells, or *in vivo*. Clearly, further work is required to determine if

NS1-mediated PI3K activity counters a host-species and/or strain specific function of the NS segment. It is not without precedent for an influenza A virus NS segment derived from one host species to encode products that function inefficiently in another host (Basler *et al*. 2001, Treanor *et al*., 1989).

It is worth noting that there are 6 amino-acid differences (2 conservative) between the NEP proteins of Ud and WSN, and 26 amino-acid differences (12 conservative) between the NS1 proteins of Ud and WSN. In addition, the NS1 protein of Ud has a 7 amino-acid extension at its C-terminus. A recently described phenotypic difference between the Ud and WSN NS1 proteins has been attributed to some of these differences; namely, their nuclear localisation within both infected and transfected human tissue-culture cells (Melen *et al*., 2007). Thus, whilst Ud/NS1 is distributed throughout the nucleus, WSN/NS1 appears to be excluded from intra-nuclear regions likely to be nucleoli (Melen *et al*., 2007). Furthermore, given the striking difference with regards WSN/NS1 localisation during infection of MDCK cells (nuclear) and primary murine epithelial cells (cytoplasmic) [Newby *et al*., 2007], it is possible that this protein displays cell-type and/or species specific functions. These data are not incompatible with the notion that WSN/NS1 has lost a function in certain cell-types that requires counter-balance by PI3K signalling. It will be intriguing to determine whether a specific nucleolar function of Ud/NS1 is countered by NS1-activated PI3K.

### *4.2.3 PI3K and influenza A virus-induced apoptosis.*

The PI3K/Akt signalling pathway is commonly activated by viruses in order to limit apoptosis during infection (see **Introduction**). Thus, it was not surprising to find that the rUd NS1-Y89F virus induced the cleavage of PARP slightly faster than WT rUd in HEp2 cells. However, given the diverse array of PI3K-regulated physiological

processes [reviewed in (Manning & Cantley, 2007, Vanhaesebroeck *et al.*, 2005)], it is possible that other apoptosis-independent, cell-type specific consequences of NS1-mediated PI3K activation exist. Nevertheless, other groups have confirmed the observation made here that markers of apoptosis are more prominent during influenza A virus infections when PI3K/Akt signalling is blocked (Ehrhardt *et al*., 2007a, Shin *et al*., *in press*, Zhirnov & Klenk, 2007). Thus, a functional model has been proposed whereby NS1-activated PI3K causes the phosphorylation and activation of Akt, which further leads to the phosphorylation and inactivation of pro-apoptotic GSK-3β, and the inhibition of several pro-caspase cleavage events (Ehrhardt *et al*., 2007a).

Interestingly, given the influenza A virus-induced kinetics of Akt phosphorylation in MDCK cells (i.e. increasing to peak at ~8h p.i. before declining; Zhirnov & Klenk, 2007), it may be that NS1 only activates PI3K in order to limit virus-induced cell-death at early/mid times post-infection (Zhirnov & Klenk, 2007). Indeed, the apparent dephosphorylation of Akt observed after 8h p.i. correlates well with increased activation of p53 signalling and the induction of apoptosis (Zhirnov & Klenk, 2007). It is not entirely clear what is responsible for causing the dephosphorylation of Akt during infection, but data from this work indicate that negative-regulation of PI3K signalling by PTEN does not play a major role. Although dephosphorylation of Akt was not observed in infected 1321N1 cells, a host-cell factor need not be responsible, as it has not yet been determined if influenza A viruses replicate productively in these cells. Thus, specific expression of another viral protein later in infection may contribute in some way to dephosphorylation of Akt. Alternatively, non-specific build-up of all viral proteins at later times post-infection may overwhelm the cell, and induce an apoptotic stress response which begins with the cell-mediated "switching-off" of PI3K/Akt signalling.

An intriguing observation is that an engineered influenza A virus unable to express NS1 (PR8delNS1) not only fails to induce Akt phosphorylation during infection, but also fails to activate p53 signalling (Zhirnov & Klenk, 2007). This is surprising given that p53 has been proposed to have roles in influenza A virus-induced IFN responses and/or cell-death (Turpin *et al*., 2005), both processes that are likely to be significantly increased upon infection with PR8delNS1 (Egorov *et al*., 1998, Garcia-Sastre *et al*., 1998, Talon *et al*., 2000a, Wang *et al*., 2000, Zhirnov *et al*., 2002a). As PI3K/Akt activation efficiently down-regulates p53 activity [reviewed in (Manning & Cantley, 2007)], it might have been expected that p53 signalling would be increased (and occur earlier) during infection with a virus unable to activate PI3K. However, the inability of PR8delNS1 (unlike WT PR8) to stabilise p53 by inducing its Ser15 phosphorylation (Zhirnov & Klenk, 2007) suggests that NS1 has a direct effect on p53 activity. Indeed, previous work has hinted at an interaction between NS1 and p53 during virus infection (Luke *et al*., 2005).

One hypothesis to account for these observations is that NS1 may act as a "dynamic switch" in cell survival and cell-death signalling. Thus, at early times post-infection NS1 binds and activates PI3K, thus limiting virus-induced apoptosis by suppression of (among other things) p53 signalling. Later, NS1 may dissociate from PI3K (or this complex becomes inactive), and an alternative NS1 population interacts with and activates p53, leading to defined cell-death. This could account for the apparently conflicting data on the role of NS1 for apoptosis induction (Schultz-Cherry *et al*., 2001, Zhirnov *et al*., 2002a). This hypothesis does not exclude the possibility that p53 activation subsequent to attenuation of NS1-mediated PI3K signalling occurs indirectly via the potentially toxic effects of NS1 limiting host-cell mRNA processing. Work is currently underway to determine if NS1 remains complexed with

p85β late in infection, and if NS1 thereby constantly activates PI3K. It will also be of interest to determine the kinetics of NS1 interacting with and modulating p53 activity.

### *4.2.4 PI3K and other stages of the virus replication cycle?*

#### *4.2.4.1 NS1-activated PI3K and cytokine responses.*

It is of note that NS1 has recently been shown to limit the induction of numerous specific transcriptional events associated with the maturation of human dendritic cells (DCs) in response to virus infection (Fernandez-Sesma *et al*., 2006). In particular, expression of IL-12 p35 (a subunit of the proinflammatory and immunoregulatory cytokine IL-12) was found to be inhibited in DCs infected with recombinant heterologous viruses expressing NS1 (Fernandez-Sesma *et al*., 2006). IL-12 is a key regulator of host innate and adaptive immune responses during infection: it enhances the cytolytic activity of natural killer cells and $CD8^+$ T-cells, and contributes to the optimal production of IFNγ (Trinchieri, 1995). As IL-12 synthesis by DCs has previously been shown to be negatively controlled by PI3K signalling (Fukao *et al*., 2002), it will be of great interest to determine if NS1-mediated PI3K activation is responsible for down-regulating IL-12 production during influenza A virus infection of DCs. Likewise, the effect of NS1-activated PI3K for TLR-mediated innate-immune responses may be worth investigating.

#### *4.2.4.2 NS1-activated PI3K and mRNA translation.*

For some viruses (particularly HCV), PI3K activation has been linked not only to reducing apoptosis, but also to enhancing translation of viral mRNAs by upregulating mTORC1 (Mannova & Beretta, 2005). As stated in the **Introduction**, activation of mTORC1 inhibits 4E-BP1 by direct phosphorylation, which subsequently leads to the activation of eIF4E, and the promotion of cap-dependent mRNA translation (Mamane

*et al*., 2004). Although NS1 has been shown to play a role in enhancing influenza A virus translation, this is likely due to its interactions with the cellular proteins eIF4GI and PABI (Aragon *et al*., 2000, Burgui *et al*., 2003), rather than the activation of PI3K. Indeed, a recent study has shown that influenza A virus mRNA translation is largely independent of eIF4E activity, and is functionally substituted by the virus-encoded polymerase complex (Burgui *et al*., *in press*). Viral mRNA translation occurred normally in cells treated with the general mTOR inhibitor, rapamycin, and in cells expressing eIF4E shRNA (Burgui *et al*., *in press*). Additionally, transfection of a dominant-negative, non-phosphorylatable mutant of 4E-BP1 had no effect on influenza A virus replication (Burgui *et al*., *in press*). Thus, if NS1 recruits the mTOR/4E-BP1 arm of PI3K signalling during virus infection, it is not likely to be essential for efficient mRNA translation, and may have an as yet unidentified function.

#### *4.2.4.3 NS1-activated PI3K and ENaC function.*

It should be stressed that PtdIns(3,4,5)$P_3$ generated by class IA PI3K can recruit and activate ~20 different PH domain-containing substrates, many of which may be activated in a cell-type specific manner (Krugmann *et al*., 2002). Although Akt is very well characterised, activation of PI3K by NS1 may result in downstream signalling to a number of other Akt-independent effectors. As an example, preliminary unpublished work done in collaboration with Michael Gallacher and Dr. Stuart Wilson (University of Dundee, U.K.) has shown that NS1-mediated PI3K may also induce the activation of SGK1, a serum- and glucocorticoid- induced kinase that is directly activated downstream of PI3K-PDK1. Furthermore, in polarised H441 human epithelial airway cells, transient expression of wild-type PR8/NS1, but not PR8/NS1-Y89F, caused the activation of epithelial sodium channels (ENaCs), a

process which (*in vivo*) would result in the diffusion of water from the apical (air) surface of the lung into the bloodstream (Michael Gallacher, *personal communication*). SGK1 activity is known to increase ENaC function in at least two ways: overall stimulation of ENaC mRNA transcription; and phosphorylation and inhibition of Nedd4-2, an E3-ligase that would otherwise cause the ubiquitination and proteasome-mediated degradation of ENaC [reviewed in (Lang *et al*., 2006)]. Further work to characterise a role for NS1-activated ENaC in influenza A virus infections is currently under way.

## 4.3 Outlook.

### *4.3.1 PI3K as a novel anti-influenza drug target.*

Antivirals may provide an important initial defence against rapidly emerging and antigenically novel strains of influenza A virus (De Clercq, 2006). Targeting virus-activated signalling cascades (such as PI3K) could be an attractive strategy for anti-influenza drugs (De Clercq, 2006). As shown here, PI3K inhibition (using LY294002 or wortmannin) greatly reduces the yield of influenza A virus under tissue-culture conditions (**Fig. 3.10B**). Various PI3K inhibitors are already under investigation as potential long-term therapies for cancer and chronic inflammatory disorders, thus it may be possible to develop similar compounds for use as short-term antivirals.

The advantage of targeting a host-cell pathway is that treatments may be less susceptible to virus mutation and drug resistance. However, unknown toxic side-effects on uninfected tissues could be cause for concern, and inhibitors such as wortmannin and LY294002 clearly affect multiple essential cellular events [see **Introduction**; (Gharbi *et al*., 2007, Sarkar *et al*., 2004)]. An alternative strategy would be to specifically inhibit the activation of PI3K by NS1. However, as this is a

virus-specific target there is potential for the development of drug resistance. Nevertheless, this approach is very attractive as Tyr89 of NS1 appears wholly conserved among human and avian influenza A viruses, thus compounds targeting this virus-host interaction may be effective against many influenza A virus strains.

### *4.3.2 PI3K and live-attenuated influenza vaccines.*

The potential use of recombinant influenza A viruses with truncated/mutated NS1 proteins as live-attenuated vaccines has been demonstrated (Ferko *et al*., 2004, Richt *et al*., 2006, Talon *et al*., 2000b). Such viruses are partially debilitated in their ability to counteract the host IFN-response, yet can grow efficiently to high titres in suitable IFN-deficient systems, such as Vero cells or embryonated chicken eggs (<6 days old) (Talon *et al*., 2000b). However, as the viruses are replication competent in the host (unlike delNS1 viruses, which are overwhelmed by innate immunity), they can be administered intranasally. Together, these properties mean NS1-truncated viruses elicit antibody, cell-mediated, and mucosal protective immune-responses (Ferko *et al*., 2004, Talon *et al*., 2000b).

#### *4.3.2.1 Virus design.*

As another strategy to generate live-attenuated influenza vaccines, residues of NS1 involved in PI3K activation could be targeted (together with residues involved in IFN-antagonism or other functions) to generate mutant viruses expressing full-length "designer" NS1 molecules. Viruses could thus be specifically constructed in such a way as to possess the optimal balance of replication ability vs. *in vivo* attenuation/immunogenicity. Although, influenza A viruses with specific point mutations are likely to be unstable and prone to genotypic/phenotypic reversion (Falcon *et al*., 2005, Schickli *et al*., 2001), it may be advantageous to have a vaccine

virus possessing a full-length NS segment in which NS1 function is finely "tuned". Despite this, the use of NS1-truncated viruses as potential vaccines has most commonly been evaluated, and no comparison between NS1-truncated and NS1-designer viruses has yet been done. The obvious benefit of NS1-truncated viruses is that they are likely to display increased genetic stability, a property ascribed to the multiple stop codons and/or large nucleotide deletions/insertions they contain (Ferko *et al*., 2004, Quinlivan *et al*., 2005, Richt *et al*., 2006, Schickli *et al*., 2001, Talon *et al*., 2000b).

#### *4.3.2.2 Cell-line design.*

Propagation of live-attenuated influenza A viruses in 6-day old embryonated chicken eggs for 2 days may not yield industrial quantities of vaccine. Thus, cell-based production of NS1-truncated mutants in cell-lines with phenotypes similar to Vero cells (i.e. lacking IFNα/β genes) may be more commercially viable. With this in mind, it is interesting to note that influenza A viruses lacking NS1 expression are attenuated ~10-100-fold in Vero cells as compared with wild-type viruses (Garcia-Sastre *et al*., 1998, Kochs *et al*., 2007b), demonstrating that NS1 has functions independent of classical IFN-antagonism. As shown in this thesis, one such additional function of NS1 is PI3K activation. Indeed, most NS1-truncated viruses used to date have large deletions in the C-terminal effector domain of NS1, which is essential for binding p85β. Additionally, such viruses have been shown to be poor inducers of Akt phosphorylation (Ehrhardt *et al*., 2007a). Thus, if Vero cells could be engineered to have constitutively active PI3K signalling, it may be that this cell-line would augment the growth characteristics of NS1-truncated influenza A viruses. This approach could of course be extended to any cell-line (or identified function of NS1) with the aim of increasing live-attenuated influenza A virus vaccine yields.

### *4.3.3 PI3K and influenza A virus as an oncolytic therapy.*

Previously, PR8delNS1 has been shown to replicate efficiently and cause cell-death in cells expressing high levels of oncogenic Ras, a known enhancer of PI3K function (Bergmann *et al*., 2001). Conversely, growth of PR8delNS1 is restricted in non-malignant cells. This suggested that PR8delNS1 can act as a conditionally replicating oncolytic virus in cell-lines with active Ras (Bergmann *et al*., 2001). The reason for this was originally ascribed to the ability of Ras to down-regulate PKR activity (Bergmann *et al*., 2001, Mundschau & Faller, 1992), which might otherwise inhibit PR8delNS1 replication. However, it is also possible that PR8delNS1 virus was able to replicate efficiently in Ras-overexpressing cells due to aberrant PI3K activation. The ability to activate PI3K would be lacking in PR8delNS1, but could be complemented by Ras acting directly on p110 (**Fig. 1.15**). Thus, given that deregulation of the PI3K pathway is commonly associated with numerous human cancers [reviewed in (Nicholson & Anderson, 2002)], it may be that influenza A viruses expressing NS1 truncations/mutants could be developed to specifically target tumour cells with constitutively active PI3K.

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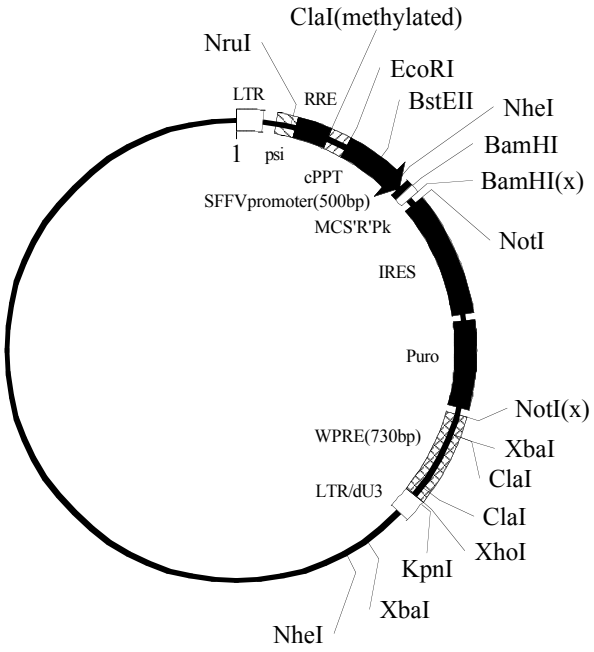
# Appendices

## A.1 Plasmid map for the pdl'MCS(C'V5) vector:




- Plasmid generated by Dr. Yun-Hsiang Chen, University of St. Andrews, U.K.
- ~10710bp in size.
- Ampicillin resistance.
- Multiple cloning site (MCS):

  5'-ggatct*gcggccgc*at*accggt*gc*actagt*cg*acgcgt*ga*catatg*ttcc*ggatcc*-V5-3'

  ·····*Not*I·····*Age*I·····*Spe*I·····*Mlu*I·····*Nde*I·····*Bam*HI·····

- *Not*I and *Age*I (underlined) are not unique.
- *Spe*I, *Mlu*I, *Nde*I and *Bam*HI are recommended cloning sites.
- V5 epitope sequence (immediately downstream of MCS):

  ·····**G···K··P···I···P···N···P··L··L·G···L··D··S··T···*···*···*·····

  5'-MCS-*ggatcc*ggaaagccgatcccaaaccctctattaggtctggactccacctaatagtgagatcc-3'

  ·····*Bam*HI·····

  The 5' *Bam*HI site shown above (V5 sequence) is also shown at the 3' end of the MCS.

## A.2 Publications:

**Hale, B.G.**, *et al*. (2006). Influenza A virus NS1 protein binds p85β and activates phosphatidylinositol-3-kinase signaling. *Proc Natl Acad Sci U S A* **103**, 14194-9.

**Hale, B.G.** & Randall, R.E. (2007). PI3K signalling during influenza A virus infections. *Biochem Soc Trans* **35**, 186-7.

**Hale, B.G.** *et al*. (2008). Binding of influenza A virus NS1 protein to the inter-SH2 domain of p85β suggests a novel mechanism for PI3K activation. *J Biol Chem* **E-pub**.

## A.3 Presentations:

"*Influenza A virus NS1 protein binds p85β and activates PI3K/Akt signalling*" – **3rd Focused Meeting on PI3K Signalling and Disease** (Bath, U.K., Nov. 2006).

"*Activation of the PI3K/Akt signalling pathway during influenza A virus infection*" – **12th Annual Glasgow Virology Workshop** (Glasgow, U.K., Feb. 2007).

"*Activation of PI3K by the NS1 protein of influenza A virus*" – **160th Society for General Microbiology Meeting** (Manchester, U.K., March 2007).

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