Role of ubiquitin-like modification during adenovirus infection

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DECLARATION

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ABSTRACT

SUMO-1 is a small ubiquitin like protein that can be covalently linked to lysine residues in target substrates with a range of biological consequences. Following viral infection, various cellular mechanisms are subverted. In particular, it has been demonstrated that in adenovirus infected cells SUMO-1 conjugated proteins accumulate. This is exemplified by the cellular substrate RanGAP where an increase in the SUMO-1 conjugated form of the protein is detected. In addition it has been shown that two adenovirus gene products, E1B-55kDa and protein V, undergo SUMO-1 conjugation. Indeed, from in vivo and in vitro experiments, it appears that E1B-55kDa is mono-SUMO-1 modified, while protein V seems to be a substrate for multiple SUMO-1 conjugation. Immunofluorescence studies have indicated that there are sites in the nucleus where E1B-55kDa and SUMO-1, and protein V and SUMO-1, colocalise. To establish the basis for the observed changes in SUMO-1 modification the metabolism and cellular localisation of the enzymes involved in SUMO-1 conjugation were examined. While the total amount of SUMO-1 activating enzyme was not altered by adenovirus infection it was noted that a number of higher molecular weight species of the protein were induced by the virus. However the cellular localisation of the protein was not altered by adenovirus infection. Western blotting indicated that Ubch9 was not modified, but after infection the cellular localisation of the protein was altered from diffuse nuclear to a highly punctuate distribution. These changes suggest that adenovirus infection alters the activity and/or cellular localisation of the enzymes involved in SUMO-1 conjugation. It was also noted that adenovirus infection induced activation of the transcription factor NF-kB. This appeared to result

from a dramatic increase in the ubiquitination and turnover of the IkBα inhibitor protein. After adenovirus induced nuclear translocation, NF-kB was sequestered into discrete subnuclear sites, which may alter the activity of the protein. Together these data indicate that ubiquitin-like protein modifications are important cellular functions that are targeted by viral activities after infection.

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I am indebted to my supervisor, Prof. R. T. Hay, for giving me this opportunity, for his advice and for his important part in my vocational training. I have been so fortunate in finding a supervisor who trusted me and gave me such unfailing help and encouragement.

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ABBREVIATION

Ad2	Adenovirus type 2
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
bp	Base pairs
BSA	Bovine serum albumin
CMV	Cytomegalovirus
D-MEM	Dulbecco s modified essential medium
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
DUB	Deubiquitination enzymes
E. coli	*Eschericia coli*
E1	Ubiquitin activating enzyme
E2	Ubiquitin conjugating enzyme
E3	Ubiquitin ligase
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetracetic acid
FCS	Foetal calf serum
GST	Glutathione S-transferase
HA	Haemagglutinin
HCl	Hydrochloric acid
HSV	Herpes symplex virus

Ig	Immunoglobulin
IkB	Inhibitor kappa B
IPTG	Isopropyl-b-D-thiogalactopyranoside
IVTT	*In vitro* transcription translated
KCl	Potassium chloride
KDa	Kilo Dalton
LB	Luria broth
m.o.i	multiplicity of infection
MW	Molecular weight
NF-kB	Nuclear factor kappa B
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PVDF	Polyvinylidene difluoride
RNA	Ribonucleic acid
SAE1	SUMO-1 activating enzyme subunit 1
SAE2	SUMO-1 activating enzyme subunit 2
SCF	Skp1-Cdc53/ Cul1-F-box protein E3 complex
SDS	Sodium dodecyl sulphate
SSPs	SUMO-1 specific proteases
SUMO-1	Small ubiquitin modifier
TNFa	Tumor necrosis factor alpha

Tris	2-amino-2-(hydroxymethyl)propane-1,3-diol
Ubch9	SUMO-1 conjugating enzyme
UCH	Ubiquitin C-terminal hydrolase
UV	Ultra violet

1. INTRODUCTION

1.1 Adenoviridae Family

The Adenoviridae family is divided into two genera depending on the natural host of the virus: Adenoviruses and Mastadenoviruses.

Human adenoviruses were first isolated as distinct viral agent in 1953 (Rowe et al., 1953). So far, 47 human adenoviruses serotypes have been identified and all of these viruses contain a linear double-stranded DNA genome encapsidated in an icosahedral protein shell. The most common targets of human adenoviruses are the terminally differentiated epithelial cells of the bronchial tract. It has been found that adenoviruses are responsible of a large number of acute febrile respiratory syndromes among military recruits, only of a small proportion of acute respiratory disease in the general population and about 5-10% of respiratory illness in children. Besides respiratory disease, adenoviruses cause epidemic conjuntivites (Jawetz, 1959) and they have been associated with the infection of the gastro-intestinal tracts.

In 1962 it was shown for the first time that a human virus, adenovirus type 12, was able to induce oncogenesis in rodents (Trentin et al., 1962), but no evidence has been reported for the involvement of adenovirus in Human tumors. Nevertheless, the ability to induce tumors in animals and to transform cells in culture are peculiarities that make adenovirus an important model for cancer studies.

1.2 Replicative cycle

As with all other viruses, the adenovirus genome only has a limited coding capacity and must therefore parasitize components of the host-cell synthetic machinery for the synthesis of virus specific macromolecules.

Adenoviruses are internalised via receptor mediated endocytosis, where the head domain of the fibre protein interacts with high affinity with specific receptors on the cellular membrane (Bergelson et al., 1999; Bergelson et al., 1997; Louis et al., 1994; Philipson et al., 1968; Svensson, 1985; Xia et al., 1995). In particular, two glycoproteins of 40 and 42 KDa have been identified as the major components of the receptor site for adenovirus (Defer et al., 1990; Svensson et al., 1981). Moreover the penton base proteins binds to specific members of a family of heterodimeric cell surface receptors, named integrins (Wickham et al., 1993). During the course of internalisation viruses are subject to several sequential uncoating steps and this allows them to translocate from the cellular to the nuclear membrane (Greber et al., 1993; Svensson and Persson, 1984). At this point the uncoating process is completed by releasing the viral DNA into the nucleus where transcription can then begin.

The replication cycle is divided into two phases separated by the onset of viral DNA replication: early and late events. Early events are those triggered as soon as the virus interacts with the host cell and they include absorption, penetration, transcription and translation of an early set of genes.

1.3 Adenovirus transcription: early viral genes

There are three main goals for early adenovirus gene expression and they are to induce the host cell to enter the S phase of the cell cycle (providing optimal environmental for viral replication), to set up viral systems that antagonise a variety of host antiviral defences, and to synthesise viral gene products that are essential for viral DNA replication.

The first transcription units to be expressed after the infection are the immediate early (E1A), delayed early (E1B, E2A, E2B, E3 and E4), and intermediate genes (IVa2 and IX), whereas the maximum level of late gene expression (L1 to L5) is revealed at 18 hours post infection (Lucas and Ginsberg, 1971) (Fig.1).

E1A is the first viral transcription unit to be translated after the viral DNA reaches the nucleus. This region codes for five mRNA species generated by alternative splicing of which only two are expressed during the early stage of the infection: E1A13s and E1A12s. The E1A proteins activate transcription of all viral early genes, are responsible of the activation of the MLP (major late promoter) and they bind to certain cellular proteins and modify their functions.

One of the cellular targets of E1 gene products is the product of the retinoblastoma tumor suppressor gene, pRB. pRB is a negative regulator of cell proliferation, and its inactivation has been shown to be an important step in the development of many human cancers. In transformed cells constitutively expressing E1A proteins, the E1A products bind to and inactivate pRB. This functionally mimics the loss of pRB often seen in human tumors. pRB forms a complex with the transcriptional factor E2F which results in the inhibition of E2F mediated transactivation. Most of the genes controlled by E2F

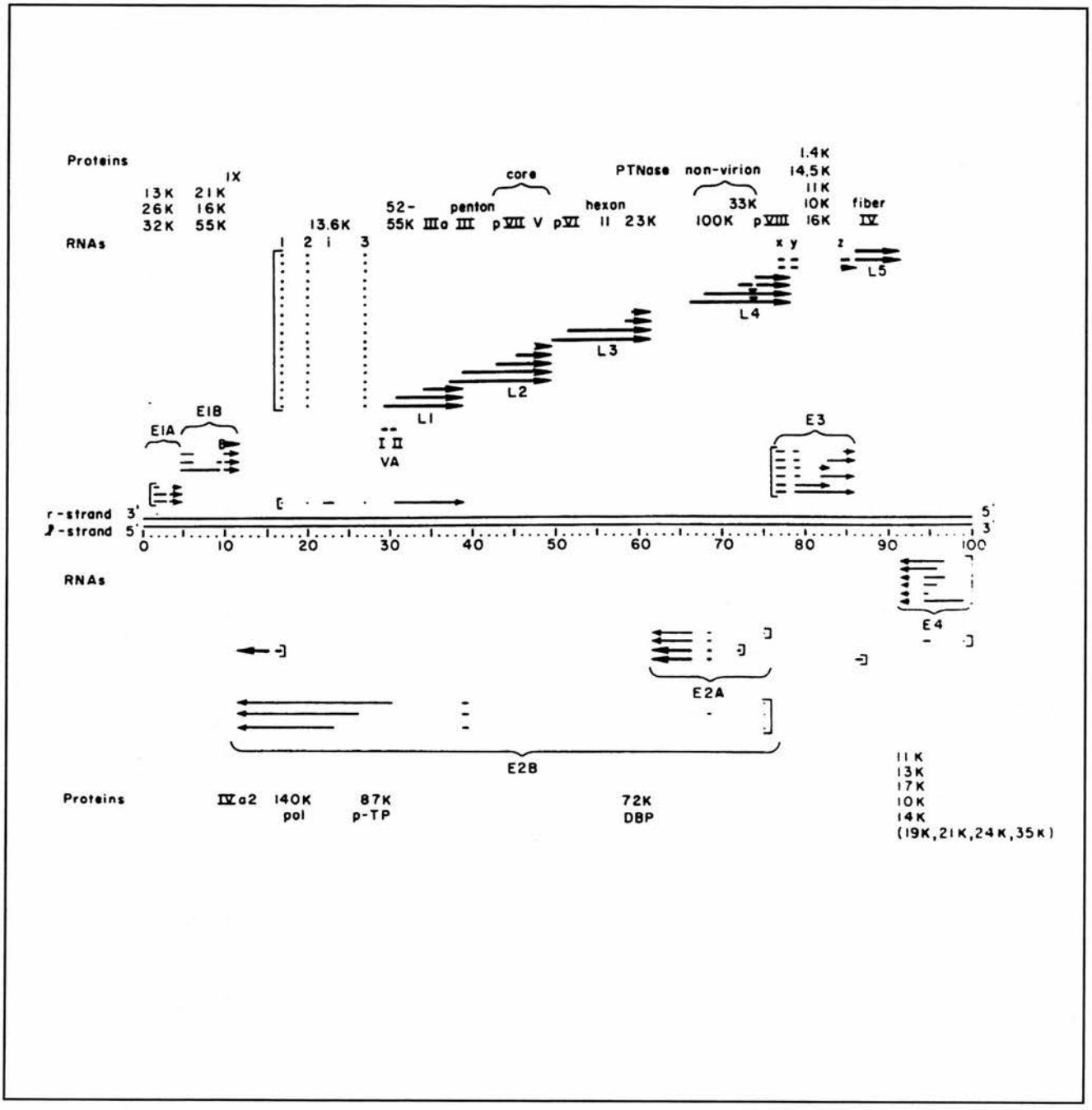


Figure 1. Transcription and translation map of adenovirus type 2. Thin lines indicate early mRNA species (E), while thick lines indicate late mRNAs (L). Polypeptides are identified by the conventional number system for the virion structural components and by size (KDa) for non-structural proteins.

play an important role in the regulation of cell proliferation. During cell cycle progression pRb can exist in the cell in both a phosphorylated and/or an unphosphorylated state, and the phosphorylation seems to change its conformation preventing its interaction with E2F (Dyson, 1994; Kaelin, 1999). Moreover pRb represses E2F dependent transcription by recruitment of histone deacetylase HDAC1 or HADC2 (Brehm et al., 1998; Luo et al., 1998). It is thought that the histone deacetylase is recruited by pRb to E2F target promoters through a direct protein/protein interaction, which involves the A/B domain of pocket proteins (pRb) and an LXCXE motif in the histone deacetylase (Magnaghi-Jaulin et al., 1998). The interaction between HDACs and pRb is inhibited by transforming proteins such as human papillomavirus E7 (Brehm et al., 1998) and adenovirus E1A (Brehm et al., 1999; Ferreira et al., 1998).

Indeed in adenovirus infected or transformed cells, E1A binds to pRB disrupting its association with HDACs and E2F, thus promoting expression of E2F dependent genes, leading to unrestricted growth. E1A proteins therefore induce cell proliferation which results in p53 accumulation and apoptosis (White, 1995). In fact, once the transcription via E2F is activated, this results in the induction of ARF. In turns, ARF binds to MDM2 and promotes its rapid degradation, leading to the stabilisation and accumulation of p53 (Kaelin, 1999; Zhang et al., 1998) (Fig.2).

In order to have a productive adenovirus infection the apoptosis triggered by p53 accumulation is inhibited by two other viral proteins, i.e. the products of the E1B transcriptional region.

The E1B-55KDa protein blocks apoptosis by interacting with p53 and altering its functions (Rao et al., 1992; Teodoro and Branton, 1997). In turn p53 apoptosis can also

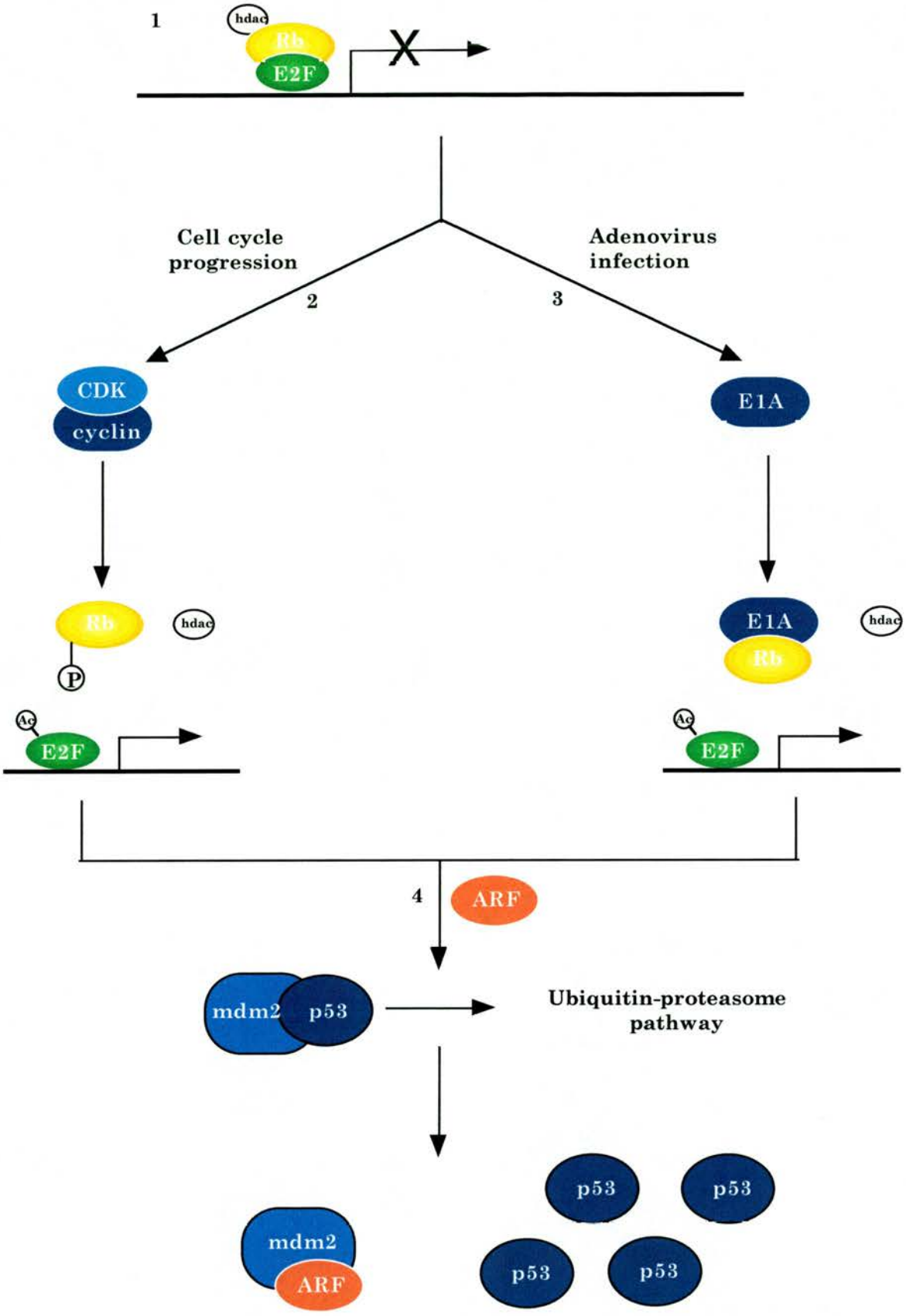


Figure 2.(1) Unphosphorylated Rb binds to E2F inhibiting its transcriptional activity. (2) During cell cycle Rb is phosphorylated by cyclin dependent kinase (CDK) and this leads to the release of E2F which is required for transactivation of S-phase genes. (3) Following adenovirus infection E1A products binds to and inactivate pRb, obviating the requirement for Rb phosphorylation. In both cases, after the dissociation of the complex E2F/Rb/histone deacetylase (hdac), E2F becames substrate of histone acetylase. (4) E2F induces the production of ARF, ARF binds to mdm2 and blocks its rapid degradation, leading to the stabilisation of p53.

be inhibited by the E1B-19KDa, a protein similar in structure and function to the antideath gene Bcl-2 (Hansen and Braithwaite, 1996; Rao et al., 1992).

Apoptosis, then, represents a sort of cellular response to the viral infection, that can potentially inhibit viral growth; therefore viruses have evolved inhibitory mechanism in order to antagonise these cellular defences. Adenovirus is not the only case of a virus that codes for genes able to block apoptosis. Indeed many viruses have been reported to encode proteins with this function (Clem et al., 1991; Jerome et al., 1999).

Thus the E1A and E1B adenovirus proteins are oncoproteins and they cooperate to transform cultured cells. The oncogenic transformation is related to the ability of these proteins to interfere with the normal function of tumor suppressor proteins.

As regards the E1B-55KDa, it has also been shown to be involved in the control of the accumulation of viral and cellular mRNA. In particular, during the late phase of adenovirus infection, the nuclear export of most cellular mRNA is inhibited while the export of viral mRNA species is promoted and they are accumulated in the cytoplasm. This process has been attributed to two adenovirus proteins: E1B-55KDa and E4-34KDa (E4Orf6) (Pilder et al., 1986). The formation of a complex between E1B-55KDa and E4-34KDa relocalise the E1B-55KDa to the nucleus; so during the late phase of the infection the E4-34KDa directs the E1B-55KDa to the periphery of the viral replication-transcription centres (Ornelles and Shenk, 1991). How the control of mRNA export/import happens is not yet known, it has been reported that the complex E1B-55KDa/E4-34KDa continuously shuttles between the nucleus and the cytoplasm, so it may works as a nucleocytoplasmatic transporter for viral m-RNA. Furthermore the

ability to direct nuclear export and import has been attributed to the E4-34KDa protein (Dobbelstein et al., 1997).

It has been shown that the E4Orf6 protein enhances the transformation of primary rodent cells induced by the E1 proteins via a more complete inactivation of p53 tumor suppressor function (Nevels et al., 1999). Indeed the E4Orf6 is able to bind p53 and together with the E1B-55KDa target p53 for degradation (Konig et al., 1999; Steegenga et al., 1998).

The E4 region codes for a number of different polypeptides, corresponding to seven different translational open reading frames (ORF s). The E4orf3 protein, like E4Orf6, has been shown to interact with the E1B-55KDa and the result of this interaction is again a relocalisation of E1B-55KDa from the cytoplasm to the nucleus (Konig et al., 1999; Leppard and Everett, 1999); these three early adenovirus proteins are involved in regulating late viral gene expression.

The E4Orf3 protein is also responsible for the disruption of the PML nuclear bodies (Carvalho et al., 1995; Doucas et al., 1996). It has been recently reported that the E4orf3 protein can cooperate with adenovirus E1 proteins to transform cells (Nevels et al., 1999).This ability of E4Orf3 to promote oncogenic cell growth is not linked to a modulation of p53 function, but it seems to be related to its localisation to the PML nuclear bodies where it induces a reorganisation of these structures (Nevels et al., 1999).

The E4 Orf6/7 protein transactivates the E2A gene by binding to the E2F cellular factor and increasing its affinity for the E2A promoter (Neill et al., 1990). As mentioned above, pRB interacts with E2F and inhibits its ability to activate transcription. During

infection, the E1a proteins disrupt this interaction allowing E2F to bind adenovirus E2 promoters along with the E4Orf6/7 protein resulting in a marked stimulation of transcription. mRNA transcribed from the E2A and E2B regions code for several products, i.e. the DNA binding protein (DBP), the preterminal protein (pTP) and DNA polymerase (pol), all of which are required for DNA replication (Hay et al., 1995).
As regard the E3 region, during the early phase of the infection it encodes for a number of proteins, which are not essential for the virus replication but are involved in modulating the host response to the infection (Elsing et al., 1998; Wold and Gooding, 1991).

The last stage of the early viral transcription known as intermediate transcription, occurs at 6-8 hours post infection, and results in the expression of the structural protein IX and IVa2. The IVa2 protein has been found to be a late stage specific transcriptional activator of the major late transcription unit (MLTU) promoter after the onset of DNA synthesis (Lutz and Kedinger, 1996).

As E2 products accumulate and the infected cells enter the S phase of the cell cycle, the optimal environment for viral DNA replication has been created. The initiation of replication requires the formation of a large nucleoprotein complex at the origin of DNA replication. Adenovirus DNA replication begins with the formation of a preinitiation complex, which consists of three viral proteins encoded by the E2 region (DBP, pTP and pol) and two cellular transcription factor (NFI and NFIII/Oct-1), which stabilise the complex and stimulate the initiation (Verrijzer et al., 1991; Verrijzer et al., 1992).

Following the onset of DNA replication there is a change in the transcriptional programme of adenoviruses and expression of a new set of late viral genes is initiated which eventually leads to assembly of viral progeny.

1.4 Late gene transcription

Concomitant with the onset of DNA replication there is a marked switch in gene expression. At this time there is the activation of late genes which is in turn accompanied by a reduction of the expression of early genes.

The late region is organised in a single large transcription unit, which is processed by differential poly(A) site selections and splicing to generate a number of distinct mRNAs. All the products of this region are grouped in five families, L1 to L5., and their expression is controlled by the major late promoter MLP (Gustin et al., 1998; Fessler et al., 1999). The late genes encode all of the structural proteins.

The MLP promoter has a low level of activity during the early stage of infection, but its activity increases substantially at late stages. An important event governing the switch from the early phase to the late phase is believed to be the process of DNA replication and not simply the increase in copy number of the template. Furthermore, during DNA replication, the intermediate product IVa2 seems to act as a transcriptional regulator that can promote the activity of the MLP promoter (Lutz and Kedinger, 1996; Tribouley et al., 1994). Accumulation of late mRNAs is not only dependent on activation of the MLP promoter but also requires alleviation of the premature termination that maintains a low the level of late transcription during the early phase of the replicative cycle. Indeed the accumulation of the late transcripts is also regulated at

the level of the polyadenylation (poly(A)) site selection (Larsson et al., 1992). Early in the infection only the L1 transcript is accumulated, whereas late in the infection the other sites for the poly(A) are recognised by 3 processing factors which directs the accumulation of the other late mRNAs.

When DNA replication begins and the full pattern of late mRNAs is produced, the cytoplasmic accumulation of cellular mRNA is blocked. This block is mediated by the E1B-55KDa/E4-34KDa complex; which beyond the inhibition of cellular mRNA cytoplasmic accumulation, is believed to promote the accumulation of viral transcripts to the cytoplasm. It is not known how these proteins influence the export/import of both cellular and viral mRNA, but it has been hypothesised that this viral complex could bind to a cellular factor required for mRNA export and relocate it to the viral replication centres, limiting, in this way, the availability of this factor for the cellular mRNA.

1.5 Virion assembly and release from the cell

Once the viral genome has been replicated and genes for all the structural proteins expressed virion assembly can then commence.

The first step of virus assembly is the formation of trimeric hexon capsomers, which is facilitated by the interaction of the L4-100KDa protein with the hexon monomers. At the same time penton capsomers, consisting of a pentameric penton base and trimeric fibre, are also assembled. These steps take place in cytoplasm, but hexons and pentons are subsequently translocated to the nucleus where virion assembly occurs. After construction of the hexon shell, assembly of viral particles proceeds via a well defined series of events. Initially, an assembly intermediate can be identified where the virion is

associated with the capsid proteins pVI, pVII, pIIIa and pIX. Subsequently the viral genome is taken into the virion left end first and the viral DNA condensed into a core structure by the viral proteins pV, pVII and possibly pmu. After DNA packaging virion proteins undergo a series of cleavage reactions, catalysed by the L3 coded protease, which are required for the development of infectivity (Greber et al., 1996; Cotton et al., 1995; Matthews et al.,1995).

After the penetration of the virus into the host cell, a sequential process of uncoating takes place and it seems that the core proteins enter the nucleus along with the DNA. The sequence of events that transports the virus genome to the nucleus is not known, but it has been postulated that core proteins would play a role in this compartmentalisation since they would be exposed to, and in contact with, the cellular environment. In this perspective the role of the core protein V has been investigated. Protein V interacts with both protein VI and VII, which seems to serve a bridging function between the nucleoprotein core and the capsid (Matthews and Russell, 1998). The evidence of a translocation of pV from the cytoplasm to the nuclear membrane during the course of the infection, and the presence of a nuclear localisation signal (NLS) in its primary structure has suggested that this protein could have additional functions regards the adenovirus cell cycle beyond its structural role.

Recently it has been shown that pV interacts with the cellular protein p32 (Matthews and Russell, 1998). p32 has a cytoplasmic localisation in close association with the mitochondria, although it also seems to be present in the nucleus as granules and tubules. The subcellular localisation of p32 and pV has been followed during adenovirus infection and it has been shown that as infection proceeds pV rapidly enters

the nucleus and the p32 distribution shifts from cytoplasmic to nuclear. Thus, late in infection the interacting p32 and pV are both found in the nucleus.

A possible interpretation for these results is that pV could be delivered to the nucleus along with p32, which is thought to be a component of a system that transports proteins between the mitochondria and the nucleus, as has been suggested for other proteins (Bereiter-Hahn, 1990). This will fit the hypothesis that the sequence of events that directs the infecting adenovirus genome to the nucleus is not random; indeed it seems more rational to suppose that the virus would parasitize some of the normal cellular transport system utilising the efficiency and the rapidity that characterise this process.

1.6 Regulation of p53 level and transcriptional activity by adenovirus proteins E1B-55KDa and E4Orf6.

The cellular protein p53 acts as a tumor suppressor and inactivation of this function is the most prevalent alteration found in human and animal tumors. In normal cells p53 is present at a low level, as it has a very short half life under these circumstances. Exposure to DNA damaging agents, such as UV light or ionising radiation, induces a marked increase of p53 level as a result of protein s stabilisation. In particular, p53 acts at level of G1/S checkpoint, in order to arrest damaged cells before entering S phase. Furthermore p53 is involved in triggering apoptosis in response to external stress, such as viral gene expression (Debbas and White, 1993; Lowe and Ruley, 1993).

Following adenovirus infection, transcription of the early gene E1A triggers, through the pRb/arf/E2F system, p53 dependent apoptosis by stabilising p53 and increasing its level in the infected cell (Fig.2). In normal cells the Mdm2 oncoprotein

inhibits p53 response by inhibiting p53 dependent transcription of targets genes and inducing the degradation of p53 via the ubiquitin-proteasome pathway. However p53 activates expression of the Mdm2 gene and as a consequence expression of E1A products will not only lead to an increase of the level of p53, but also it will promote the expression of Mdm2. Despite of this, it has been reported that adenovirus infection prevents the Mdm2 mediated degradation of p53, suggesting that the stabilisation of p53 proceeds via a modification of either p53 or Mdm2 which renders p53 resistant to the ubiquitin-proteasome mediated degradation (Nakajima et al., 1998).

Under normal circumstances a high level of p53 leads to apoptosis, but adenoviruses have evolved a strategy to stop this happening. We have already mentioned that the p53 dependent apoptosis is inhibited by the expression of the E1B products. Indeed the E1B-55KDa protein is able to bind p53 and to repress its transcriptional activity, not only by blocking stereochemically the p53 transactivation domain, but also acting as a repressor for p53 dependent transcription. It has been showed that an other early protein is able to bind p53, i.e. the E4Orf6 protein (Querido et al., 1997) (Querido et al., 1997). The E4Orf6 protein is known to form a complex with the E1B-55KDa protein and this complex functions in the transport and stabilisation of viral mRNA and in shutoff of host cell protein synthesis. Moreover the block of p53 accumulation, crucial for a productive infection, is not due to the shutoff of the host cell metabolism but results from the formation of a multiple complex between p53, E1B-55KDa and E4Orf6 that targets p53 for degradation (Konig et al., 1999; Steegenga et al., 1998). It has been also purposed that this degradation may proceed, via the ubiquitin-proteasome pathway, in a way parallel to the Mdm2 mediated degradation although this has yet to be established.

1.7 The ubiquitin-proteasome pathway

Turnover of proteins by degradation is a highly complex, temporally and spatially controlled, and tightly regulated process, which plays physiologically important roles in a wide range of cellular processes. Indeed it is often necessary for intracellular protein levels to change during development, during the cell cycle or in response to environmental stimuli. For example, misfolded proteins must be eliminated because they are not functional and prone to aggregation. Moreover protein degradation via ubiquitination represents an efficient system which the cell commonly employs to modulate crucial regulatory factors. A clear example for this role is given by the regulation of cyclins during the cell cycle. The progression through the cell cycle is directed by a complex control system which consist of cyclins, cyclin-dependent kinase (CDK) and cyclin/CDK inhibitors (Pines, 1994; Sherr and Roberts, 1995). Both the cyclins and the cyclin/CDK inhibitors have been found to undergo ubiquitination (Barinaga, 1995).

Ubiquitination is a post-translational modification in which the C-terminus of the small protein ubiquitin is covalently coupled to lysine groups in the target proteins. The modification of target protein by ubiquitin is the result of a complex multistep process, each of which is catalysed by a specific set of enzymes, and proceeds via a thiolester cascade (Fig. 3). The first step in linking ubiquitin to an other protein is the activation of the ubiquitin α-carboxyl group and its linkage to a thiol group of the activating enzyme, known as E1. In particular this step consist of two phases, a first one in which there is the activation of Ub C-terminus via an ATP dependent adenylation,

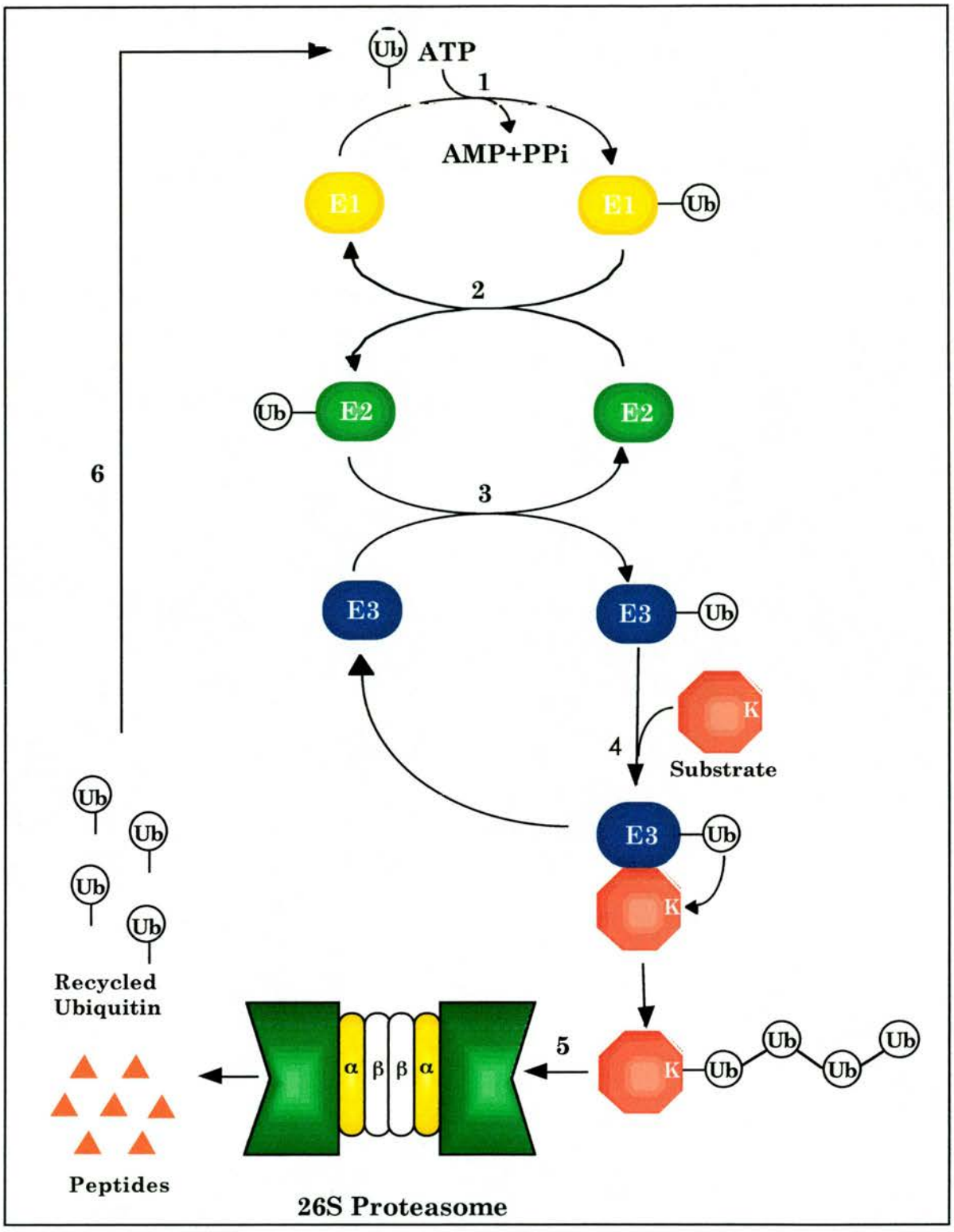


Figure 3: The Ubiquitin-proteasome pathway.
(1) Activation of Ubiquitin (Ub) by the Ubiquitin activating enzyme, E1, in an ATP dependent manner. (2) Transfer of activated ubiquitin from E1 to the active cysteine residue of a member of the ubiquitin conjugating enzyme family, E2. (3) Transfer of activated Ubiquitin to a substrate-specific E3 ligase. (4) Formation of a substrate-E3 complex and biosynthesis of a substrate-anchored polyubiquitin chain. (5)Binding of the polyubiquitinated substrate to the ubiquitin receptor subunit in the 19S complex of the 26S proteasome and degradation of the substrate to short peptides by the 20S complex. (6) Recycling of ubiquitin via the action of isopeptidases.

and a second phase in which there is the formation of an high energy thiolester bond between the C-terminal Glycine residue of Ub and a cysteine residue in the protein E1 accompanied by the release of AMP. In successive transesterification reactions the Ub molecule is transferred from the Ub activating enzyme E1 to an other enzyme, the Ub conjugating enzyme E2, which in turn transfers the ubiquitin to an E3 ubiquitin protein ligase. This last enzyme catalyses the formation of an isopeptide bond between the C-terminus of Ub and the ε-amino group of a lysine in the target protein.

At this point further Ub monomers can be attached to a lysine residue within Ub itself; as result proteins are tagged by poly-Ub chains, which probably act as a recognition signal for the 26S proteasome. Indeed mono-ubiquitinated proteins are not degraded.

Obviously, this process must be highly specific and selective in order to prevent the degradation of proteins still functional in the cell. It seems that the high specificity of the process can be attributed to the different E3 ligase activities. Some proteins can be directly recognised by the specific E3 via a primary motif present in the amino acid sequence; the class of enzymes involved in this direct recognition is called N-end rule , because usually the motif to be recognised is at the N-terminus of the substrate. However in most cases the recruitment of substrate by E3 is not direct and proceeds via multi-subunit complex and/or modification of the substrate. Thus E3 proteins seem to function as adapters that recruit the target protein to a complex containing also an E2 activity. A clear example of the combinatorial control of E3 ligase specificity is the SCF (**S**kp1-**C**dc53/Cullin-**F**box proteins) family. SCFs are multi-protein complexes containing adapter subunits, called F-box proteins that recognise different substrates through specific protein-protein interaction domains. Moreover F-box proteins interact

with a catalytic core composed of Skp1, Cdc53 and the E2 ubiquitin conjugating enzyme Cdc34. The latter appears to be recruited to this multi-complex via Rbx1, an E3 associated protein, which stabilises the interactions between Cdc34/Cdc53 and it seems that all this network of protein-protein interaction functions to stimulate the catalytic activity of the ub-conjugating enzyme (Seol et al., 1999; Skowyra et al., 1999; Tyers and Willems, 1999). One of these ligase complexes is involved in the degradation of IkBα. Different studies have shown that the β-TrCP protein, a mammalian homologue of the drosophila Slimb protein (Jiang and Struhl, 1998), is a component of the IkB ubiquitin ligase. In particular β-TrCP functions as the F-box of an SCF complex containing Skp1 and Cdc53/Cul1, that then recognises and recruits IkBα; but a productive interaction between IkBα and the SCF specific complex, through β-TrCP, requires the phosphorylation of two residue of Ser, 32 and 36, in IkBα. This mean that the targeting of IkBα for degradation depends on a signalling pathway, triggered by specific stimuli that leads to its phosphorylation and a direct effect of the recruitment of IkBα by the SCF complex is the activation of NF-kB dependent transcription.

1.8 De-Ubiquitination enzymes

An other important class of enzyme, which take part in the ubiquitin pathway, is represented by the de-ubiquitinating enzymes.

Ubiquitination, like phosphorylation, results in a post-translational modification that can modulate and control the activity of target proteins. Specific substrate recognition and subsequent modification is carried on by the co-operation of E2 and E3 enzymes in the case of Ubiquitination, while phosphorylation is mediated by kinase activity. However,

in both cases, it is a dynamic process, so that ubiquitination or the phosphorylation of a substrate depends on the balance of the activity of two antagonistic enzymes: kinases vs. phosphates and E2/E3 vs. de-ubiquitinating enzymes.

De-ubiquitinating enzymes are cysteine proteases (Hochstrasser, 1996; Wilkinson, 1997) that specifically cleave ubiquitin from ubiquitin conjugated products. Release of ubiquitin is essential in two different but complementary processes. One is the degradation process, where it is important to release ubiquitin from terminal proteolytic products, to recycle the polyubiquitin chains and to check targeting of the correct substrates. On the other end, they play an important role in the biosynthesis of ubiquitin, where free Ub molecules are released following the cleavage of the polyubiquitin precursor (Ciechanover, 1998).

In particular this class of enzymes is divided in two subgroups: Ubiquitin C-terminal proteases (UCHs) and Ubiquitin specific proteases (USPs). UCHs are involved in co-translational processing of pro-ubiquitin gene products and in the cleavage of ubiquitin from adducts with small molecules, while USPs catalyse release of ubiquitin from conjugates with cellular proteins or from free polyubiquitin chains.

Obviously, these enzymes can act at different stages in the ubiquitination pathway, and depending on the stage where they carry on their action, they may promote or inhibit the degradation of a certain substrate (Ciechanover and Schwartz, 1998) Indeed the hydrolysis of the isopeptide bond between ubiquitin and a mistakenly tagged protein results in the inhibition of proteolysis and the prevention of damage to the cell; while release of free ubiquitin from its precursor in order to supply E1/E2/E3 with available ubiquitin results in the stimulation of the proteolytic process.

1.9 Oncogenic effects of the ubiquitin machinery

Considering the wide range of substrates and processes regulated in some way by the ubiquitination, it is not surprising to find a correlation between alteration in the ubiquitin pathway and a number of disease states, including cancer.

The fact that a number of oncogene products have been discovered to be substrates for ubiquitination (Ciechanover et al., 1991; Ciechanover et al., 1994) has suggested that a possible mechanism for oncogenic transformation could be the alteration of the ubiquitin dependent degradation process, which would in normal circumstances modulate the proto-oncogene functions. In fact it has been shown that c-Jun but not its transforming counterpart v-Jun, is efficiently ubiquitinated and degraded. c-Jun is a part of a regulatory system that governs cell growth. It seems that the presence of the extra δ domain in c-jun is destabilising the protein, while the lack of this domain in v-Jun stabilises the product and may in part explain its transforming activity (Isaksson et al., 1996).

It has also been demonstrated that a low level of p53 is associated with cervical carcinoma caused by HPV. The tumor suppressor is, in fact, a substrate for the E6 oncoprotein of HPV, which in combination with the cellular E6-AP targets it for degradation via the ubiquitin proteasome pathway. Blocking of tumor suppressor activity by viral oncoproteins seems to be a general theme recurring in cell transformation following infection with DNA viruses, which replicate in the nucleus of the cell.

Moreover accumulation of ubiquitin conjugates and subsequent formation of aggregates have been reported in the pathologic lesions of some neurodegenerative disease, such as Alzheimer s and Parkinson s diseases (Johnson WG, 2000; Mukaetova et al., 2000).

1.10 Ubiquitin pathway and viruses

The interaction between some viruses and the ubiquitin pathway leads the virus to bypass the immune defences of the host cell. Nuclear antigen I of Epistein-barr, a member of the herpesvirus family, is resistant to Ub-mediated degradation as a consequence of a Gly-Ala C-terminal repeat sequence (Levitskaya et al., 1997). While this antigen is ubiquitinated it can not be processed, thus blocking antigen presentation of and inhibiting the immune response to virus infection (Weber et al., 1999).

An other example is given by the human virus cytomegalovirus (CMV). This virus encodes two proteins (US2 and US11) resident in the endoplasmic rethiculum, where they induce degradation of the MHC class I molecules. When these viral proteins are expressed the MHC is transported back to the cytoplasm and degraded in a proteasome dependent manner (Wiertz et al., 1996). By disrupting the MHC molecules, the virus blocks the presentation of viral antigens on the cell, thus evading the immune response.

1.11 Ubiquitin like proteins

In recent years many proteins related to ubiquitin, known as Ubiquitin-like proteins have been identified. Some of these, such as Parkin, implicated in the pathogenesis of a form of Parkinson s disease (Kitada et al., 1998), are bigger than Ub and are characterised by the presence of ubiquitin-like domains, but they lack the C-terminal Gly and so cannot generate conjugation products.

However other proteins have been characterised and they show significant similarity to ubiquitin in critical regions and which suggests that they can assume an ubiquitin-like fold. All these protein are synthesised with carboxyl terminal extension peptides that are cotranslationally processed to expose the mature glycine dipeptide conserved in ubiquitin. Interestingly, they are also involved in covalent post-translational modification of target proteins.

The mechanism by which these ubiquitin-like proteins are conjugated to target protein appears to be similar to ubiquitin conjugation, but they do not seem to be involved in targeting modified substrates for degradation (Hochstrasser, 1998).

As an example, UCRP is an interferon-inducible protein that contains two ubiquitin-like domains; it is conjugated to a number of subcellular proteins and seems to be involved in targeting proteins to cytoskeleton (Haas et al., 1987; Loeb and Haas, 1994).

In particular we focus our attention on one of these Ub-like proteins: SUMO-1 (**S**mall **U**biquitin-like **Mo**difier).

1.12 SUMO-1 and function of SUMO-1 conjugation

SUMO-1 is a small protein of 11.5 KDa with high structural homology to Ubiquitin (Bayer et al., 1998). This similarity in the 3dimensional structure reflects a similar function, and both proteins modify target substrates via formation of a covalent isopeptide bond (Fig. 4). Indeed SUMO-1 is responsible for the post-translational modification of target proteins such as RanGAP1, PML, Sp100, p53 and IkBα (Desterro et al., 1998; Kamitani et al., 1997; Matunis et al., 1996; Rodriguez et al., 1999; Saitoh et al., 1997). For example, SUMO-1 conjugation of RanGAP1 seems to be necessary for its translocation to the nuclear envelope, where it binds RanBP2, a cytoplasmic component of the nuclear pore complex (NPC) (Mahajan et al., 1998). RanBP2 is a GTPase required for the transport of proteins across the NPC, and its GTP/GDP cycle is regulated by RanGAP1. Interestingly a necessary requirement for the functional interaction of RanGAP1 with RanBP2 is the SUMO-1 modification of RanGAP1 (Mahajan et al., 1998).

An other important case of SUMO-1 conjugation involved in the regulation of cellular proteins is represented by IkBα, the inhibitor of NF-kB. NF-kB is an important transcription factor in the cell, which regulates the inducible expression of a large group of genes. The transcriptional activity of NF-kB depends on its subcellular localisation. In particular, the inactive form of NF-kB is a cytoplasmic complex with its inhibitor IkBα. Following stimulation by various factors IkBα undergoes phosphorylation and subsequent ubiquitination. Once IkBα is polyubiquitinated, it becomes a substrate of the 26S proteasome and it is degraded. At this point the uncomplexed NF-kB is free to translocate to the nucleus, where it binds specific DNA target sequences and activates

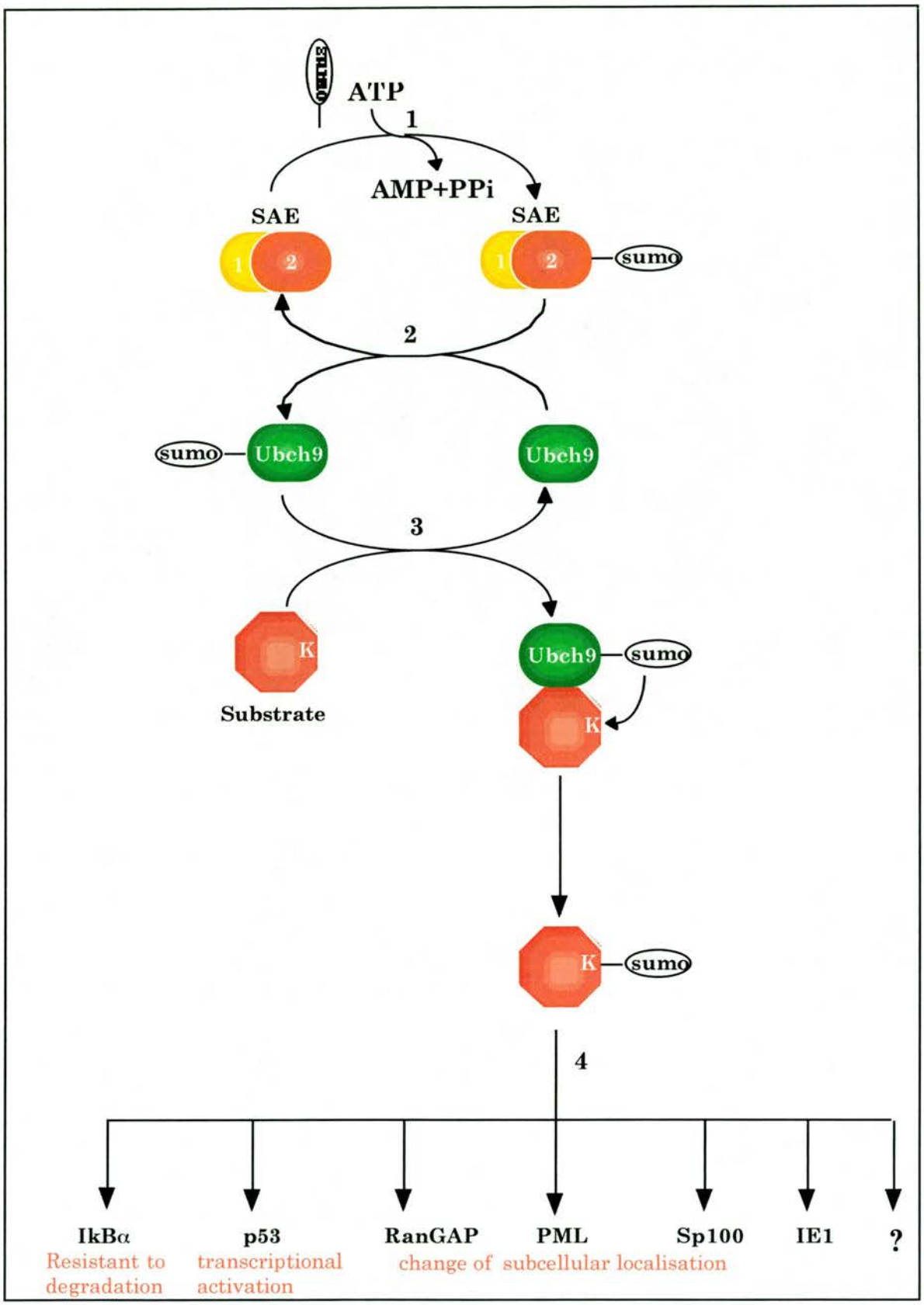


Figure 4: The SUMO-1 conjugation pathway.
(1) Activation of SUMO by the Sumo activating enzyme, SAE, in an ATP dependent manner. (2) Transfer of activated SUMO from SAE to the active cysteine residue of the Sumo conjugating enzyme, Ubch9. (3) Conjugation of a single moiety of SUMO to a target substrate. (4) Substrates for SUMO-conjugation .

the transcription of responsive genes. Which role does SUMO-1 play in this context? It is suggested that a single molecule of SUMO-1 is conjugated to the non-phosphorylated form of IkBα on the same lysine (lys21) which in the phosphorylated form is targeted by ubiquitin. The SUMO-1 modified form of IkBα cannot be ubiquitinated and is therefore resistant to proteasome mediated degradation. Thus NF-kB bound to SUMO-1 modified IkBα cannot be activated by the normal pathway of signal induced degradation (Desterro et al., 1998). These facts therefore suggest a crucial role of SUMO-1 in regulating the transcriptional activity of NF-kB and underline the importance of SUMO-1 modification in cellular functions.

1.13 Enzymes involved in SUMO-1 conjugation

If the covalent modification of target proteins by SUMO-1 is very similar to that of ubiquitin, there may exist three kind of enzymatic activity involved in this system: E1 activating enzyme; E2 conjugating enzyme; E3 protein ligase enzyme.

In particular it seems that the specificity of the ubiquitination process is due to the last two enzymes. E1 does not seem to play a crucial role in recognition of substrates for ubiquitination. However the peculiarity of E1 is that, even although it is not directly involved in the discrimination of the target protein, it can distinguish between Ubiquitin and Ubiquitin-like proteins, activating only its specific substrate.

Apart from the E1 involved in the ubiquitin conjugation, other kinds of E1 activity have been described (Johnson et al., 1997; Lammer et al., 1998; Liakopoulos et al., 1998) although our particular interest is in the SUMO-1 activating enzyme.

Recently, indeed, a new enzyme has been isolated (Desterro et al., 1999; Okuma et al., 1999). This enzyme catalyses the ATP dependent activation of SUMO-1 protein; the first step in the SUMO-1 conjugation pathway. The other enzyme which has been shown to take part in this process is the so called Ubch9 (Desterro et al., 1997; Johnson and Blobel, 1997) (Saitoh et al., 1998; Schwarz et al., 1998) and it represents the SUMO-1 conjugating enzyme, which is the equivalent of the E2 activity in the ubiquitination pathway. The three-dimensional structure of ubiquitin-conjugating enzyme 9 (Ubch9) has been obtained to a resolution of 2.8 A by X-ray diffraction (Giraud et al., 1998). Although Ubch9 is homologous to ubiquitin conjugating enzymes, it is known to conjugate SUMO-1 but does not conjugate ubiquitin (Desterro et al., 1997). The structure of Ubch9 shows significant differences compared with the structures of known ubiquitin-conjugating enzymes. Ubch9 has a different recognition surface compared to ubiquitin-conjugating enzymes, which is likely to play an important role in SUMO/ubiquitin discrimination.

Concerning the SUMO-1 activating enzyme (SAE), it has been shown to catalyse not only the ATP dependent activation of SUMO-1, but also to transfer SUMO-1, once activated, to the Ubch9 enzyme. In contrast with the ubiquitin E1 which is a monomeric enzyme, SAE is a heterodimeric enzyme; it consists of two subunits, respectively of 38 (SAE1) and 72 (SAE2) kDa. In particular, comparing the sequence of these two activating enzymes (E1 for ubiquitination and for SUMO-1 conjugation), it s clear that SAE1 is homologous to the N-terminal region of ubiquitin activating enzyme, while SAE2 is homologous to the C-terminus of the ubiquitin activating enzyme. The Cysteine residue which interacts with the C-terminal Glycine of SUMO-1 via a

thiolester bond is localised in the sequence of SAE2, but what is important is that neither of the two subunits, SAE1 or SAE2, alone is able to form a thiolester linkage with SUMO-1; this means that the activation of SUMO-1 is due effectively to the heterodimeric enzyme SAE1/SAE2.

The similarity between the ubiquitin E1 and SAE suggests a similar function for these two enzymes, even if their specificity is directed to different substrates with different consequences on the cellular events.

Once the E1 and E2 for the SUMO-1 conjugation pathway have been identified, the next step was addressed to the identification of an eventual E3 ligase activity that was responsible for the physical transfer of the SUMO-1 molecule from the E2 to the cellular substrate. It has been shown that in the in vitro reconstituted system for the SUMO-1 conjugation of RanGAP1 and IkBα (Desterro et al., 1999) the only two enzymatic activity required were SAE (E1) and Ubch9 (E2). Thus SUMO-1 ligase protein was not required. Probably since Ubch9 can bind RanGAP1 and IkBα, it may work as SUMO-1 ligase in this system (Desterro et al., 1999; Okuma et al., 1999). Of course, it cannot be excluded that a SUMO-1 ligase could act *in vivo*, increasing the rate and the efficiency of this process.

1.14 SUMO-1 deconjugating enzyme

The level of free SUMO-1 inside the cell is very low, and the major part of this protein is present as conjugation products (Matunis et al., 1996). This indicates that the availability of free SUMO-1 is tightly controlled by a dynamic equilibrium between

SUMO-1 conjugating and de-conjugating enzymes. The presence of two antagonistic enzymes also for the SUMO-1 modification agrees perfectly with the strategy adopted by the cell to modulate other post-translational modifications as ubiquitination and phosphorylation.

In this respect a new enzymatic activity concerning SUMO-1 modification has been recently discovered. This enzyme, named Ulp1, can process both SUMO-1 linked proteins and SUMO full length (Li and Hochstrasser, 1999). Indeed, like ubiquitin, SUMO is produced as a precursor that needs to be cleaved to expose a reactive C-terminal Gly that is conjugated to target substrates. This discovery, of course, establishes the basis for further studies, which will allow to gain a better understanding of the regulation of SUMO-1 modification and the possible consequences deriving from its malfunctions.

1.15 SUMO-1, PML and viral proteins

The PML protein was first identified as part of a fusion product with the retinoic acid receptor alpha, resulting from the t(15;17) chromosomal translocation associated with acute promyelocytic leukemia (Grimwade et al., 1997). PML is tightly bound to the nuclear matrix and is concentrated in defined subnuclear structures (NB, nuclear bodies) that are disorganised in certain human disease, such as leukemia, neurodegenerative disorder and viral infections. It has been found that PML, like another component of the NB, Sp100, undergoes SUMO-1 conjugation; and it has been suggested that this modification is an important factor for the localisation of these proteins to the NB (Duprez et al., 1999; Muller et al., 1998). Moreover it seems that the

modification of PML and Sp100 is regulated during the cell cycle. In particular the SUMO-1 conjugated isoforms of PML and Sp100 characteristic of the interphase, were completely eliminated during mitosis (Everett et al., 1999). It has been suggested that as mitosis begins, SUMO-1 is deconjugated from PML, probably this deconjugation is mediated by the recently discovered Ulp1, and a portion of PML is phosphorylated.

Further studies have shown that some of the events, which lead to mitosis, are also induced as a consequence of adenovirus infection. Adenovirus infection, in fact, causes a drastic change in NB morphology. In particular the early adenovirus protein E4Orf3 induces a redistribution of PML from spherical nuclear bodies to fibrous structures, where both the proteins are found to colocalise (Carvalho et al., 1995; Doucas et al., 1996; Leppard and Everett, 1999). Following adenovirus infection the SUMO-1 modified isoforms of PML disappear and a PML form of very similar electrophoretical mobility to the mitotic form accumulates (Leppard and Everett, 1999).

It has also been shown that herpes virus (HSV-1) infection cause a reorganisation of the nuclear bodies. In particular the HSV-1 regulatory protein Vmw110 induces the degradation of PML and Sp100, mainly of the SUMO-1 conjugated forms, in a proteasome dependent manner (Chelbi-Alix and de The, 1999; Everett et al., 1998; Muller and Dejean, 1999).

Like HSV-1, CMV also induces a disruption of the nuclear bodies. In this case the CMV protein IE1 is responsible for the loss of SUMO-1 conjugated forms of PML and Sp100. Interestingly, it has been shown that the IE1 itself is a substrate for SUMO-1 conjugation (Muller and Dejean, 1999), representing the first viral protein found to undergo this type of post-translational modification. However there is no evidence that

this modification addresses the IE1 protein to the NB, while it seems more likely that the interaction between PML and IE1 could be implicated in the initial targeting of the viral protein to the NB. At this point, because SUMO-1 modification has been shown to be also involved in the stabilisation of target protein by generating conjugated products resistant to degradation (Desterro et al., 1998), it could be speculated that the SUMO-1 modification of IE1 affects the stability of this protein.

1.16 Aims of the project

Post-translational modifications, generally, modulate protein function by altering its activity, subcellular localisation and, at least, the ability to interact with other proteins. They thus represent an extremely selective and valid means for the cell to modulate protein function, to trigger cellular response and to control a crucial equilibrium that assures the survival of the cell.

In this context, the aim of this work has been to analyse the role of SUMO-1 conjugation during adenovirus infection. This information will help us to understand the role of this post-translational modification in the cell and to investigate how the virus subverts this cellular process.

2. MATERIAL and METHODS

2.1 Antibodies

For protein detection in western blot analysis and/or immunofluorescence analysis, the monoclonal antibody 2A6 anti-E1b-55KDa was used at a dilution of 1:10 (Sarnow et al., 1982).

SV5 Pk tag monoclonal antibody (Hanke et al., 1992) was obtained from R.E. Randall, University of St. Andrews and was used to immunodetect (1:2000) proteins containing either an N-terminal or C-terminal SV5 epitope tag of 14 aminoacids (GKPIPNPLLGLDST) of the protein P of Simian Virus 5 (SV5).

HA-tagged proteins were detected using a polyclonal antibody, specific for a 9 amino acid HA peptide sequence (YPYDVPDYA) from influenza HA, obtained from Babco. It was used in immunofluorescence at a dilution of 1:200.

Monoclonal antibody DO-1 (Vojtesek et al., 1995) recognises the N-terminus of human p53 and used at 1:1000 dilution in western blotting.

Monoclonal anti-SUMO-1 antibody was purchased from Cambridge Bioscience and use in western blotting and immunofluorescence at a ratio, respectively of 1:500 and 1:50.

2.2 Expression and purification of GST-SAE1

GST-SAE1 construction (Desterro et al., 1999) was expressed in *Escherichia coli* strain B834. Induction of expression, glutathione agarose affinity chromatography and thrombin cleavage of fusion proteins was as described (Jaffray et al., 1995).

2.3 Quantitation of protein

Protein concentrations were determined using Bradford s method (Bradford, 1976). Protein samples were mixed with Bradford s reagent (Biorad) and the absorbance at 595 nm was measured on a spectrophotometer. Protein absorbances were converted to mg/ml concentrations using a standard curve constructed by measuring the absorbances of a range of bovine serum albumin (BSA) concentrations.

2.4 Affinity purification of SAE1 and Ubch9 antibody

Primary antibodies to SAE1 and Ubch9 were raised in sheep (Scottish Antibody Production Unit, Carluke) and were antigen affinity purified. NHS Hi-Trap^{TM} affinity columns were used for the purification of the antibodies from the crude sheep serum . The columns were washed with 10 volumes of coupling buffer (200mM $NaHCO_3$/ 500mM NaCl, pH 7.8). Following this, the appropriate ligand was bound to the column by recirculation of 5mg of recombinant protein (GST-SAE1 or Ubch9) over the column for 2 hrs at RT. In order to deactivate any excess groups that have not coupled ligand and to wash out not-bound ligand, the columns were washed in buffer A (500mM NaCl/ 500mM Ethanolamine, pH 8.3) and buffer B (100mM CH_3COONa/ pH 4.0). After columns preparation, a 1:10 dilution (in PBS) of sheep serum was recirculated over the column for 16 hrs. Then the column were washed with 20 volumes of 10mM Tris/ 500mM NaCl/ pH 7.5 and eluted with 100mM Glycine pH 2.25. 500 µl fractions were collected in 50µl of 1M Tris pH 8.0 in order to neutralise the acidic eluate and preserve antibodies activity. The antibodies so purified were then stored in 50% Glycerol/

1mgml^{-1}BSA/0.01% NaN_3 at -20ßC. The anti-SAE1 was used in western blotting at 1:3000 dilution and 1:300 in Immunofluorescence; while the anti-Ubch9 was used in western blot analysis at 1:1000 dilution and 1:100 in Immunofluorescence.

2.5 Cell culture and transfection

293 cells, which complements Adenovirus E1a and E1b functions, and HeLa cells were maintained in exponential growth in Dulbecco s modified Eagle s medium (DMEM) containing respectively 10% and 5% fetal calf serum.

For 293 cells, 12 µg of plasmid DNAs were transfected for 14 hours in subconfluent 75 cm^3 flasks using Lipofectamine™ according to instructions provided by the manufacturer (Gibco). After 36 hours of expression, cells were washed in PBS and cellular extracts prepared for further analysis.

HeLa cells were transfected by electroporation (950mF, 200V, Equibio Easyject plus) as described (Arenzana-Seisdedos et al., 1997). 10 µg of plasmid DNA encoding the HA-SUMO-1 (Desterro et al, 1998) was transfected into 5 $X10^6$ cells. After transfection, cells were seeded in 75 cm^3 flasks and incubation continued for a further 36h.

2.6 Virus stock and Adenovirus infection

Adenovirus type 2 was obtained from Prof. R.T. Hay.

For western blot analysis, about 1 x 10^6 cells in 60mm tissue culture plates were infected by adding an amount of virus calculated to give a multiplicity of 20 p.f.u. per cell in 0.4 ml of serum-free DMEM. After 1 hr at 37ßC, 4 ml of DMEM containing 2%

fetal calf serum and antibiotics were added. Cells were then incubated at 37ßC/5% CO_2 for different time points. For immunofluorescence studies, 0.25 x 10^6 HeLa cells were grown for each coverslip and were infected with a multiplicity of 2 p.f.u. per cell in a final volume of 0.2 ml of serum-free DMEM.

2.7 Preparation of cell extract

Cell extracts were prepared by lysis in SDS sample buffer (5% SDS, 0.15 M Tris HCl pH 6.7, 30% glycerol) diluted 1:3 in RIPA buffer (25 mM Tris/pH8.2, 50 mM NaCl, 0.5% Nonidet P40, 0.5% Deoxycholate, 0.1% SDS, 0.1% Azide), containing 10 mM Iodoacetamide and complete¤ protease inhibitor cocktail (Boehringer Mannheim). 20 µl of each lysate was fractionated on either 8.5%, 10% or 12.5% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Sigma). Protein expression was checked by Western blotting.

2.8 Purification of 6XHIS-tagged SUMO-1- conjugates

Cells transfected with 6His-tagged SUMO-1 (or 6His-tagged Ubiquitin) (Rodriguez et al., 1999; Treier et al., 1994) and HeLa stably transfected with a plasmid expressing 6His-tagged SUMO-1 (gift from Peter O'Hare, unpublished) were infected with Ad2 in a time course experiment. Cells from a 75 cm^3 flask were lysed in 4 ml of 6 M guanidinium-HCl, 0.1 M Na_2HPO_4/NaH_2PO_4 , 0.01 M Tris/HCl , pH 8.0 plus 5mM imidazole and 10 mM beta-mercaptoethanol. After sonication, to reduce viscosity, the lysates were mixed with 50 ml of Ni^{2+}-NTA-agarose beads prewashed with lysis buffer and incubated for 2 hours at room temperature. The beads were successively washed with the following: 6 M guanidinium-HCl, 0.1 M Na_2HPO_4/NaH_2PO_4 , 0.01 M Tris/HCl

, pH 8.0 plus 10 mM b-mercaptoethanol; 8 M Urea, 0.1 M Na_2HPO_4/NaH_2PO_4 , 0.01 M Tris/HCl , pH 8.0, 10 mM b-mercaptoethanol; 8 M Urea, 0.1 M Na_2HPO_4/NaH_2PO_4 , 0.01 M Tris/HCl , pH 6.3, 10 mM b-mercaptoethanol (buffer A) plus 0.2% Triton X-100; buffer A and then buffer A plus 0.1% Triton x-100. After the last wash with buffer A the beads were eluted with 200 mM imidazole in 5% SDS, 0.15 M Tris HCl pH 6.7, 30% glycerol, 0.72 M beta-mercaptoethanol. The eluates were subjected to SDS-PAGE analysis and the proteins transferred to a polyvinylidene difluoride membrane (Sigma). Western blotting was performed with antibodies against adenovirus E1B-55KDa, RanGAP, Adenovirus pV and p53.

2.9 SDS/PAGE and Western Blot analysis

Protein samples were resuspended in disruption buffer (1X: 20 mM Tris/ HCl pH6.8, 2% SDS, 5% b-mercaptoethanol, 2.5% glycerol and 2.5% bromophenol blue) and denaturated at 100°C for 5 minutes before loading on a 8.5-12.5% SDS-polyacrylamide gel (acrylamide percentage appropriate for the size of proteins to be separated). Bio-Rad mini gel equipment was used in accordance with the manufacters instructions. New England Biolabs protein molecular weight markers were used as standards to establish the apparent molecular weights of proteins resolved on SDS-polyacrylamide gels. Separated polypeptides were either stained with Comassie Blue (0.2% Comassie brilliant blue R250; 50% methanol; 10% acetic acid) for 30 minutes and then destained (20% methanol; 10% acetic acid) or tranferred to a polyvinylidene difluoride membrane (Sigma) using a wet blotter (Biorad Systems). The membranes were blocked with PBS containing 5% skimmed milk powder and 0.1% Tween 20 then

incubated with monoclonal or polyclonal antibodies diluted in blocking buffer. Horseradish peroxidase conjugated anti-mouse IgG, anti-rabbit IgG (Amersham) and anti-sheep IgG were used as secondary antibodies. Western blotting was performed using ECL detection system. After ECL detection and when necessary western blots were stripped as described (Roff et al., 1996).

2.10 In vitro transcription-translation

In vitro transcription/translation was performed using 1-2 mg of plasmid DNAs (pV and E1B constructs in pcDNA3) and a TNT Coupled Wheat Germ Extract System (Promega) according to the instructions provided by the manufacter. ^{35}S-methionine (Amersham) was used in the reactions to generate radiolabelled proteins.

2.11 In vitro SUMO-1 conjugation assay

^{35}S-methionine labelled in vitro transcribed/translated proteins (1 l) were incubated with 2 l of HeLa cell fraction containing SUMO-1 E1 (SAE1/2) activity (fr II.4) (Desterro et al., 1999) in a 10 l reaction including an ATP regenerating system (50 mM Tris pH 7.6, 5 mM $MgCl_2$, 2 mM ATP, 10mM creatine phosphate, 3.5 U/ml of creatine kinase and 0.6 U/ml of inorganic pyrophosphatase), SUMO-1 (1 mg/ml), Ubch9 (60 g/ml). Reactions were incubated at 37^{0}C for 2 h. After terminating the reactions with SDS sample buffer containing mercaptoethanol, reaction products were fractionated by SDS-PAGE (8.5%) and the dried gels analysed by phosphoimaging (Fujix BAS 1500, MacBAS software).

2.12 Immunofluorescence

Cells were grown on coverslips and infected with a multiplicity of 2pfu per cell for varying times post infection. Following this, cells were carefully washed in PBS/50mM $MgCl_2$/50mM$CaCl_2$, fixed for 10 min with a solution of 3% paraformaldehyde in PBS and fixation quenched by 2 x 10 minutes incubation in 0.1M Glycine/ PBS. Then cells were permeabilised with a 10 minutes treatment in 0.1% Triton X-100 and cells were blocked (before antibody addition) by incubation in 0.2%BSA/PBS. The appropriate dilution of primary antibodies was prepared in 0.2%BSA/PBS and subsequently the cells were incubated at room temperature for 1h. Immobilised antibody/antigen complexes were detected using secondary antibodies (anti-mouse Texas Red and anti-rabbit FITC) at a concentration of 1:200. Then cells were visualised and photographed using immunofluorescence microscopy. The images obtained were processed by Adobe Photoshop 5.0 and annoted in Adobe Pagemaker 6.0.

2.13 Luciferase assay

HeLa 57A (Rodriguez et al., 1999) were infected with Ad2 with a m.o.i of 20 p.f.u. per cell, and as control uninfected cells were activated with TNF (10ng/ml final in medium) for 6 hours. For each time point, cells were collected by trypsinisation and centrifuged at 1-2000 rpm for 5 minutes. The cells were then washed once with 1 ml of PBS, followed by lysis in 50 µl of luciferase lysis buffer (25mM Tris phosphate pH 7.8, 8mM $MgCl_2$, 1mM DTT, 1% Triton-X-100 and 15% glycerol) to yield the cytoplasmic extract. Then luciferase activity was assayed in luciferase buffer (25mM luciferin, 1mM

ATP, 1% BSA) made up in lysis buffer. Luciferase activity was measured using the MicroLumat (LB96P) plate reader.

Data obtained were processed using Cricket graph program and the standard error was calculated as a function of the standard deviation.

3. RESULTS

3.1 Effects of adenovirus infection on the SUMO-1 conjugation pathway

In order to explore the effects of adenovirus infection on the SUMO-1 conjugation pathway, HeLa cells were infected with Adenovirus type 2 (Ad2) with a multiplicity of infection of 20 p.f.u. per cell. Whole cell extracts were prepared at 0, 12, 24, 36 and 48 hours post-infection. The extracts were then fractionated by SDS-PAGE, transferred to a Polyvinylidene difluoride (PVDF) membrane and analysed with a mouse monoclonal antibody to SUMO-1. As infection proceeds there is a general increase in the amount of SUMO-1 conjugation and an increase in the number of species conjugated to SUMO-1 (fig. 5). Indeed the antibody anti-SUMO-1 is detecting all the different SUMO-1 conjugated products, while unconjugated SUMO-1, a molecule of about 11.5 KDa, is not usually detected because the amount of free SUMO-1 in the cell is very low, it is almost only present in conjugated products. The same samples were analysed using an anti mouse anti alfa-tubulin antibody to confirm that equal amounts of cell extracts were loaded in each lane.

To monitor the progress of infection, cell extracts were also analysed for the presence of viral proteins. In particular, antibodies against the early adenovirus protein E1B-55KDa (fig. 6A) and the late adenovirus protein pV (fig. 6B) were used for this purpose. In both cases the sample corresponding to uninfected cells is negative to presence of viral proteins, and consistively with the adenovirus transcription events, the early E1B-55KDa protein is detected since 12 hours post infection, while the late product pV is detectable from 24 hours post infection.

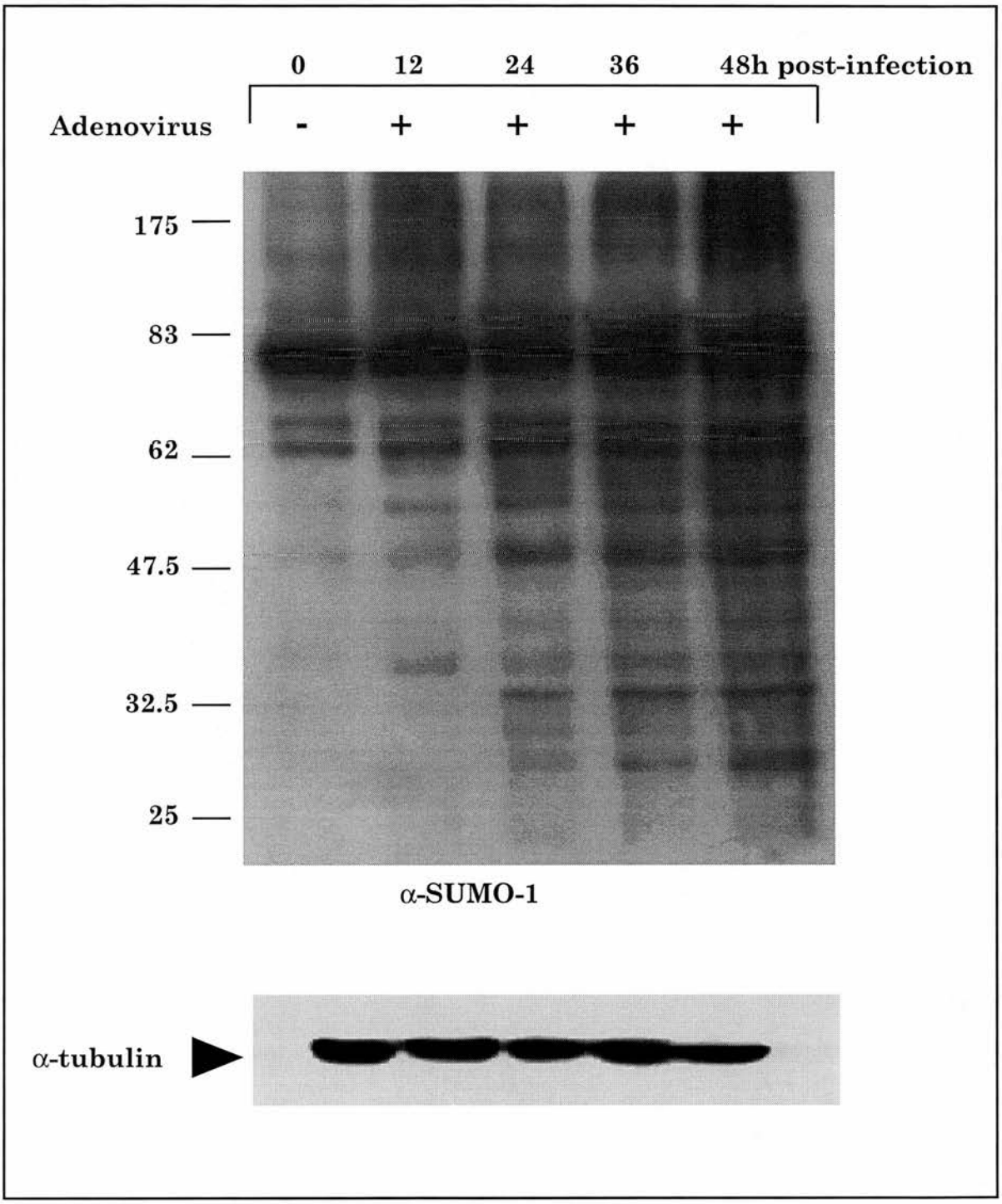


Figure 5. Effect of adenovirus infection on the general SUMO-1 conjugation pattern
1 x 10^6 Hela cells were infected with Ad2 at 37°C and total cell extracts prepared at 0, 12, 24, 36 and 48 hours post infection. Extracts were separated by SDS-PAGE on a 8.5% gel, blotted and probed for SUMO-1. The molecular weight markers are indicated on the left. The extracts were also probed with a monoclonal antibody against α-tubulin, in order to verify a similar protein content in all the lanes.

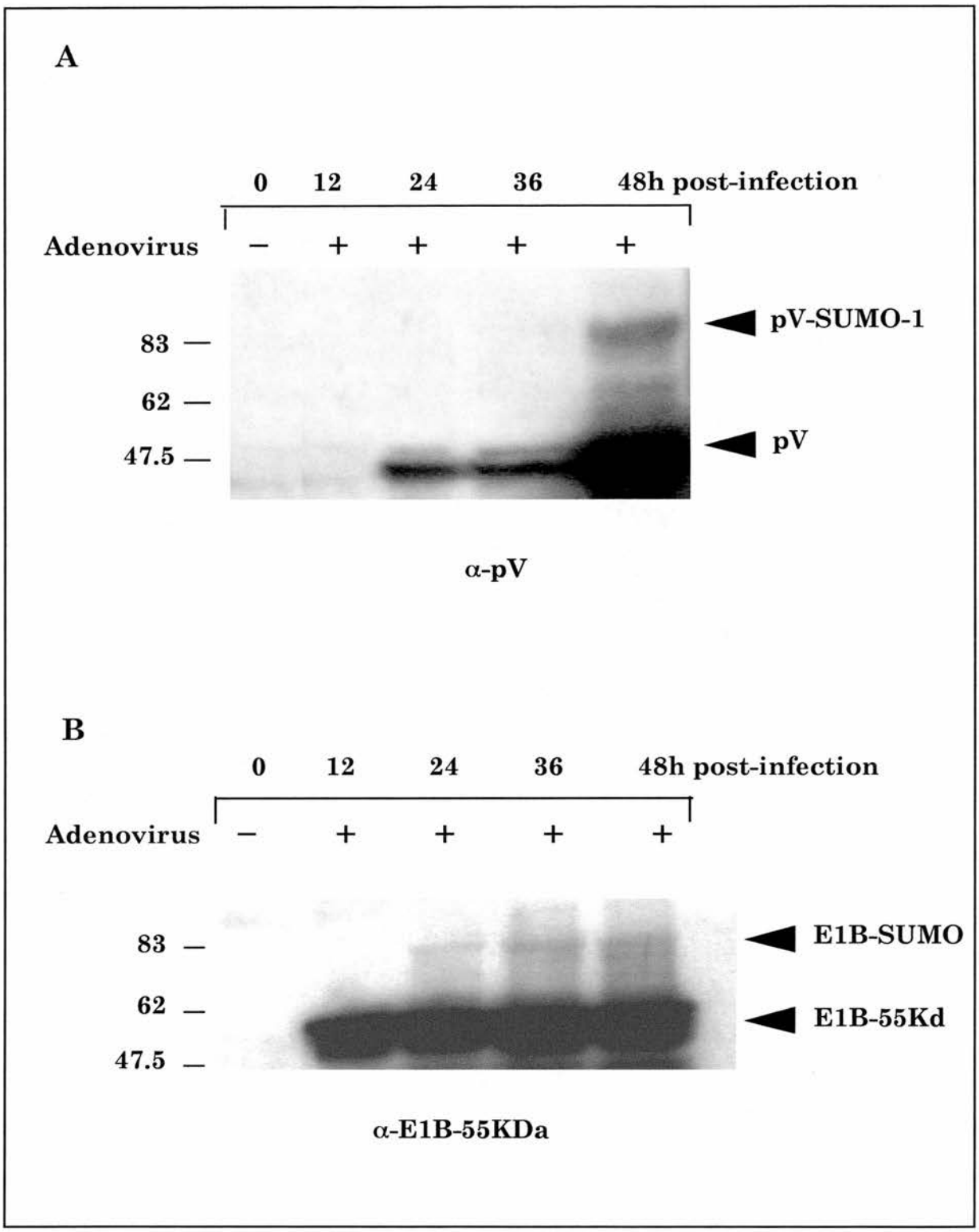


Figure 6. Expression of Adenovirus protein during infection.

(A) Hela cells were infected with Ad2 with a multiplicity of 20 p.f.u. per cell. Sample were collected for different times post-infection. The cell extracts were then fractionated by SDS-PAGE and pV analysed by Western blotting, using a pV specific polyclonal antibody. **(B)** The same samples, obtained as described above, were tested for the presence of E1B-55KDa adenovirus protein, using a specific E1B-55KDa monoclonal antibody.

In both cases, the presence of a band of higher molecular weight could indicate that E1B-55KDa and pV were undergoing a covalent modification. Given that SUMO-1 modification is increased after infection and that the modified species were approximately 20KDa larger than the predicted molecular weight of the primary translation product, it was suspected that the more slowly migrating species could represent SUMO-1 modified species.

3.2 SUMO-1 modification and Ubiquitination of E1B-55KDa *in vivo*

To test the hypothesis that the E1B-55KDa protein was a substrate for SUMO-1 modification, COS7 and 293 cells were transfected with a vector expressing a His-tagged SUMO-1 protein. These two cell lines were used because of the efficiency of transfection, and in particular 293 are constitutively expressing the E1B protein, so that it should be possible to detect also small amount of modified protein. After being transfected, cells were also infected for 24 hours with Ad2. At 0 and 24 hours post-infection, cells were lysed in 6M guanidine-HCl and the extracts were subjected to affinity purification with nickel-charged agarose beads to recover proteins covalently bound to 6his SUMO-1 or ubiquitin. Materials bound to the beads were then separated by SDS-PAGE and analysed by Western blotting with the anti-E1B-55KDa antibody (fig. 7). Uninfected COS-7 cells serve as a negative control. With both cell lines (lanes 5, 6 and 8) a 6His SUMO-1 modified species of apparent molecular weight 80KDa is detected. This species is present at very high level in both uninfected and infected 293 cells (lanes 5 and 6). This indicates that in adenovirus infected HeLa cells and in

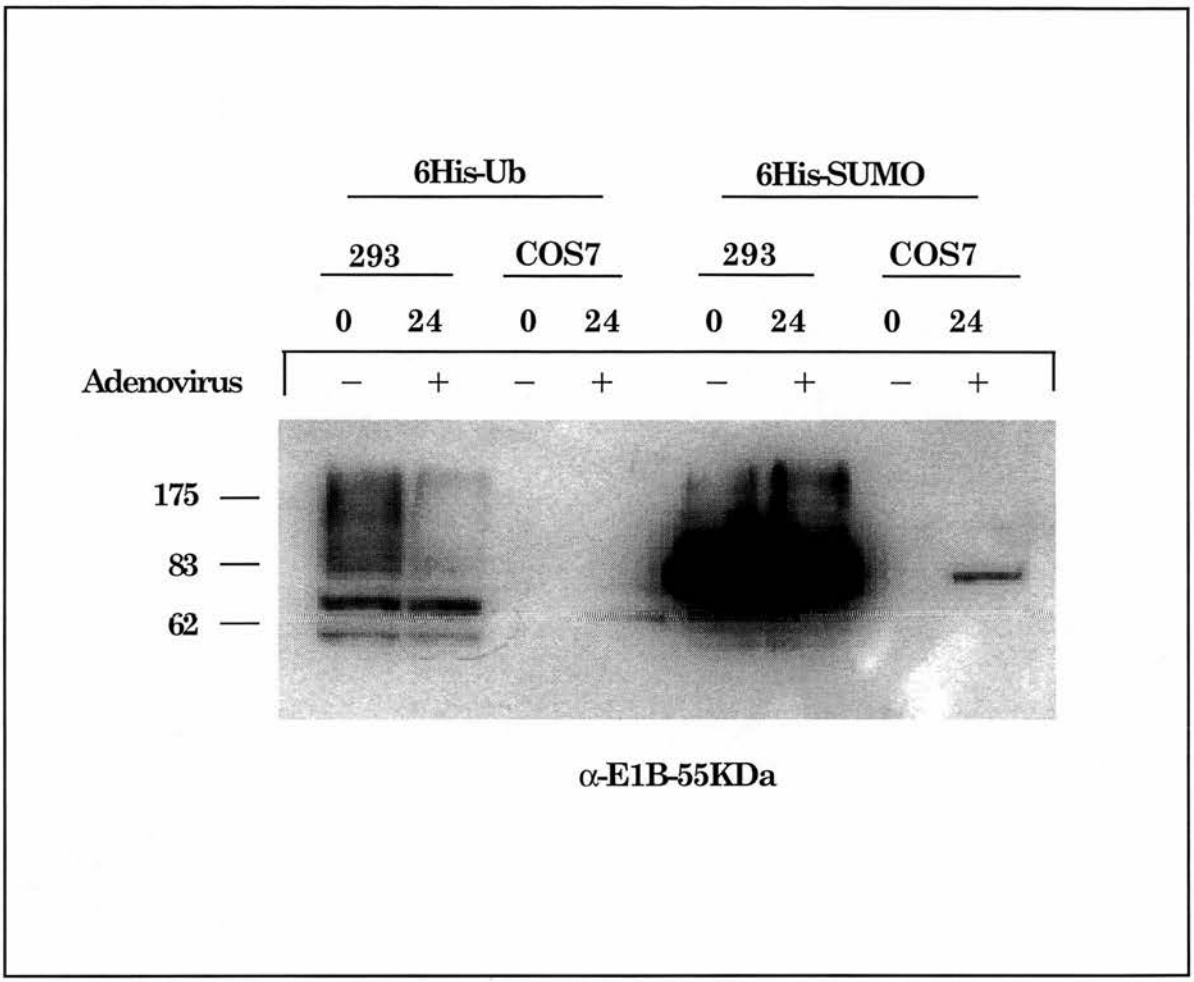


Figure 7. SUMO-1 modification and Ubiquitination of E1B-55KDa in 293 and COS-7 cells

6His-tagged SUMO was conjugated to E1B-55KDa *in vivo*. 293 and COS-7 cells were transfected with 6His-tagged SUMo and 6His-tagged Ubiquitin as indicated. Transfected cells were then infected with Ad2 for 24 hours. Cells were lysed in guanidine-HCl buffer, and proteins linked to 6His-tagged SUMO (or Ubiquitin) were purified using Ni-agarose beads and after exstensive washing eluted with 200mM imidazole. Eluted proteins were fractionated by SDS-PAGE and transferred to a PVDF membrane. 6His-SUMO (or Ub) conjugates were detected by Western blotting using an anti-E1B55KDa antibody. Molecular weight markers are shown on the left.

adenovirus transformed cells the early adenovirus E1B-55KDa protein is a substrate for SUMO-1 conjugation.

As E1B-55KDa has been reported to be involved with E4Orf6 in targeting p53 for degradation via the proteasome pathway, His-tagged Ubiquitin was also transfected in 293 and COS-7 cells. The samples were processed as described above. Western blot analysis indicates that E1B-55KDa is undergoing substantial ubiquitination in uninfected 293 cells and this is reduced after adenovirus infection (lanes 1, 2).

It has been shown that E1B-55KDa is a member of a multiprotein complex that targets p53 for degradation thus blocking an apoptotic response following infection. It was therefore of interest to determine the Ubiquitination and SUMO-1 conjugation status of p53, in the same conditions used for E1B-55KDa. In this case the different p53 adducts were detected with a monoclonal p53 antibody, DO-1 (Fig. 8). For different cell lines p53 basal level was detected; COS-7 and 293 cells, being transformed respectively with SV40 T-antigen and E1 adenovirus proteins, showed an higher level of p53 with respect to HeLa cells (fig. 8B). In particular p53 level in 293 cells appears to be higher with respect to COS-7 cells, and after adenovirus infection p53 level is decreased as expected.

As already shown, p53 is a substrate of Ubiquitin mediated degradation (Maki et al., 1996) and of SUMO-1 conjugation (Rodriguez et al., 1999). The 293 cells show a higher level of SUMO-1 modified p53 with respect to COS-7, which may be related to the fact that 293 are transformed cells already expressing the adenovirus E1A and E1B proteins. Indeed it is the E1A protein that causes an increase of p53 level following infection. However after infection, in order to avoid an apoptotic response, the increase

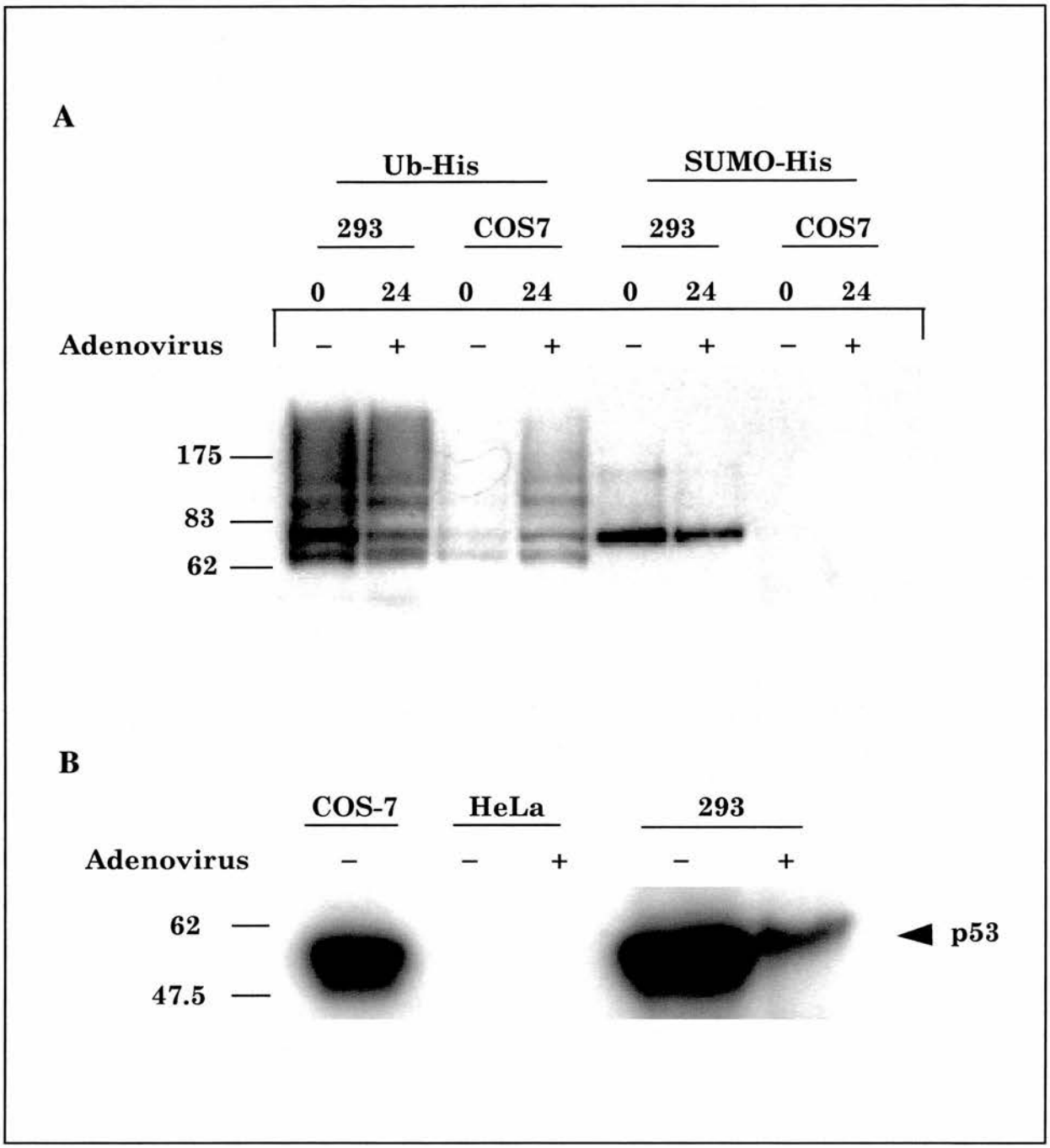


Figure 8. SUMO-1 modification and Ubiquitination of p53 in 293 and COS-7 cells
(A) 293 and COS-7 cells were transfected with 6His-tagged SUMO and 6His-tagged Ubiquitin as indicated. Transfected cells were then infected with Ad2 for 24 hours. Cells were lysed in guanidine-HCl buffer, and proteins linked to 6His-tagged SUMO (or Ubiquitin) were purified using Ni-agarose beads and after extensive washing eluted with 200mM imidazole. Eluted proteins were fractionated by SDS-PAGE and transferred to a PVDF membrane. 6His-SUMO (or Ub) conjugates were detected by Western blotting using an anti-p53 antibody. Molecular weight markers are shown on the left. **(B)** Cell extracts from COS-7, HeLa and 293 cells were fractionated by SDS-PAGE and analysed by Western Blotting to check the different level of p53 in these cell lines.

in production of p53 is abrogated by other adenovirus proteins, like E4Orf6 and E1B-55KDa, leading to a degradation of p53. This may explain why after infection the SUMO-1 modified p53 form is decreasing. As regards ubiquitination, an increase in ubiquitination is expected as a consequence of the triggered p53 degradation. Indeed, after infection the level of p53 ubiquitination in COS-7 cells is much higher with respect to uninfected cells. However, when 293 cells were transfected with His-tagged Ubiquitin and subsequently infected, analysis of the proteins eluted from the Ni-agarose beads showed a slight decrease in the ubiquitination of p53. The other protein required for p53 degradation in infected cells is E4Orf6, this protein is expressed only after infection of 293 cells, so it is possible that the expression of this product may, in same way, regulate the p53 adenovirus induced degradation.

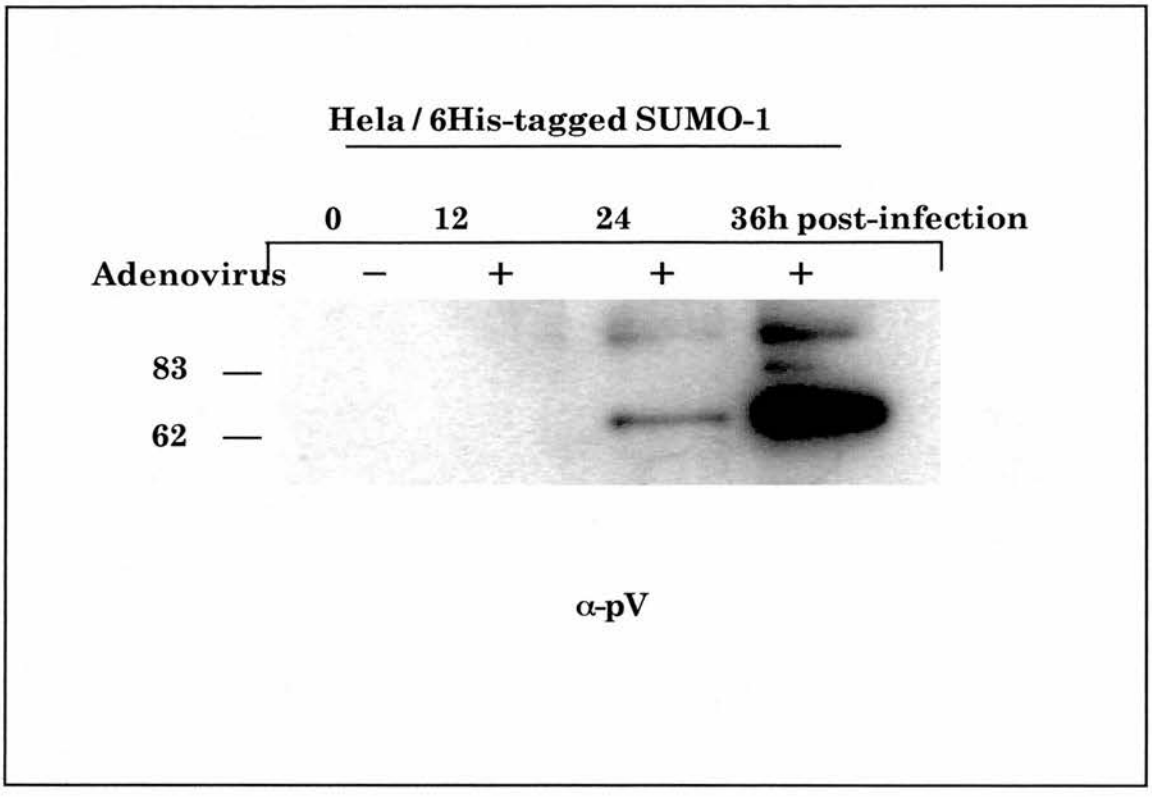


Figure 9. pV is SUMO-1 modified *in vivo*

Hela cells transformed with a plasmid expressing 6His-tagged SUMO-1 were infected with Ad2 with a multiplicity of 20 p.f.u. per cell and cells were lysed in a guanidine-HCl buffer at 0, 12, 24 and 36 hours post-infection. Proteins covalently linked to 6His-SUMO-1 were purified using Ni^{2+}-NTA-agarose beads and after extensive washing eluted with 200mM imidazole. Eluted proteins were then separated by SDS page on a 10% gel, transferred to a PVDF membrane and probed for pV.

3.3 SUMO-1 conjugation of pV *in vivo*

To verify that the adenovirus late protein pV was also covalently coupled to SUMO-1 in vivo, a particular HeLa cell line, stably expressing a 6His-tagged form of SUMO-1, was infected with adenovirus. Cells were lysed in guanidinium HCl and 6-His containing proteins eluted from Ni-agarose with imidazole. Western blotting with an antibody to pV (fig. 9) indicated that pV is also a substrate for SUMO-1 conjugation. Moreover the presence of higher molecular weight species with respect to the mono-SUMO-1 modified form suggests that pV could undergo multi-SUMO-1 modification. Indeed analysis of pV sequence is indicates that 3 regions of the protein contain the sequence ΨKxE, which represents the SUMO-1 modification consensus sequence. This hypothesis has then been tested in an *in vitro* system.

3.4 *In vitro* modification of pV and E1B-55KDa by SUMO-1

To investigate the nature of SUMO-1 modification of E1B-55KDa and pV, these two new substrates were tested in an *in vitro* system for SUMO-1 modification (Desterro et al., 1998). ^{35}S-labelled *in vitro* translated E1B-55KDa and pV were incubated with a source of SUMO-1 activating enzyme (SAE1/2) (Desterro et al., 1999) and SUMO-1 conjugating enzyme (Ubch9) (Desterro et al., 1997) in the presence of SUMO-1 and ATP. Under these conditions ^{35}S-labelled E1B-55KDa and pV were converted to a more slowly migrating form that is consistent with SUMO-1 modification. To confirm that this species was indeed a SUMO-1 modified product, GST-SUMO-1 was substituted for SUMO-1 in the reaction, resulting in the detection of a modified species with altered electrophoretic mobility (fig. 10/ 11). SUMO-1

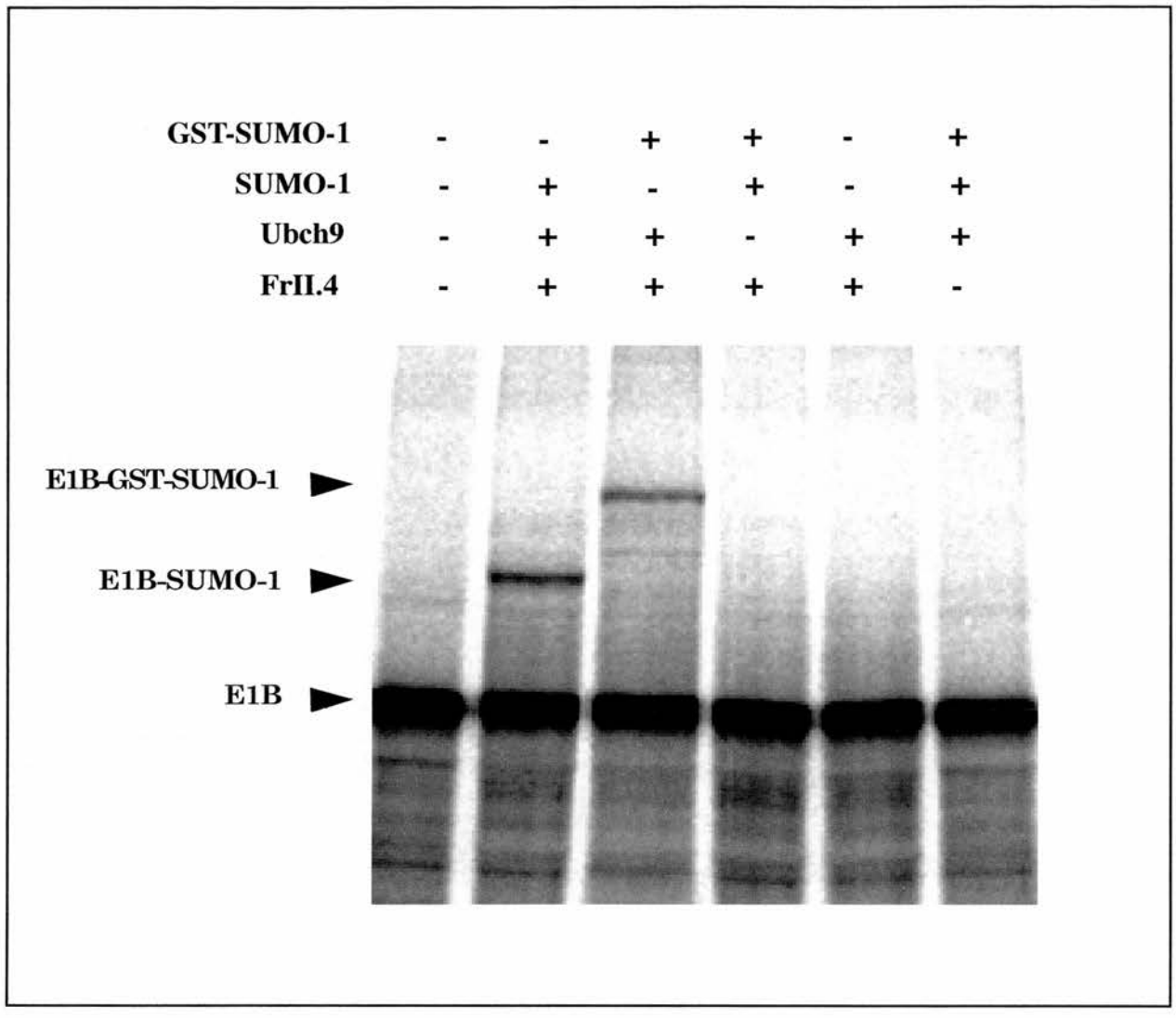


Figure 10. In vitro conjugation of SUMO-1 to E1B-55KDa
In vitro expressed and ^{35}S labelled E1B-55KDa was incubated with ATP, recombinant SUMO-1 or GST-SUMO-1, Ubch9, and Hela FrII.4 containing SUMO-1 E1 activity (SAE) as indicated. Reaction products were fractionated by SDS-PAGE, and the dried gel was analysed by phosphorimaging. The positions of E1B, the SUMO-1 conjugated form of E1B and the GST-SUMO-1 conjugated form of E1B are indicated.

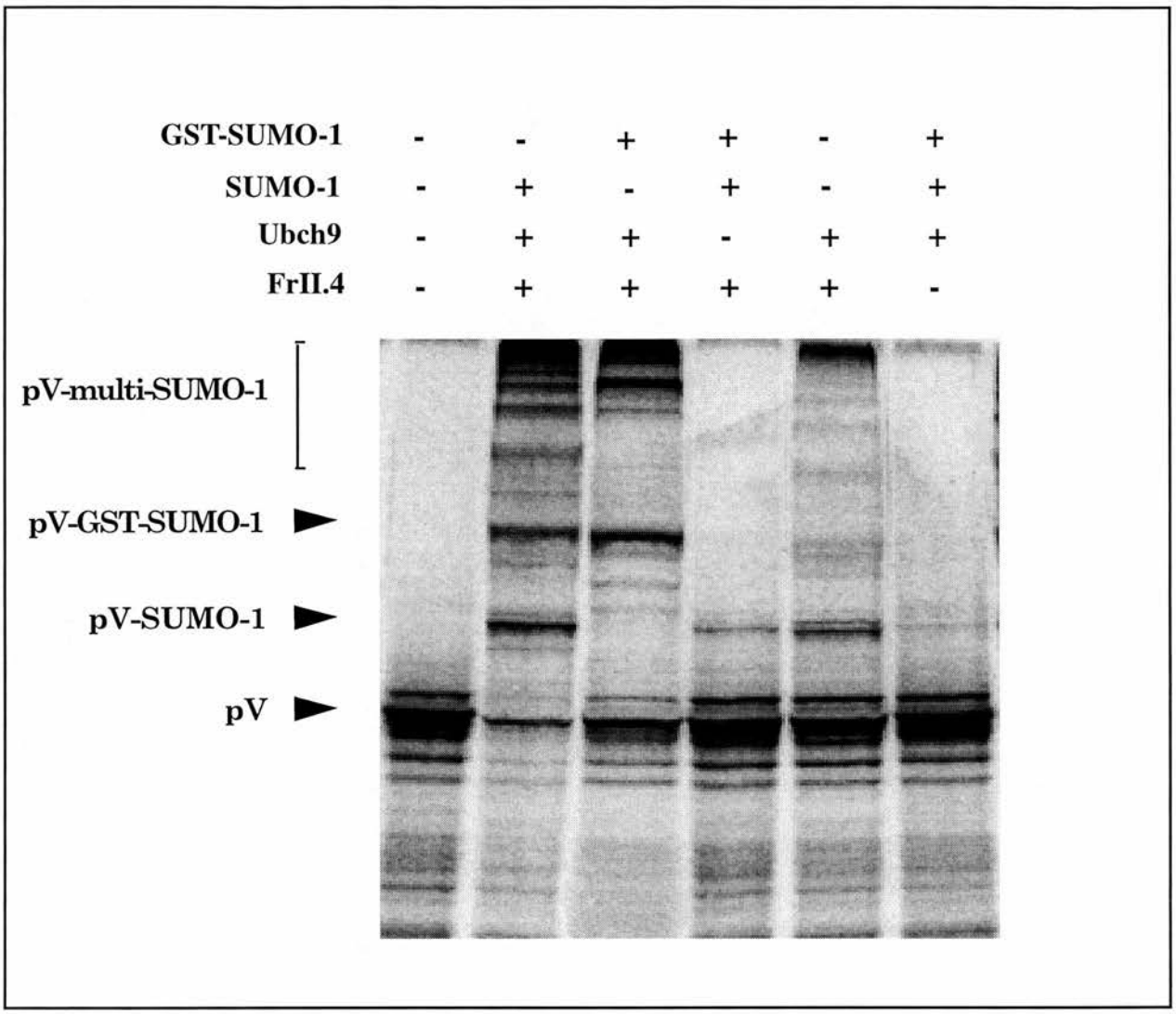


Figure 11. *In vitro* conjugation of SUMO-1 to pV
In vitro expressed and ^{35}S labelled pV was incubated with ATP, recombinant SUMO-1 or GST-SUMO-1, Ubch9, and Hela FrII.4 containing SUMO-1 E1 activity (SAE) as indicated. Reaction products were fractionated by SDS-PAGE, and the dried gel was analysed by phosphorimaging. The positions of pV, the mono-SUMO-1 conjugated form of pV and the GST-SUMO-1 conjugated form of pV are indicated.

modification was abolished if either SUMO-1, SAE, Ubch9 or ATP was omitted from the reaction. While E1B-55KDa (fig. 10) is clearly mono-SUMO-1 modified, pV appears to be conjugated to multiple SUMO-1 molecules *in vitro* (fig. 11). This indicates that pV, like PML, is a substrate for multiple SUMO-1 conjugation. Whether this multiple conjugation is due to covalent linkage of a poly-SUMO-1 chain or to the modification of many different lysine residues is not known. As there is no precedent for formation of a poly SUMO-1 chain, it seems likely that more lysine residues could be sites for SUMO-1 conjugation. Indeed analysis of the pV aminoacid sequence indicates that three different lysine residues (Lys 7, Lys 23, Lys 162) conform to the consensus sequence (-YKxE-) for this modification. In the E1B-55KDa primary structure, there is, indeed, only one lysine residue matching with the SUMO-1 conjugation consensus sequence, Lys 104.

3.5 Level of SUMO-1 modified cellular proteins during infection: RanGAP

Following Adenovirus infection, a general increase in the amount of SUMO-1 conjugation is observed. The finding that two viral protein, E1B-55KDa and pV, were themselves substrates for SUMO-1 modification, is consistent with this observation, but it is unlikely that this alone could be sufficient to amount for the increase in SUMO-1 conjugation. To address this question and to further investigate the observations regarding the effects of the infection on this cellular process, it is essential to examine what happens to cellular protein involved directly (as enzymes) or indirectly (as substrates) in this process.

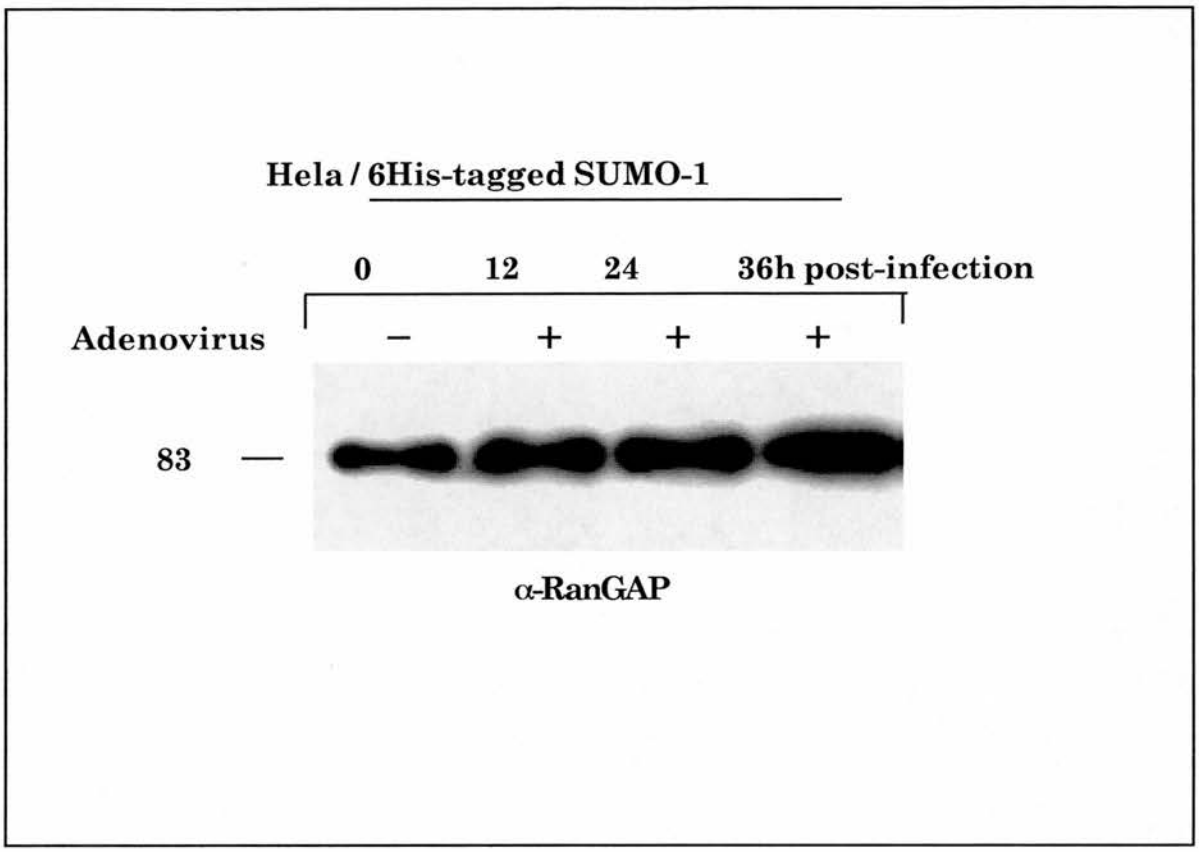


Figure 12. Level of SUMO-1 modified RanGAP during adenovirus infection
Hela cells stabley transformed with a plasmid expressing 6His-tagged SUMO-1 were infected with Ad2 in a time course experiment and cells were lysed in a guanidine-HCl buffer at 0, 12, 24 and 36 hours post-infection. Proteins covalently linked to 6His-SUMO-1 were purified using Ni^{2+}-NTA-agarose beads and after extensive washing eluted with 200mM imidazole. Eluted proteins were then separated by SDS page on a 10% gel, transferred to a PVDF membrane and probed using an anti-mouse monoclonal antibody specific for RanGAP. It is evident that the level of the SUMO-1-RanGAP adduct is increasing gradually during the infection.

In the cell, one of the major substrates for SUMO-1 conjugation is RanGAP. To test if and how the level of the SUMO-1 modified form of RanGAP varies during the infection, a HeLa cell line stably transformed with a plasmid expressing 6His-tagged SUMO-1 was infected with Ad2. Proteins covalently attached to the tagged version of SUMO-1 were purified as described above, and samples were analysed by Western blotting using a specific monoclonal antibody for RanGAP (fig. 12). The time course experiment clearly reveals an increase in the amount of SUMO-1 conjugated RanGAP, showing that the general increase in the SUMO-1 conjugation pattern is on one hand due to the availability of new substrates after the infection, and on the other is due to the increase of the level of modification of substrates already present.

3.6 Enzymes involved in SUMO conjugation and Adenovirus infection

While it has been established that adenovirus infection alters the pattern of SUMO-1 modification, the mechanism by which this is achieved remains to be determined. One possibility is that the levels, activity or subcellular localisation of the SUMO-1 activating enzyme (SAE) and SUMO-1 conjugating enzyme (Ubch9) are modified during infection.

To test this possibility extracts prepared from infected HeLa cells at 0, 12, 24, 36 and 48 hours p.i., were separated on a 10% gel, blotted and probed for SAE (fig. 13A) and Ubch9 (fig. 14). Although there is no evidence for variation in the basal level of these two enzymes during the infection, it is important to underline, in the case of SAE (fig.13A), the presence of additional higher molecular weight immunoreactive species, that are not present in the uninfected extract. To show that these higher molecular

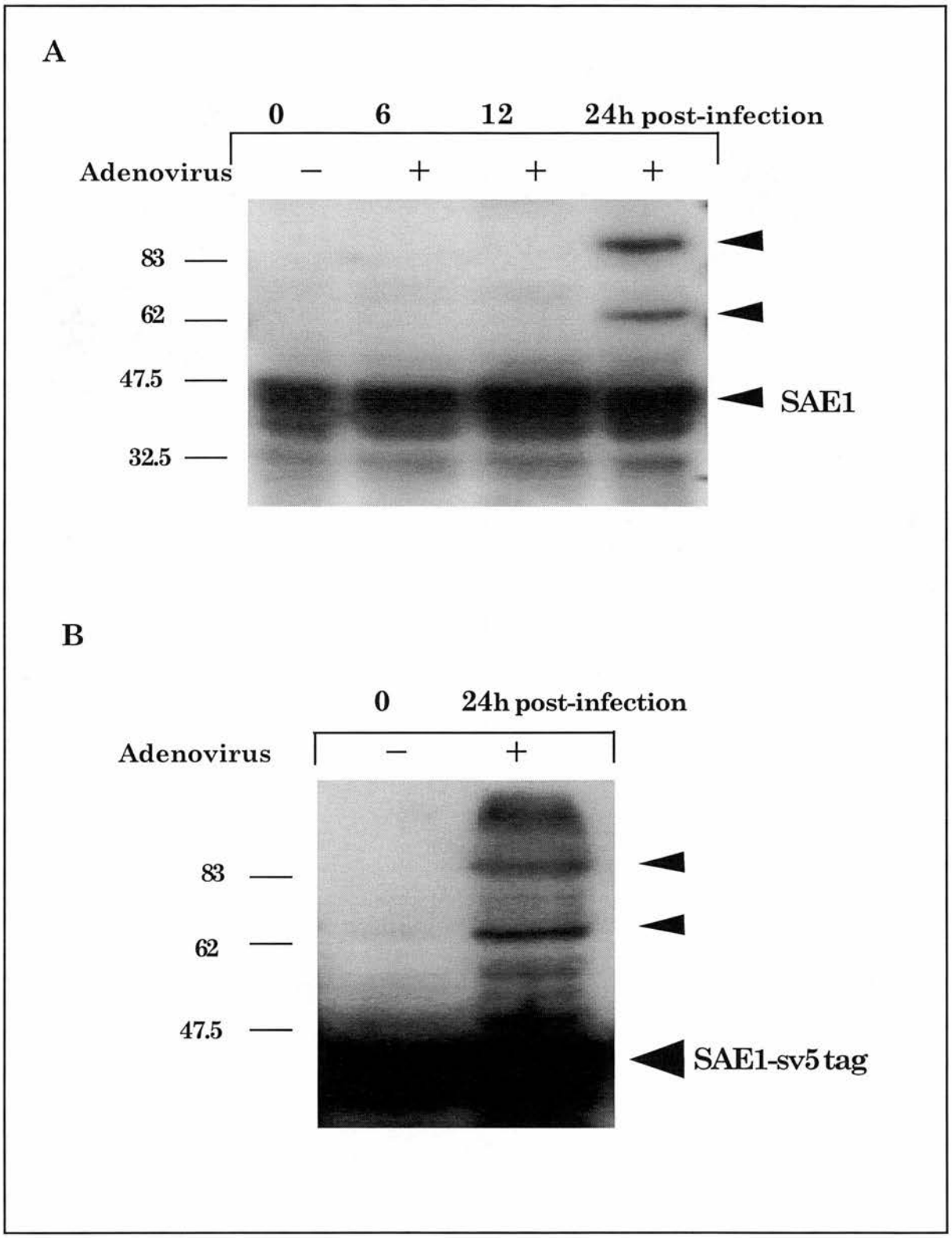


Figure 13. Level of SAE1 during adenovirus infection

(A) Hela cells were infected with Ad2 and total cell extracts were prepared at 0, 6 12 and 24 hours post infection. Extracts were then fractionated by SDS-PAGE and analysed by Western blotting, using a specific antibody against the 35KDa subunit of the Sumo activating enzyme (SAE). The same experiment was done in 293 cells, reporting the same results (data not shown). **(B)** 293 cells were transfected with SV5-tagged SAE1 and infected with Ad2. Total cell extracts were prepared at 0 and 24 hours post-infection, and the samples were analysed by Western blotting using a monoclonal antibody against the SV5 tag.

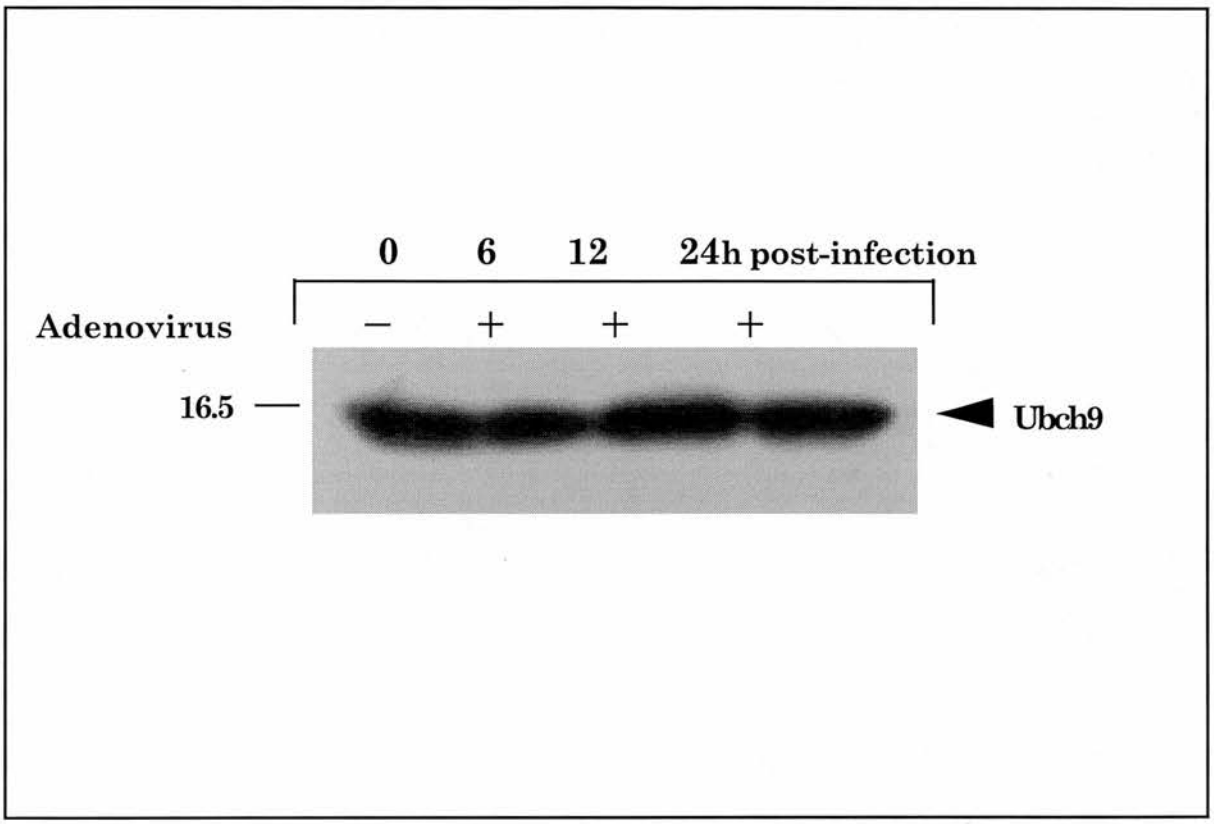


Figure 14. Levels of Ubch9 during adenovirus infection

1 x 10^6 Hela cells, for each time point, were infected with Ad2 and total cell extracts were prepared at 0, 6 12 and 24 hours post infection. Extracts were then fractionated by SDS-PAGE and analysed by Western blotting, using a specific antibody against the SUMO-1 conjugating enzyme, Ubch9.

weight species are related to SAE, cells were transfected with a vector containing SV5-tagged SAE1. Transfected cells were subsequently infected and total cells extracts were analysed as described above, using this time an antibody against the SV5 tag (fig. 13B). After infection it is possible to note the appearance of additional higher molecular weight species, which have a molecular weight comparable to, that found for the species detected with the antibody against SAE1, indicating that they are the same species. It is likely that these new species of SAE1 represent covalently modified forms of the protein, which may be linked to the increase shown in the SUMO-1 conjugation pattern.

3.7 Localisation of SAE1 and Ubch9 in adenovirus infected cells

To investigate the cellular localisation of the SUMO-1 activating (SAE) and conjugating (Ubch9) enzymes, HeLa cells grown on coverslip were infected with Ad2, at different times post-infection cells were fixed and stained with anti-SAE1 or anti-Ubch9 antibodies.

In both cases the proteins seems to have a nuclear localisation in uninfected cells. As for SAE1, there is no variation in the localisation after 12 or 24 hours of infection; indeed the protein showed a nuclear stain without any particular localisation to subnuclear compartments (fig. 15). The Ubch9 protein has a nuclear stain both in uninfected and infected cells, but at late time of infection it is possible to detect peculiar subcellular structures very similar to the viral replicative foci (Fig. 16).

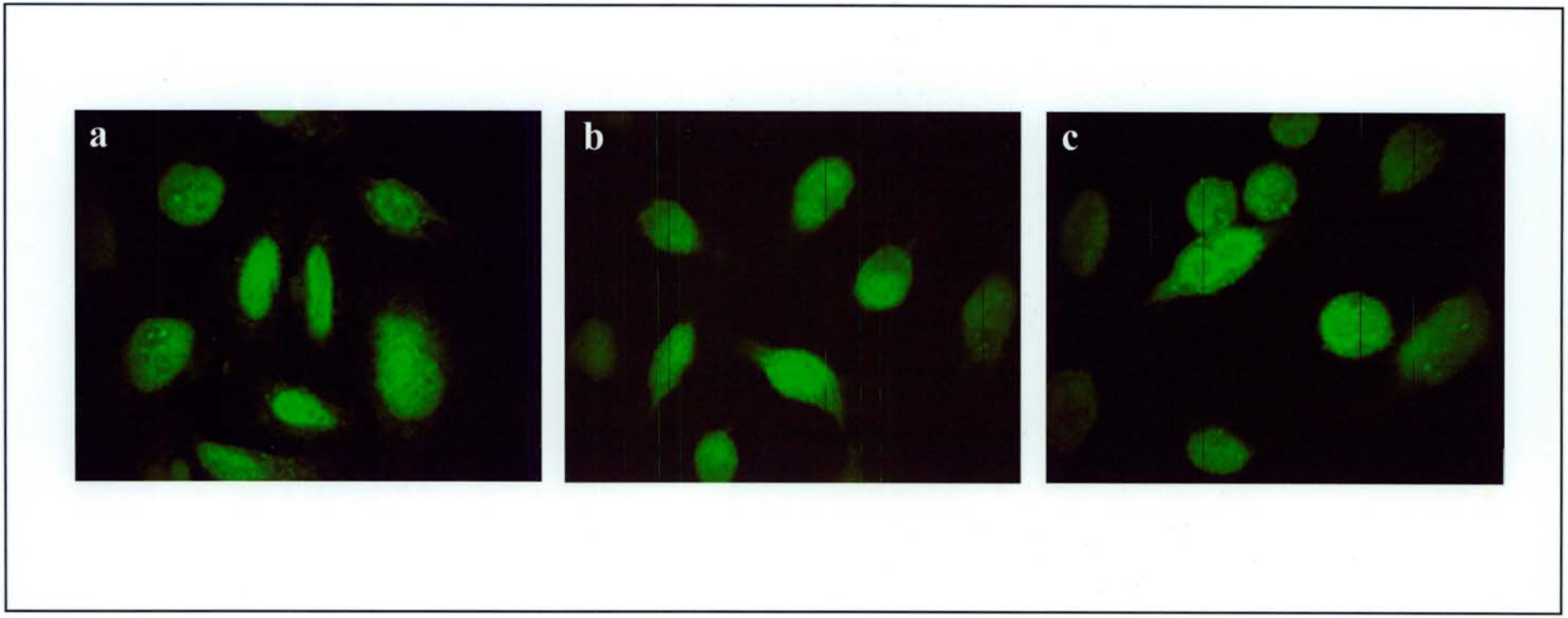


Figure 15. Localisation of SAE1 in adenovirus infected cells
HeLa cells were infected with Ad2, at 0, 12 and 24 hours post-infection cells were fixed and stained with a specific antibody recognising SAE1. This specific signal was detected using as secondary antibody an FITC-conjugated anti-sheep Ig. **a)** Uninfected cells. **b)** 12 hours post-infection. **c)** 24 hours post-infection.

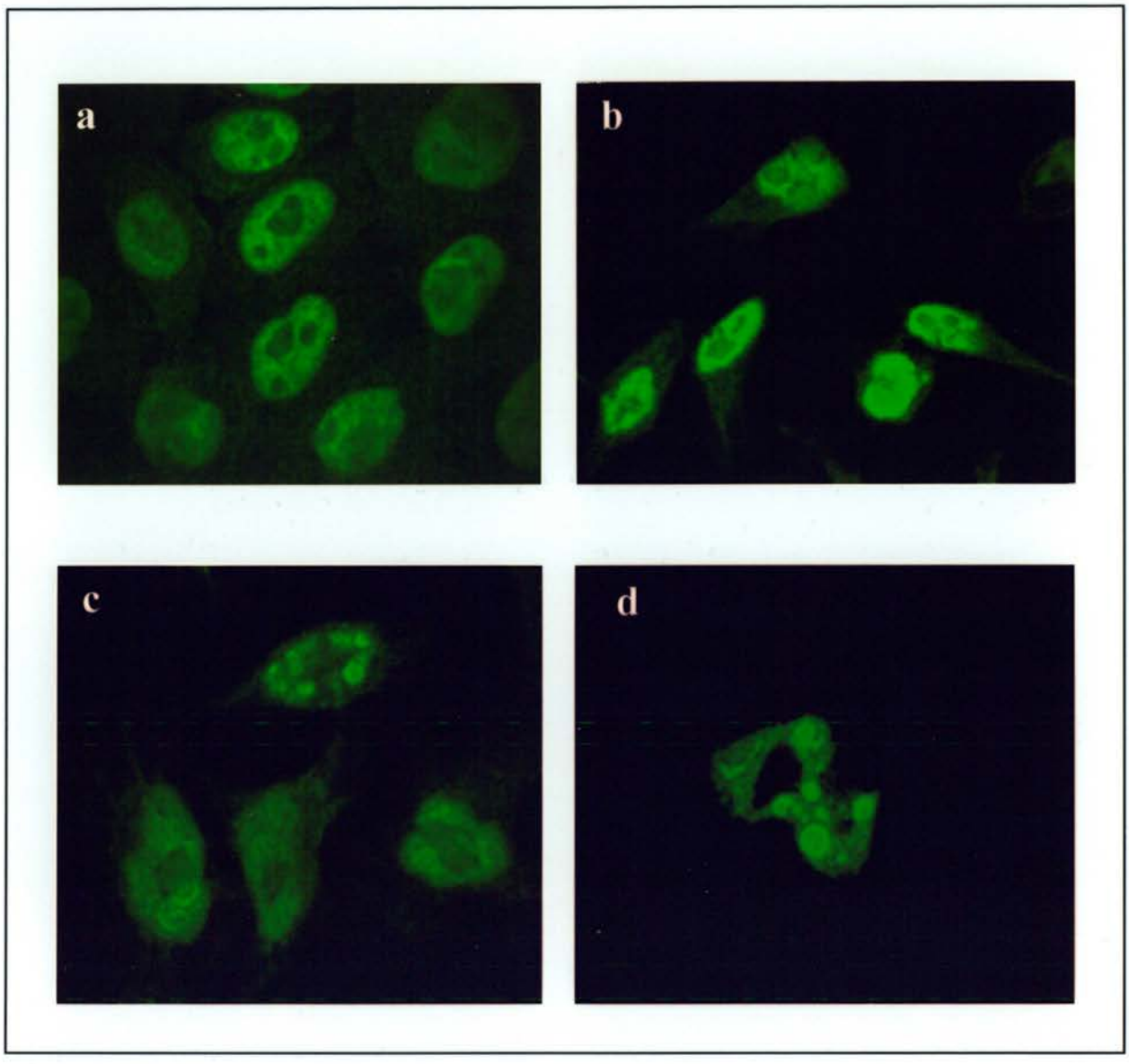


Figure 16. Localisation of Ubch9 in adenovirus infected cells
HeLa cells were infected with Ad2. At 0, 12 and 24 hours post-infection cells were fixed and stained with a specific antibody recognising Ubch9. The signal was detected using as secondary antibody an FITC-conjugated anti-sheep Ig. **a)** Uninfected cells. **b)** 12 hours post-infection. **c)** and **d)** 24 hours post-infection, it seems that at this stage the Ubch9 is reorganised in a peculiar "ring" structure into the nuclei.

3.8 Co-localisation of E1B-55KDa and pV with SUMO-1

Since E1B-55KDa and pV are substrates for SUMO-1 conjugation *in vivo* and *in vitro*, it was of interest to determine if E1B-55KDa, or pV, and SUMO-1 are localised to identical sites in Ad2 infected cells.

For this purpose, HeLa cells were transfected with HA-tagged SUMO-1 and infected with Ad2 with a m.o.i of 2 p.f.u. per cell. Successively cells were fixed and stained with both anti-E1B-55KDa and polyclonal HA (to visualised transfected SUMO) (fig. 17). Upon merging of the two signals, it can be seen that the two signals overlap, as indicated by the yellow signal shown in fig. 17c.

Moreover, during adenovirus infection a transient association of E1B-55KDa with reorganising PML bodies has been reported previously (Doucas et al., 1996; Leppard and Everett, 1999). The finding that E1B is SUMO-1 modified could then be related to this association, since some of the proteins localised to this structure are SUMO-1 modified and for some of them it has been hypothesised a correlation between the SUMO-1 modification and the localisation to the PML bodies.

To test a co-localisation between E1B-55KDa and one of the PML component of these bodies, cells were infected with Ad2 a double stain with a monoclonal anti-E1B and a polyclonal PML antibody has been performed. The collected data (Fig. 19) showed that only a portion of the E1B present in the cell is associated to PML tracks. This is in agreement with what has already been reported upon infection; in fact PML is reorganised from a well defined foci structure of uninfected cells into a set of tracks.

As regards pV, infected cells were double stained with a monoclonal anti-SUMO-1 antibody and a polyclonal anti-pV. The merging of the two signals reveals a

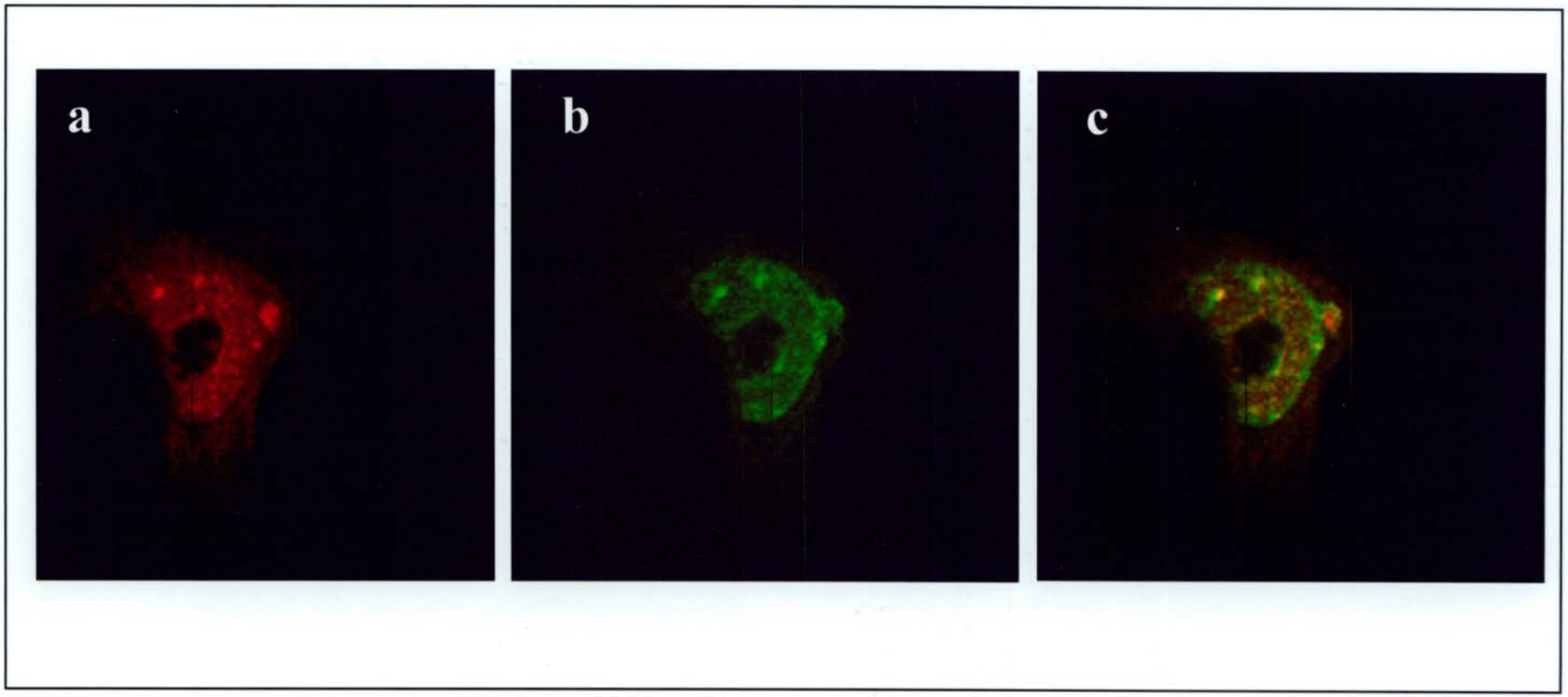


Figure 17. Colocalisation of E1B-55KDa and SUMO-1

HeLa cells were transfected by electroporation with HA-tagged SUMO-1 and infected with Ad2. At 12 hours post-infection, cells were fixed and double stained with a monoclonal anti E1B-55KDa and a polyclonal anti-HA. **a)** E1B-55KDa staining detected using as secondary antibody a TexasRED-conjugated anti-mouse Ig. **b)** HA (SUMO) staining detected using as secondary antibody an FITC-conjugated antirabbit Ig. **c)** Merge of the two signals, the yellow colour indicates co-localisation.

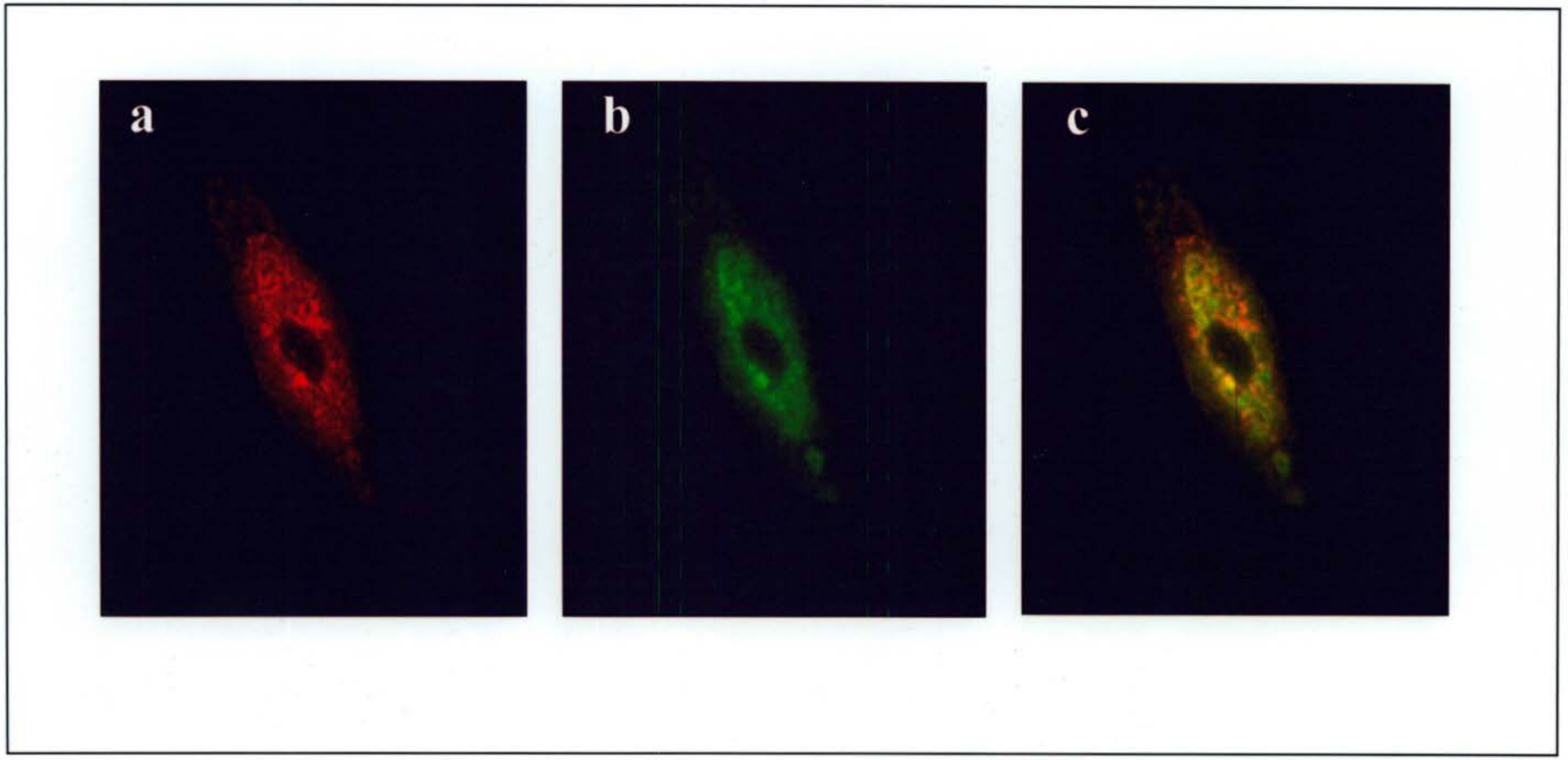


Figure 18. Colocalisation of V and SUMO-1

HeLa cells were infected with Ad2 with a multiplicity of 2p.f.u. per cell. At 24 hour post infection cells were fixed and double stained with a monoclonal antibody against SUMO-1 and a polyclonal antibody recognising adenovirus protein V. **a)** SUMO-1 stain detected using as secondary antibody a TexasRED-conjugated anti-mouse Ig. **b)** pV stain detected using as secondary antibody an FITC-conjugated anti-rabbit Ig. **c)** Merge of the two signals, the yellow colour indicates co-localisation.

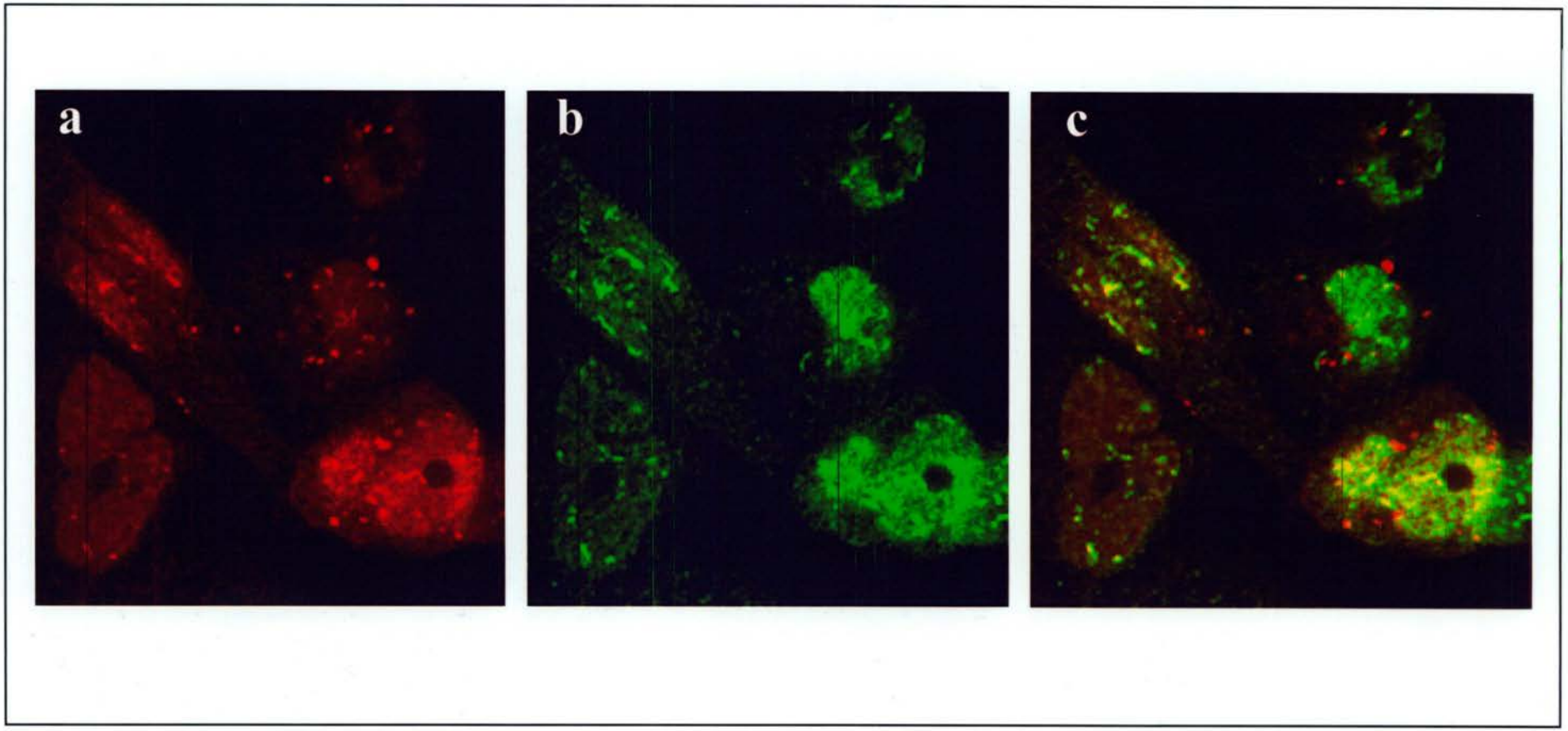


Figure 19. Colocalisation between E1B-55KDa and PML

HeLa cells were infected with Ad2 with a m.o.i of 2 p.f.u. per cell. After 12 hours of infection cells were fixed and double stained with a monoclonal anti E1B-55KDa and a polyclonal anti-PML. **a)** E1B-55KDa signal detected with a TexasRED-conjugated anti-mouse Ig. **b)** PML signal, detected with an FITC-conjugated anti-rabbit Ig. **c)** Merge of the two signals, the yellow indicates co-localisation. It is evident that just a portion of E1B-55KDa co-localises with PML.

co-localisation between these two proteins (Fig. 18), supporting the *in vivo* and *in vitro* observations already collected.

3.9 Adenovirus activates NF-kB response

It has been reported that a variety of adenovirus proteins are involved in modulating the activity of the transcriptional factor NF-kB (Schmitz et al., 1996) (Limbourg et al., 1996). In unstimulated cells NF-kB is held in an inactive state by its inhibitor IkBα. The activation of this transcription factor is dependent on signal induced degradation of IkBα. Moreover it has been recently discovered that IkBα undergoes SUMO-1 conjugation and that this new form is resistant to regulation (Desterro et al., 1998). Then the balance between Ubiquitination and SUMO-1 conjugation seems to be a key event through NF-kB activation.

In particular the E1A 13S gene product has been shown capable of activating the DNA-binding form of NF-kB and of enhancing NF-kB p65-dependent transactivation (Schmitz et al., 1996). Both these activating effects of E1A were counteracted by the E1B-19KDa protein (Limbourg et al., 1996).

To investigate the eventual activation of NF-kB following adenovirus infection, the cell line HeLa 57A which contains an integrated NF-kB dependent luciferase reporter, was infected with Ad2. At each time point, cells were lysed and NF-kB activity determined in a luciferase assay. Stimulation with TNF for 6 hours is used as a positive control. The data collected (Fig. 20), show an increase of NF-kB activity from 0 until 36 hours post-infection of 20 fold (100 fold for TNF stimulation). To determine if this activation was related to virus induced degradation of IkBα, HeLa cells were

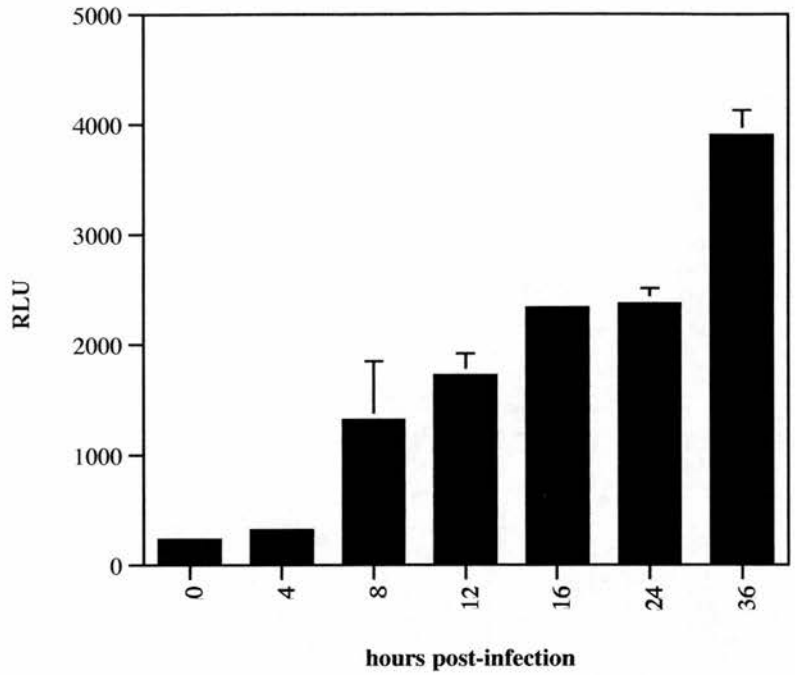


Figure 20. NF-kB activation after adenovirus infection

Hela 57A were infected with Ad2 for the times indicated and luciferase activity measured. Results were plotted as a measure of relative light units (RLU) against time of post-infection with Ad2. Error bars for 0, 4 and 16 hours post-infection were too small to be shown graphically.

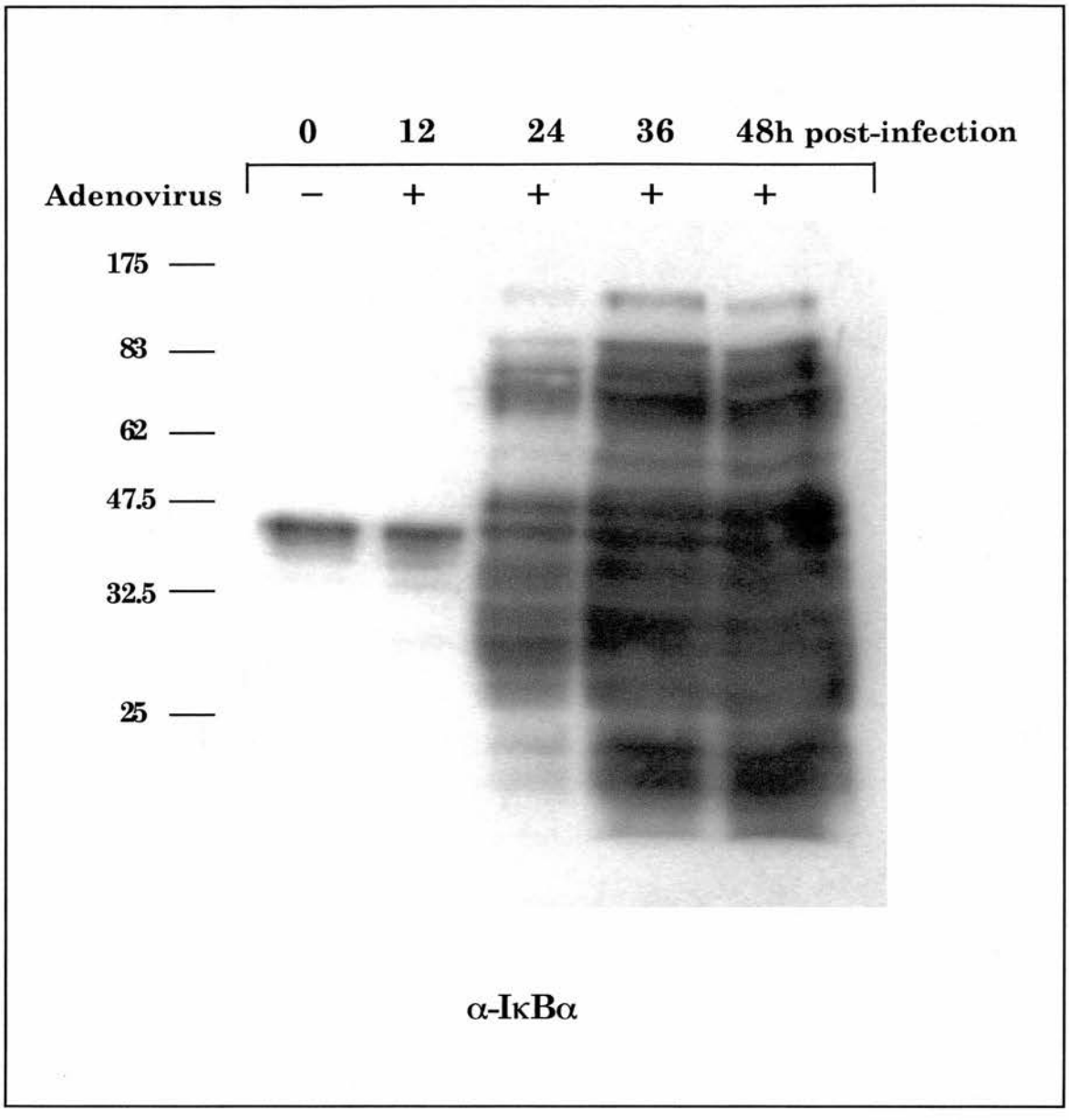


Figure 21. Effect of adenovirus infection on IκBα

1 x 10^6 HeLa cells for each time point were infected with Ad2 and total cells extracts were prepared at 0, 12, 24, 36 and 48 hours post-infection. The samples were then fractionated on a 10% polyacrylamide denaturing gel, transferred to a PVDF membrane and probed for IκBα.

infected with Ad2 and cells extracts were examined for the presence of IkBα, using a polyclonal antibody for IkBα (C-21) able to recognise both the SUMO-1 conjugated and ubiquitinated forms (fig. 21). After 12 hours of infection there is a reduction in the steady state level of IkBα, but this is followed by a drastic increase in IkBα species that appear to be covalently modified form of the protein.

3.10 The nuclear distribution of p65 is altered after adenovirus type 2 infection

To investigate a possible reorganisation of NF-kB in the cells after infection, immunofluorescence experiments were set up. HeLa cells were infected with Ad2, fixed and stained with antibodies to p65 and DBP (adenovirus DNA binding protein).

As a positive control cells on coverslip were treated with TNF for 30 minutes, indeed after TNF treatment it is possible detect a translocation of NF-KB from the cytoplasm to the nucleus (fig 22).

Cells infected and fixed at different times post-infection were stained for p65. It is evident that p65 progressively reorganised during the infection. Indeed while at 0h it is mainly localised into the cytoplasm, after 12h of infection a nuclear translocation, similar to that one induced with TNF, is detectable. But at 24 hours post-infection p65 appears completely reorganised into spherical subnuclear structures (fig. 22). To check that this redistribution is related to the infection, the same kind of experiment has been repeated, and the cells were double stained with p65 and an antibody against a viral protein, DBP, as a control for infection. Clearly, the phenomena observed are related to adenovirus infection (data not shown).

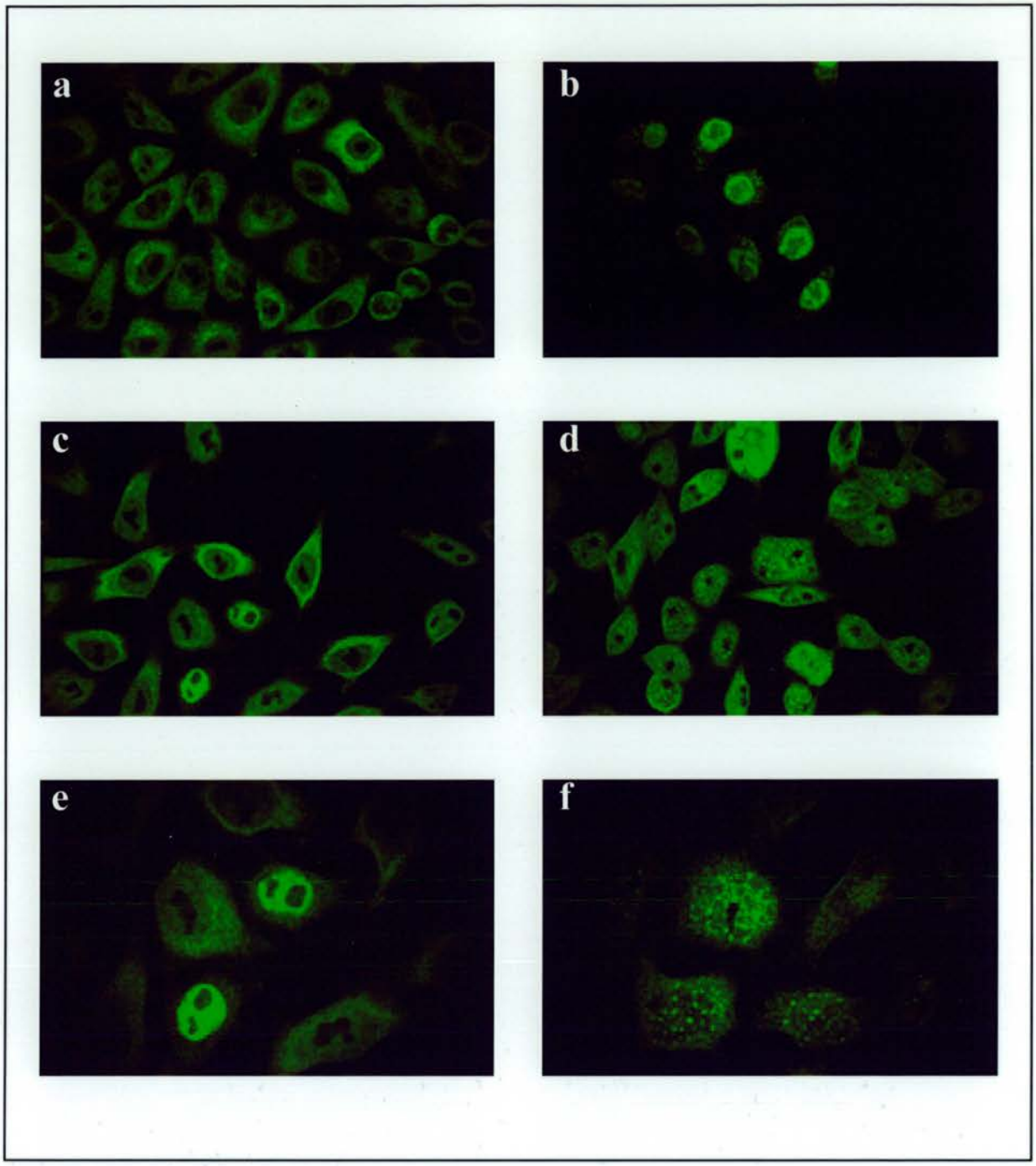
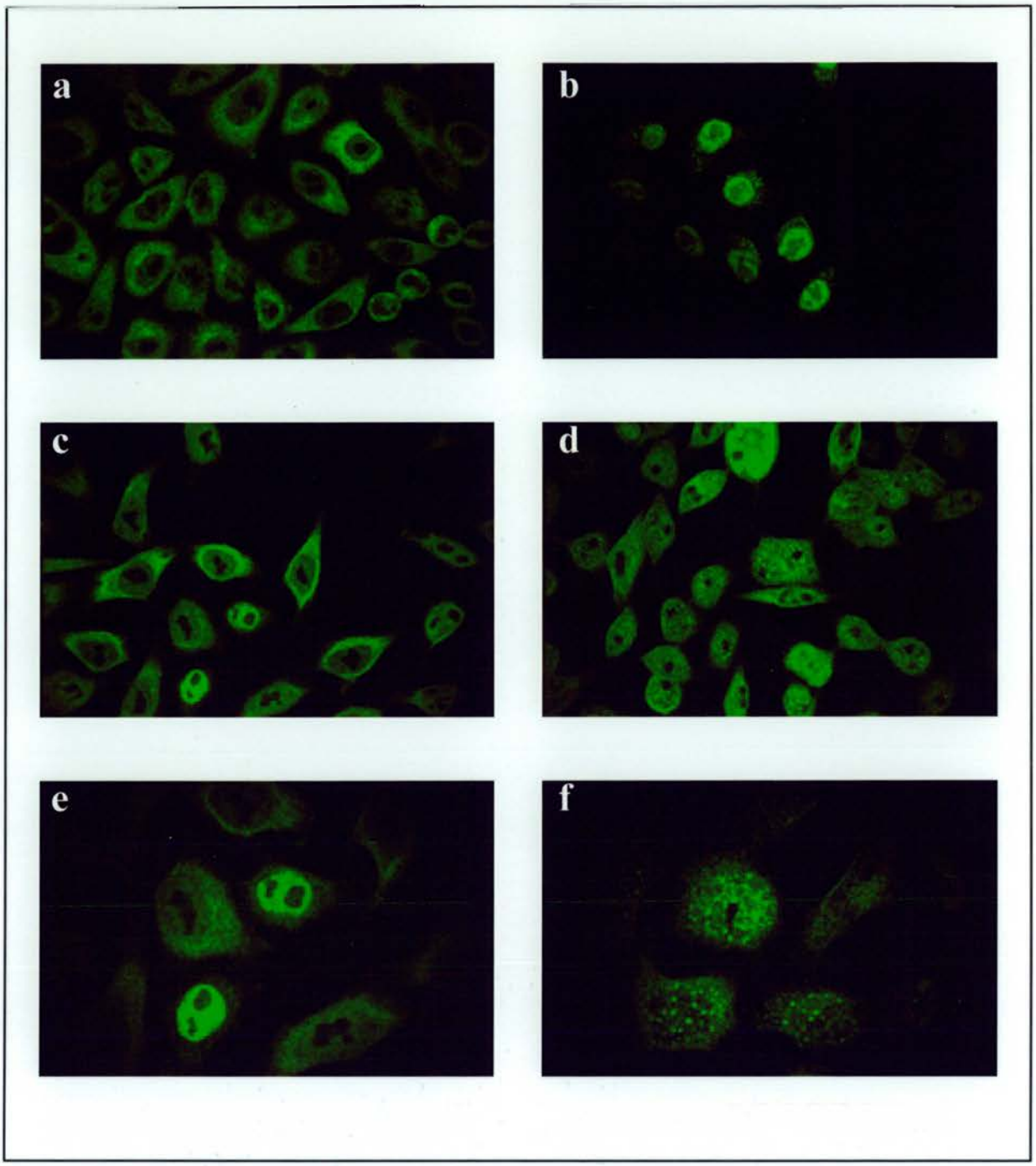


Figure 22. Distribution of p65 in adenovirus infected cells

HeLa cells were infected with Ad2 and cells were fixed and stained with an anti-p65 polyclonal antibody at 0, 12 and 24 hours post infection. **a)** Uninfected cells. **b)** Cells were treated with TNF for 30 minutes, fixed and stained as described. **c)** Cells at 12 hour post-infection, p65 is relocalised into the nuclei. **d)** Cells at 24 hour post-infection, p65 is reorganised in characteristic nuclear foci. **e)** and **f)** images corresponding respectively to c. and d. panels using an objective with a higher magnification to better visualise subcellular structures.

The similarity of these adenovirus induced foci of p65 to that observed with PML and/ or E1B-55KDa, lead us to check if there was any co-localisation between p65 and these two proteins. For this purpose, HeLa cells were infected with Ad2, fixed and double stained for E1B/ p65 in one case and for PML/p65 in the other. The data collected after 24 hours of infection, when it is possible to observe a redistribution of p65 into the nucleus, shown a partial co-localisation of p65 with PML tracks (fig. 23) and with E1B foci (fig. 24), comparable with the level of co-localisation of E1B-55KDa and PML. These findings indicate, in agreement with previous observations (Carvalho et al., 1995), that the subnuclear domains containing PML represent a preferential target for DNA viruses, either for a localisation of viral protein, or for a redistribution of cellular factors to these bodies. This could imply a key role of PML in regulate Adenovirus (and DNA virus, in general) replication.



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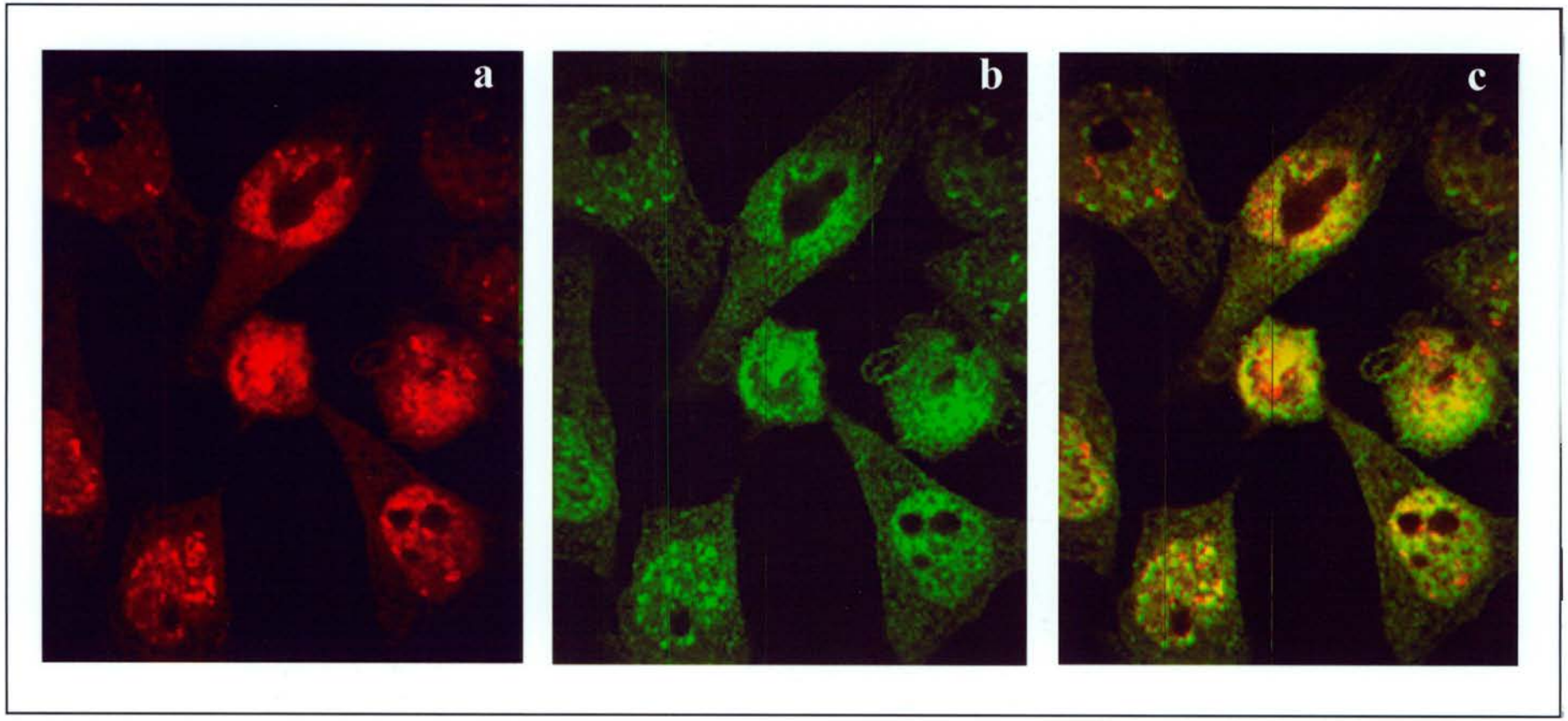

Figure 23. Co-localisation between p65 and PML in adenovirus infected cells
HeLa cells were infected with Ad2, at 24 post-infection cells were fixed and double stained with a monoclonal anti-PML and a polyclonal anti-p65 **a)** PML signal, detected using as secondary antibody a TexasRED-conjugated anti-mouse Ig. **b)** p65 signal, detected using as secondary antibody ar FITC-conjugated anti-rabbit Ig. **c)** Merge of the two signals, yellow colour indicates co-localisation; as for E1B and PML only a partial co-localisation is detectable.

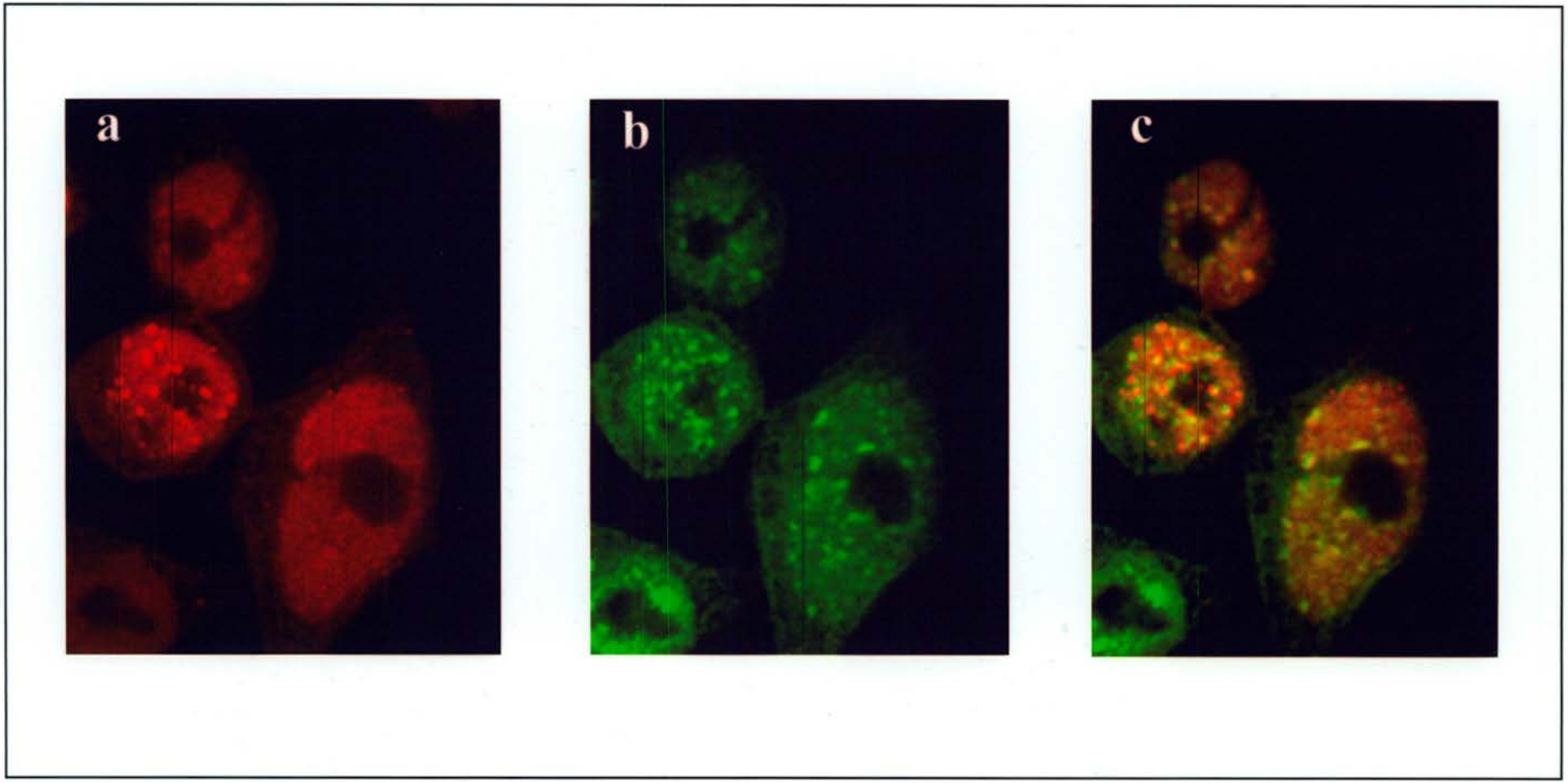

Figure 24. Co-localisation between p65 and E1B-55KDa in adenovirus infected cells
HeLa cells were infected with Ad2, at 24 hours post-infection cells were fixed and double stained with a monoclonal anti-E1B55KDa and a polyclonal anti-p65 antibody. **a)** E1B-55KDa signal, detected using as secondary antibody a TexasRED-labelled anti-mouse Ig. **b)** p65 signal, detected using as secondary antibody an FITC-conjugated anti-rabbit Ig. **c)** Merge of the two signals, yellow colour indicates co-localisation; it is possible to detect a partial co-localisation.

4. DISCUSSION

4.1 Adenovirus and SUMO-1 conjugation

To date, SUMO-1 modification has been shown to be involved in the targeting of substrate proteins to a specific subcellular compartment (as for RanGAP or PML) and in altering stability of target proteins so that they become resistant to ubiquitin-mediated degradation (as for IkBα). In yeast SUMO-1 conjugation is required for entry into mitosis (Li and Hochstrasser, 1999; Seufert et al., 1995). Moreover there are evidence that SUMO-1 conjugation of PML and sp100 are regulated in a cell cycle dependent manner (Everett et al., 1999). Thus it is likely that SUMO-1 conjugation represents a dynamic process by which the cell controls several processes, including cell cycle, differentiation and virus infection.

Viruses, in general, subvert various cellular processes to the advantage of a productive infection. In this work we have studied which are the effects of adenovirus infection on the SUMO-1 conjugation machinery.

Following adenovirus infection, it is possible to observe a redistribution of several proteins, both viral and cellular. For example, a dynamic redistribution of nuclear matrix proteins is detected (Carson et al., 1999), and subcellular localisation of late Ad2 non-structural 100 KDa and 33 KDa is altered with an increase in an accumulation of these two proteins into the nucleus (Gambke and Deppert, 1981). Moreover changes in nuclear morphology, with the appearance of new structures containing proteins and nucleic acid has already been reported (Reich et al., 1983; Voelkerding and Klessig, 1986). A typical example is indeed the formation of

replicative foci were cellular factors, as hnRNP, are shuttled and this may be involved in the block of accumulation of host mRNA. It is likely, then, that Adenovirus induces a structural reorganisation inside the cell.

As already mentioned, adenovirus infection causes a drastic redistribution of PML from spherical nuclear bodies into fibrous structures. The product encoded by adenovirus E4Orf3 is responsible for this reorganisation and it is shown to localise with PML into these fibres. As we have seen and as it has been described another viral protein is associated, even if transiently, with PML: E1B-55KDa (Doucas et al., 1996; Leppard and Everett, 1999). It has been reported that following adenovirus infection the SUMO-1 modified isoforms of PML disappear (Everett et al., 1998), this in some way correlates the disruption of PML bodies with the deconjugation of SUMO-1-PML adducts. On this basis, our founding that two viral proteins, pV and E1B-55KDa, undergo SUMO-1 modification could be coupled with the deconjugation of SUMO-1 from PML so that more competitive substrates are modified.

E1B is localised to the PML bodies by its interaction with E4Orf3. In light of this fact SUMO-1 conjugation of E1B could represent more than the driving force for the colocalisation to these structures, as it is for other substrates (PML) (Ishov et al., 1999), a mechanism for PML bodies disruption. Indeed, because deconjugated PML forms are associated with disrupted PML bodies, it is possible that once E1B-55KDa is recruited to the PML bodies by E4Orf3, SUMO-1 is deconjugated from PML, probably via a SUMO-1 deconjugating enzyme, and conjugated, via the usual pathway to E1B. Indeed, the level of free SUMO-1 in the cell is very low, and it is likely that SUMO-1 is continuously recycled via deconjugation and conjugation to target proteins, as PML.

Thus when an other substrate for this modification becomes available after infection, this sort of equilibrium could be altered by conjugation of the new substrate. Recent observations (Rodriguez, personal communication) seem to indicate that the SUMO-1 modification occurs in the nucleus. When E1B-55KDa is synthesised, it has a cytoplasmic localisation, but when the E4Orf3 protein is expressed E1B is transported from the cytoplasm to the nucleus, in particular to the PML bodies, where it is eventually SUMO-1 modified. Later in infection E1B is recruited into a complex with E4Orf6, which seems to be involved in modulating the cytoplasmic accumulation of both host and viral mRNA late in the infection (Ornelles and Shenk, 1991) and in targeting p53 for degradation.

The evidence of the increase in the SUMO-1 conjugation pathway following adenovirus infection are, then, the increase of the SUMO-1 modified form of the cellular substrate RanGAP and the presence of the two new substrates, E1B and pV. To investigate the causes and/ or the molecules involved in the alteration of the SUMO-1 conjugation process, we have analysed the effects of adenovirus infection on the enzymes involved in the SUMO-1 conjugation process: SAE and Ubch9.

For both enzymes there was no a detectable change in the basal level, but for the SAE it was possible to detect, after infection, the presence of higher molecular weight species. On these bases, one of the hypotheses could be that this represents more active forms of the SUMO-1 activating enzyme. Furthermore we looked at the cellular localisation of the SAE1 (35KDa subunit of the SUMO-1 activating enzymes) during adenovirus infection. These data indicate that the SAE1 is essentially nuclear both in

uninfected and infected cells, but there is no evidence of any reorganisation or translocation following adenovirus infection.

As regards the SUMO-1 conjugating enzyme, Ubch9, immunofluorescence studies revealed also a nuclear localisation in both uninfected and infected cells, but in this case adenovirus has an effect on the localisation of Ubch9. Indeed, after 24 hours of infection, using an antibody against Ubch9, new structures are detected into the nucleus. It is remarkable that Ubch9 undergoes a sort of reorganisation and these new structures are very similar to the replicative viral centres (Luis et al., 1993). Because a similar localisation was not observed for SAE1 it is difficult to postulate whether or not this is related to the SUMO-1 conjugation process, but an hypothesis could be that some of the proteins, such as splicing factors, presents in these viral replicative foci, undergoes SUMO-1 modification and this could represent an other important role for this new post-translational modification.

4.2 Adenovirus and NF-kB activation

It has been reported that, in transformed cells, the E1A 13S can activate the human transcription factor NF-kB, and this activation seems to be dependent on the phosphorylation of IkBα at ser32 and 36, followed by its degradation. Here, we have shown, that in adenovirus infected cells it is possible to detect an activation of NF-kB, corresponding to a 20 fold increase after 36 hours of infection with respect to the 0 time (uninfected cells). As regards IkBα, when we analyse the level of this protein in infected cells, we found that there is a stage in the infection were the production of IkBα is switched on and at the same time it is targeted for ubiquitin mediated

degradation. This is coherent with an activation of NF-kB, as the gene for IkBα is NF-kB dependent. Whether this activation is dependent or not on the phosphorilation at ser32 and 36 it is not clear and further experiments need to be done. Even if this activation was not related to the phosphorilation of those residues, it will not be surprising that the virus uses an alternative mechanism to induce the same effects, so to avoid cellular control.

The intracellular distribution of the transcription factor NF-kB has been examined in E1A transformed cells. Immunofluorescence studies have showed that the main component of this transcription factor, p65, was constitutively localised to the nucleus of these cells (Darieva et al., 1999).

We have examined the localisation of this component in HeLa cells after adenovirus infection. In uninfected cells p65, as expected, is predominantly localised into the cytoplasm, while after 30 minutes of TNF stimulation, p65 is translocated to the nucleus as a result of signal induced degradation of its inhibitor IkBα via the ubiquitin-proteasome pathway. Proceeding through the infection p65 undergoes an intracellular redistribution. At 12 hours post-infection p65 is clearly relocalised into the nucleus, while after 24 hours it is present in punctuate structures inside the nucleus. The activation of NF-kB transcriptional activity in the reporter cell line fits well with a relocalisation of p65 to the nucleus following IkBα degradation.

As regards the characteristic nuclear foci present after 24 hours of infection, one could hypothesise that at late stage of infection all p65 translocated into the nucleus is reorganised in these peculiar structures that could represent, then, a deposit of p65. On the other side the presence of cells with these characteristic structures and cells that

showed an higher level of p65 in the nucleus might indicate that after p65 activation there is a phase where p65 is inactivated and redistributed into these subnuclear structures.

The partial co-localisation found between E1B-55KDa and the punctuate structures of p65, could be an indication of a role for this viral protein in the redistribution and/or activation of p65. An alternative hypothesis could be that other proteins associated with subnuclear structures such as E4Orf6 or E4Orf3 are implicated in this process. Moreover, the fact that the structures in which p65 is reorganised are found to partially co-localised with PML bodies, lead us to speculate on a possible role of the PML bodies as storage sites for proteins and nuclear factors, which are successively released when their activity is required for cellular processes such as transcription or cell division.

5. CONCLUSION

Adenovirus infection affects several cellular mechanisms. We have shown that after adenovirus infection there is an increase in SUMO-1 conjugation both in cellular substrates, as RanGAP and viral substrates such as E1B-55KDa and pV.

Moreover we have reported that in adenovirus infected cells it is possible to observe an activation of the transcription factor NF-kB, even if the mechanism of this activation is not known. These results give evidence for the activation of NF-kB in adenovirus infected cells, confirming the data already published on transformed and/or transfected systems

More experiments need to be done to understand the mechanism that lead to the effects we have registered, but these data underline the importance of the SUMO-1 modification for regulatory events into the cell.

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