Quantitative SUMO-1 Modification of a Vaccinia Virus Protein Is Required for Its Specific Localization and Prevents Its Self-Association

Silvia Palacios,^{*†} Laurent H. Perez,^{*†} Sonja Welsch,[‡] Sibylle Schleich,^{*}
Katarzyna Chmielarska,[§] Frauke Melchior,[§] and Jacomine Krijnse Locker^{*}

^{*}European Molecular Biology Laboratory, Cell Biology and Biophysics Programme, 69117 Heidelberg, Germany; [†]Faculty of Medicine, Institute for Hygiene, University of Heidelberg, 69120 Heidelberg, Germany; and [§]Department of Biochemistry, University of Goettingen, 37073 Goettingen, Germany

Submitted November 16, 2004; Revised February 24, 2005; Accepted March 21, 2005
Monitoring Editor: Peter Walter

Vaccinia virus (VV), the prototype member of the Poxviridae, a family of large DNA viruses, carries out DNA replication in specialized cytoplasmic sites that are enclosed by the rough endoplasmic reticulum (ER). We show that the VV gene product of A40R is quantitatively modified by SUMO-1, which is required for its localization to the ER-enclosed replication sites. Expression of A40R lacking SUMO-1 induced the formation of rod-shaped cytoplasmic aggregates. The latter likely consisted of polymers of nonsumoylated protein, because unmodified A40R interacted with itself, but not with the SUMO-1-conjugated protein. Using a bacterial sumoylation system, we furthermore show that unmodified A40R is mostly insoluble, whereas the modified form is completely soluble. By electron microscopy, the A40R rods seen in cells were associated with the cytosolic side of the ER and induced the apposition of several ER cisternae. A40R is the first example of a poxvirus protein to acquire SUMO-1. Its quantitative SUMO-1 modification is required for its proper localization to the viral “mini-nuclei” and prevents its self-association. The ability of the nonsumoylated A40R to bring ER membranes close together could suggest a role in the fusion of ER cisternae when these coalesce to enclose the VV replication sites.

INTRODUCTION

Posttranslational modification of proteins is an important mechanism for the regulation of their function, activity, or localization. The structurally related modifications ubiquitination and sumoylation are unusual because the modifier is a small polypeptide. Although ubiquitination is predominantly involved in protein turnover (Hershko and Ciechanover, 1998), the exact function of SUMO conjugation is less clear. A number of cellular proteins have been reported to acquire SUMO-1, most of which reside in the nucleus. Sumoylation of these proteins can alter their intracellular localization, their activity, their stability, and their interaction with other proteins (Melchior, 2000; Muller *et al.*, 2001; Johnson, 2004). In mammalian cells, three SUMO family members have been described: SUMO-1, SUMO-2, and SUMO-3 (Melchior *et al.*, 2003). Human SUMO-1, a 101-amino acid polypeptide, shares ~50% sequence identity with SUMO-2/3 and with the yeast *Saccharomyces cerevisiae* protein Smt3. Although the pathway of sumoylation is mechanistically similar to ubiquitination, the enzymes are different. SUMO-1 is activated in an ATP-dependent manner by an E1-activating enzyme, which consists of a heterodimer

of the Aos1 and Uba2 proteins. Activated SUMO is transferred to the E2-conjugating enzyme Ubc9, which catalyzes the formation of an isopeptide bond between the C terminus of SUMO-1 and the ϵ -amino group of a lysine residue of the target protein (Melchior, 2000; Muller *et al.*, 2001; Johnson, 2004). Unlike ubiquitin conjugation, SUMO-1 modification in vitro is not strictly dependent on an E3 protein ligase, although E3 ligase activity is usually required to increase the efficiency of modification in vitro and in vivo (Johnson and Gupta, 2001; Sachdev *et al.*, 2001; Pichler *et al.*, 2002; Kagey *et al.*, 2003). A growing number of viral proteins have been described that are either SUMO-1 conjugated themselves or that interfere with the sumoylation machinery (reviewed in Wilson and Rangasamy, 2001). These proteins are invariably encoded by DNA viruses that replicate in the nucleus, and interfering with the sumoylation of these proteins interferes with their localization to the nucleus.

Vaccinia virus (VV), the prototype member of the Poxviridae, was used successfully to eradicate variola virus, the cause of smallpox. Poxviridae are large DNA viruses with a double-stranded DNA genome encoding for ~200 proteins. They are unique among DNA viruses because DNA replication occurs entirely in the cytoplasm rather than in the nucleus of the infected host cell. The cytoplasmic life cycle of VV involves virion entry, transcription, replication, and the assembly and egress of virions (Moss, 2001). VV replication is known to occur in discrete cytoplasmic structures called “factories” (Cairns, 1960). These sites become gradually enveloped by the rough endoplasmic reticulum (ER) resembling cytoplasmic “mini-nuclei,” a process that facilitates viral replication (Tolonen *et al.*, 2001). Cellular inner nuclear

This article was published online ahead of print in *MBC in Press* (<http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E04-11-1005>) on March 30, 2005.

[†] These authors contributed equally to this work.

Address correspondence to: Jacomine Krijnse Locker (krijnse@embl.de).

Figure 1. Characterization of a newly raised antibody to A40R. (A and B) HeLa cells were infected with a multiplicity of infection of 25, fixed at 3 h postinfection, and double labeled with the anti-A40R antibody (B) and Hoechst (A). (C) Thawed cryosections prepared from HeLa cells fixed at 3 h postinfection were labeled with anti-A40R followed by protein A coupled to 10-nm gold particles. The image shows a replication site (RS) surrounded by ER membranes (large arrows). The central part, but not the ER membrane, is decorated with gold particles. M, mitochondrion. Bar, 250 nm. (D) HeLa cells were infected at a multiplicity of infection of 10, and cell lysates prepared at the indicated times postinfection. Equal amounts of total protein were analyzed by Western blotting with the anti-A40R antibody.

membrane proteins, known to bind to DNA, are, however, not recruited to the viral replication sites. We have therefore proposed that the recruitment of ER cisternae to the viral DNA sites is mediated by viral (ER-resident) membrane proteins. To find such proteins, we have previously tagged viral genes of unknown function having a putative transmembrane domain with green fluorescent protein (GFP) and selected these by their localization to the DNA replication sites upon expression in infected cells. In this manner, we identified an early VV membrane protein, the gene product of E8R, and based on its behavior in infected cells, we have proposed that this protein is a possible candidate that mediates ER recruitment (Tolonen *et al.*, 2001; Doglio *et al.*, 2002).

In this same GFP screening, the gene product of A40R, a protein with a single putative transmembrane domain at its N terminus, also localized to the viral replication sites. The aim of the present study was therefore to characterize this protein and to define its potential role in the ER-wrapping process. Here, we show that A40R is modified by SUMO-1, both *in vivo* and *in vitro*. We identify the residue in A40R required for sumoylation and show that the modification is essential for its proper localization to the DNA replication sites. Specifically, overexpression of A40R lacking the SUMO-1 modification, induces electron-dense cytoplasmic structures that mediate the apposition of ER cisternae.

This report thus represents the first example of a poxvirus protein that is SUMO-1 modified and the first example of a DNA virus for which this modification does not lead to nuclear localization but instead is a prerequisite for its location to the cytoplasmic viral mini-nuclei.

MATERIALS AND METHODS

Cells, Viruses, and Antibodies

HeLa cells (ATCC C13) were grown as described previously (Sodeik *et al.*, 1993). VV strain western reserve (WR) or VVT7 were propagated and semi-purified as described previously (Pedersen *et al.*, 2000). The polyclonal antibody to A40R was raised in rabbit by using the peptide corresponding to amino acids 71–86 of the A40R sequence according to the manufacturer instructions (Neosystems, Strasbourg, France). The goat antibodies to SUMO-1 and SUMO-2/3 have been described previously (Pichler *et al.*, 2002). The mouse antibodies to myc and hemagglutinin (HA) were from American Type Culture Collection (Manassas, VA) and BAbCO (Richmond, CA), respectively; anti-GMP-1, used for Western blotting, was from Zymed Laboratories, South San Francisco, CA). The antibodies to the VV proteins H5R and A14L have been described previously (Salmons *et al.*, 1997; Tolonen *et al.*, 2001), and anti-protein disulfide-isomerase (PDI) was a kind gift of Stephen Fuller (Oxford University, Oxford, United Kingdom).

DNA Constructs

A40R was amplified by PCR from the VV WR genome by using the following primers: 5'-cggaagcttctaaccgaagtagtgga-3' (forward) and 5'-cggaagcttctaaccgaagtagtgga-3' (reverse). After digestion with *NotI* and *BamHI*, it was cloned into the pGEX-KG vector (Amersham Biosciences, Piscataway, NJ) cut with the same enzymes. For the cloning of A40R in pCDNA-3.1 (Invitrogen, Carlsbad, CA), pRSET (Invitrogen) and pFTX5 (a kind gift of Dr. Angel Nebreda, Spanish National Cancer Centre, Madrid, Spain), A40R was extracted from pGEX-KG by *BamHI*/*XhoI* digestion and cloned on the *BamHI*/*XhoI* sites of the digested vectors. The generation of an N-terminal six histidines A40R was performed by cloning A40R in the pHAT2 vector (*BamHI*/*HindIII*).

The site directed mutagenesis of the K⁹⁵ to A was done using the QuikChange mutagenesis kit (Stratagene, La Jolla, CA) with the following oligonucleotides: 5'-tccaaattcgatcttaattgcgatagactcacaac-3' (forward) and 5'-gtttggagctctatcgcaattagatcggaatttga-3' (reverse). Glutathione S-transferase (GST)-Ulp1 was kindly provided by Dr. Mark Hochstrasser (Yale University, New Haven, CT; Li and Hochstrasser, 1999). Cloning, expression, and purification of the recombinant E1 (His-Aos1 and Uba2) and E2 (Ubc9) enzymes and SUMO-1 were described previously (Pichler *et al.*, 2002).

Infections, Transfections, Immunofluorescence, and Electron Microscopy

HeLa cells were infected with VV WR or VVT7 for 45 min at 37°C and fixed at the indicated times. Transfections using Lipofectin (Invitrogen) were done as described previously (Mallardo *et al.*, 2001). Immunofluorescence was essentially carried out as described previously (Den Boon *et al.*, 1991), except that the cells also were labeled with 0.5 mg/ml Hoechst (Sigma-Aldrich, St. Louis, MO) to visualize viral and cellular DNA. For electron microscopy, HeLa cells infected for the indicated times, were fixed and processed for cryosectioning (van der Meer *et al.*, 1999) or Epon embedding (Griffiths, 1993). Thawed cryosections were labeled with affinity-purified antibodies followed by 10-nm protein A gold, and images were taken using a Zeiss EM10 electron microscope.

Sample Preparation, Immunoprecipitation, and Western Blotting

For Western blotting, HeLa cells were scraped from the dishes with ice-cold phosphate-buffered saline, pelleted, resuspended in 100 μ l of lysis buffer (1% NP-40 in 10 mM Tris-Cl, pH 9), and incubated for 30 min on ice. The nuclei were pelleted by centrifugation, and the cleared supernatant was mixed with sample buffer. The preparation of postnuclear supernatants (PNSs), Na₂CO₃, and TX-114 treatment were performed as described previously (Jensen *et al.*, 1996). For immunoprecipitation, cell pellets were resuspended in radioimmunoprecipitation assay (RIPA) buffer (10 mM sodium-phosphate buffer, pH 7.2, 150 mM NaCl, 1% sodium-deoxycholate, 1% Triton X-100, 0.1% SDS, 1 mM dithiothreitol, protease inhibitors mix). Equal amounts of total protein extract, usually 500 μ g, were precleared and immunoprecipitated 2 h at 4°C with 1 μ g of anti-myc monoclonal antibody (mAb) immobilized on agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA). The beads were washed four times with RIPA buffer and then resuspended sample buffer. The extracts or immunoprecipitates were separated by SDS-PAGE and blotted on to nitrocellulose as described previously (Perez *et al.*, 2002).

In Vitro Translation, SUMO-1 Modification of A40R, and GST Pull-Down

In vitro translation of A40R was done according to the instructions of the manufacturer (Promega, Madison, WI). In vitro modification assay using cell extracts was performed as described previously (Mahajan *et al.*, 1997). After incubation with cell extracts, A40R was immunoprecipitated with anti-myc and digested with GST-Ulp1 for 30 min at room temperature (RT). In vitro modification assays using the recombinant enzymes (Pichler *et al.*, 2002) were performed in a total volume of 20 μ l with 150 ng of Aos1/Uba2, 200 ng of Ubc9, 500 ng of SUMO-1, and 1 μ l of the *in vitro*-translated protein. The reactions, containing 1 mM ATP, 0.05% of Tween 20 and 0.2 mg/ml ovalbumin, were incubated at 30°C for 60 min and stopped by addition of sample buffer. For the GST pull-down, the reticulocyte lysate (20 μ l) was diluted 1:10 in immunoprecipitation (IP) buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 20 mM NaF, 5 mM EDTA, 5 mM EGTA, 100 μ M sodium-vanadate, 1% NP-40), supplemented with microcystin, protease inhibitors, and 1% bovine serum albumin and incubated for 60 min at 4°C with 1 μ g of GST or GST-A40R, isolated from *Escherichia coli*, prebound to 5 μ l of glutathione beads (New England Biolabs, Beverly, MA). The beads were washed four times in IP buffer and resuspended in sample buffer. All samples were analyzed by SDS-gels followed by autoradiography. For SUMO-1 modifica-

Figure 2 (facing page). Modification of A40R modification occurs only upon expression in eukaryotic cells. (A) A40R tagged with 6His or with myc was translated *in vitro*, and migration in SDS-gels of the tagged proteins was analyzed by autoradiography. Control is the *in vitro* transcription/translation mix without DNA. The numbers on the left refer to the approximate molecular mass of 18 and 20 kDa, inferred from the migration of molecular mass markers. (B) Expression of A40R tagged with GST or an HA epitope was induced in bacteria. Bacteria were lysed in sample buffer 3 h after the addition of IPTG. The samples were analyzed by Western blotting with the anti-A40R antibody. (C) Same myc-tagged A40R construct used in A was transiently expressed in VVT7-infected HeLa cells. Extracts of infected/untransfected cells (lane 1) or infected/transfected cells (lane 2) were prepared at 16 h posttransfection and analyzed by Western blots with anti-myc. Note that both the predicted molecular mass form (asterisk) and the higher molecular mass form (double asterisk) are present under this condition. (D) A40R was transiently expressed in uninfected cells (lane 3) and detected by Western blot with the anti-A40R antibody. Lane 1 is from uninfected and nontransfected cells, and lane 2 is from infected cells and serves as a negative and positive control, respectively.

Figure 2.

Figure 3. A40R can acquire SUMO-1 modification in vitro and is SUMO-1 modified in eukaryotic cells. (A) In vitro-translated A40R was incubated with HeLa cell extracts supplemented with 1 mM ATP for 0, 30, and 60 min at RT. (B) A40R was translated in vitro and incubated with (+) or without (–) HeLa cells extracts for 30 min. Half of the reaction incubated with cellular extract was immunoprecipitated with the anti-myc antibody and digested for 30 min with Ulp-1. (A and B) Degradation product of the SUMO-1–modified form that occurs after the incubation with cell extracts is marked with an asterisk. (C) HeLa cells were infected with VVT7, transfected with mycA40R, or mock transfected. After 16 h of expression, cells were processed for immunoprecipitation with anti-myc. Samples were resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and probed with the rabbit anti-A40R antibody (right) or the mouse anti-SUMO-1 GMP-1(left). The IgG light chain is marked with two asterisks.

tion of A40R in bacteria, BL21 (DE3) competent cells were transformed with either pHAT2-A40R alone or cotransformed with pT-E1E2S1 (expressing SUMO-1, Ubc9, and Aos1/Uba2, a kind gift of Drs. Hisato Saitoh and Yasuhiro Uchimura; Uchimura *et al.*, 2004). A single colony was selected and transferred to 100 ml of LB media containing chloramphenicol and the appropriate antibiotics. Bacterial cultures were kept at 37°C with shaking for 8 h until an OD₆₀₀ of at least 1.0. Isopropyl- β -D-thiogalactopyranoside (IPTG) was then added at a concentration of 0.2 mM. After incubation for another 6 h at 30°C, the bacteria were collected by centrifugation and lysed by incubation with lysozyme for 30 min on ice followed by sonication. The same protein quantity of supernatant and pellet was run on a 15% SDS-PAGE and unmod-

ified or SUMO-1–modified A40R was detected by Western blotting by using anti-His mAb (Sigma-Aldrich).

RESULTS

The Gene Product of A40R Acquires a Modification That Increases Its Predicted Molecular Mass

The A40R gene product has previously been described as a membrane protein that is N-glycosylated and transported to

Figure 4. Sumo-1-modified proteins are recruited to the VV DNA replication sites. Uninfected HeLa cells (A, B, E, and F) or HeLa cells infected with VV for 3h (C, D, G, and H) were fixed and double labeled with Hoechst (A, C, E, and G) and SUMO-1 (B and D) or SUMO-2/3 (F and H) antibodies. Note that only SUMO-1- (D) but not SUMO-2/3-conjugated proteins are recruited to the VV DNA replication sites.

the cell surface (Wilcock *et al.*, 1999). However, our localization studies using GFP-tagged A40R showed that the protein colocalized with the viral replication sites. A newly raised antibody to A40R labeled the Hoechst-positive DNA replication sites at early (3 h postinfection; Figure 1, A and B) and late times (6 h) postinfection (our unpublished data), confirming our previous results. By electron microscopy, EM A40R did not localize to the ER membranes around the replication sites, as expected of a membrane protein (Figure 1C). Instead, the antibody labeled the central part of the

replication sites, a feature shared by VV proteins lacking a transmembrane domain such as the gene product of H5R (Tolonen *et al.*, 2001; see below). On Western blots, the antibody detected a single band from 3 h postinfection onwards and the expression level increased during the infection (Figure 1D). This band also was seen in the presence of hydroxy-urea, an inhibitor of viral replication, indicating that A40R is an early protein (our unpublished data). Importantly, A40R, which has a predicted molecular mass of 18 kDa, migrated at ~38 kDa in SDS-gels at all times postin-

Figure 5.

fection (Figure 1D). An 18-kDa form was sometimes seen in lysates of cells infected for 6 h and after overexposure of the blot (our unpublished data).

Thus, A40R is an early protein that, in contrast to the results from Wilcock *et al.* (1999), localizes to the DNA replication sites. The migration of A40R in SDS-gels suggested that the protein acquired a modification that resulted in a 20-kDa increase of its predicted molecular mass.

The Modification of A40R Depends on Eukaryotic Expression but Is Independent of Vaccinia Virus Infection

When A40R was translated in vitro as a fusion protein with a 6His- or a myc-tag, it migrated with the predicted molecular mass of 18 kDa (Figure 2A). Furthermore, expression of A40R in bacteria as a fusion protein with a GST- or an HA-tag showed that A40R migrated with its predicted molecular mass and did not acquire the modification seen in infected cells (Figure 2B). When the same myc-tagged A40R construct, resulting in a single band of 18 kDa upon in vitro translation (Figure 2A), was expressed in infected cells, two bands migrating with an apparent molecular mass of 38 and 18 kDa were detected (Figure 2C). Detection of the latter form indicated that upon overexpression of the protein the mechanism that modifies A40R probably became saturated. The A40R modification was independent of VV infection, because in transiently transfected uninfected cells A40R migrated with its higher molecular mass form of 38 kDa (Figure 2D). This result demonstrates that modification of A40R is dependent on its expression in eukaryotic cells, but it is independent of VV infection.

A40R Is Sumoylated In Vitro and In Vivo

The sequence of A40R predicts three putative *N*-glycosylation sites, several putative phosphorylation sites, and one putative sumoylation site. In the study by Wilcock *et al.* (1999), it also was noticed that A40R migrates in gels with a molecular mass that is higher than predicted from its sequence and was attributed to the apparent acquisition of *N*-linked glycosylation. However, treatment of VV-infected cells with tunicamycin to inhibit *N*-glycosylation or of cell lysates with λ -phosphatase (that dephosphorylates serine, threonine, tyrosine, and histidine residues) did not alter the migration of A40R, demonstrating that neither *N*-glycosylation nor phosphorylation could explain its higher molecular mass (our unpublished data).

Sumoylation of proteins generally does not occur upon their expression in bacteria or upon in vitro translation, but can sometimes be reconstituted by incubating the expressed

protein with cell extracts in presence of ATP. Myc-tagged A40R was therefore synthesized in vitro and incubated with cell extracts of infected cells in presence of ATP. Within 30 min of incubation, about half of the protein had shifted from the 18-kDa to the 38-kDa form, and this percentage did not increase with time (Figure 3A). The A40R modification occurred with equal efficiency upon incubation with extracts of uninfected cells (our unpublished data), consistent with the fact that the modification of A40R is dependent on expression in eukaryotic cells but independent of VV infection.

To confirm that the 20-kDa size shift was due to the attachment of SUMO, the same experiment was repeated but analyzed with or without prior digestion with purified recombinant Ulp-1, a SUMO-specific protease from *S. cerevisiae* (Li and Hochstrasser, 1999). Ulp-1 digestion resulted in the quantitative loss of the higher molecular mass form of A40R, demonstrating that in vitro synthesized A40R acquires SUMO-1 upon incubation with cellular extracts (Figure 3B).

Myc-tagged A40R was then expressed in infected cells, and the protein was immunoprecipitated using anti-myc and analyzed by Western blots with anti-A40R or anti-SUMO-1 antibodies. Anti-myc immunoprecipitates of lysates of infected/transfected cells revealed two bands by Western blots with the anti-A40R antibody, corresponding to the 18- and 38-kDa forms, respectively, that were not seen in untransfected cells (Figure 3C, left). The anti-SUMO-1 antibody only recognized the 38-kDa form in infected/transfected cells, whereas, as expected, this band was not seen in myc-immunoprecipitates of untransfected cells (Figure 3C, right). Thus, the higher molecular mass of A40R made in vivo also was due to the attachment of SUMO-1.

A SUMO-1 Antibody Decorates the DNA Replication Sites

We next asked whether the general pattern of SUMO-1-conjugated proteins was altered by the VV infection. By immunofluorescence, a SUMO-1-specific antibody revealed a faint, diffuse staining in the cytoplasm and a concentration in a number of discrete nuclear dots in uninfected cells (Figure 4B), as described previously (Sapetschnig *et al.*, 2002; Eskiw *et al.*, 2003). Because the same nuclear labeling pattern was seen in infected cells (Figure 4D), VV infection did not obviously affect the general nuclear SUMO-1 labeling pattern. Furthermore, analysis of lysates of infected and uninfected cells by Western blots with anti-SUMO-1, revealed that the overall pattern of SUMO-1-conjugated proteins was not altered upon infection (our unpublished data). The anti-SUMO-1 antibody also decorated the viral DNA replication sites at early (Figure 4D) and late times (our unpublished data) postinfection. The general pattern of SUMO-2/3-conjugated proteins was similar to the SUMO-1-conjugated proteins, with a diffuse staining in the cytoplasm and a typical concentration in nuclear dots (Figure 4F). The SUMO-2/3 antibody did, however, not label the DNA replication sites in infected cells (Figure 4H). Because A40R is a relatively abundant protein, as assessed by immunofluorescence and EM, it is possible that the SUMO-1 labeling of the replication sites is due to the localization of (SUMO-1-modified) A40R to these sites. These results also indicate that the VV infection does not significantly affect the normal SUMO-1 and SUMO-2/3 nuclear pattern.

Lysine 95 of A40R Is the Residue Modified by SUMO-1

One relatively well established consensus sequence for SUMO-1 modification consists of four amino acids with the sequence ψ KXE, where ψ is a large hydrophobic amino acid,

Figure 5 (facing page). A40R-A95 does not acquire SUMO-1 and does not localize to the VV replication sites. (A) mycA40R and mycA40R-A95 were translated in vitro and then incubated with 150 ng of Aosl/Uba2, 200 ng of Ubc9, and 500 ng of SUMO-1 in presence (+) or absence (–) of 1 mM ATP. The 35 S-labeled proteins were analyzed by SDS-PAGE and autoradiography. (B) mycA40R and mycA40R-A95 were expressed by transfection in VVT7-infected cells. After 5 or 16 h of expression, PNSs were prepared and the proteins were analyzed by Western blot with anti-myc. The position of A40R and sumoylated A40R (A40R-SUMO-1) is indicated. (C–F) mycA40R and mycA40R-A95 were expressed as in B, the cells were fixed at 5 h of infection (4 h of expression), and then double labeled with Hoechst (to label DNA) and anti-myc (to label A40R). The replication sites labeled with Hoechst (C and E), and the distribution of myc-A40R (D) and myc-A40R-A95 (F) was followed by immunofluorescence microscopy. Note that mycA40R colocalizes with the DNA replication sites, whereas the A40R-A95 localizes to structures that do not colocalize with the Hoechst-positive replication sites (D).

K is the lysine residue modified by SUMO-1, X is any amino acid, and E is glutamic acid. The A40R sequence predicts a putative sumoylation site between the amino acids 94–97 with the sequence I⁹⁴KIE⁹⁷. To investigate whether this was the site for SUMO-1 modification, the lysine at position 95 was mutated to an alanine in the wild-type myc-tagged A40R construct. We chose to use an alanine for substitution as this relatively small amino acid is unlikely to significantly affect the structure of the protein.

An assay that reconstitutes the SUMO-1 modification *in vitro* by using purified recombinant SUMO-1, Uba2/Aos1 (the SUMO-1-activating enzymes), and Ubc9 (the SUMO-1-conjugating enzyme) was used to determine whether Lysine 95 of A40R was required for SUMO-1 modification. *In vitro*-translated wild-type A40R incubated with the purified proteins was partially converted to the 38-kDa form in the presence, but not in the absence, of ATP (Figure 5A). In contrast, A40R-A⁹⁵ was not converted to the 38-kDa form under any of the conditions, indicating that Lysine 95 is the residue modified by SUMO-1. Wild-type mycA40R and mycA40R-A⁹⁵ were then expressed in VV-infected cells, and lysates were analyzed at 5 and 16 h posttransfection. As shown above (Figure 2C), overexpression of wild-type A40R resulted in the 38-kDa form after 5 h of expression, as well as the 18-kDa form at late times posttransfection (Figure 5B). On transfection of mycA40R-A⁹⁵, the 38-kDa form was not seen, and instead the 18-kDa form accumulated at all times of expression, showing that the Lysine 95 residue also is required for SUMO-1 modification of A40R *in vivo* (Figure 5B).

Sumoylation of A40R Is Necessary for the Localization of A40R to the DNA Replication Sites

We next investigated whether the SUMO-1 modification of A40R had an effect on its intracellular distribution. The transiently expressed wild-type myc-tagged A40R, in VVT7-infected cells, localizes to the DNA replication sites, similar to the wild-type protein (Figure 5, C and D), whereas mycA40R-A⁹⁵ localizes to distinct structures spread over the cytoplasm (Figure 5, E and F). Similar structures were observed upon overexpression of the wild-type protein at later times posttransfection, when nonsumoylated A40R was detected by Western blots (Figures 2C and 5B; our unpublished data).

By EM, with sections of conventional resin-embedded cells, these structures looked like dense, rod-shaped aggregates (Figure 6, A–C). The aggregates resulted in the apposition of membrane cisternae, typical of the ER. Typically, the electron-dense material was aligned by two cisternae on each side (Figure 6, A and C), but, occasionally, multiple layers of cisternae, interspaced by electron-dense rods, were seen (Figure 6B). On cryosections, the structures were labeled with anti-myc, implying that they were induced by the overexpressed mutant protein (Figure 6, D and E). Anti-PDI labeling, a marker of the ER, confirmed that the cisternae that enclosed the rod-shaped aggregates were of ER origin (Figure 6, F and G). Again, identical structures were observed by EM at late times of wild-type A40R overexpression, when significant amounts of unmodified A40R started to accumulate (our unpublished data). Together with the immunofluorescence observations, these results strongly argued that the accumulation of the aberrant A40R aggregates was due to the lack of sumoylation rather than the lysine-to-alanine substitution in the mutant protein. Because the wild-type and mutant proteins were expressed in the background of a normal VV infection, the overexpressed proteins did not significantly affect the normal biogenesis of the

replication sites, including the ER wrapping process (our unpublished data).

Thus, the SUMO-1 modification of A40R is required for its proper localization to the ER-enclosed VV replication sites. Deletion of the SUMO-1 site or overexpression of the unmodified wild-type protein resulted in the formation of distinct aggregates that induced the tight apposition of ER cisternae.

A40R Is Not an Integral Membrane Protein

The sequence of A40R predicts a stretch of 22 hydrophobic amino acids (amino acids 7–29) that can adopt an α -helical configuration, a property of membrane-spanning domains (Tolonen *et al.*, 2001). The EM images, however, suggested that the SUMO-1-modified form behaved as a soluble protein (Figure 1C), whereas the non-SUMO form apparently associated peripherally with membranes (Figure 6).

PNSs were therefore subjected to Na₂CO₃ treatment, and membranes and integral membrane proteins were separated from peripherally associated and soluble proteins by centrifugation. A characteristic of VV proteins that lack transmembrane domains, such as the gene product of H5R, is their ability to pellet in absence of carbonate treatment and to become solubilized after this treatment, whereas integral membrane proteins, such as A14L, pellet irrespective of this high pH treatment (Doglio *et al.*, 2002). We have proposed two possible explanations for the behavior of VV proteins lacking transmembrane domains. VV-soluble proteins can either form large complexes that are dissolved upon high pH treatment. Alternatively, soluble VV proteins may be peripherally associated with membranes, because carbonate treatment is commonly used to extract such proteins from membranes (Fuijki *et al.*, 1982).

In untreated extracts, the soluble protein H5R, the membrane protein A14L (Salmons *et al.*, 1997), and A40R were all found in the pellet (Figure 7A). After the Na₂CO₃ treatment, A14L remained associated with the membrane pellet, whereas both A40R and H5R partitioned exclusively in the supernatant, indicating that A40R did not behave as an integral membrane protein. Furthermore, when A40R from infected cells or translated *in vitro* was extracted with TX-114, to separate hydrophobic from hydrophilic proteins, the protein partitioned in the aqueous phase (Figure 7, B and C), indicating it behaved as a hydrophilic protein. The combined results thus significantly differ from those shown by Wilcock *et al.* (1999) because A40R behaved as a hydrophilic protein both *in vivo* and *in vitro* and is not an integral membrane protein in infected cells.

Sumoylation of A40R Prevents It from Interacting with Itself

The EM images implied that overexpression of unmodified A40R induced the formation of aggregates, probably consisting of polymers of the overexpressed protein interacting with itself. Furthermore, cryosections labeled with a panel of antibodies to both viral and cellular proteins failed to label the rod-shaped structures, arguing that they were predominantly composed of A40R (our unpublished data). We therefore tested whether A40R was able to interact with itself. A40R was translated *in vitro* and incubated with HeLa cell extracts to obtain both the unmodified and the sumoylated form of A40R (Figure 3, A and B). Radioactively labeled A40R was then mixed with purified GST-tagged A40R or GST alone, bound to glutathione beads, and bound proteins were analyzed by SDS-gels. As shown in Figure 8A, GST-tagged A40R was able to interact with the unmodified, *in vitro*-translated, A40R but not with the sumoylated form.

Figure 6. Overexpression of A40R-A95 results in the apposition of ER cisternae. (A–G) HeLa cells were infected with VVT7, transfected with mycA40R-A95, and fixed for EM at 7 h postinfection (6 h posttransfection). (A–C) Sections of Epon-embedded cells show electron-dense rods aligned on both sides by a membrane cisterna typical of the ER. The arrow in A points to a rod structure that is associated with the nuclear envelope. (B) Occasionally, the electron-dense rods and ER cisternae align in stacks. The arrows in C point to the ER. (D–G) Fixed cells were subjected to cryosectioning, and thawed sections were labeled with anti-myc (D and E) or anti-PDI (F and G). The arrows in D show the electron-dense rods as they look in thawed cryosections. (F and G) Cisternae that align the rods are abundantly labeled with anti-PDI. M, mitochondria; Nu, nucleus. Bars, 200 nm.

Figure 7. Gene product of A40R is not an integral membrane protein. (A) Postnuclear supernatants of uninfected cells (–Inf) or of cells infected for 16 h were incubated or not incubated with Na₂CO₃ and subjected to ultracentrifugation to separate membrane (M) from the soluble proteins (S). All fractions were analyzed by Western blotting with antibodies to A40R, H5R and A14L. In the absence of Na₂CO₃ treatment, A40R, H5R, and A14L are found in the membrane fraction and not in the supernatant. After the Na₂CO₃ treatment, H5R and A40R are in the supernatant, whereas A14L is in the membrane fraction. (B) Cells infected for 16 h were extracted with TX-114, and the detergent (D) and the aqueous phase (A) were analyzed by Western blots with the antibodies indicated on the left. The two bands observed with the anti-A14L antibody represent the monomeric and disulfide-bonded forms of the protein. The dimeric, disulfide-bonded form of A14L, which can sometimes be seen in SDS-gels, is known to be relatively resistant to reduction by β -mercaptoethanol. (C) A40R and A14L were translated in vitro, extracted with TX-114, and the untreated protein (T) or the detergent (D) and the aqueous (A) phases were analyzed by autoradiography.

While purifying GST-tagged A40R from bacteria, we noticed that the protein was highly insoluble and that the protein yields were very low. To address whether the lack of SUMO-1 modification accounted for this property, we made use of a system that allows for sumoylation in bacteria. It consists of coexpressing a protein of interest, such as A40R, with the vector pT-E1E2S1, encoding for the SUMO-1 machinery (Uchimura *et al.*, 2004). On coexpression A40R was resolved into two bands of equal intensity, whereas the higher molecular mass form was absent if A40R was expressed alone (Figure 8B). Importantly, whereas unmodified A40R was highly insoluble upon expression in bacteria, the SUMO-1-modified form seemed entirely soluble (Figure 8B). The combined data thus strongly suggests that SUMO-1

modification of A40R prevents its aggregation and its ability to interact with itself.

DISCUSSION

A40R Acquires SUMO-1, a Modification That Is Unusually Stable

The present study demonstrates that the VV gene product of A40R acquires SUMO-1 that is required for its localization to the viral DNA replication sites. In a previous study, the VV E3L gene was shown to interact with SUMO-1 in a yeast two-hybrid system, an interaction that was not confirmed at the biochemical level (Rogan and Heaphy, 2000). It also was

Figure 8. Unmodified A40R interacts with itself. (A) Myc-tagged A40R was translated in vitro and incubated with or without HeLa cell extracts for 90 min at RT. The in vitro-translated protein was incubated with GST or GST-A40R purified from *E. coli*, bound to glutathione beads. After extensive washing, the beads with bound proteins were analyzed by SDS-PAGE and autoradiography (top) (³⁵S A40R). The first two lanes without GST or GST-A40R incubation represent 10% of the total in vitro translation reaction analyzed directly and incubated with or without cell extract. Bottom, corresponding Coomassie staining of the same gel, showing the migration of GST alone or GSTA40R. M, molecular mass marker, the size of some of the bands is indicated on the left. (B) His-tagged A40R was either expressed alone or together with pT-E1E2S1 in bacteria. The bacteria were lysed, and the soluble fraction (S) and the pellet (P) were separated by centrifugation. Equal amounts of protein (measured by Bradford) were separated by SDS-gels, and the proteins were detected by Western blots with anti-His. The positions of unmodified A40R (A40R) and the modified form (A40RSUMO-1) are indicated with the arrows on the right. The approximate position of the marker proteins of 20 and 37 kDa are indicated on the left.

noted previously that the I7L gene of VV showed significant homology to the yeast protease Ulp-1 (Li and Hochstrasser, 1999). Thus, although a role for SUMO-1 in the life cycle of poxviruses is not completely unexpected, the present data are the first report of a VV protein, the A40R gene product, to acquire SUMO-1 modification both in vivo and in vitro. In vitro, SUMO-1 modification could be reconstituted with rel-

ative efficiency, by using two known cellular factors, the Aos1/Uba2 SUMO-1-activating and the Ubc9-conjugating enzyme, indicating that SUMO-1 modification of A40R is mediated by cellular components. Consistently, A40R expressed in uninfected cells was efficiently and quantitatively SUMO-1 modified, implying that viral factors are not required for its modification.

The SUMO-1 modification of A40R showed two unique features not previously seen for other SUMO-1 targets. First, under normal infection conditions the protein was quantitatively converted to the SUMO-1 modified form, as assessed by Western blots. Second was the extreme stability of its modification. The majority of SUMO-conjugates are subjected to the constant interplay between the SUMO-1-conjugating and -deconjugating machinery. Generally, special precautions are required to inactivate the cysteine proteases that desumoylates SUMO-1-conjugated proteins, before cell lysis. Therefore, the amount of the SUMO-1-modified form that can be detected in SDS-gels for most SUMO-1 targets often does not exceed 5–10% of the total amount of protein detected (Melchior, 2000; Johnson, 2004). An exception is the cytoplasmic RanGTPase-activating protein RanGAP that is associated with the nuclear envelope. As for RanGAP, the reasons for the unusual stability of the SUMO-1 modification of A40R are not clear at present. The protein could be folded or interact tightly with other protein(s) in such a way that SUMO-1-deconjugating proteases have no access to the SUMO-1 site. Another possibility is that A40R and SUMO proteases localize to different subcellular structures, preventing the desumoylation of A40R.

The A40R gene product has previously been described as a membrane glycoprotein made early infection that is transported to the cell surface (Wilcock *et al.*, 1999). Our data differ from those previously published in almost every respect. We speculate therefore that the antibody raised by Wilcock *et al.* (1999) recognizes a different viral protein that happens to migrate with a similar molecular mass.

SUMO-1 Modification Is Required for Its Localization to the Viral Mini-Nuclei

We have identified the Lysine 95 as the sumoylation site of A40R. This lysine is located in the sequence I⁹⁴KIE⁹⁷ consistent with the experimentally determined SUMO-1 conjugation motif ψ KXE (Rodriguez *et al.*, 2001; Sampson *et al.*, 2001). Accordingly, mutation of the Lysine 95 to Ala results in a protein that can no longer acquire SUMO-1 in vitro and in vivo. SUMO modification of proteins has different effects depending on the target protein, one of which is to determine the subcellular localization of the protein (Matunis *et al.*, 1996; Mahajan *et al.*, 1997; Sternsdorf *et al.*, 1997; Muller *et al.*, 1998; Zhong *et al.*, 2000). Along this line, we found that the SUMO-1 modification of A40R was required for its specific localization. The majority of SUMO-conjugates are located in the nucleus, and it seems that sumoylation and nuclear localization are linked (Rodriguez *et al.*, 2001; Muller *et al.*, 2004). Consistently, all viral proteins described so far that acquire SUMO-1 are localized to the nucleus. Without exception, they are encoded by DNA viruses that replicate in the nucleus and blocking their SUMO-1 modification invariably interfered with their nuclear localization (reviewed in Wilson and Rangasamy, 2001). It is therefore intriguing that the specific localization of the SUMO-1-modified A40R are the cytoplasmic ER-enclosed sites of poxvirus replication, that morphologically and functionally resemble the nucleus (Tolonen *et al.*, 2001).

A Putative Role for the SUMO-1 Modification of A40R in the VV Life Cycle

Overexpression of the nonsumoylated protein resulted in the formation of protein aggregates associated with the cytosolic side of the ER and induced ER cisternae to become apposed. Under normal infection conditions, however, A40R is quantitatively and stably SUMO-1 modified, and these aggregates are not seen. These observations together argue strongly that the SUMO-1 modification prevents the formation of A40R aggregates, a notion that was supported by the fact that nonsumoylated A40R was able to interact with itself, but not with the SUMO-1-modified protein. This was furthermore confirmed by expression in *E. coli*, showing that SUMO-1 modification prevented aggregation of A40R. It is unlikely that the sole purpose of the quantitative SUMO-1 modification of A40R is to prevent its self-association. We therefore speculate that during the VV life cycle, small (undetectable by Western blots) amounts of non-SUMO A40R may be present, perhaps transiently, with a function different from the SUMO-1 form. We have previously shown that at the onset of VV replication, individual ER cisternae are first recruited to the replication sites and then subsequently fuse to form a sealed envelope surrounding the replication site (Tolonen *et al.*, 2001). Because the nonsumoylated protein specifically interacted with the cytosolic side of the ER, the same membranes that wrap the replication sites, we speculate that this form of A40R may play a role during this ER wrapping process. We suggest that a small pool of non-SUMO A40R could perhaps aid at bringing ER membranes together, before these fuse to generate the ER envelope that surrounds the replication site. After accomplishing this task, this pool would then be sumoylated, as excess unmodified A40R would lead to protein aggregation, ER zippering, perhaps inhibiting fusion rather than promoting it. This suggestion leaves open the putative role of the SUMO-1-modified form. Its specific localization strongly suggests a role in VV replication itself or in the transcription of late genes, known to occur on these sites. We are currently addressing this question genetically and biochemically, by looking for putative interacting partners of SUMO-1-modified A40R, because we expect that both would tell us more about its role during VV morphogenesis.

ACKNOWLEDGMENTS

We thank Gareth Griffiths for critical reading of the manuscript and Damian Brunner for suggesting the possible SUMO-1 modification of A40R. Dr. Mark Hochstrasser is acknowledged for the kind gift of GST-Ulp-1, Drs. Hisato Saitoh and Yasuhiro Uchimura for the pT-E1E2S1 vector, and Angel Nebreda and laboratory members for providing numerous reagents. This work was supported by a Marie Curie individual fellowship (to S. P.) and a European Union fifth framework network grant (to J.K.L.).

REFERENCES

Cairns, H.J.F. (1960). The initiation of vaccinia infection. *Virology* 11, 603–623.

Den Boon, J. A., Snijder, E. J., Krijnse Locker, J., Horzinek, M. C., and Rottier, P.J.M. (1991). Another triple-spanning envelope protein among intracellular budding RNA viruses: the torovirus E protein. *Virology* 182, 655–663.

Doglio, L., De Marco, A., Schleich, S., Roos, N., and Krijnse Locker, J. (2002). The Vaccinia Virus E8R gene product; a viral membrane protein that is made early in infection and packaged into the virions' core. *J. Virol.* 76, 9773–9786.

Eskiw, C. H., Dellaire, G., Mymryk, J. S., and Bazett-Jones, D. P. (2003). Size, position and dynamic behavior of PML nuclear bodies following cell stress as a paradigm for supramolecular trafficking and assembly. *J. Cell Sci.* 116, 4455–4466.

Fuijki, Y., Hubbard, A., Fowler, L. S., and Lazarov, P. B. (1982). Isolation of intracellular membranes by means of sodium carbonate treatment. Application to endoplasmic reticulum. *J. Cell Biol.* 93, 97–102.

Griffiths, G. (1993). *Fine Structure Immunocytochemistry*, Heidelberg, Germany: Springer.

Hershko, A., and Ciechanover, A. (1998). The ubiquitin system. *Annu. Rev. Biochem.* 67, 425–479.

Jensen, O. N., Houthaeve, T., Shevchenko, A., Cudmore, S., Mann, M., Griffiths, G., and Krijnse Locker, J. (1996). Identification of the major membrane and core proteins of vaccinia virus by two-dimensional electrophoresis. *J. Virol.* 70, 7485–7497.

Johnson, E. S. (2004). Protein modification by SUMO. *Annu. Rev. Biochem.* 73, 355–382.

Johnson, E. S., and Gupta, A. A. (2001). An E3-like factor that promotes SUMO conjugation to the yeast septins. *Cell* 106, 735–744.

Kagey, M. H., Melhuish, T. A., and Wotton, D. (2003). The polycomb protein Pc2 is a SUMO E3. *Cell* 113, 127–137.

Li, S. J., and Hochstrasser, M. (1999). A new protease required for cell-cycle progression in yeast. *Nature* 398, 246–251.

Mahajan, R., Delphin, C., Guan, T., Gerace, L., and Melchior, F. (1997). A small ubiquitin-related polypeptide involved in targeting RanGAP1 to nuclear pore complex protein RanBP2. *Cell* 88, 97–107.

Mallardo, M., Schleich, S., and Krijnse Locker, J. (2001). Microtubule-dependent organization of vaccinia virus core-derived early mRNAs to distinct cytoplasmic structures. *Mol. Biol. Cell* 12, 3875–3891.

Matunis, M. J., Coutavas, E., and Blobel, G. (1996). A novel ubiquitin-like modification modulates the partitioning of the Ran-GTPase-activating protein RanGAP1 between the cytosol and the nuclear pore complex. *J. Cell Biol.* 135, 1457–1470.

Melchior, F. (2000). Sumo-non classical ubiquitin. *Annu. Rev. Cell Dev. Biol.* 16, 591–626.

Melchior, F., Schergaut, M., and Pichler, A. (2003). SUMO: ligases, isopeptidases and nuclear pores. *Trends Biochem. Sci.* 28, 612–618.

Moss, B. (2001). Poxviridae: the viruses and their replication. In: *Fields Virology*, 4th ed., ed. B. N. Fields, D. M. Knipe, P. M. Howley, R. M. Chanock, M. S. Hirsch, J. L. Melnick, T. P. Monath, and B. Roizman, Philadelphia, PA: Lippincott-Raven Publishers, 2849–2883.

Muller, S., Hoege, C., Pyrowolakis, G., and Jentsch, S. (2001). SUMO, ubiquitin's mysterious cousin. *Nat. Rev. Mol. Cell. Biol.* 2, 202–210.

Muller, S., Ledl, A., and Schmidt, D. (2004). SUMO: a regulator of gene expression and genome integrity. *Oncogene* 23, 1998–2008.

Muller, S., Matunis, M. J., and Dejean, A. (1998). Conjugation with the ubiquitin-related modifier SUMO-1 regulates the partitioning of PML within the nucleus. *EMBO J.* 17, 61–70.

Pedersen, K., Snijder, E. J., Schleich, S., Roos, N., Griffiths, G., and Krijnse Locker, J. (2000). Characterization of vaccinia virus intracellular cores: implications for viral uncoating and core structure. *J. Virol.* 74, 3525–3536.

Perez, L. H., Antonio, C., Flament, S., Vernos, I., and Nebreda, A. R. (2002). Xkid chromokinesin is required for the meiosis I to meiosis II transition in *Xenopus laevis* oocytes. *Nat. Cell Biol.* 4, 737–742.

Pichler, A., Gast, A., Seeler, J. S., Dejean, A., and Melchior, F. (2002). The nucleoporin RanBP2 has SUMO1 E3 ligase activity. *Cell* 108, 109–120.

Rodriguez, M. S., Dargemont, C., and Hay, R. T. (2001). SUMO-1 conjugation in vivo requires both a consensus modification motif and nuclear targeting. *J. Biol. Chem.* 276, 12654–12659.

Rogan, S., and Heaphy, S. (2000). The vaccinia virus E3L protein interacts with SUMO-1 and ribosomal protein L23a in a yeast two hybrid assay. *Virus Genes* 21, 193–195.

Sachdev, S., Bruhn, L., Sieber, H., Pichler, A., Melchior, F., and Grosschedl, R. (2001). PIASy, a nuclear matrix-associated SUMO E3 ligase, represses LEF1 activity by sequestration into nuclear bodies. *Genes Dev.* 15, 3088–3103.

Salmons, T., Kuhn, A., Wylie, F., Schleich, S., Rodriguez, J. R., Rodriguez, D., Esteban, M., Griffiths, G., and Krijnse Locker, J. (1997). Vaccinia virus membrane proteins p8 and p16 are co-translationally inserted into the rough ER and retained in the intermediate compartment. *J. Virol.* 71, 7404–7420.

Sampson, D. A., Wang, M., and Matunis, M. J. (2001). The small ubiquitin-like modifier-1 (SUMO-1) consensus sequence mediates Ubc9 binding and is essential for SUMO-1 modification. *J. Biol. Chem.* 276, 21664–21669.

Sapetschnig, A., Rischitor, G., Braun, H., Doll, A., Schergaut, M., Melchior, F., and Suske, G. (2002). Transcription factor Sp3 is silenced through SUMO modification by PIAS1. *EMBO J.* 21, 5206–5215.

- Sodeik, B., Doms, R. W., Ericsson, M., Hiller, G., Machamer, C. E., van't Hof, W., van Meer, G., Moss, B., and Griffiths, G. (1993). Assembly of vaccinia virus: role of the intermediate compartment between the endoplasmic reticulum and the Golgi stacks. *J. Cell Biol.* 121, 521–525.
- Sternsdorf, T., Jensen, K., and Will, H. (1997). Evidence for covalent modification of the nuclear dot-associated proteins PML and Sp100 by PIC1/SUMO-1. *J. Cell Biol.* 139, 1621–1634.
- Tolonen, N., Doglio, L., Schleich, S., and Krijnse Locker, J. (2001). Vaccinia virus DNA-replication occurs in ER-enclosed cytoplasmic mini-nuclei. *Mol. Biol. Cell* 12, 2031–2046.
- Uchimura, Y., Nakamura, M., Sugawara, K., Nakao, M., and Saitoh, H. (2004). Overproduction of eukaryotic SUMO-1- and SUMO-2-conjugated proteins in *Escherichia coli*. *Anal. Biochem.* 331, 204–206.
- van der Meer, Y., Snijder, E. J., Dobbe, J. C., Schleich, S., Denison, M. R., Spaan, W.J.M., and Krijnse Locker, J. (1999). The localization of mouse hepatitis virus nonstructural proteins and RNA synthesis indicates a role for late endosomes in viral replication. *J. Virol.* 73, 7641–7657.
- Wilcock, D., Duncan, S. A., Traktman, P., Zhang, W. H., and Smith, G. L. (1999). The vaccinia virus A4OR gene product is a nonstructural, type II membrane glycoprotein that is expressed at the cell surface. *J. Gen. Virol.* 80, 2137–2148.
- Wilson, V. G., and Rangasamy, D. (2001). Viral interaction with the host cell sumoylation system. *Virus Res.* 81, 17–27.
- Zhong, S., Muller, S., Ronchetti, S., Freemont, P. S., Dejean, A., and Pandolfi, P. P. (2000). Role of SUMO-1-modified PML in nuclear body formation. *Blood* 95, 2748–2752.