SUMO-1 modification regulates the protein stability of the large regulatory protein Rep78 of adeno associated virus type 2 (AAV-2)

Stefan Weger*, Eva Hammer, Regine Heilbronn

Institut für Infektionsmedizin, Abteilung Virologie, Charité Campus Benjamin Franklin, Freie Universität Berlin, 12203 Berlin, Germany

Received 17 June 2004; returned to author for revision 9 September 2004; accepted 21 September 2004

Abstract

The large Rep proteins Rep78 and Rep68 of the helper-dependent adeno associated virus type 2 (AAV-2) are essential for both site-specific integration of AAV DNA in the absence of helpervirus and productive AAV replication in the presence of helpervirus. We have identified UBC9, the E2 conjugating enzyme for the small ubiquitin-related polypeptide SUMO-1, as binding partner of the large Rep proteins in yeast two-hybrid analysis and in GST pulldown assays. Modification of the large Rep proteins with SUMO-1 could be demonstrated in immunoblot analysis and in immunoprecipitations, with the lysine residue at amino acid position 84 serving as the major attachment site. The largely sumolation-deficient Rep78 lysine to arginine point mutant showed a strongly reduced half-life as compared to the wild-type protein. This finding implicates a role for sumolation in the regulation of Rep78 protein stability that is assumed to be critical for the establishment and maintenance of AAV latency.

© 2004 Elsevier Inc. All rights reserved.

Keywords: AAV; Adeno associated; Rep; SUMO; Modification; Stability; Latency

Introduction

Adeno associated virus type 2 (AAV-2) is a human parvovirus that has been assigned to the genus Dependovirus because it typically relies on a coinfecting helpervirus such as adenovirus or herpes virus for productive replication (Berns and Linden, 1995). Infection in the absence of helpervirus usually results in a latent state of AAV-2 by site-specific integration of the viral DNA into human chromosome 19 (Samulski, 1993). The integrated provirus can then be reactivated by superinfection with a helpervirus (Berns and Linden, 1995).

The coding region of the 4.7-kb single-stranded AAV-2 DNA genome (Srivastava et al., 1983) is flanked by two 145-base-pair inverted terminal repeats (ITRs) and consists of two open reading frames, rep and cap. Cap encodes the three structural proteins VP1, VP2, and VP3 (Becerra et al., 1988; Cassinotti et al., 1988) expressed from a promoter located at map position 40 (p40). Rep encodes four overlapping regulatory (Rep) proteins (Mendelson et al., 1986), which are expressed from two promoters located at map positions 5 (p5) and 19 (p19). The p5 promoter initiates the unspliced and the single-spliced transcripts encoding the large Rep proteins Rep78 and Rep68, respectively. These are essential for AAV-2 DNA replication in the presence of helpervirus (Herononat et al., 1984; Tratschin et al., 1984) and site-specific integration in the absence of helpervirus (Balague et al., 1997; Samulski, 1993; Weitzman et al., 1994). Both functions involve ATP-dependent helicase, site- and strand-specific endonuclease, and sequence-specific DNA binding activities of Rep78 and Rep68 (Im and Muzyczka, 1990, 1992). The p19 proteins Rep52 and Rep40 lack the 224 amino terminal residues of Rep78 and Rep68 involved in site-specific DNA binding (Owens et al., 1993). Through complex formation with Rep78 and Rep68 (Dubielzig et al., 1999), they mediate the translocation of the single-stranded AAV progeny DNA into the preformed capsid (King et al., 2001).
While Rep78 and Rep68 inhibit all three AAV-2 promoters in the absence of helpervirus (Horner et al., 1995; Kyostio et al., 1994; Trempe and Carter, 1988), they activate the p19 and p40 promoters after coinfection with adenovirus. This activation depends upon the Rep binding sites in the ITRs (ITR-RBS) and the p5 promoter as well as additional elements in the p19 and p40 promoters (McCarty et al., 1991; Pereira and Muzycka, 1997a, 1997b; Pereira et al., 1997; Weger et al., 1997). The p5 promoter is both activated by Rep78/Rep68 in the presence of adenovirus mediated by the ITR-RBS and repressed by Rep78/Rep68 mediated by the p5RBS (Pereira et al., 1997).

The Rep proteins can bind to a variety of cellular proteins. Interaction of Rep78 and Rep68 with the transcription factor Sp1 is involved in the activation of p19 and p40 gene expression in the presence of adenovirus (Pereira and Muzycka, 1997b). Through their C-terminal domain, Rep78 and Rep52 interact with and inhibit cAMP-dependent protein kinase A (PKA) and its homolog PrKX (Chiorini et al., 1998). The Rep78/Rep52 mediated decrease in PKA activity inhibits adenovirus replication and is necessary to preserve AAV2 replication fitness during an Ad co-infection (Di Pasquale and Chiorini, 2003). Other cellular interaction partners of the Rep proteins include the high-mobility chromosomal protein HMG1 (Costello et al., 1997), the TATA box binding protein TBP (Hermonat et al., 1998), the tumor suppressor protein p53 (Batchu et al., 1999), the transcriptional coactivator PC4 (Weger et al., 1999), and the topoisomerase I binding RS-rich protein Topors (Weger et al., 2002).

Not much is known yet about posttranslational modifications of the AAV Rep proteins, which might regulate their enzymatic activities and their interaction with cellular proteins. All four Rep proteins have been found to be phosphorylated at serine residues after AAV/adenovirus coinfection (Collaco et al., 1997) and hyper-phosphorylation of the Rep78 protein inhibits its binding to the AAV terminal repeats and its helicase activity in vitro (Narasimhan et al., 2002). The phosphorylation of Rep68 at serine residue 535 is critical for its association with 14-3-3 proteins ε and γ (Han et al., 2004).

Small ubiquitin-like modifier (SUMO) proteins are ubiquitously expressed in eukaryotic cells and covalently attached to a variety of cellular and viral proteins through the E1-activating enzyme Aos1/Uba2 and the E2-conjugating enzyme Ubc9 (Melchior, 2000; Muller et al., 2001). Several E3 ligases, which enhance sumolation of specific target proteins, have been identified recently (Johnson and Gupta, 2001; Kabyo et al., 2001; Sachdev et al., 2001; Schmidt and Muller, 2002). Sumolation has been shown to modulate protein–DNA or protein–protein interactions to alter the intracellular localization of the target protein or to protect it from ubiquitin-mediated degradation (Melchior, 2000).

In this study, we report the identification of the human SUMO-1-conjugating enzyme UBC9 as an interaction partner of the large Rep proteins Rep78 and Rep68. Furthermore, we demonstrate the covalent modification of the large Rep proteins by SUMO-1 in vivo, with the lysine residue at amino acid position 84 as the major SUMO-1 attachment site. The strongly reduced half-life observed for a largely sumolation-deficient mutant Rep78 protein suggests a critical role for SUMO-1 modification in the regulation of the stability of the large Rep proteins.

Results

Rep68 interacts with the SUMO-1-conjugating enzyme UBC9 protein in the yeast-two hybrid system

We have previously identified the transcriptional coactivator PC4 and a DNA Topoisomerase I binding Protein named Topors (for topoisomerase I-binding RS protein) as interaction partners of the large nonstructural AAV-2 proteins Rep78 and Rep68 in yeast based two-hybrid screens (Weger et al., 1997, 2002). In one of these screens with a construct harboring Rep amino acids 172–530 (M172/520, Fig. 1A) as bait and a target library from noninfected HeLa cells, two additional positive clones were identified. Both contained the complete open reading frame of UBC9 (human ubiquitin conjugating enzyme 9). UBC9 alone did not activate reporter gene expression in yeast in the absence of the bait protein. UBC9 is comprised of 158 amino acids and functions as an E2 enzyme in the conjugation of the SUMO (small ubiquitin like modifier) family of proteins to cellular and viral target proteins (Johnson and Blobel, 1997; Schwarz et al., 1998; Tatham et al., 2001). We analyzed the Rep domains involved in interaction with UBC9 in more detail (Fig. 1B). While the first 172 amino acids of the large Rep proteins were not required, deletion of Rep amino acids 172–224 abolished the interaction (Fig. 1B, compare M172/530 and Rep40). Rep amino acid residues 482–530 were dispensable for Rep–UBC9 interaction (Fig. 1B, M1/481). Deletion of further carboxy terminal amino acids led to a stepwise reduction of reporter gene activation (Fig. 1B, M1/369 and M1/243). The finding that a construct harboring Rep amino acids 172–243 scored negative seems to contrast the results obtained with Rep68 and M172/530. However, the lack of a detectable interaction may be due either to low expression levels or improper folding of the rather small fusion protein. A well-characterized point mutation in the nucleotide binding of the Rep proteins, which abrogates their enzymatic activities, demonstrates that this motif is dispensable for the interaction with UBC9 (Fig. 1B, M1/369 and M1/243). The finding that a construct harboring Rep amino acids 172–243 scored negative seems to contrast the results obtained with Rep68 and M172/530. However, the lack of a detectable interaction may be due either to low expression levels or improper folding of the rather small fusion protein. A well-characterized point mutation in the nucleotide binding of the Rep proteins, which abrogates their enzymatic activities, demonstrates that this motif is dispensable for the interaction with UBC9 (Fig. 1B, M1/369 and M1/243).
Fig. 1. Identification of UBC9 as a Rep-interacting protein. (A) Schematic representation of the AAV2 genome, the Rep68 and Rep40 proteins and the Rep deletion mutant M172/530 used as bait in the yeast two-hybrid-screen. The inverted terminal repeats (ITRs) are represented by hatched boxes, the three promoters at map units 5, 19, and 40 are indicated by right-angled arrows and the common polyadenylation (polyA) site for all transcripts at map position 96 is indicated by the vertical arrow. Closed and differently shaded boxes indicate the Rep78/Rep68 specific region, the region common to all 4 Rep proteins and the Rep68/Rep40 specific region. Characteristic amino acid positions are given above the boxes. (B) Analysis of Rep domains required for interaction with full-length UBC9 in the yeast two-hybrid system. The Rep regions fused to the Gal4 DNA binding domain are shown schematically on the left and indicated by amino acid numbers. The respective $\beta$-galactosidase ($\beta$-gal) activities expressed in Units are shown on the right and are corrected for the values obtained in control cotransformations of the respective Rep constructs with pGAD424, which were all below 1.0 Unit. (C) Analysis of UBC9 domains required for interaction with Rep-M1/481 in the yeast two-hybrid system. The UBC9 regions fused to the Gal4 transactivation domain are shown schematically on the left and indicated by amino acid numbers. The respective $\beta$-gal activities expressed in units are shown on the right and are corrected for the values obtained in control cotransformations of the respective UBC9 constructs with pGHT9, which were all below 1.0 $\beta$-gal Units. (B, C) The mean and standard deviation are presented for at least four assays performed with independent transformants.
interaction with Rep, respectively (Fig. 1C). The function of UBC9 as a SUMO-conjugating enzyme relies on the active site cysteine residue at amino acid position 93 (Tong et al., 1997), which is engaged in thioester formation with the carboxy group of a terminal glycine residue of the SUMO proteins generated by proteolytic cleavage (Mahajan et al., 1998). Point mutation of this active site cysteine residue to serine did not affect the interaction with Rep (Fig. 1C, UBC9C93S). Thus, the Rep–UBC9 interaction is independent of UBC-SUMO thioester formation.

**In vitro binding of 35S-labeled Rep78 to a GST–UBC9 fusion protein**

To confirm that the Rep–UBC9 interaction detected in the yeast two-hybrid system was due to a direct binding of Rep to UBC9, GST pulldown assays with in vitro transcribed/translated Rep proteins were performed. While Rep78 specifically bound to a GST fusion protein of full-length UBC9 (Fig. 2, upper panel), the corresponding small Rep52 protein showed no binding (Fig. 2, lower panel). These findings correspond to the results obtained in the yeast two-hybrid analysis.

**Rep78 and Rep68 can be covalently modified by SUMO-1**

Since UBC9 functions as a SUMO E2 conjugating enzyme, we examined a possible SUMO-1 modification of the large AAV-2 Rep proteins Rep78 and Rep68. For this purpose, Rep expression constructs were transfected into HeLa cells together with a YFP (yellow fluorescent protein) tagged SUMO-1 fusion protein. As a control, cells were cotransfected with the Rep expression constructs and a construct encoding the YFP protein only. Cell extracts were prepared under denaturing conditions and subjected to Western blot analysis with a monoclonal anti-Rep antibody. After coexpression of Rep78 or Rep68 with the YFP-tagged SUMO-1 protein, additional Rep isoforms at 120 or 110 kDa, respectively, could be detected (Fig. 3A, lanes 4 and 6). These Rep isoforms, which were not observed in the control transfections of the large Rep proteins together with the YFP protein (Fig. 3A, lanes 3 and 5), are consistent with a covalent modification of the respective Rep protein by a single YFP-SUMO-1 moiety, which has a size of 40 kDa. Several low-molecular-weight bands probably representing degradation products of Rep78 or Rep68 were detected in all Rep-expressing cell lysates, regardless of the presence or absence of YFP-SUMO (Fig. 3A, lanes 3–6). No Rep reactive bands were detected in control cells expressing YFP (Fig. 3A, lane 1) or YFP-SUMO-1 (Fig. 3A, lane 2) excluding the possibility of a cross-reaction of the Rep antibody with cellular proteins. To confirm that the bands at 120 and 110 kDa did indeed correspond to YFP-SUMO-1 modified Rep proteins, whole cell extracts of HeLa cells transfected as described above were subjected to immunoprecipitation with a Rep-antiserum. Immunoprecipitates were subsequently analyzed for YFP-SUMO-1 conjugates of Rep78 and Rep68 by Western analysis using an anti-YFP antibody (Fig. 3B). YFP reactive bands at 120 or 110 kDa corresponding in size to the bands observed in the anti-Rep Western could be detected after coexpression of Rep78 and Rep68 with YFP-SUMO-1 (Fig. 3B, lanes 4 and 6). These bands were not observed in the absence of YFP-SUMO-1 (Fig. 3B, lanes 3 and 5) or Rep protein expression (Fig. 3B, lanes 1 and 2). These experiments clearly demonstrate that an exogenous SUMO-1 protein can covalently modify both Rep78 and Rep 68.

**Rep lysine residue 84 is the major acceptor site for SUMO-1 modification**

An acceptor consensus sequence aKX(E,D), where “K” is the modified lysine residue and “a” represents a large hydrophobic amino acid, has been depicted from the analysis of the acceptor sites in a variety of SUMO-1 target proteins (Melchior, 2000). No KXD motif is present within the open reading frames of Rep78 or Rep68, whereas both proteins share three common KXE motifs. These KXE motifs are located at Rep78/Rep68 amino acid positions 84, 447, and 463 (Fig. 4A). Only the lysine residue at amino acid position 447 is directly preceded by a hydrophobic amino acid. To identify the Rep SUMO-1 acceptor site, lysine residues 84, 447, and 463 were mutated to arginine residues, either individually or in various combinations. The
replacement of lysine by arginine was chosen to maintain a basic charged residue at the respective amino acid position. The mutant Rep78 proteins were then analyzed for SUMO-1 conjugation after coexpression with the YFP-tagged SUMO-1 protein as described above. Both the K447R mutant and the K463R mutant could still be conjugated with YFP-SUMO-1 to a similar extent as the wild-type Rep78 protein (Fig. 4B, compare lanes 2, 6, and 8). In contrast, modification by YFP-SUMO-1 was almost completely abolished for the K84R mutant (Fig. 4B, lane 4). In line with this finding, a strong reduction in sumolation was also observed for the K84R/K447R double mutant (Fig. 4C, lane 4) and no sumolation was detected for the triple mutant with all three lysine residues mutated to arginine (Fig. 4C, lane 6). These experiments identify Rep amino acid 84 as the major acceptor site for SUMO-1 modification. The lysine residue at amino acid position 84 is not directly preceded by a hydrophobic amino acid and thus does not seem to fit into the sumolation consensus sequence. However, three phenylalanine residues and one valine residue are found in amino acid positions −2 to −6 with respect to this lysine residue (Fig. 4A).

Influence of SUMO-1 modification on Rep78 protein stability

SUMO-1 modification has been implicated in the regulation of protein–protein interactions, of protein stability, and in the determination of the intracellular localization of the respective target protein (Melchior, 2000). When we examined the intracellular localization of the largely sumolation-deficient Rep78 K84R mutant in comparison to the wild-type protein by immunofluorescence analysis, we observed no differences between the two proteins. Either

Fig. 4. Mapping of the SUMO-1 attachment site of Rep78. (A) Sequence alignment of the minimal sumolation consensus motif and the three KXE motifs found in the Rep78 reading frame. (B, C) Analysis of SUMO-1 modification of wild-type Rep78 protein and different Rep78 lysine to arginine point mutants. HeLa cells were cotransfected with the respective pKEXRep78 expression construct, pKEX-UBC9 expressing full-length UBC9 under control of the HCMV promoter, and either the YFP (lanes marked “−”) or the YFP-SUMO-1 expression construct (lanes marked “+”). Whole cell extracts were analyzed by immunoblotting with the monoclonal Rep antibody 303.9. Arrows indicate unmodified and YFP-SUMO-1 modified forms of the Rep78 proteins.

Fig. 3. Rep78 and Rep68 can be covalently modified by SUMO-1. (A, B) HeLa cells were cotransfected with pEYFP-SUMO encoding an YFP-SUMO-1 fusion protein and Rep expression plasmids pKEXRep78 or pKEXRep68 as indicated. As controls (lanes marked “−”), pEYFP-C2 encoding the YFP protein or the empty expression vector pKEX, respectively, were cotransfected. Filled dots indicate YFP-SUMO-1 modified forms of Rep78 and Rep68. (A) Unmodified and YFP-SUMO-1-modified forms of the Rep proteins in whole cell extracts were analyzed by immunoblotting with the monoclonal Rep antibody 303.9. (B) Whole cell extracts were subjected to immunoprecipitation with a polyclonal anti-Rep antiserum and precipitated proteins were analyzed by immunoblotting with an anti-GFP polyclonal rabbit antibody, which is also reactive with the YFP protein. The left-handed arrow indicates the position of the IgG heavy chains of the Rep-antiserum used for the immunoprecipitation.
protein localized homogenously in the nucleus with the exclusion of nucleoli (data not shown). A possible contribution of SUMO-1 modification to Rep78 protein stability was examined in pulse-chase experiments. HeLa cells transfected with wild-type Rep78 or the Rep78-K84R mutant were labeled with $^{35}$S-methionine and $^{35}$S-cysteine for a 2-h pulse from 22 to 24 h post transfection and cells were chased for 2, 12, 24, or 36 h. No difference was observed in the synthesis of the wild-type Rep78 protein and the K84R mutant during the initial labeling period (Figs. 5A, B, 0 h). Thus, differential autoregulatory effects of the two Rep proteins on the CMV promoter driving their expression or major differences in translation efficiencies can be excluded. While the amount of labeled Rep78 wild-type protein showed only a minor, about two-fold decrease over a 24-h time period (Fig. 6B), a 20-fold reduction in the amount of labeled K84R-Rep78 protein was observed over the same time period. The respective half-lives were calculated to 14.5 h for the wild-type Rep78 protein compared to only 4.7 h for the mutant Rep78-K84R protein.

Sumolation of the large AAV Rep proteins is not required for productive AAV replication

Since sumolation obviously is involved in the regulation of Rep78 protein stability, we wondered whether this modification is essential for the productive replication cycle of AAV-2 in the presence of helper virus. To address this question, a mutant AAV genome carrying the lysine to arginine point mutation at amino acid position 84 of the Rep proteins in a plasmid backbone was generated (pTAV-K84R). pTAV-K84R and the corresponding wild-type AAV plasmid (pTAV2-0) were transfected into HeLa cells, which were subsequently infected with adenovirus type 2 (Ad2) at a multiplicity of infection (MOI) of 10. Expression of Rep and Cap proteins was monitored in Western blot analysis at 18, 26, 40, or 50 h post infection. In parallel, AAV DNA replication was assayed at 18 or 26 h post infection and the amount of infectious AAV particles was determined at 20, 32, or 44 h post infection. Mutating the SUMO-1 acceptor site had no major effect on accumulation of Rep and Cap proteins (Fig. 6A) or on AAV DNA replication (Fig. 6B). The mutant AAV genome also generated similar amounts of AAV particles as the wild type AAV genome. At no time point after transfection did the titer of infectious virus obtained for AAV wild type exceed that obtained for the mutant virus by more than one log (Fig. 6C). Similar results were obtained when the corresponding virus preparations were used for reinfection of new cells at equal MOIs (data not shown). These data show that the stability of the Rep78 protein does not limit productive AAV replication in cell culture in the presence of adenovirus as a helper.

Discussion

The large Rep proteins Rep78 and Rep68 of AAV-2 are multifunctional proteins that are essential for AAV DNA replication and regulation of AAV gene expression (Berns, 1990; Carter et al., 1990). Using a yeast-based two-hybrid system, we have identified UBC9, the conjugating enzyme for the SUMO family of ubiquitin-related polypeptides, as an interaction partner of the large Rep proteins. Direct binding of UBC9 to Rep78 was confirmed by an in vitro binding assay. We could demonstrate that Rep78 and Rep68 are subject to SUMO-1 modification at amino acid position 84 as the main acceptor site. A largely sumolation-deficient Rep78 mutant exhibits a strongly reduced half-life, implicating SUMO-1 modification in the regulation of Rep protein stability.

UBC9 physically interacts with various cellular and viral proteins and mediates the attachment of SUMO-1 to one or more lysine residues in the target protein (Johnson and Blobel, 1997). In analogy to the ubiquitin system, several SUMO-1 E3 ligases that enhance the sumolation of specific substrates have been identified recently (Pichler et al., 2002; Sachdev et al., 2001). Although UBC9-mediated alterations...
in nuclear localization and transcriptional activity independent of sumolation have also been reported for a small subset of UBC9 binding proteins (Hahn et al., 1997; Kurtzman and Schechter, 2001; Poukka et al., 1999), most of the UBC9-interacting proteins have subsequently been identified as targets for SUMO modification (Melchior, 2000). In line with the function of UBC9 as a SUMO-1-conjugating enzyme, Rep isoforms corresponding in size to the addition of a single SUMO-1 moiety could be detected after coexpression of an exogeneous YFP-tagged SUMO-1 protein both in Western blot analysis and in immunoprecipitation. The denaturing conditions that were employed in these experiments confirm the covalent nature of the interaction. Only a small fraction of the total amount of the large Rep proteins was found to be posttranslationally modified by SUMO-1. The same phenomenon has also been observed for many other SUMO-1 target proteins like the tumor suppressor protein p53 (Gostissa et al., 1999; Rodriguez et al., 1999) or the transcription factor Sp3 (Ross et al., 2002; Sapetschnig et al., 2002). Sumolation is a reversible modification and the actual proportion of the sumolated form of the respective target protein may depend upon the relative activities of the enzymes involved in SUMO conjugation and SUMO specific isopeptidases, which have been found in a variety of cellular compartments (Gong et al., 2000; Kim et al., 2000; Nishida et al., 2000). Alternatively, only a subpopulation of the large Rep proteins, which fulfills specific functions within the cell, may be subject to sumolation. These possibilities are not mutually exclusive. The SUMO-1 modification of the large Rep proteins observed after transient expression of both proteins raises the question whether Rep78 and Rep68 are also modified by endogenous SUMO-1 after coinfection of cells with AAV-2 and helper virus. Although we observed some anti-Rep reactive protein bands migrating slower than the major Rep78 and Rep68 bands after AAV-2/adenovirus coinfection, we were not able to definitely prove that these bands correspond to SUMO-1-modified forms of the large Rep proteins (data not shown). The levels of exogenous YFP-SUMO-1 obtained after cotransfection of the CMV-driven expression construct are probably quite high as compared to the level of endogenous SUMO-1 protein, which may lead to an overstatement of the fraction of the Rep proteins actually modified by SUMO-1 under physiological conditions. In line with this argumentation, we favor the possibility that modification of the AAV Rep proteins by endogenous SUMO-1 may be below the detection limits of the commonly available SUMO-1 antibodies.

The major SUMO-1 lysine acceptor residue is located in the N-terminal region of Rep78/Rep68 at amino acid position 84 of the large Rep proteins. (A–C) HeLa cells were transfected either with the AAV wild-type plasmid pTA2-0 (wt) or with plasmid pTA2K84R (mu) carrying the lysine to arginine point mutation and overinfected with adenovirus type 2 at an MOI of 10. At the indicated time points post infection, cells were monitored (A) for Rep and Cap expression by immunoblot analysis with monoclonal antibodies 303.9 and B1, respectively, (B) for AAV DNA replication by Southern blot analysis and (C) for production of infectious virions by dot blot analysis.

Fig. 6. Replication of a mutant AAV genome harboring a lysine to arginine point mutation at amino acid position 84 of the large Rep proteins. (A–C) HeLa cells were transfected either with the AAV wild-type plasmid pTA2-0 (wt) or with plasmid pTA2K84R (mu) carrying the lysine to arginine point mutation and overinfected with adenovirus type 2 at an MOI of 10. At the indicated time points post infection, cells were monitored (A) for Rep and Cap expression by immunoblot analysis with monoclonal antibodies 303.9 and B1, respectively, (B) for AAV DNA replication by Southern blot analysis and (C) for production of infectious virions by dot blot analysis.
position 84. Some residual modification was seen after point mutation of this lysine residue to arginine, which may point to the presence of additional, alternative acceptor sites.

Similar findings have been made with p53, c-Jun (Schmidt and Muller, 2002), TEL (Chakrabarti et al., 2000), and Sp100 (Pichler et al., 2002). Rep lysine residue 447 may be one candidate for an alternative acceptor site, since point mutation of this lysine residue further reduced sumolation. From a variety of SUMO-1 target proteins, a sumolation consensus site, KxE (where ψ represents a large hydrophobic amino acid), has been depicted (Hay, 2001; Melchior, 2000). Whereas the amino acids surrounding Rep lysine residue 447 fit into the sumolation consensus sequence, those surrounding the major Rep sumolation site at lysine residue 84 do not. However, nonconsensus SUMO-1 acceptor sites have also been reported from other target proteins (Hoeger et al., 2002; Kagey et al., 2003; Kim et al., 1999). Of note, a total of four hydrophobic amino acids are found at position −2 to −6 with respect to Rep amino acid 84, which may compensate for the lack of a hydrophobic amino acid at position −1.

Whereas the intracellular localization of the largely sumolation-deficient Rep78-K84R mutant was not altered, it showed a markedly reduced half-life as compared to the wild-type Rep78 protein. The mechanisms underlying the regulation of the stability of the large Rep proteins by SUMO-1 modification remain to be elucidated. For the inhibitor protein I-B, which sequesters the transcription factor NFκB in the cytoplasm, a competition of sumolation and ubiquitination for the same lysine residue was demonstrated (Desterro et al., 1998). By this mechanism, SUMO-1 modification prevents polyubiquitination and subsequent proteasome-mediated degradation of IκB. Nothing is known yet about polyubiquitination of the large Rep proteins. However, our results confirm those of earlier studies (Redemann et al., 1989) that the AAV-2 Rep proteins are rather long-lived proteins and probably do not undergo extensive polyubiquitination. An AAV genome harboring largely sumolation-deficient Rep78/Rep68 proteins was clearly not impaired for productive AAV infection in cell culture in the presence of adenovirus. This was not an unexpected finding since it has been shown that the concentration of the large Rep proteins is not the limiting factor for AAV replication (Weger et al., 1997). On the contrary, a modest reduction in the levels of the large Rep proteins may even enhance AAV DNA replication and capsid protein expression (Li et al., 1997). Concerning the importance of Rep sumolation for the AAV life cycle, one may speculate that the stability of large Rep proteins plays a role in the maintenance of the latent state of AAV in the absence of helper virus. During AAV latency in cell culture, only a very low expression of AAV genes can be detected (Laughlin et al., 1986; Walz and Schlehofer, 1992). This low expression level is most probably due to down regulation of all three AAV promoters by the large Rep proteins (Horer et al., 1995, 2002). Plasmid pGAD424-UBC9 (1–158) encoding a fusion protein of the Gal4 transactivation domain with full-length UBC9 corresponds to one of the original clones obtained in the yeast two-hybrid screen for Rep interacting protein. All other UBC9 two-hybrid constructs were obtained from pGAD424-UBC9 (1–158) through PCR amplification of the indicated parts of the UBC9 coding region or by site-directed mutagenesis (QuikChange Site-Directed Mutagenesis Kit, Stratagene).

The GST-UB9 expression vector pGEX-UBC9 was obtained by subcloning of the UBC9 coding region and 3′ nontranslated region from pGAD24-UBC9 (1–158) into plasmid pGEX-4T-3 (Promega).

Plasmids pKEXRep78 and pKEXRep68 expressing AAV-2 Rep78 or Rep68, respectively, under control of the human cytomegalovirus early promoter and plasmid pEYFP-SUMO-1 expressing the yellow fluorescent protein with the complete open reading frame of the Rep open reading frame have been described (Weger et al., 1999, 2002). Plasmid pGAD424-UBC9 (1–158) encoding a fusion protein of the Gal4 transactivation domain with full-length UBC9 corresponds to one of the original clones obtained in the yeast two-hybrid screen for Rep interacting protein. All other UBC9 two-hybrid constructs were obtained from pGAD424-UBC9 (1–158) through PCR amplification of the indicated parts of the UBC9 coding region or by site-directed mutagenesis (QuikChange Site-Directed Mutagenesis Kit, Stratagene).

A Gal4-based yeast two-hybrid screening system (Clontech) was used to isolate cDNAs, which encode proteins that are able to interact with the central part of the AAV-2 large Rep proteins. Yeast two-hybrid screening of a cDNA library from noninfected HeLa cells was performed essentially as described in Weger et al. (1999) using HF7c yeast cells.
Quantitative two-hybrid interaction studies were performed in yeast strain SFY526 (Clontech) as described in Weger et al. (2003).

**GST pull-down assays**

GST pull-down assays with a GST-UBC9 fusion protein and $^{35}$S labeled Rep78 and Rep52 were performed as described in Weger et al. (1999). Bound Rep proteins were eluted with SDS sample buffer, loaded on a 10% polyacrylamide gel (SDS-PAGE) and visualized by autoradiography.

**Immunoprecipitation and Western blot analysis**

HeLa cells transfected with the indicated expression constructs were lysed directly in the culture dishes with 1 ml RIPA buffer (50 mM Tris–HCl pH 8.0, 150 mM NaCl, 0.5% DOC, 0.1% SDS and 1% NP40) containing 10 mM iodoacetamide at 4°C. Constructs were lysed directly in the culture dishes with 1 ml RIPA buffer (50 mM Tris–HCl pH 8.0, 150 mM NaCl, 0.5% DOC, 0.1% SDS and 1% NP40) containing 10 mM iodoacetamide. Immunoprecipitated proteins were analyzed by Western blot using a polyclonal antiserum from guinea pig and subsequently 30 μl Protein A Sepharose beads in RIPA buffer (10%, w/v) were added for 1 h at 4°C. Beads were washed five times with RIPA buffer containing 10 mM iodoacetamide. Immunoprecipitated proteins were analyzed by Western blot using a polyclonal anti-GFP antiserum from goat (Biotrend, Köln, Germany).

**Pulse-chase analysis**

Transfected cells were metabolically labeled with 50 μCi of $^{35}$S-methionine and $^{35}$S-cysteine (in vitro cell labeling mix, Amersham) for 2 h. Labeling medium was removed and cells were washed three times with 1 ml of DMEM containing unlabeled methionine and cysteine. After further incubation for the indicated time periods, cells were harvested in 1 ml RIPA buffer and subjected to immunoprecipitation with an anti-Rep antiserum as described above. Immunoprecipitated Rep proteins were analyzed by SDS-PAGE and subsequent autoradiography.

**Extraction of viral DNA and Southern blotting**

Extraction of viral DNA by a modified Hirt procedure and Southern blotting were performed as described in Weger et al. (1997). After treatment with DpnI to digest input DNA, the DpnI-resistant replicated pAAV-DNAs were detected with a 1.59-kb $^{32}$P labeled HindII fragment from plasmid pTAV2-0 (Heilbronn et al., 1990) harboring a large part of the cap open reading frame.

**Dot blot analysis of infectious AAV virions**

Cells transfected with infectious AAV genomes and overinfected with adenovirus type 2 (MOI= 10) were lysed by three freeze–thaw cycles. Cellular debris was removed by centrifugation for 10 min at 5000 ×g and 4°C and the supernatant was incubated for 1 h at 56°C to inactivate the adenovirus. HeLa cells in 96-well plates (1 × 10⁶ cells/well) were infected with 10 μl each of serial dilutions of the supernatants and coinfected with adenovirus type 2 (MOI = 10). After 3 days, cells were subjected to three freeze–thaw cycles, transferred to a nylon membrane, and hybridized with the 1.59-kb $^{32}$P labeled HindII pTAV2-0 fragment described above.

**Acknowledgment**

This work was supported by a grant from the Deutsche Forschungsgemeinschaft (SFB 506).

**References**


Hoeger, C., Pfander, B., Moldovan, G.L., Pyrowolakis, G., Jentsch, S., 2002. RAD6-dependent DNA repair is linked to modification of PCNA by ubiquitin and SUMO. Nature 419 (6903), 135–141.


