Bovine Papillomavirus E1 Protein Is Sumoylated by the Host Cell Ubc9 Protein*

Received for publication, May 8, 2000, and in revised form, June 21, 2000 Published, JBC Papers in Press, June 27, 2000, DOI 10.1074/jbc.M003898200

Dhandapani Rangasamy and Van G. Wilson‡

From the Department of Medical Microbiology and Immunology, Texas A&M University System Health Science Center, College Station, Texas 77843-1114

Papillomavirus E1 protein is the replication initiator that recognizes and binds to the viral origin and initiates DNA strand separation through its ATP-dependent helicase activity. The E1 protein also functions in viral DNA replication by recruiting several cellular proteins to the origin, including host DNA polymerase α and replication protein A. To identify other cellular proteins that interact with bovine papillomavirus E1, an HeLa cDNA library was screened using a yeast two-hybrid assay. The host cell sumoylating enzyme, Ubc9, was found to interact specifically with E1 both in vitro and in vivo. Mapping studies localized critical E1 sequences for interaction to amino acids 315–459 and strongly implicated leucine 420 as critical for E1-Ubc9 complex formation. In addition to binding E1, Ubc9 catalyzed the covalent linkage of the ubiquitin-like protein, SUMO-1, to E1. An E1 mutant unable to bind Ubc9 showed normal intracellular stability, but was impaired for intranuclear distribution. Failure to accumulate in appropriate nuclear subdomains may account for the previously demonstrated replication defect of a human papillomavirus 16 E1 protein that was also unable to bind Ubc9 and suggests that sumoylation is a functionally important modification with regulatory implications for papillomavirus replication.

Bovine papillomavirus (BPV)† is a valuable model for the study of eukaryotic DNA replication. A characteristic feature of BPV is the ability to remain stably maintained in the host cell nucleus as an extrachromosomal replicon of relatively constant copy number (1). This regulated replication of the viral genomes undoubtedly requires a complex interaction of both viral and host cell proteins (2). The E1 and E2 proteins are the only two viral proteins required to replicate the viral genome, with the rest of the replication machinery being supplied by the host cell (2, 3). E1 is a multifunctional, nuclear protein whose properties include ATP-dependent DNA helicase activity, sequence-specific DNA binding to the origin of replication, and initiation of viral DNA replication (3–7). For initiation of viral DNA synthesis in vitro, only the E1 protein is required, indicating that the E2 protein does not supply a requisite replication function (7). Instead, association of E1 with the E2 transactivator enhances the binding of E1 proteins on the viral origin to form the active initiation complex (8–10). In addition to the E2 protein, E1 interacts with host cell DNA polymerase α and replication protein A (11–13) and presumably recruits these replication factors to the viral origin. The E1 protein also has regions of sequence and functional homology with the well-studied viral initiator protein, SV40 large T antigen, including the domains necessary for DNA helicase activity, ATPase activity, and DNA polymerase binding (14). Furthermore, there are some intriguing similarities in structural organization of the DNA binding domains for T antigen and E1 that suggest they possess related three-dimensional structures (15).

Several additional host cellular proteins have been found to interact with the E1 protein including histone H1 (16), SW1/SNF5 (17), cyclin E/cdk (18), Hsp40/Hsp70 (19) and Ubc9 (20) though the functional significance of all these interactions has not been completely characterized. To identify additional cellular proteins that interact with E1, the yeast two-hybrid approach was used to find E1 partners. We demonstrate that the BPV E1 protein interacts with a host cell protein designated Ubc9. A previous study with HPV16 E1 (20) also noted an interaction between E1 and Ubc9 and found that reduction in Ubc9 binding correlated with a viral replication defect. However, no data were presented pertaining to possible mechanistic pathways by which viral replication was affected. Ubc9 is now known to be related to E2-type ubiquitin-conjugating enzymes (21). Instead of ubiquitin, however, Ubc9 catalyzes the modification of target proteins by covalent addition of a small ubiquitin-like modifier known as SUMO-1 (22).

The conjugation of SUMO-1 to cellular proteins has been implicated in multiple vital cellular processes, including nuclear transport, cell cycle control, oncogenesis, and the response to viral infection (23–25). Unlike ubiquitin, attachment of SUMO-1 does not appear to target proteins for rapid degradation and instead has been proposed to change the ability of modified protein to interact with other cellular proteins (26–28). The 1xα protein was reported to be modified by SUMO-1 at the same residue as the one used for ubiquitylation, thus rendering the protein resistant to proteasomal degradation (29). In the case of p53, a well-established substrate of the ubiquitin/proteasome system, the covalent linkage of SUMO-1 enhances both the stability and the transactivation ability of the p53 protein (30–32). SUMO-1 has also been shown to be covalently linked to RanGAP1, the activating protein of RanGTPase involved in the regulation of nucleocytoplasmic trafficking. Conjugation of SUMO-1 to RanGAP1 targets the protein from its otherwise cytosolic localization to the nuclear pore complex (27, 33). In addition, promyelocytic leukemia-associated (PML) protein and Sp100 are two important SUMO-1-conjugated proteins of the so-called PML nuclear bodies, also known as ND10s (28, 34, 35). ND10s are targeted for destruc-

* This work was supported by American Cancer Society Grant VM-183. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1794 solely to indicate this fact.

‡ To whom correspondence should be addressed: Tel.: 979-845-5207; Fax: 979-845-3479; E-mail: v-wilson@tamu.edu.

† The abbreviations used are: BPV, bovine papillomavirus; HPV, human papillomavirus; PML, promyelocytic leukemia protein; 3-AT, 3-amino-1,2,4-triazole; DBD, DNA binding domain; GAD, GAL4 activation domain; GFP, green fluorescence protein; GST, glutathione S-transferase; HA, hemagglutinin; PCR, polymerase chain reaction.

This paper is available on line at http://www.jbc.org

30487
**Sumoylation of BPV E1 Protein**

**GST Fusion Protein Expression and GST Pull-down Assay**—The Ubch-cDNA was amplified by PCR from the pGAL4-GH library clone and ligated into the pGEX-5X-1 vector (Amersham Pharmacia Biotech, Piscataway, NJ) between the Smal and BamHI sites for expression of the GST-Ubc9 fusion protein in *E. coli*. Both GST-Ubc9 and GST alone were purified by affinity chromatography using Glutathione Sepharose 4B (Amersham Pharmacia Biotech) as described previously (15). The various wild-type and mutant E1 proteins were expressed from the pRSET clones using the T7-coupled rabbit reticulocyte lysate system in the presence of [35S]methionine according to the manufacturer’s instructions (Promega, Madison, WI). For the binding assay, ~2 μg of GST alone or GST-Ubc9 fusion proteins were prebound to glutathione-Sepharose beads by incubating the beads with agitation for 15 min at 4 °C, then washing with 0.5 ml of binding buffer (10 mM Tris-HCl, pH 7.4, 50 mM NaCl, 2% bovine serum albumin). Three μl of [35S]labeled protein was then added, and incubation was continued for at least 2 h. The beads were washed five times with 0.5% Nonidet P-40 in 10 mM Tris-HCl, pH 8, 140 mM NaCl, and 0.025% NaN₃, buffer. Labeled protein bound on the beads was recovered by heating at 70 °C in 10 μl of SDS-sample buffer (150 mM Tris-HCl, pH 6.7, 4% SDS, 30% glycerol) and was analyzed by SDS-polyacrylamide gel electrophoresis. Radiolabeled bands were visualized by autoradiography and were quantitated by PhosphorImager analysis (Molecular Dynamics).

**In Vitro SUMO-1 Conjugation Assay**—To test for SUMO-1 modification, a HeLa cell extract containing SUMO-1-activating enzymes, UBA2AOS1 (25), was prepared as described previously (39). [35S]-La beled protein (10–20 μg) was translated in vitro using rabbit reticulocyte lysate supplemented with 5 μl of HeLa cell extracts in a 25–μl reaction, including an ATP-regenerating buffer (50 mM Tris-HCl, pH 7.6, 5 mM MgCl₂, 2 mM ATP, 10 mM creatine phosphate, 3 units/ml creatine kinase, and 0.5 unit/ml inorganic pyrophosphatase), 6 μg of purified SUMO-1, and 1 μg of Ubch9. Reactions were incubated at 37 °C for 2 h. Control reactions had one or more of the components omitted and replaced by additional buffer. After terminating the reaction with SDS-sample buffer, reaction products were analyzed by SDS-polyacrylamide gel electrophoresis followed by phorsphorimaging.

**Transfection and Coimmunoprecipitation**—For transfections, COS-1 cells were routinely cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and 50 units/ml each of streptomycin and penicillin. For transient transfection, 5 x 10⁵ cells were seeded onto 60-mm dishes 24 h prior to transfections, and 2 h before the addition of DNA the cells received fresh Dulbecco’s modified Eagle’s medium with 0.1% fetal bovine serum and full growth supplements. DNA consisting of 1.5 μg of pcDNA3.1-IA-E1, 1 μg of pcDNA3.1-SUMO-1, and 0.5 μg of pcDNA3.1-Ubc9 was cotransfected into cells using LipofectAMINE Plus reagent according to the manufacturer’s instructions (Life Technologies, Inc.). Parental pcDNA3.1 vector with no insert was used as a control for mock transfection. At 36 h after transfection, cells were harvested and washed once in ice-cold phosphate-buffered saline. The cells were lysed directly with 200 μl of SDS-sample buffer and diluted 1.3 in radiimmunoprecipitation buffer (25 mM Tris-HCl, pH 8.0, 50 mM NaCl, 0.2% Nonidet P-40, 0.5% deoxycholate, 1 mM phenylmethylsulfonyl fluoride) containing 10 mM iodoacetamide and complete protease inhibitor mixture (Sigma). The lysate was sonicated briefly and then cleared by centrifugation at 10,000 x g for 20 min at 4 °C. For immunoprecipitation, cell extracts (~500 μg) were preclarred with 50 μl of immunopreipitin (Promega, Madison, WI) and then incubated with 5 μl of anti-SUMO-1 or 5 μl of epitope affinity-purified HA antibody for 3 h at 4 °C. For samples with anti-SUMO-1, 50 μl of 10% (v/v) protein A-Sepharose (Sigma) was added and the mixtures were further incubated for 2 h with mild agitation. The beads were collected and washed five to six times with 1-mlü aliquots of 50 mM Tris-HCl, pH 8.0, 200 mM NaCl, 0.5% Nonidet P-40, 0.5% deoxycholate and 1 mM phenylmethylsulfonyl fluoride. By using this protocol, protein A-Sepharose precipitation with anti-HA, 30 μl of preblocked Protein G Plus/Protein A-agarose suspension (Novagen, Madison, WI) was used to collect the antigen-antibody complexes instead of protein A-Sepharose. The beads were collected and washed five times with the buffer described in the manufacturer’s protocol (Novagen, Madison, WI). The bound proteins were solubilized by addition of 20 μl of 2x SDS-sample buffer and Western blotting was performed according to standard procedures us-

**Experimental Procedures**

**Materials**—Protease inhibitor mixture and 3-amino-1,2,4-triazole (3-AT) were obtained from Sigma (St. Louis, MO). Anti-E1 rabbit polyclonal antibody raised against a polypeptide corresponding to amino acids 2–75 of BPV E1 was described previously (5). Anti-SUMO-1 and anti-beta-galactosidase antibodies (C27 and S277, respectively) were purchased from Zymed Laboratories Inc. (South San Francisco, CA). Purified anti-Ubc9 was a kind gift from Gregory J. Kota, The Johns Hopkins University. Mouse monoclonal anti-GAL4 AD, anti-GAL4 DBD, and horseradish peroxidase-conjugated secondary antibodies were from Upstate Biotechnology (Lake Placid, NY). Rhodamine- and fluorescein-conjugated secondary antibodies for indirect immunofluorescence were obtained from Southern Biotechnology (Birmingham, AL). Plasmid pGEX-Ulp1 was a gift of Mark Hochstrasser, University of Chicago.

**Plasmids**—The construction of the wild-type GAL4 DNA binding domain (GAL4-DBD)-E1 fusion construct in the pGFB vector, designated pGFB9-E1, has been described previously (38). Truncated forms of E1 were made by insertion of multiple translational stop codons as described previously (5). The substitution mutants of E1 were generated using PCR-based site-directed mutagenesis (27), and the respective DNAs were cloned into BamHI-digested pGFB9 or into pRSET (Invitrogen, Carlsbad, CA). To construct pcDNA3.1-IA-E1, the full-length E1 coding region was excised from pGFB9 by BamHI-XhoI digestion and cloned into the pcDNA3.1-IA vector (Invitrogen). The resulting construct expressed E1 protein with the HA epitope (YPYDVPDYA) fused to the N terminus of E1. Human SUMO-1 (gift of Joana Desterro, University of St. Andrews, Scotland) and the Ubc9 cDNA were inserted into pcDNA3 at the BamHI site of pGFP-C2 (CLONTECH, Palo Alto, CA). All PCR-amplified products were fully sequenced to exclude the possibility of second site mutations.

**Yeast Two-hybrid Screen**—The pGFB9-E1 plasmid was transformed using the standard LiAc procedure into YRG2 yeast transformed with the pGBT9 plasmid, an HeLa cell Matchmaker cDNA library in the pGAD-GH vector (CLONTECH) at 1×10⁶ transformants (1 ml) per 10⁵ cells. To test for SUMO-1 modification, a HeLa cell extract containing SUMO-1-activating enzymes, UBA2AOS1 (25), was prepared as described previously (39). [35S]-Labeled protein (10 μg) was translated in vitro using rabbit reticulocyte lysate supplemented with 5 μl of HeLa cell extracts in a 25–μl reaction, including an ATP-regenerating buffer (50 mM Tris-HCl, pH 7.6, 5 mM MgCl₂, 2 mM ATP, 10 mM creatine phosphate, 3 units/ml creatine kinase, and 0.5 unit/ml inorganic pyrophosphatase), 6 μg of purified SUMO-1, and 1 μg of Ubch9. Reactions were incubated at 37 °C for 2 h. Control reactions had one or more of the components omitted and replaced by additional buffer. After terminating the reaction with SDS-sample buffer, reaction products were analyzed by SDS-polyacrylamide gel electrophoresis followed by phosphorimaging.

**Western Blotting**—Western blotting was performed according to standard procedures using 50 μg of protein samples, transferred to nitrocellulose membranes, and probed with affinity-purified HA antibody diluted 1:3000. Membranes were then incubated with goat anti-rabbit antibody conjugated with horseradish peroxidase (Promega, Madison, WI) and visualized with the chemiluminescent reagent Western Lightning (Perkin Elmer, Norwalk, CT) according to manufacturer’s instructions.
ing the following primary antibodies: anti-HA, anti-SUMO-1, and affinity-purified anti-E1.

Fluorescence Microscopy—COS-1 cells were cultured on four-well chamber slides (Nunc, Naperville, IL), and 1 × 106 cells/chamber were transfected with either 300 ng of pEGFP-E1 (wild-type) or the pEGFP-E1 420/421 mutant DNA using LipofectAMINE Plus reagent according to the manufacturer’s instructions (Life Technologies, Inc.). At 24, 36, and 48 h post-transfection, cells were fixed with 2% paraformaldehyde in phosphate-buffered saline for 15 min at room temperature. The fixed cells were washed twice in phosphate-buffered saline, and the DNA was stained by brief incubation in 0.5 μg/ml Hoechst 33258 (Polysciences, Warrington, PA) prior to mounting in 90% glycerol containing 0.1% p-phenylenediamine. GFP and Hoechst staining were visualized on a Nikon Eclipse E800 microscope and photographed in the same field by changing filter sets. For protein quantification, 6 × 105 cells were seeded onto 60 mm dishes and transfected with either 3 μg of pEGFP-E1 (wild-type) or the pEGFP-E1 420/421 mutant DNA using LipofectAMINE Plus reagent as described above. Transfected cells were directly lysed with SDS-sample buffer and were analyzed by Western blotting using anti-E1 antibody.

RESULTS

Identification of Ubc9 as an E1-interacting Protein—To identify proteins that interact with the BPV E1 protein, a yeast two-hybrid screen of an HeLa cDNA library was performed. A bait expression vector was constructed by fusing the pGBT9-encoded GAL4-DNA binding domain (GAL4-DBD) to the full-length E1 protein (amino acids 1–605). The pGBT9-E1 bait was transformed into the yeast strain YRG2, which contains two GAL4-induced reporter genes, HIS3 and LacZ. Expression of the E1 protein in the yeast cells transfected with pGBT9-E1 was confirmed by Western blot analysis using an anti-GAL4-DBD antibody (data not shown). YRG2 yeast cells transformed with pGBT9-E1 alone or cotransfomed with pGBT9-E1 and the activation domain vector, pGAD424, did not activate transcription from either the HIS3 or LacZ reporter genes in the presence of 20 mM 3-AT. Thus, further experiments were performed to screen an HeLa cell cDNA library for proteins that interact with E1. Approximately 1.5 × 108 yeast transformants were screened, and two His+ colonies were ultimately obtained that were also positive in the β-galactosidase filter assay. The two clones expressing putative E1-interacting proteins were characterized further by sequence analysis and retransformation into YRG2 for verification of true positives.

Sequence analysis of the two clones identified above revealed that both clones encoded the same gene and were fused in-frame with the GAD sequence; only the length of 3′-untranslated region differed in the two clones (data not shown). Both clones contained 93 base pairs of a presumably 5′-untranslated region prior to the first methionine codon. The subsequent open reading frame encoded a 158-amino acid protein with a calculated molecular mass of 19 kDa. A GenBank™ search revealed 100% homology with the human ubiquitin-conjugating enzyme, Ubc9 (23). Strikingly, this clone is identical to the gene that was identified previously as a partner for HPV16 E1 protein (20). As shown in Fig. 1A, the protein encoded by the pGAD-Ubc9 clones interacted specifically with the E1 protein and did not interact with the unfused GAL4-DBD protein expressed from the parental pGBT9 vector.

To further explore the interaction between E1 and Ubc9, we mapped the domain of E1 that interacts with Ubc9 in vivo using the two-hybrid assay in yeast. As shown in Fig. 1B and C, deletion analysis revealed that the C-terminal region of E1 (amino acids 315–605) showed strong interaction with Ubc9, whereas the N-terminal half of E1 (amino acids 1–311) showed no interaction. Furthermore, an E1311–459 construct lacking the C-terminal 146 amino acids was still capable of interacting with Ubc9. The combined results from the various deletions suggest that the region from residues 315–459 is critical for E1-Ubc9 interaction. The specificity of the Ubc9 interaction with truncated E1 proteins was confirmed by lack of detectable β-galactosidase activity when the Ubc9 construct was replaced with the pGAD424 vector alone.

Ubc9 Binds to the E1 Protein in Vitro—To address whether Ubc9 also interacts with the E1 protein in a context other than in yeast, we performed GST fusion pull-down assays with a bacterially expressed GST-Ubc9 fusion protein and 35S-labeled full-length E1 and E1 deletion mutants (Fig. 2A). An equal amount of 35S-labeled protein was incubated with 2 μg each of GST alone or GST-Ubc9 fusion proteins bound to glutathione-Sepharose beads. After extensive washing, the 35S-labeled protein bound to the beads was extracted and analyzed by autoradiography. Consistent with the in vivo results, full-length E1 protein (amino acids 1–605), as well as E11–409 and E1311–605, associated with GST-Ubc9 but not with GST alone (Fig. 2B).

FIG. 1. Yeast two-hybrid analysis of E1-Ubc9 interaction. A, the indicated plasmid pairs were cotransfected into the YRG2 yeast strain, and three independent transformants were isolated for each plasmid pair. Liquid cultures of each transformant were prepared and tested for β-galactosidase activity; there was little variation between independent clones of the same transformation pair. Shown are the average units for triplicate assays with representative clones for each plasmid pair. B, schematic diagram of the E1 protein depicting mapped domains of major functional activities and the structures of various truncation mutants tested in C. Numbers refer to E1 amino acid residues. C, yeast two-hybrid quantitation of β-galactosidase activity generated by E1 truncation mutants. The plasmid pairs are indicated to the left and each was assayed as in A.
Neither of the N-terminal clones, E11–311 or E1121–311, was capable of binding with GST-Ubc9. At least 14% of the input E1 protein was immobilized on the beads for the positive constructs, indicating efficient E1–Ubc9 interaction. These in vitro results correlated well with protein-protein interactions detected with the yeast two-hybrid system and confirmed that the region from E1 amino acids 315 to 459 was critical for Ubc9 interaction.

Mapping the Ubc9 Binding Site Region of the E1 Protein—To further study the amino acid residues of E1 that are involved in Ubc9 interactions, we made several site-directed mutants of E1 within amino acids 315–459 (Fig. 3A) and tested each for Ubc9 binding. Although several proteins have been recently shown to interact with Ubc9, the consensus sequence for Ubc9 binding is not yet defined. Analysis of sequences within the interaction domains of several Ubc9 binding proteins, including transcriptional proteins such as steroid activator (41), ETV6 (42), and E2A (43) suggested that Ubc9 binding occurs at hydrophobic regions that may contain either an LK and/or KL dipeptide (44, 45). Interestingly, there are 2 LK and 2 KL pairs found in E1 within amino acids 315–459: residues 387/388, 416/417, 417/418, and 420/421. Each of these sites was mutated in the context of full-length E1 and tested for Ubc9 interaction by the yeast two-hybrid system (Fig. 3B). The results of these experiments indicated that the double mutants 387/388, 416/418, and 417/418 had no significant effect on Ubc9 binding. In contrast, two different substitution mutants of residues 420/421 completely abolished E1 interaction to Ubc9, confirming the importance of this region identified by the deletion analysis.

A previous study with HPV16 E1 indicated that a mutation at amino acid Ser-330 also failed to interact with Ubc9 (20). However, the amino acid corresponding to Ser-330 in BPV E1 is Thr-286, which lies outside of the region required for Ubc9 binding as indicated by our deletion analysis (Figs. 1C and 2B). To investigate this discrepancy, the BPV E1 residue Thr-286 was mutated into alanine and tested in the yeast system. Surprisingly, the T286A mutant did have a significantly lower response with Ubc9 as determined by the quantitative β-galactosidase assay (Fig. 3B). However, in contrast to the double
mutants, the expression level of the T286A mutant protein in yeast was much lower than wild-type E1 as judged by immunoblot analysis of whole yeast protein extracts (data not shown), suggesting that this change destabilized the GAL4DBD-E1 fusion protein. Thus, the decrease in β-galactosidase activity was at least in part the result of the quantitative reduction in E1 expression with this mutant, and this precluded a simple assessment of the direct role of this residue in Ubc9 binding in vivo.

To further examine the physical interaction of the E1 mutants with Ubc9, in vitro binding assays were performed using GST fusion proteins. In vitro translated E1 mutant proteins were incubated with glutathione-Sepharose beads prebound with GST alone or GST-Ubc9 protein. As in vivo, the 420/421 double mutant showed no significant interaction with Ubc9 while the other three double mutants were effectively retained on the GST-Ubc9 beads compared with the GST protein alone beads (Fig. 3B). The percentage of input mutant protein bound to the GST-Ubc9 beads varied somewhat between the functional double mutants and in all cases was slightly less than the amount bound for wild-type E1, consistent with the results obtained in the β-galactosidase assays. Interestingly, although the T286A mutant protein was still more impaired than the double mutants, it did exhibit significant binding to Ubc9 in vitro, indicating that this residue was not absolutely essential for Ubc9 interaction. In combination, the in vivo and in vitro deletion and point mutation data are most consistent with the T286A mutation affecting the overall structure of E1 rather than being involved in direct contact with Ubc9. In contrast, the extreme defectiveness of both the LK 420/421 double mutants implicates one or both of these E1 residues as critical for interaction with Ubc9. Note that the leucine at BPV residue 420 is more conserved than lysine 421 and may be the more critical determinant for interaction, although we have not yet tested this by separate single mutations (Fig. 3C). Finally, we compared the E1 sequence from 315 to 459 with known Ubc9-binding domains of other proteins to find similarities that would allow us to predict a consensus sequence for a Ubc9 binding site. While this comparison did not yield a specific consensus sequence present in all proteins examined (data not shown), limited sequence similarities are present within the regions that are critical for Ubc9 interaction in some proteins (Fig. 3D). However, although the similarities between c-Jun and E1 are statistically nonrandom (Match-Box Web Server 1.3, Molecular Biology Research Unit, The University of Namur, Belgium), those between E2A and c-Jun or E1 are not, so the functional significance of these alignments is uncertain.

SUMO-1 Is Conjugated to E1 Protein

Ubc9 was recently shown to act as an E2-type conjugating enzyme that conjugates SUMO-1 to target proteins rather than ubiquitin (29, 46). Based on comparison with known SUMO-1-conjugating sites (29, 34, 47), there are from one to three potential sites on E1 (Lys-155, Lys-288, and Lys-514) at which SUMO-1 could be added. To investigate whether E1 is a substrate for SUMO-1 modification by Ubc9, an in vitro system that could accurately recapitulate the in vivo sumoylation process was developed.
protein generated by in vitro translation in the presence of [35S]methionine was used as a substrate in the presence or absence of purified Ubc9, SUMO-1 (deleted for four amino acids at the C terminus of SUMO-1 to expose Gly-97 for full activity), and a partially purified protein extract containing the SUMO-activating enzymes, UBA2/AOS1 (39, 46). After incubation with the various reaction components, the E1 protein was analyzed by SDS-polyacrylamide gel electrophoresis and a slower migrating form of E1 with an apparent molecular mass of ~102 kDa was observed (Fig. 4A). The appearance of the slower migrating form of E1 was strictly dependent on the presence of Ubc9, SUMO-1, and the SUMO-activating enzymes; removal of any component eliminated formation of the 102-kDa product (Fig. 4A and data not shown). Furthermore, immunoprecipitation by anti-E1 antibody followed by Western blotting with anti-SUMO-1 confirmed that this 102-kDa protein contained both E1 and SUMO-1 (data not shown). Finally, the 102-kDa product was eliminated by treatment with the GST-Ulp1 protease (Fig. 4B), which specifically cleaves SUMO-1 and not ubiquitin from proteins (40). There was no reduction in the amount of the 102-kDa product upon incubation with GST alone (not shown). Ulp1 treatment did not affect the unmodified E1 as expected for a protease that is specific for the substrate-SUMO-1 linkage. Taken together, the above results establish that E1 is a substrate for in vitro SUMO-1 conjugation.

E1 Is Covalently Modified by SUMO-1 in Vivo—To demonstrate that E1 is also modified by SUMO-1 in vivo, COS-1 cells were transiently transfected with vectors expressing SUMO-1, Ubc9, and HA-tagged E1. The transfected cells were directly lysed in SDS-sample buffer containing iodoacetamide and were analyzed by Western blotting with an anti-E1 antibody. As shown in Fig. 5A, a minor band was visible above the primary E1 band and its apparent molecular mass (~102 kDa) was consistent with that of E1 protein covalently modified by SUMO-1 in vitro. To confirm that the 102-kDa product represented SUMO-1 modification of E1 in vivo, cell extracts were subjected to immunoprecipitation with an anti-HA antibody, followed by Western blot analysis with antibodies to detect E1 or SUMO-1. As seen in Fig. 5 (B–D), the Western blots demonstrated that both anti-HA and anti-E1 detected two bands with apparent molecular masses of 82 and 102 kDa. Only the 102-kDa band was detected with an anti-SUMO-1 antibody indicating that it contained both E1 and SUMO-1 (Fig. 5E). In contrast, neither band was detected in control IgG immunoprecipitates. To further establish that the 102-kDa protein was the SUMO-1-modified form of E1, a converse experiment was performed where cell extracts were immunoprecipitated with anti-SUMO-1 antibody and then blotted with anti-HA. As shown in Fig. 5 (F and G), a 102-kDa band was specifically immunoprecipitated by the anti-SUMO-1 antibody and was also recognized by the anti-HA antibody. Again, no 102-kDa product was observed in precipitates either from control extract or from immunoprecipitation with preimmune serum. Also note that there was a very faint band in Fig. 5G, lane 2, migrating more slowly than the 102-kDa product. This larger product was inconsistently observed but may represent a small population of E1 molecules with an additional SUMO-1 moiety attached. The results in Fig. 5, combined with the in vitro data, clearly indicate that E1 is covalently modified with SUMO-1 both in vitro and in vivo.

Subcellular Localization of E1—Recently it was shown that sumoylation of the nuclear dot-associated proteins, PML and Sp100 (34, 35), is involved in targeting these proteins to the nuclear body. Because E1 is a nuclear protein (48, 49), we tested whether loss of Ubc9 binding by E1 influenced the subcellular distribution of E1 protein, because sumoylation should be prevented or greatly reduced. A GFP-E1 construct and a GFP-E1 420/421 mutant, in which critical residues for Ubc9 binding were substituted, were expressed in COS-1 cells and...
analyzed by fluorescence microscopy at 24, 36, and 48 h post-transfection (Fig. 6A and data not shown). In GFP-E1-transfected cells, E1 localized in a large number of discrete nuclear clumps distributed on a diffuse background fluorescence throughout the nucleus. In contrast, GFP-E1 420/421-transfected cells predominantly exhibited the diffuse nuclear fluorescence without punctuate accumulation or with fewer clumps in a less widely distributed pattern; there also appeared to be more accumulation of signal around the nuclear periphery. No change in these distribution patterns was observed during the period examined (24–48 h). It is unlikely that this difference in intranuclear accumulation pattern was due to different expression levels of the mutant and wild-type proteins. As demonstrated by Western blot analysis of total E1 levels (Fig. 6B), there were similar amounts of WT and mutant E1 expressed, particularly at later times. Consequently, it appears that reduced Ubc9 binding, and presumably reduced sumoylation of E1, disrupts normal intranuclear distribution. However, it is not yet clear whether redistribution of E1 protein is directly linked to changes in interaction with nuclear bodies, and subsequent work will be required to address this issue.

**DISCUSSION**

Using the yeast two-hybrid system, we identified Ubc9, a SUMO-1-conjugating enzyme, as a protein that interacts specifically with BPV E1 protein. The interaction between E1 and Ubc9 was demonstrated both in vivo and in vitro. A previous study with human papillomavirus 16 E1 also found that Ubc9 binds E1 and that elimination of Ubc9 interaction with E1 mutants correlated with reduction in replication activity (20). However, the mechanistic basis for why the Ubc9-E1 interaction was necessary for E1 replication function was not defined. Furthermore, at the time of the previous study the role of Ubc9 in sumoylation was not known, so the possible post-translation modification of E1 protein by SUMO-1 conjugation was not investigated. In the present study, we demonstrated that BPV E1 protein not only binds Ubc9 but is also a substrate for sumoylation. Larger forms of E1 have been observed in vivo in some studies, which is consistent with naturally occurring sumoylation, although the biochemical composition of these species was not determined (50). Failure to consistently observe these larger E1 forms may be explained by the recent observations for IκBα (29) and p53 (30) that the presence of endogenous SUMO-1-specific proteases, such as Ulp1 (40) and SENP1 (51), rapidly cleaves SUMO-1 from substrate proteins in cell extracts unless these proteases are inactivated by addition of SDS/iodoacetamide. Our in vitro experiments confirmed that the E1-SUMO-1 bond can be cleaved by exogenous Ulp1, indicating that the linkage is biochemically typical of other known sumoylations. These results suggest that sumoylated E1 would be unstable in cell extracts under standard immunoprecipitation conditions, which likely prevented the detection of this modified species in most previous studies.

Both in vivo and in vitro, the apparent molecular mass of the E1-SUMO-1 conjugate was ~20 kDa larger than the input E1. Although this molecular mass difference potentially represents addition of two of the ~11-kDa SUMO-1 moieties, we cannot rule out the possibility that the 102-kDa species contains only a single SUMO-1 group for three reasons: 1) we have never observed under any condition an intermediate product that would represent singly modified E1, 2) mono-addition is more
typical of sumoylation, although PML does contain multiple sumoylation sites (21), and 3) addition of a single SUMO-1 group to PML or RanGAP1 proteins results in an increase in apparent molecular mass of ~20 kDa (33, 35). Consequently, the stoichiometry of the E1-SUMO-1 complex is not yet certain. Additional studies are in progress to map the number and location of the sumoylation sites on E1 by site-directed mutagenesis of potential SUMO-1 acceptor lysines.

Truncation mapping studies were consistent with the involvement of E1 protein residues 315–459 in the interaction with Ubc9. Moreover, further analysis of the C terminus revealed that E1 residues 420 and/or 421 were essential for interaction, because double substitution mutants completely abolished Ubc9 binding. Examination of E1 amino acid sequences of other papillomaviruses indicated that leucine 420 of BPV E1 is more highly conserved than lysine 421, suggesting that the leucine residue may be providing a critical recognition function for Ubc9 interaction. The potential importance of leucine 420 is also consistent with the observation that a single amino acid substitution changing leucine 122 of adenovirus type 5 E1A protein to an isoleucine completely abrogated binding to Ubc9 (44). However, broader comparison of the Ubc9 binding region of E1 with the corresponding regions of other known Ubc9 binding proteins did not yield a specific consensus sequence for Ubc9 interaction, although limited homology does exist between E1 and a subset of other Ubc9 targets. This lack of a strong consensus may reflect that other features besides primary sequence are involved in Ubc9-target interaction. For example, the crystal structure of hUbc9 suggests that electrostatic interactions with target proteins will also be important for Ubc9 recognition, and most target proteins have significant regions of overall negative charge (52). Less dependence on strict primary sequence and greater reliance on more global structural features might account for the large substrate range of Ubc9. Interestingly, a recent study of homeodomain-interacting protein kinase 2 noted that most proteins that bind Ubc9, including BPV E1, PML, E1A, HPV16 E1, and Ran-GAP1, contain PEST sequences (53). It will be important in future studies to determine the relationship between PEST sequences and Ubc9 binding and/or sumoylation of target proteins.

The precise functional role of SUMO-1-conjugated E1 is not yet clear. However, based on known effects of sumoylation on other substrates, it is possible to envisage a number of models by which SUMO-1 conjugation to E1 could influence E1 activities. Three known effects of sumoylation, antagonism of ubiquitin-mediated degradation, enhancement of nuclear uptake, and modulation of intranuclear distribution, were compared for wild-type E1 and a Ubc9-nonbinding double mutant. The 420/421 double mutant was tested, because the inability to bind Ubc9 should greatly reduce or eliminate sumoylation of E1 in vivo. Under our culture conditions, a degradation protective role of sumoylation was not observed, because the steady-state level of the E1 double mutant protein did not differ significantly from that of the wild-type E1 protein. Likewise, there was no specific sumoylation requirement for nuclear uptake, because both the wild-type and mutant proteins were located primarily in the nucleus. In contrast, the transfection experiments indicated that wild-type E1 protein exhibited discrete nuclear accumulations, whereas the E1 420/421 mutant presented a more diffuse nuclear staining with reduced numbers of punctate bodies. These results are consistent with sumoylation influencing the intranuclear distribution of E1. A similar effect has been observed for the intranuclear distribution of homeodomain-interacting protein kinase 2 (53).

Recent models have proposed that proteins involved in transcription and/or replication, including viral proteins, localize to specific intranuclear regions that may serve as deposition sites for accumulation and assembly of functional multiprotein complexes (37). The signaling mechanisms that control these dynamic structures are poorly understood, but sumoylation has been implicated, particularly for ND10s (28, 54). Like other DNA viruses, papillomavirus replication is at least partially dependent on ND10 structures (55) suggesting that the punctate accumulations of E1 observed in this study reflect association of E1 with these nuclear bodies. The decreased association of the E1 mutant with these bodies implies that sumoylation of E1 is necessary for proper intranuclear distribution. Failure of unmodified E1 to adequately localize within nuclear substructures could account for the previously observed replication defect of a Ubc9-nonbinding E1 protein (20). Construction and characterization of E1 mutants specifically lacking sumoylation sites will be necessary so that the functional role of this modification can be more definitively assigned.

REFERENCES

1. Law, M. F., Lowy, D. R., Dworetzky, I., and Howley, P. M. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 2727–2731
2. Melendy, T., Sedman, J., and Stenlund, A. (1995) J. Virol. 69, 7857–7867
3. Ustav, M., and Stenlund, A. (1991) EMBO J. 10, 449–457
4. Yang, L., Mohr, I., Fouts, E., Lim, D. A., Nobahle, M., and Botchan, M. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 5086–5090
5. Wilson, V. G., and Lades-Meyers, J. (1991) J. Virol. 65, 5314–5322
6. Ustav, M., Ustav, E., Szymanski, P., and Stenlund, A. (1991) EMBO J. 10, 4321–4329
7. Bonne-Andrea, C., Santucci, S., and Cleriant, P. (1995) J. Virol. 69, 3201–3205
8. Seo, Y.-S., Müller, P., Laskey, M., Gibbs, E., Kim, H.-Y., Phillips, B., and
