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Casein Kinase II Phosphorylation-induced Conformational Switch Triggers Degradation of the Papillomavirus E2 Protein*

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The major phosphorylation sites of the bovine papillomavirus E2 transactivator protein are two serine residues, 298 and 301, that are located in a flexible hinge region between the DNA binding and transactivation domains. Phosphorylation of serine residue 301 promotes ubiquitination and rapid degradation of the E2 protein by the proteasome pathway. To understand the mechanism through which phosphorylation regulates the intracellular levels of this unique papillomavirus regulatory protein, we have carried out an extensive mutational analysis of the region surrounding the phosphorylation sites of the E2 protein. Our results indicate that casein kinase II phosphorylates serine 301. However, phosphorylation of serine 301 is not a sufficient recognition motif for proteasomal degradation; other residues that directly surround the phosphorylation sites are crucial for E2 degradation. The phenotypes of E2 proteins mutated in this region indicate that phosphorylation of serine 301 induces a conformational change that leads to degradation of the E2 protein. In support of this model, circular dichroism studies of the conformational tendencies of peptides from this region indicate that phosphorylation at position 301 decreases the local thermodynamic stability of this region. Thus, this region appears to have evolved to display a marginal local thermodynamic stability that can be regulated by phosphorylation, leading to targeted degradation of the E2 protein.

Papillomaviruses are small DNA viruses that infect epithelia and cause persistently infected lesions known as papillomas or warts. The viral genome is maintained as a multicopy episome in the infected, dividing basal cells. The productive stage of the viral life cycle is highly dependent on epithelial differentiation; genome amplification, late gene expression, and capsid assembly occur only in the more differentiated cells of the epithelium. A subset of human papillomaviruses are associated with development of cervical carcinoma; however, bovine papillomavirus, type 1 (BPV-1),[1] has historically served as a molecular prototype for genetic analysis of the virus.

BPV-1 encodes for several E2 gene products. These proteins are crucial for the viral life cycle, participating in several functions such as transcriptional regulation, viral DNA replication, and genome maintenance and segregation. The E2 protein activates transcription by binding to enhancer sequences in the viral genome (1) and supports initiation of viral DNA replication by cooperatively binding with the E1 protein to the viral origin of replication (2). E2 mediates viral genome segregation by binding the viral genomes and tethering them to the cellular mitotic chromosomes (3–5). The full-length E2 protein contains two conserved functional domains as follows: the N-terminal transactivation domain of ~200 amino acids, and a C-terminal DNA binding and dimerization domain contained in the last 100 amino acids (reviewed in Ref. 6). These domains are separated by a flexible hinge region that is not well conserved among papillomavirus E2 proteins. The E2 protein has two major phosphorylation sites, mapped to serines 298 and 301, located in the region of the hinge that is directly adjacent to the DNA binding and dimerization domain (7). A minor phosphorylation site has also been mapped in the hinge at serine 235 (8). The major phosphorylation sites, 298 and 301, are found in a sequence of E2 that scores highly as a PEST sequence. These sequences are rich in proline, glutamate, aspartate, serine, and threonine and are found in many proteins with short half-lives and thus are believed to play a role in protein turnover (9). Phosphorylation of serine 301 targets E2 for rapid turnover by the ubiquitin-proteasome pathway (10). Substitution of serine 301 to alanine results in a protein with an increased half-life and reduced levels of ubiquitination (10). This, in turn, results in a virus that maintains episomal viral genomes at a much higher copy number than that of the wild type genome (11).

The ubiquitin-proteasome pathway plays an important regulatory role in the cell by specifically degrading proteins. Proteins are marked for degradation by the addition of ubiquitin moieties to specific lysine residues (reviewed in Ref. 12). Three main classes of enzymes are involved in the conjugation of ubiquitin to a substrate: an E1 ubiquitin activation enzyme, an E2 ubiquitin carrier protein, and an E3 ubiquitin ligase. The E3 ubiquitin ligase confers specificity to the system by its ability to recognize protein substrates for targeted degradation. Proteins can be constitutively recognized or conditionally targeted by the ubiquitin pathway. In some cases, the specific E3 ubiquitin ligase must be modified before it can interact with its target protein, and in others, the E3 ligase requires the use of ancillary or docking proteins to interact with their targets. An example of the latter E3 ligase is another papillomavirus protein, E6, which is required to mediate an association between the ubiquitin ligase E6-AP and the target protein, p53 (13). Finally, E3 ubiquitin ligase recognition can be triggered by post-translational modification of the substrate protein.

In the case of BPV-1 E2 protein, phosphorylation at residue 301 results in enhanced protein degradation. Phosphorylation could trigger this recognition in a number of ways: the phos-

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[1] The abbreviations used are: BPV-1, bovine papillomavirus, type 1; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; TFE, 2,2,2-trifluoroethanol; MOPS, 4-morpholinepropanesulfonic acid; CK2, casein kinase II; MAPK, mitogen-activated protein kinase.

phorylated region of the E2 protein could be directly recognized by a specific ubiquitin ligase; alternatively, phosphorylation could induce a conformational change that exposes either a ubiquitin ligase recognition site or a lysine that is normally masked for ubiquitin conjugation. In this study, we have carried out an extensive mutational analysis of the region surrounding the E2 phosphorylation sites to understand how phosphorylation targets E2 for recognition and degradation by the ubiquitin-proteasome pathway. We demonstrate that casein kinase II (CK2) can phosphorylate serine 301. However, the ubiquitin ligase responsible for recognition and degradation of E2 does not directly recognize phosphoserine 301; phosphorylation most likely induces a conformational change that is important for recognition and subsequent degradation of E2.

EXPERIMENTAL PROCEDURES

Cell Culture—CV-1-derived lines, expressing the E2 proteins from an inducible metallothionein promoter, were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. These cell lines were generated by transfection of a series of pMEP-E2 plasmids using Superfect (Qiagen). Hygromycin B-resistant colonies containing episomal plasmids were pooled and used for all experiments.

Plasmids—E2 genes expressing either wild type or mutated E2 proteins from the inducible metallothionein promoter were cloned in the pMEP-4 plasmid (Invitrogen), as described before (10). To generate these plasmids, the KpnI to StyI region of E2 was reconstructed with synthetic oligonucleotides encoding the sequence of choice in an intermediate cloning vector containing a portion of the BPV-1 genome. Mutations were made using the most conservative base changes and were verified by DNA sequencing. The KpnI to StyI region (nucleotides 3460–3535) of E2 was reconstructed with synthetic oligonucleotides to yield plasmids containing wild type E2, $E2_{R291A}$, $E2_{Q292A}$, $E2_{E293A}$, $E2_{E294A}$, $E2_{E295A}$, $E2_{E296A}$, $E2_{Q297A}$, $E2_{P299A}$, $E2_{D300A}$, $E2_{T302A}$, $E2_{E303A}$, $E2_{E304A}$, $E2_{E305A}$, $E2_{P306A}$, $E2_{V307A}$, $E2_{T308A}$, $E2_{L309A}$, $E2_{P310A}$, $E2_{A293–296}$, $E2_{\Delta 293–296}$, $E2_{A303–305}$, $E2_{\Delta 303–305}$, $E2_{A293–296,A303–305}$, $E2_{E303P}$, $E2_{E304P}$, $E2_{E305P}$, $E2_{insA299–300}$, and $E2_{insAA299–300}$. The resulting KpnI to BamHI BPV-1 fragments from the intermediate plasmids were replaced for the equivalent region in the pMEP-E2 vector, as described previously (10). In these plasmids the pMEP vectors contain BPV-1 sequences 2608–4208.

For plasmids pMEP-$E2_{S301D}$, pMEP-$E2_{S301E}$, pMEP-$E2_{S298A,E304Q}$, $E2_{EEE(S290E,S298E,S301E)}$ and $E2_{E304Q}$, the mutations were generated as described above, transferred to the BPV-1 genome, and the KpnI to BamHI fragment transferred from here to the pMEP-E2 plasmid. To generate plasmids pMEP-$E2_{S290A}$, pMEP-$E2_{S298A}$, and pMEP-$E2_{AAA\ (S290A,S298A,S301A)}$, the KpnI (nucleotide 3460) to BamHI (nucleotide 4451) fragments containing the amino acid substitutions were obtained from existing constructs (7, 11, 14). This series of DNAs contains BPV-1 sequences 2608–4451.

To generate plasmids to express E2 *in vitro* in an *Escherichia coli* lysate, the NheI to BamHI fragments from pMEP-E2, pMEP-$E2_{S301A}$, and pMEP-$E2_{S298A,E304Q}$ were sub-cloned into the corresponding sites of pET-23a *in vitro* expression plasmid (Novagen). To optimize for expression in *E. coli*, the initiation region of E2 was reconstructed with an optimal ribosome-binding site by inserting double-stranded oligonucleotides 5′-TATGGAGACAGCATG-3′ between the NdeI and SphI sites.

Protein Expression and Extraction—E2 protein expression was induced in hygromycin-resistant CV-1 cell lines by treatment for 2 h with 1 μM $CdSO_4$. For pulse-chase analysis, $CdSO_4$ was removed after induction, and cells were treated with 25 μg/ml cycloheximide and 25 μg/ml emetine. Where proteasome inhibitors were used, cells were treated with 20 μM MG132 (Calbiochem) either during induction or during the treatment with 25 μg/ml cycloheximide and 25 μg/ml emetine (Sigma). For standard SDS-PAGE analysis, cellular protein was extracted in 2% SDS, 50 mM Tris-HCl, pH 6.8, 2 mM NaF, 100 mM dithiothreitol, and 1× Complete™ protease inhibitor mixture (Roche Applied Science). Samples for isoelectric focusing were extracted in 8 M urea, 1% Triton X-100, 1 mM dithiothreitol, 30 mM NaF, and 1× Complete™ protease inhibitor mixture. Total protein concentrations were measured using a BCA protein assay (Pierce). The stability of each E2 protein was assayed at least three times.

SDS-PAGE, Two-dimensional Isoelectric Focusing, and Western Blotting—For standard SDS-PAGE analysis, 10 μg of protein was separated on a 10% SDS-polyacrylamide gel and transferred onto Immobilon P membranes (Millipore). For two-dimensional isoelectric focusing, 20 μg of protein was separated on pH3–7NL Immobilon dry strips using the IPGphor system (Amersham Biosciences) using standard procedures. Following isoelectric focusing, the Immobilon strips were equilibrated in 50 mM Tris, pH 6.8, 6 M urea, 30% glycerol, 2% SDS, and separated in the second dimension, as described above. Western blotting was performed following standard protocols using anti-E2 monoclonal antibody B201 (obtained from Elliot Androphy) followed by peroxidase-conjugated anti-mouse IgG (Pierce). E2 proteins were detected using chemiluminescence reagent Super Signal Dura (Pierce). Images were collected on a Kodak Imagestation 440CF using Kodak One-dimensional Image Analysis software. The phosphorylation status of each protein was assayed at least three times.

Kinase Assays—*In vitro* kinase assays were carried out using various E2 substrates. Synthetic peptides spanning residues 283–311 of the E2 protein were kinased for 30 min with either CK1 or CK2 (Upstate Biotechnology, Inc.) in a reaction containing 0.2 mM peptide, 10 μCi of [γ-^{32}P]ATP, 50 μM ATP, 20 mM MOPS, pH 7.2, 25 mM glycerol phosphate, 5 mM EGTA, 1 mM sodium orthovanadate, 1 mM dithiothreitol. Similar assays were carried out with GSK-3, cdc-2, MAPK, and DNA-dependent protein kinases. Full-length E2 proteins translated *in vitro* from pET23-E2 plasmids in *E. coli* extracts (Roche Applied Science RTS system) were also used as substrates for CK2, as was E2 protein expressed in mammalian CV-1 cells from pPAVA viruses as described previously (7) using conditions similar to those described for synthetic peptides. Phosphorylation of full-length E2 protein was analyzed by SDS-PAGE and autoradiography. Incorporation of ^{32}P into peptides was analyzed after separation on 20% polyacrylamide SDS/Tris-Tricine gels. Phosphorylated peptides were detected using a PhosphorImager and ImageQuant software (Amersham Biosciences). Fifty micrograms of unlabeled peptides were separated on the same gel and detected by silver stain.

Peptide Synthesis and Purification—Peptides representing amino acids 283–311 of E2 were obtained through the NIAID peptide facility (National Institutes of Health) and were purified by reverse phase-high pressure liquid chromatography. All peptides were dissolved in water to a concentration of ~500 μM and stored at −70 °C. Quantification of the peptides was carried out by absorbance at 220 nm in HCl. The peptides are named according to the phosphorylated residue ($E2_{283–311}$, $E2_{283–311}S298^{P}$; and $E2_{283–311}S301^{P}$).

Circular Dichroism—Circular dichroism spectra of peptides were obtained using a Jasco J-815 instrument. Cell paths were either 0.1 or 1 mm, and protein concentration, pH, and temperature are indicated in the experiment. Five far-UV scans were averaged, and the buffer base line was subtracted from all spectra registered. Where indicated, 2,2,2-trifluoroethanol (ICN) was added. Temperature was kept within ±0.1 °C, using a peltier device. For the fraction of α-helical secondary structure (f_α) content of the peptides we used Equation 1 (15).

$$f_\alpha = \frac{[\theta]_{222}^{exp} - [\theta]_{222}^{coil}}{[\theta]_{222}^{max} - [\theta]_{222}^{coil}} \quad \text{(Eq. 1)}$$

where $[\theta]_{222}^{exp}$ is the measured ellipticity value at 222 nm; $[\theta]_{222}^{coil}$ is the tabulated ellipticity for random coil, and $[\theta]_{222}^{max}$ is the ellipticity corresponding to a 100% helical peptide (16).

RESULTS

Phosphorylation of Serine 301 Targets BPV-1 E2 for Degradation by the Proteasome—The BPV-1 E2 transactivator protein has a half-life of ~40 min (10, 17). We have shown previously (10) that substitution of the phosphoacceptor serine residue 301 with alanine leads to an increase in half-life and reduced levels of ubiquitination. To assess further the stability of the E2 species, the phosphorylation status of E2 was analyzed when proteasomal degradation was blocked with the inhibitor MG132. These experiments were carried out using CV-1 cells that stably maintain Epstein-Barr virus-derived extrachromosomal vectors, which express E2 from an inducible metallothionein promoter. As described previously, these cell lines can be induced to express E2 at levels that exhibit appropriate nuclear localization and turnover of the E2 protein (10). CV-1 cells were induced to express both wild type E2 protein and E2 with a serine to alanine substitution at residue 301 (E2 S301A). Phosphorylation of E2 does not produce a mobility shift on SDS-polyacrylamide gels, and therefore phosphoryl-

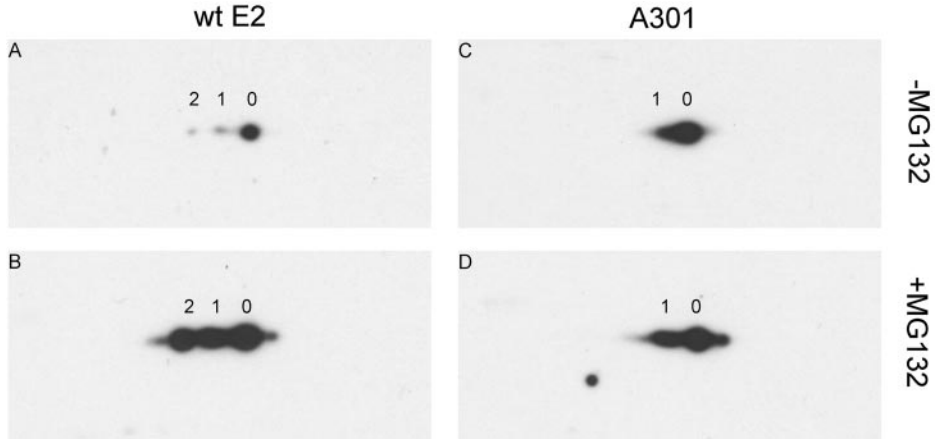

FIG. 1. **Proteasome inhibition increases stability of phosphorylated E2 species.** Expression of wild type (*wt*) E2 (*panels A* and *B*) and E2 S301A (*A301*) (*panels C* and *D*) proteins was induced in CV-1 cells for 3 h with 0.2 μM $CdSO_4$. *Panels B* and *D*, cells were also treated during induction with 20 μM MG132 to block activity of the proteasome. Unphosphorylated E2 is labeled as *0*; phosphorylation on one residue with *1*; phosphorylation of two residues with *2*.

ated species must be separated by isoelectric focusing and detected by Western blot using a monoclonal antibody directed against E2. As shown in Fig. 1, *panel A*, three species of wild type E2 protein were observed when they were separated by size and charge. The most abundant species corresponds to unmodified E2; the middle species corresponds to phosphorylation on one residue (but does not distinguish between serine 298 and serine 301), and the most acidic species furthermost to the left corresponds to E2 containing two phosphorylated residues. We have shown previously (7) by phosphate labeling that the more acidic species correspond to phosphorylated forms of E2 protein. As shown in Fig. 1, *panel C*, the number of phosphorylated species is reduced to one in the E2 S301A protein, and the abundance of both phosphorylated and unphosphorylated species is increased. Treatment with MG132 should result in the accumulation of the most rapidly turned over species, and as predicted, an accumulation of phosphorylated species was observed (Fig. 1, *panels B* and *D*). This is most dramatic in the wild type E2 protein (Fig. 1, *panel B*). This confirms that the phosphorylated species of E2 are more rapidly degraded by the proteasome than unmodified E2 and supports the hypothesis that phosphorylation of serine 301 targets E2 for degradation by the proteasome.

Mutational Analysis of the PEST Region of BPV-1 E2—To understand exactly how E2 is targeted for degradation by the proteasome, an extensive mutational analysis of the region surrounding the phosphorylation sites was carried out, and the resulting E2 proteins were assayed for phosphorylation and protein stability. For this analysis, the previously mapped serine phosphoacceptors at positions 298 and 301 were substituted with both neutral and negatively charged residues that could potentially substitute for phosphoserine. In addition, kinase consensus sequences present in this region of E2 were targeted for mutation, and stretches of negatively charged amino acids toward the N terminus and C terminus of the phosphorylation sites were deleted or substituted with alanine residues. To determine whether conformation of the region C-terminal to the phosphorylation sites is critical in determining the stability of the protein, proline substitutions were made to disrupt any inherent structure in this region. Finally, each residue from residue 286–310 was individually substituted with an alanine residue. The mutations generated are illustrated in Fig. 2. CV-1 cell lines expressing each mutated E2 protein were established, as described above. To understand how phosphorylation of serine 301 targets BPV-1 E2 for ubiquitination and subsequent degradation by the proteasome, the

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                  298 301
TVPVDLASRQEEEEQSPDSTEEEPVTLPR   WT
TVPVDLAARQEEEEQSPDSTEEEPVTLPR   S290A
TVPVDLASAQEEEEQSPDSTEEEPVTLPR   R291A
TVPVDLASRAEEEEQSPDSTEEEPVTLPR   Q292A
TVPVDLASRQAEEEQSPDSTEEEPVTLPR   E293A
TVPVDLASRQEAEEQSPDSTEEEPVTLPR   E294A
TVPVDLASRQEEAEQSPDSTEEEPVTLPR   E295A
TVPVDLASRQEEEAQSPDSTEEEPVTLPR   E296A
TVPVDLASRQEEEEASPDSTEEEPVTLPR   Q297A
TVPVDLASRQEEEEQAPDSTEEEPVTLPR   S298A
TVPVDLASRQEEEEQSADSTEEEPVTLPR   P299A
TVPVDLASRQEEEEQSPASTEEEPVTLPR   D300A
TVPVDLASRQEEEEQSPDATEEEPVTLPR   S301A
TVPVDLASRQEEEEQSPDSAEEEPVTLPR   T302A
TVPVDLASRQEEEEQSPDSTAEEPVTLPR   E303A
TVPVDLASRQEEEEQSPDSTEAEPVTLPR   E304A
TVPVDLASRQEEEEQSPDSTEEAPVTLPR   E305A
TVPVDLASRQEEEEQSPDSTEEEAVTLPR   P306A
TVPVDLASRQEEEEQSPDSTEEEPATLPR   V307A
TVPVDLASRQEEEEQSPDSTEEEPVALPR   T308A
TVPVDLASRQEEEEQSPDSTEEEPVTAPR   L309A
TVPVDLASRQEEEEQSPDSTEEEPVTLAR   P310A
TVPVDLASRQEEEEQSPDSTEQEPVTLPR   E304Q
TVPVDLASRQEEEEQAPDSTEQEPVTLPR   S298A,E304Q
TVPVDLASRQ----QSPDSTEEEPVTLPR   Δ293-296
TVPVDLASRQAAAAQSPDSTEEEPVTLPR   A293-296
TVPVDLASRQEEEEQSPDSTAAAPVTLPR   A303-305
TVPVDLASRQAAAAQSPDSTAAAPVTLPR   A293-296,A303-305
TVPVDLASRQEEEEQSPDST---PVTLPR   Δ303-305
TVPVDLASRQEEEEQSPDSTPEEPVTLPR   E303P
TVPVDLASRQEEEEQSPDSTEPEPVTLPR   E304P
TVPVDLASRQEEEEQSPDSTEEPPVTLPR   E305P
TVPVDLASRQEEEEQSPDETEEEPVTLPR   S301E
TVPVDLASRQEEEEQSPDDTEEEPVTLPR   S301D
TVPVDLASRQEEEEQEPDSTEEEPVTLPR   E298
TVPVDLAERQEEEEQEPDETEEEPVTLPR   EEE
TVPVDLAARQEEEEQAPDATEEEPVTLPR   AAA
TVPVDLASRQEEEEQSPAADSTEEEPVTLPR insAA299-300
TVPVDLASRQEEEEQSPADSTEEEPVTLPR  insA299-300
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FIG. 2. **Mutational analysis of E2 phosphorylation region.** The region surrounding the E2 phosphorylation sites is shown. The phosphorylation sites are shown in *red*, and the amino acid substitutions, insertions, and deletions are shown in *blue*. *WT*, wild type.

phosphorylation status and stability of each of the mutated E2 proteins were analyzed.

Phosphorylation Status of Mutated E2 Proteins—To determine the phosphorylation status, E2 expression was induced in CV-1 cell lines, and protein extracts were separated by two-dimensional isoelectric focusing and were detected by Western blot analysis using a monoclonal antibody directed against E2. As described for Fig. 1, the wild type E2 protein displays three species; the most basic species corresponds to unmodified protein; the middle species corresponds to phosphorylation on one residue (either serine 298 or serine 301); and the most acidic species corresponds to the fully phosphorylated protein (both serine 298 and 301 phosphorylated). The phosphorylation status of the mutated E2 proteins is shown in Fig. 3.

A CK2 Consensus Sequence Is Crucial for Phosphorylation of Serine 301—We have shown previously that substitution of serine 301 with alanine eliminates phosphorylation of this site

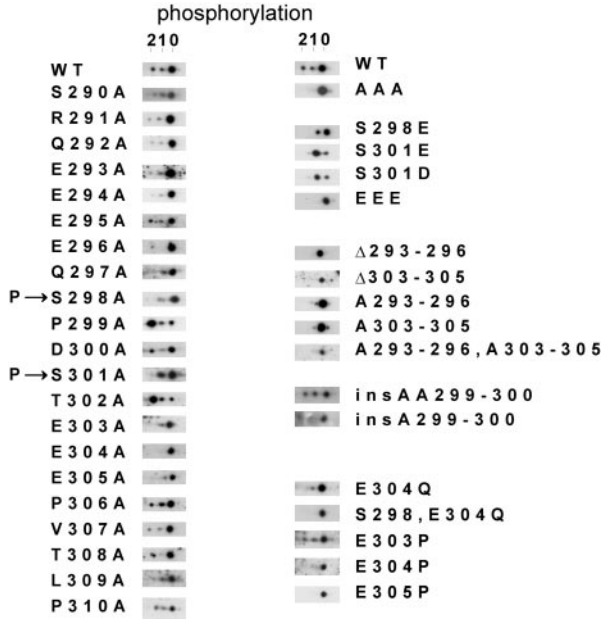

FIG. 3. **Phosphorylation analysis of mutated E2 proteins.** E2 expression was induced in CV-1 cell lines for 3 h with 0.2 μM $CdSO_4$, and protein extracts were separated by two-dimensional electrophoresis and E2 species detected by Western blot analysis by using the B201 monoclonal antibody. Charged species were aligned with reference to marker proteins, and phosphorylated and non-phosphorylated species are indicated, as described in Fig. 1. The previously mapped phosphorylation sites at serine 298 and 301 (7) are indicated by *arrows*. *WT*, wild type.

and stabilizes the protein. In this study, serine 301 was also mutated to glutamic and aspartic acid to determine whether a negatively charged side chain can substitute for phosphoserine at position 301. As shown in Fig. 3, substitution of residue 301 with alanine, glutamic acid, or aspartic acid resulted in loss of at least one phosphorylated species. The strongest consensus for well characterized protein kinases that could modify serine 301 is CK2. The CK2 consensus is (S/T)*XX*(D/E) where S/T is the phosphorylation site, and there is an absolute requirement for a negative charge at the +3 position (18). To determine whether CK2 is responsible for phosphorylation of serine 301, the kinase consensus site was destroyed by substituting glutamine for glutamate at residue 304 in the crucial +3 position ($E2_{E304Q}$). As can be seen in Fig. 3, this substitution also eliminated at least one phosphorylated E2 species, confirming that CK2 is responsible for phosphorylation of residue 301 *in vivo*. It has been shown that an environment of negatively charged residues enhances phosphorylation by CK2 (18), and notably this region of the E2 protein is strongly acidic. Deletion or substitution of the stretches of acidic residues toward the N terminus (293–296) and/or C terminus (303–305) of residue 301 was found to decrease E2 phosphorylation, providing further evidence that CK2 is responsible for modification of serine 301.

Serine 298 Can Be Phosphorylated by an Alternative Kinase—Serine 298 is also a major phosphorylation site of E2 (7). However, mutation of this residue has no phenotype in measurements of E2 function that have been tested so far (11). As shown previously, substitution of serine 298 with alanine eliminates one phosphorylated E2 species (Fig. 3) (7). However, serine 298 does not contain a strong consensus for any particular kinase. Serine 298 could be a substrate for CK2 phosphorylation if serine 301 is phosphorylated, thus providing a negative charge at the +3 position. However, $E2_{S301A}$ retains the ability to be phosphorylated at one site; therefore, at least under these conditions, residue 298 must be modified by a different kinase. Disrupting the spacing between serines 298 and 301 by inserting two alanine residues between proline 299 and aspartate 300 ($E2_{insAA299-300}$) should abolish CK2 phosphorylation of serine 298; however, this protein is fully phosphorylated. Therefore, another kinase must have the ability to phosphorylate residue 298.

Serine 298 is also a potential CK1 site, which requires a stretch of negative charges N-terminal to the phosphoaccepter site with a crucial negative charge at the −3 position (19). Alanine substitution or deletion of the glutamate stretch N-terminal to the phosphorylation sites (293–296) greatly reduces phosphorylation of E2. The decrease in phosphorylation could be the direct result of eliminating the CK1 consensus for serine 298, but this analysis does not allow us to distinguish between phosphorylation of residues 298 and 301. Another explanation for the effect of mutation of the glutamate group (residues 293–296) is that the presence of the negative charges N-terminal to the phosphorylation sites could also increase CK2 phosphorylation, which is enhanced with increasing numbers of negatively charged amino acids in the surrounding region.

Furthermore, when serine 298 is phosphorylated, serine 301 could be a potential CK1 site. CK1 was first classified as a phosphate-directed protein kinase with the requirement for a phosphorylated serine (pS) or threonine (pT) at the *n*-3 position ((pS/pT)*XX*S) (20). However, for the same reasons listed previously for synergism of CK2 phosphorylation between the phosphorylation sites, there must be additional or alternative mechanisms to phosphorylate these sites *in vivo* when the second phosphorylation site is removed by mutation or the spacing between the sites is changed.

Serine 298 is also contained in a very minimal consensus motif for cyclin-dependent kinases (an SP motif). However, alanine substitution of proline 299 should eliminate such phosphorylation, and on the contrary, phosphorylation of both sites is greatly increased. However, it remains possible that a proline-directed kinase does modify residue 298 in the wild type E2 protein, but when the proline is removed then 298 becomes phosphorylated by an alternative mechanism.

E2 Mutations That Increase Phosphorylation—Notably, some mutations resulted in dramatically enhanced levels of the doubly phosphorylated protein (the most acidic species); these were substitutions of proline 299 or threonine 302 with alanine, and to a lesser extent substitution of aspartate 300 with alanine (Fig. 3). This enhancement could be explained either as an enhancement in kinase recognition or an increase in stability of the double-phosphorylated protein so that it now becomes the predominant species. The results of the stability experiments described below (Fig. 5) indicate that the latter situation is likely, as each of these proteins has increased stability. Proteins in which the serine 301 phosphorylation site has been replaced by an acidic residue, S301E and S301D, also show enhanced phosphorylation on the remaining serine 298 site. This could also be because of increased protein stability and/or enhancement of serine 298 phosphorylation by the presence of a negative charge at the +3 position.

E2 Is Phosphorylated in Vitro by CK1 and CK2—To determine further which kinases are able to phosphorylate the E2 protein, a series of *in vitro* kinase assays were carried out by using different sources of E2 protein as substrates. To confirm that serine residue 301 is a CK2 substrate, full-length E2 protein was translated *in vitro* in *E. coli* lysates and used as a substrate for CK2. As shown in Fig. 4, *panel A*, wild type E2 was phosphorylated by CK2, and this was dependent on a serine residue at 301; substitution with alanine eliminated phosphorylation. Destruction of the serine 301 CK2 consensus site by substitution at the +3 position ($E2_{E304Q}$) also eliminated phosphorylation, as did a combination of this mutation

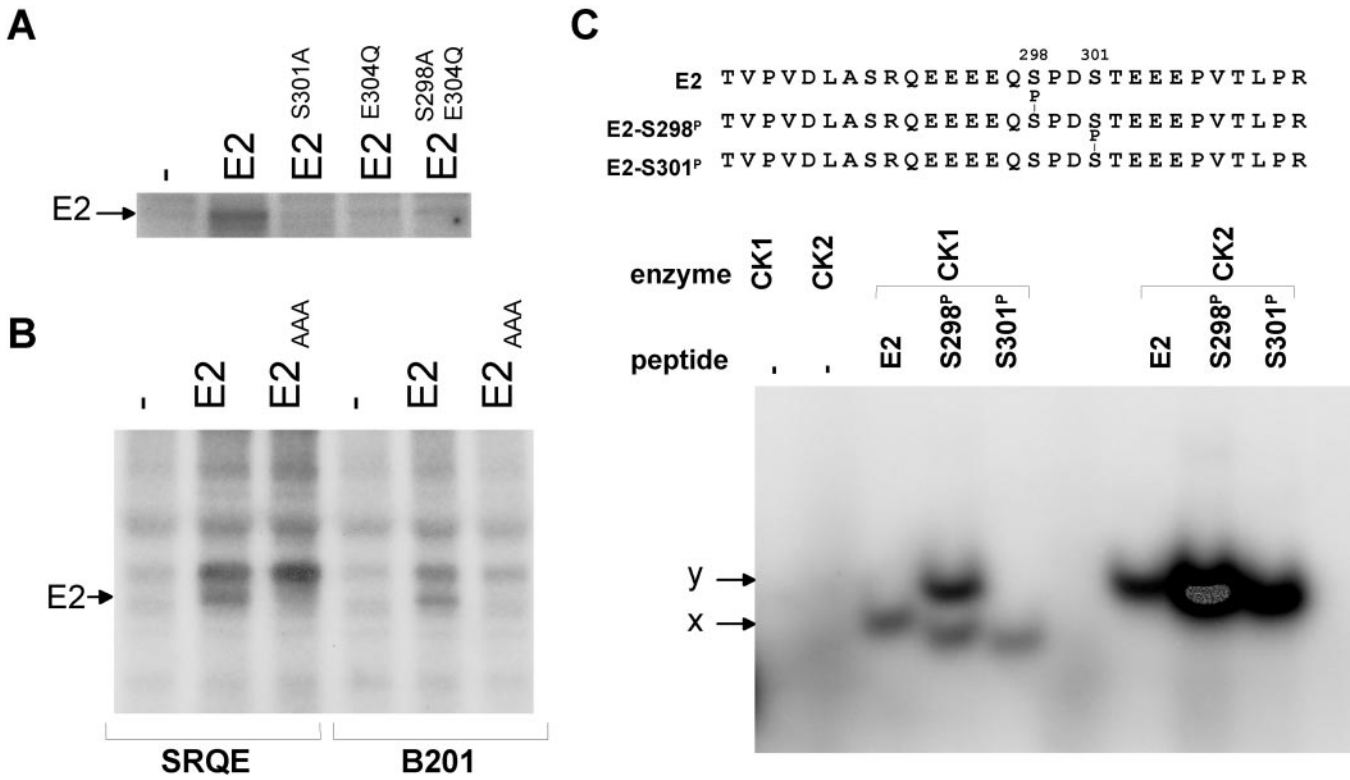

FIG. 4. ***In vitro* kinase analysis of E2 proteins and peptides.** *In vitro* kinase assays were carried out using various E2 substrates, and ^{32}P incorporation was analyzed by SDS-PAGE. *A,* full-length E2 proteins, as indicated, were translated *in vitro* in *E. coli* extracts and used as substrates for CK2. *B,* full-length E2 proteins expressed in mammalian COS-1 cells from recombinant SV40 pPAVA viruses were immunoprecipitated with either B201 or SRQE E2-specific antibodies, as indicated, and phosphorylated with CK2. *C,* the peptides shown were phosphorylated with CK1 or CK2 as indicated. Electrophoresis of unlabeled peptides separated on the same gel and detected by silver stain indicated that all three unlabeled peptides comigrated with the lower band (designated *x*). Peptides phosphorylated on both 298 and 301 residues result in a mobility shift (designated *y*).

and an alanine substitution at the other phosphorylation site ($E2_{S298A,E304Q}$). The fact that the alanine 301 substitution eliminates CK2 phosphorylation *in vitro* further demonstrates that serine 298 cannot be phosphorylated by CK2 in the absence of a negative charge at position 301. These data agree with the *in vivo* phosphorylation data and confirm that serine 301 is phosphorylated by CK2.

In addition, both wild type and $E2_{AAA\ (S290A,S298A,S301A)}$ proteins were expressed in mammalian cells and used as a CK2 substrates. In this experiment E2 proteins were expressed in COS-1 cells from recombinant SV40 viruses, as described previously (7), immunoprecipitated with E2 specific antibodies, and used as substrates in a CK2 assay. As shown in Fig. 4, *panel B*, wild type E2, but not $E2_{AAA}$, could be phosphorylated by CK2.

Finally, synthetic peptides spanning residues 283–311 were used as substrates for a range of kinases. These 29-mers were either unmodified ($E2_{283–311}$) or phosphorylated at residue 298 ($E2_{283–311}S298^P$) or 301 ($E2_{283–311}S301^P$). Commercially available peptides containing kinase consensus motifs were used as positive and negative controls (data not shown). All three E2 peptides (phosphorylated and unphosphorylated) were modified by both CK1 and CK2 (Fig. 4, *panel C*) but not by GSK-3, cdc-2, MAPK, or DNA-dependent protein kinase (data not shown). Phosphorylation of all three peptides with CK2 and of $S298^P$ with CK1 gave rise to a mobility shift that is likely due to changes in peptide conformation (designated *y* in Fig. 4, *panel C*). Electrophoresis of unlabeled peptides separated on the same gel and detected by silver stain indicated that unmodified peptide and the singly phosphorylated $S298^P$ and $S301^P$ peptides comigrated with the lower band (designated *x* in Fig. 4, *panel C*). Therefore, only peptides that are phosphorylated on both 298 and 301 residues result in a mobility shift.

CK2 was able to modify all three peptides, even when serines 298 or 301 were already phosphorylated ($S298^P$ and $S301^P$). In fact, peptide $S298^P$ was a much more efficient substrate. Therefore, either 298 or 301 can be phosphorylated by CK2, but 301 is a preferred substrate when serine 298 is already phosphorylated. Furthermore, CK2 synergistically modifies both serine 298 and 301, as peptides with the mobility of a single phosphorylated species were not observed at any incubation time in the kinase assay (data not shown).

CK1 phosphorylation was relatively inefficient compared with that of CK2. After CK1 phosphorylation, the peptide band with slower mobility that we postulate is due to double-phosphorylation of residues 298 and 301 was only observed with the $S298^P$ peptide. This is most likely due to phosphorylation of residue 301 by CK1. Phosphorylation of $S301^P$ by CK1 does not result in this shift, indicating that serine 298 is not a substrate for CK1, perhaps because of the adjacent proline residue at position 299. However, all three peptides are labeled by CK1, but this does not result in the mobility shift associated with double-phosphorylation of serine 298 and 301 (Fig. 4, *panel C, band x*). We propose that this is due to phosphorylation at position 290 (although residue 290 is not phosphorylated *in vivo*, it does fit a minimal CK1 consensus).

In conclusion, both CK1 and CK2 can phosphorylate this region of the E2 protein *in vitro*; this is in agreement with the *in vivo* results obtained with E2 proteins mutated in this region. However, although interplay can occur between the sites and can lead to enhanced phosphorylation by CK2, there are also structural constraints that prevent phosphorylation of serine 298 by CK1. Serine 298 could be modified by CK2, but the fact that E2 proteins with a serine to alanine substitution at

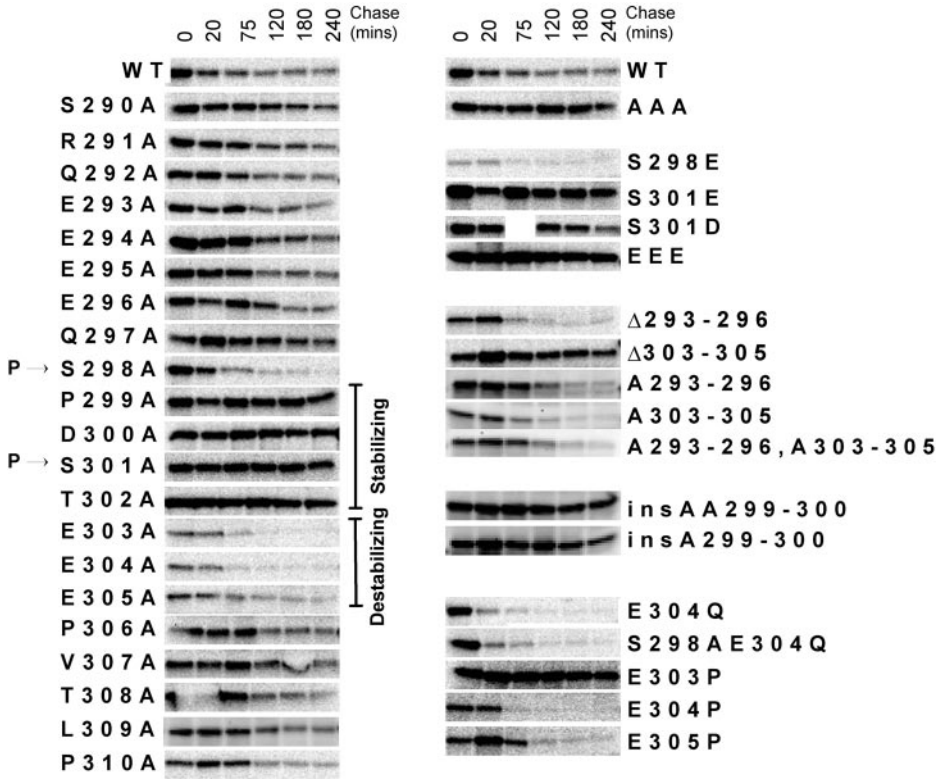

FIG. 5. **Analysis of the stability of mutated E2 proteins.** E2 protein expression was induced in pMEP-E2 CV-1 cell lines with 1 μM $CdSO_4$ for 2 h and chased by removal of the $CdSO_4$ from the medium and the addition of 25 μg/ml emetine and 25 μg/ml cycloheximide for the times indicated. E2 protein levels were analyzed by SDS-PAGE and Western blot analysis using B201 monoclonal antibody. *WT*, wild type.

301 or insertion of alanine residues between the phosphorylation sites are still modified on serine 298 indicates that other kinases are capable of phosphorylating this site *in vivo*.

Stability of E2 Proteins Mutated in the PEST Sequence—To assess the stability of the mutated E2 proteins described above, pulse-chase experiments were carried out in the pMEP-E2 CV-1 cell lines. E2 protein expression was induced in the cell lines and chased by removal of the $CdSO_4$ from the medium and addition of a mixture of protein translation inhibitors (emetine and cycloheximide). E2 protein levels were analyzed by SDS-PAGE and Western blot analysis by using a monoclonal antibody directed against E2. Fig. 5 illustrates the results of one such experiment in which E2 expression was induced for 2 h and then chased at various times up to 4 h.

As expected and observed previously, substitution of residue 301 with alanine (S301A and AAA) resulted in a protein with greatly increased stability. In many proteins a negatively charged residue such as glutamate or aspartate can functionally mimic the phosphoserine side chain. Serines 298 and 301 were substituted with negatively charged residues ($E2_{S301E}$, $E2_{S301D}$, and $E2_{EEE}$), as shown in Fig. 2. However, proteins with these substitutions at residue 301 also had increased stability, similar to that of proteins with alanine substitutions at 301. Therefore, negatively charged side chains cannot mimic phosphorylation in the degradation of E2, and any substitution of residue 301 resulted in a stable protein.

The residues immediately surrounding the phosphorylation sites are also important in determining E2 protein stability. Alanine substitutions at proline 299, glutamate 300, serine 301, and threonine 302 ($E2_{P299A}$, $E2_{D300A}$, $E2_{S301A}$, and $E2_{T302A}$) dramatically extend the half-life of the protein. Therefore, substitution of any of the residues PDST (299–302) increases E2 stability. Notably, with the exception of mutation of the phosphoacceptor serine 301, each of these substitutions leads to increased phosphorylation (Fig. 3). As discussed above, this could be due to increased phosphorylation and/or increased stability of the E2 proteins. The results of the stability experiments suggest that the latter situation is most likely, as each of these proteins has increased stability. This indicates that phosphorylation and E2 degradation are separable. Insertion of either one or two alanines between residues 299 and 300 also resulted in a more stable E2 protein, although there were no dramatic changes in phosphorylation, further confirming that the region between the phosphorylation sites is important for regulating E2 protein stability.

Conversely, it was noted that substitution of acidic residues 303, 304, and 305 could greatly destabilize the E2 protein. As described above, serine 301 is modified by CK2, and the negative charges to the C terminus of this residue (in particular glutamate 304 at the +3 position) are important for this phosphorylation. All of these unstable proteins show reduced phosphorylation, with the loss of at least one phosphorylated species. It was hypothesized that a conformational change in this region regulates the stability of the E2 protein by exposing a region of the polypeptide that is important for recognition by the proteasome pathway. The proline residue at position 299, between the phosphorylation sites, is predicted to cause a turn in the polypeptide structure just before the serine 301 residue. Depending on the structure of this region, phosphorylation of serine 301 would be predicted to either stabilize or destabilize the secondary structure of the polypeptide (21, 22). The resulting disruption or stabilization of the structure C-terminal to the phosphorylation sites could be important for recognition of E2 by components of the proteasome pathway. To test this hypothesis, proline substitutions were made in residues 303–305. Proline substitution of residues 304 and 305 resulted in E2 proteins with a protein turnover rate that was much faster than that of the wild type protein. This is to be expected because phosphorylation is required to trigger degradation of the wild type protein, and this corresponds to only a small portion of the protein pool at any specific time. In contrast, the entire pool of E304P and E305P proteins would be predicted to adopt the unstable conformation resulting in immediate degradation and a shorter half-life. Alanine substitutions of glu-

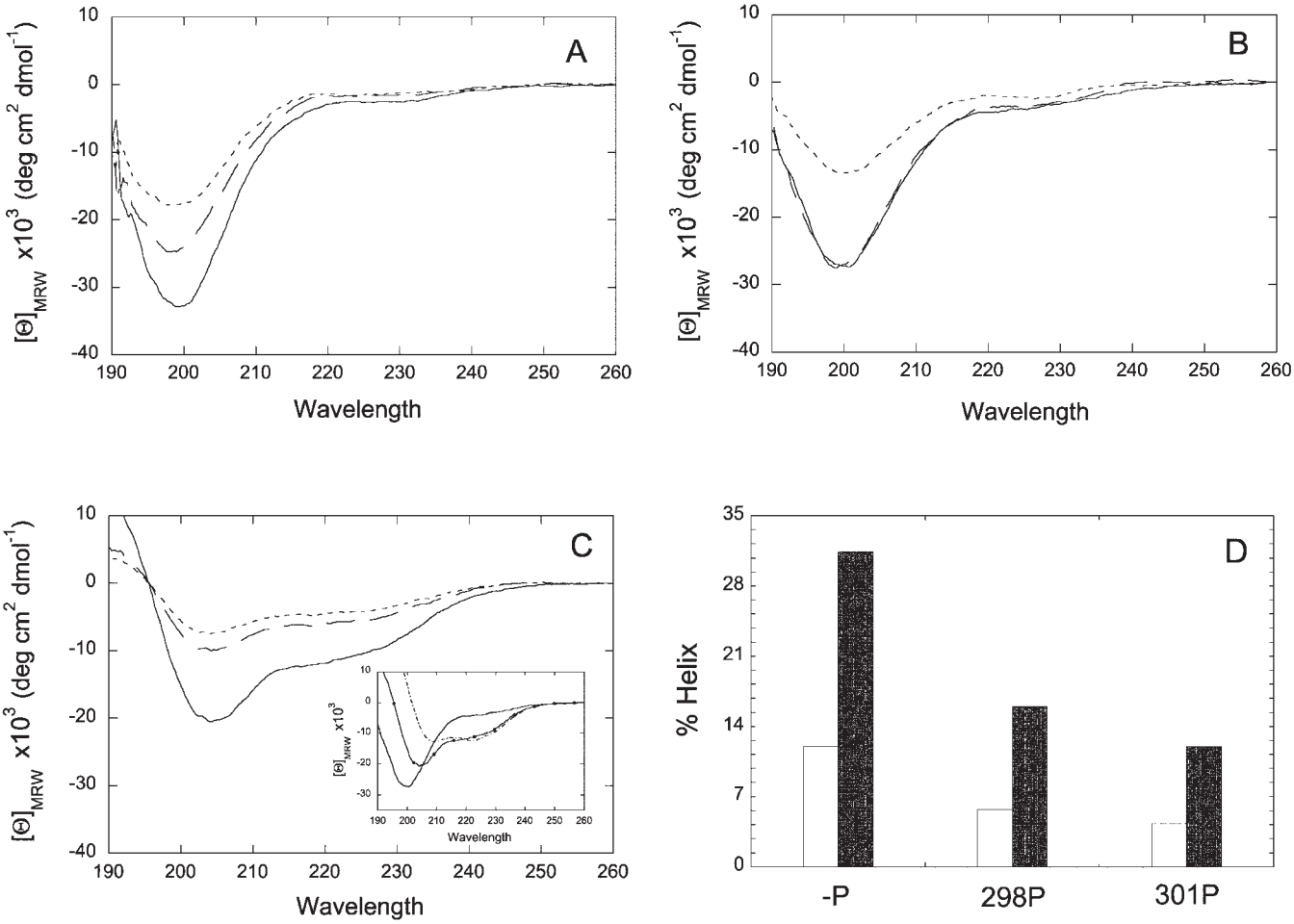

FIG. 6. **Effect of phosphorylation on hinge peptides monitored by far-UV circular dichroism.** *A*, spectra of $E2_{283–311}$ (*solid line*), $E2_{283–311}S298^P$ (*dashed line*), and $E2_{283–311}S301^P$ (*dotted line*) at 20 μM protein concentration in 20 mM Tris-HCl buffer, pH 7.5. *B*, same as *A* but in 20 mM sodium acetate buffer, pH 4.5. *C*, same as *B* but with 50% TFE added. *Inset*, spectrum of the unphosphorylated $E2_{283–311}$ peptide in TFE (*solid line* with *black dots*) compared with the same peptide in buffer (*solid lines*) and a model pure α-helical peptide (*dotted line*). *D*, α-helical content of the different peptides taken from *B* in aqueous buffer (*white bars*) or with 50% TFE added (*gray bars*).

tamates 303–305 also resulted in proteins with shorter half-lives than wild type E2, as did the substitution of 304 with glutamine.

Notably, a proline at position 303 stabilized the protein. In agreement with this, deletion of residues 303–305 had the same effect; this deletion places proline 306 immediately adjacent to threonine 302 resulting in the same linear polypeptide sequence, SPDSTP. Presumably, a proline at this position either stabilizes the backbone and prevents the conformational change necessary for degradation of E2 or interferes with substrate recognition. Otherwise, deletion or substitution of the acidic residues toward the N terminus or C terminus of this region reduced overall phosphorylation but did not greatly affect stability.

E2 Phosphorylation and Degradation Are Separable—As described above, proteins with substitutions in the PDST motif at residues 299–302 are highly phosphorylated, yet are stable. Conversely, when serine 301 phosphorylation is abolished by substitutions of glutamate residue 304 with glutamine, alanine, or proline ($E2_{E304Q}$, $E2_{E304A}$, and $E2_{E304P}$) the resulting proteins have half-lives that are shorter than the wild type E2 protein. Therefore, E2 phosphorylation and degradation are separable, and it is not the recognition of the phosphate group at position 301 *per se* that leads to degradation but more likely a conformational change caused by the addition of the phosphate group to serine 301.

In conclusion, substitutions in the region 298–305 of E2 have wide ranging effects on protein half-life. Changes immediately adjacent to and between the phosphorylation sites tend to increase the stability of E2, whereas substitutions to the C terminus of this region have a destabilizing effect. We propose that this region is a conformational switch that is modulated by phosphorylation and controls degradation of the E2 protein by the ubiquitin-proteasome pathway.

Effect of Phosphorylation on the Conformational Tendencies of Peptides Spanning the Phosphorylation Region—To test our hypothesis, the conformational tendencies of 29-mer peptides corresponding to the region spanning the phosphorylation sites (residues 283–311) were analyzed. The far-UV CD spectra of both unphosphorylated and phosphorylated peptides at pH 7.5 resemble an apparently disordered conformation, as is expected for small peptides, with a minimum below 200 nm (Fig. 6, *panel A*). However, the negative ellipticity of the non-phosphorylated peptide is much more pronounced than the Ser-298- and Ser-301-phosphorylated peptides, and this band disappears with the increase of temperature (not shown). If these spectra represented a pure random coil conformation, they should be superimposable, because the sequences are identical. A small shoulder at around 230 nm also suggested that there was residual structure in the unphosphorylated peptide. The highly acidic nature of this region indicated that its conformation could be influenced by pH, and indeed the behavior of the peptide is similar to polyglutamic acid model peptides (23). At pH 4.5, the secondary structure of unphosphorylated and $S298^P$

peptides are indistinguishable, but the S301^{p} peptide shows a much less negative minimum at 200 nm, suggesting less residual structure (Fig. 6, *panel B*).

Small peptides lacking the possibility of long range tertiary interactions are not expected to acquire substantial structure; however, their structural propensity will result from an always present equilibrium between disordered and ordered structures. This equilibrium can be shifted by changing the solvent composition, and this can be attained by using TFE as cosolvent (24, 25), which is known to stabilize α-helical structures by shifting the equilibrium. Addition of 50% TFE to all of the E2 peptides at pH 4.5 caused a large stabilization of the residual α-helical structure present in the peptides in aqueous solution (Fig. 6, *panel C*), and the percent of helical content can be calculated for peptides in TFE and in water (see "Experimental Procedures"). The minima at 222 and 208 nm are the hallmark of an α-helix; however, the minimum at 208 is shifted to 205 nm, due to the strong contribution of the band at 198 nm (Fig. 6, *panel* C, *inset*). Therefore, although an α-helix is induced, it appears to be restricted to a particular region, and other structural features persist in 50% TFE. Under these conditions, the α-helical content for the unphosphorylated peptide is 31.4%, and it is 16.8% for S298^{p} and 12.6% for the S301^{p} peptide (Fig. 6, *panel D*). At pH 4.5 and without the cosolvent, these values are 12, 10, and 5% for the same peptides. Therefore, local thermodynamic stability of this region can be disrupted by phosphorylation, in particular that of serine residue 301.

DISCUSSION

The hinge region of the E2 proteins is poorly conserved among the large number of papillomavirus types, in contrast with the strong conservation of the N-terminal transactivation and C-terminal DNA binding domains (6). The hinge region has been regarded as a flexible or unstructured link between the two functional domains. However, the hinge region has been shown to have additional diverse functions in a number of papillomaviruses. For example, the hinge region of the HPV11 E2 protein is crucial for localization of E2 to the nucleus and nuclear matrix (26), and the hinge region of HPV5 E2 can activate promoters by interaction with Sp1 (27) and can associate with splicing factors (28). As studied here, the major phosphorylation sites of the BPV-1 E2 protein have been mapped to the hinge region, and phosphorylation of serine 301 leads to degradation of the BPV-1 E2 proteins via the ubiquitin-proteasome pathway (10). We propose that this region is a conformational switch that is modulated by phosphorylation and controls recognition of E2 by the ubiquitin-proteasome pathway. Notably, the sequence central to this "switch" (SPD-ST(E/D)) is conserved among all other sequenced fibropapillomaviruses: European elk, reindeer, deer, ovine, and bovine papillomavirus type 2.

In this study we investigate the mechanisms by which phosphorylation results in recognition and targeting of E2 for degradation by the proteasome. This was done by carrying out an extensive mutational analysis of the region and analyzing the resulting mutated proteins for their stability and phosphorylation status *in vivo* and *in vitro*. This study has yielded insight into the kinases responsible for phosphorylation of this region of E2. We have shown that CK2 can phosphorylate both full-length E2 protein and derived peptides *in vitro* and that mutation of the CK2 consensus for residue 301 eliminates phosphorylation *in vivo*. CK2 is a constitutively active and pleiotropic kinase that has hundreds of substrates, most of which are involved in transcription and signal transduction and many of which are viral proteins (reviewed in Ref. 29). As proposed by Pinna in 1990 (30), CK2 phosphorylation could represent a constitutive type of post-translational modification that is regulated by dephosphorylation and/or other factors. In the case of papillomavirus E2 protein, constitutive phosphorylation by CK2 could ensure that this important regulator of viral transcription and replication is rapidly turned over. However, at different times in the viral life cycle there may be a need for the E2 protein to be stabilized. For example, we have shown previously (10) that there are very high levels of E2 protein in the differentiated cells of a papilloma that are vegetatively amplifying viral DNA, and this could represent a situation in which the constitutive degradation of E2 must be abrogated. In addition to its role as a transcription and replication factor, E2 is important for genome maintenance and segregation. The BPV-1 E2 protein tethers the viral genome to mitotic chromosomes in dividing cells to ensure its efficient segregation to daughter nuclei (3). Presumably, E2-viral genome complexes must also be stabilized throughout the length of mitosis to ensure efficient segregation. Conversely, when E2 is expressed in the absence of the viral genome or other viral proteins, we observe a distinct drop in the amount of E2 protein at mitosis[2] indicating that there is an additional regulatory event at this step. An attractive hypothesis is that E2-TA bound to viral DNA is resistant to degradation, and "free E2" is phosphorylated and targeted for degradation. Notably, the region of E2 containing the PEST region is immediately adjacent to the DNA binding domain. There is precedent for this in the case of the muscle-specific transcription factor, MyoD. MyoD is phosphorylated by cyclin-dependent kinases and ubiquitinated and degraded by the proteasome in a phosphorylation-dependent manner (31). This process is inhibited by specific MyoD DNA binding (32, 33). Phosphorylation and degradation of E2 could be further modulated by interaction with the viral E1 protein. It has been reported that the unphosphorylated form of E2 (or E2 with mutations in phosphorylation sites 235, 298, and 301) interacts with E1 with greater affinity than that of the phosphorylated form of E2 (34, 35). Furthermore, it has been proposed that phosphorylation is required to dissociate the E1-E2 protein complex to allow interaction of E2 with mitotic chromosomes (35). Therefore, there are a number of points in the viral life cycle in which E2 turnover and function might be modulated by CK2 phosphorylation.

A specific kinase for serine residue 298 remains elusive. Whereas CK2 can phosphorylate serine residue 298 *in vitro* (at least when serine 301 is already phosphorylated), it cannot be the sole kinase that phosphorylates this residue *in vivo*. For instance, one phosphorylated species remains in $E2_{S301A}$, and this cannot be due to CK2 phosphorylation of Ser-298. The presence of a proline at position 299 is also thought to be detrimental to CK2 phosphorylation of the preceding residue (29), and it is evident that substitution of this proline results in greatly increased double phosphorylation of E2 *in vivo*. However, CK2 can modify both Ser-298 and Ser-301 *in vitro*. CK1 could modify serine 301 in E2-derived peptides, but only when they were already phosphorylated on serine 298. Surprisingly, CK1 was unable to phosphorylate serine residue 298 *in vitro*. The SP motif at residues 298 and 299 is also a minimal consensus for a number of proline-directed kinases. However, MAPK and cdc2 were unable to phosphorylate peptides derived from the PEST region of E2 in *in vitro* assays, but it is possible that other proline-directed kinases can modify this region.

The E2 phosphorylation sites are spaced three residues apart. The presence of a negative charge at position -3 or $+3$ is important for phosphorylation by CK1 and CK2, respectively. Therefore, it is expected that phosphorylation of one site would affect phosphorylation of the other. However, the pres-

[2] K. Penrose and A. McBride, unpublished data.

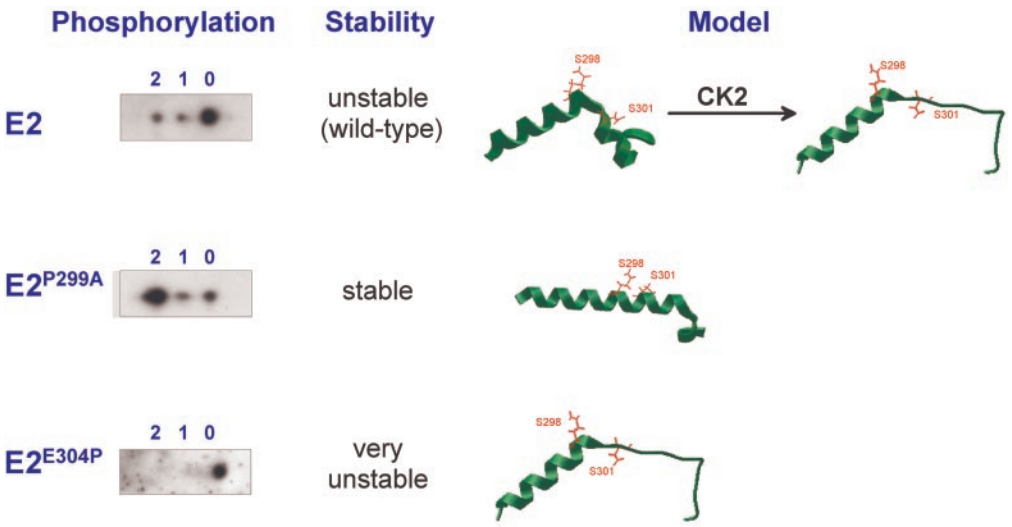

FIG. 7. **Summary of separation of phosphorylation and degradation.** Examples are shown of mutated proteins that demonstrate the separation of E2 phosphorylation and protein stability and a model of hypothetical conformational changes within this region.

ence of a proline residue in the intervening sequence between the serine residues could disrupt the alignment of the serine side chains and modulate the interplay between serine 298 and serine 301 phosphorylation. In this study we have evidence that phosphorylation at one site does affect phosphorylation of the other. For example, mutation of the intervening proline results in an enhancement of doubly phosphorylated E2. This indicates that without the structural distortion imposed by the proline residue, interplay between phosphorylation sites is enhanced. In addition, a peptide already phosphorylated on residue 298 is a much more efficient substrate for CK2 phosphorylation of serine 301 *in vitro*. However, this spacing is not crucial for phosphorylation *in vivo* as insertion of either one or two alanine residues between residues 299 and 300 did not eliminate doubly phosphorylated E2, indicating that serine 298 and 301 must also be able to be phosphorylated independently.

Investigation of the structure of a peptide spanning the phosphorylation region showed that, even though it lacks tertiary interactions that would stabilize a cooperative and independent fold, it does have residual structure. This behavior resembles that of polyglutamic acid models. The peptide is likely to have at least some regions of polyproline-type (PII) structure, with a characteristically enhanced minimum at 198 nm, well below that of a disordered backbone (23, 36). Similarly, a decrease in pH caused protonation of the multiple negative charged side chains and decreased repulsive interactions, with the consequent potential for stabilizing local structure. Phosphorylation of Ser-301 clearly destabilizes this type of residual structure, which appears to be less affected by phosphorylation of Ser-298. The helical region within the peptide can be stabilized by the addition of TFE, but the extent of helix attained is restricted to 9 residues (31% of 29 residues). However, the spectrum of the unphosphorylated peptide in TFE shows a strong contribution from the PII-type of structure, in addition to the helical region. Phosphorylation of either serine residue affects the structure substantially, with phosphorylation of Ser-301 having a more drastic effect. Because phosphorylation of this residue disrupts a structure, possibly PII-type, in the absence of the cosolvent, the fact that a larger effect on the overall structure is observed in TFE suggests that there could be interaction between the α-helix and the PII-type structure present in the peptide.

In summary, our results clearly show that local thermodynamic stability of the PEST sequence correlates with the biological half-life of the E2 protein. Fig. 7 shows a model of putative conformational changes within this region. This region has some propensity to form an α-helical structure. We predict that phosphorylation of residue 301 disrupts the local stability of the structure and that this disruption is dependent on residues both N-terminal and C-terminal to the phosphorylation site. Proline residue 299 allows a marginally stable local structure to be affected by phosphorylation, providing an important regulation point of the turnover of this key viral protein. Substitution of proline 299 with alanine is predicted to stabilize the local structure of the phosphorylation region and it does, in fact, increase the biological half-life of the E2 protein. Conversely, substitution of residues to the C terminus of serine 301 can either stabilize or destabilize the E2 protein *in vivo*. For example, in the case of E2 E304P we predict that, even without phosphorylation, this substitution would render the structure unstable enough to be recognized by the ubiquitin-proteasome pathway. This is in line with the fact that phosphorylation of Ser-301 has a more drastic effect on peptide conformation than Ser-298 and that the region around this residue is determinant for overall stabilization.

Notably, the region that regulates E2 turnover is centered around a proline residue. Proline residues are often associated with conformational changes because the amide bond between the proline and the preceding amino acid is unique in that it can undergo *cis-trans* isomerization. As described above, the SP motif at residues 298 and 299 is also a minimal consensus for a number of proline-directed kinases, and phosphoserine-proline is a substrate for the prolyl isomerase Pin1 (37). Pin1 recognizes this motif and isomerizes the Ser-Pro bond between the *cis* and *trans* configuration, thereby changing protein conformation. We have found that E2-derived peptides that are phosphorylated on serine 298 are bound by Pin1, and E2 proteins expressed *in vivo* are recognized by mpm2,[3] an antibody that recognizes mitosis-specific phosphoserine-proline motifs in a manner similar to Pin1 (38). Therefore, it is possible that a conformational change based on isomerization of this serine-proline bond is also important in the regulation of E2 phosphorylation and degradation at specific stages of the viral life cycle. Notably, CK2 and Pin1 form a specific complex in mitosis (39).

It is evident from this analysis that phosphorylation and degradation by the ubiquitin-proteasome pathway are separable. As summarized in Fig. 7, we have generated E2 proteins

[3] A. McBride, unpublished data.

that are unphosphorylated but extremely unstable and highly phosphorylated proteins that have a greatly extended half-life. Therefore, it is very unlikely that phosphoserine 301 is directly recognized by a ubiquitin ligase; instead phosphorylation is predicted to induce a conformational change that leads to recognition and targeting of E2 to the proteasomal degradation pathway. Such disruption of local structure could expose a recognition motif for a specific ubiquitin ligase or a lysine that functions as a ubiquitin acceptor. Zetina (40) has proposed that a reversible "unfolding motif" exists in proteins that contain a region of extended conformation. These regions have a high content of proline and acidic residues, and the motif has a strong similarity to CK2 sites (40). As mentioned above, phosphorylation of serine residues can either disrupt or stabilize local protein structure (21, 22), and in agreement with our data, negatively charged amino acids are often unable to substitute for phosphoserine in this disruption of polypeptide configuration (21).

This study concludes that the region between residues 298 and 305 is crucial for the stability and turnover of the E2 protein. This region contains both major phosphorylation sites, which are separated by a proline residue. Mutations in this region have wide ranging effects on E2 protein stability, and we postulate that it functions as a conformational switch to regulate the half-life of the E2 protein.

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