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E3-Ubiquitin Ligase/E6-AP Links Multicopy Maintenance Protein 7 to the Ubiquitination Pathway by a Novel Motif, the L2G Box*

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Ubiquitin ligases are generally assumed to play a major role in substrate recognition and thus provide specificity to a particular ubiquitin modification system. The multicopy maintenance protein (Mcm) 7 subunit of the replication licensing factor-M was identified as a substrate of the E3-ubiquitin ligase/E6-AP by its interaction with human papillomavirus-18E6. Mcm7 is ubiquitinated *in vivo* in both an E6-AP-dependent and -independent manner. E6-AP functions in these reactions independently of the viral oncogene E6. We show that recognition of Mcm7 by E6-AP is mediated by a homotypic interaction motif present in both proteins, called the L2G box. These findings served as the basis for the definition of substrate specificity for E6-AP. A small cluster of proteins whose function is intimately associated with the control of cell growth and/or proliferation contains the L2G box and is thereby implicated in an E6-AP and, by default, HPV-E6-dependent ubiquitination pathway.

Selective proteolysis represents a fast and irreversible way for the control of the regulation of transition states in biology and is commonly employed from bacteriophages to human cells (1). Post-translational modification of a lysine residue from an acceptor protein by ϵ-amidation with the C-terminal glycine residue of a poly-ubiquitin chain serves as a signal for selective proteolysis by the 26 S proteasome (2). This modification is a particularly effective form of regulation for many cellular control processes where a certain unidirectional and irreversible sequential order of events is crucial for the fidelity of a system, such as the regulation of S-phase entry or the anaphase cell cycle transition (3). Ubiquitin is transferred onto substrate proteins by an enzymatic cascade (4). The ubiquitin is first activated as a thiol ester on a ubiquitin-activating enzyme in an ATP-dependent reaction, and the ubiquitin thiols are then transferred to the ubiquitin-conjugating E2[1] enzyme and finally ligated to the target protein in concert with the E3 specificity factors. These E3 components provide substrate recognition and are thus generally considered to give specificity to the ubiquitin-mediated proteolysis system. E6-AP in association with the oncogenic E6 proteins from the human papillomavirus (HPV) resembles an E3 enzyme in that it targets the cellular tumor suppressor p53 for ubiquitin-mediated degradation (5–7). In contrast with other E3 proteins, such as the anaphase-promoting complex (3), or the S-phase entry specific SKP1, Cdc53, F box protein complex (8, 9), E6-AP functions not only as an adapter between a ubiquitin acceptor substrate protein and the E2 enzyme but has intrinsic ubiquitin ligase activity. Thus E6-AP is a thiol ubiquitin acceptor from its E2 enzyme, selects substrate, and serves as the ultimate ubiquitin donor that directly couples activated ubiquitin to target proteins (10). Besides its E6-associated function, E6-AP mutations have been linked with Angelman syndrome, and this serves as the first example of a genetic disorder associated with the ubiquitination pathway in mammals (11–13). Despite its E6-dependent association with p53 in HPV pathology, the role for the E6-AP ligase function in cell growth and proliferation is poorly understood. Very little is known about other cellular substrates of E6-AP, but the link with Angelman syndrome suggests the existence of additional, essential targets for the E6-AP E3-ligase system.

A sequential order of cell cycle transitions ensure that DNA replication takes place only once per cell cycle. This regulation is provided by the replication licensing factor (RLF) that is activated on exit from metaphase, and as a consequence, cells become competent for the initiation of DNA replication. Before replication is ultimately initiated at the G_1/S transition, however, a series of checkpoint controls during G_1-phase have to ensure the fidelity of the entire cell for duplication. Once started, the competence for the initiation of DNA replication is erased by inactivating RLF (14–16). Differential polyethylene glycol precipitations and subsequent purification from *Xenopus* egg extracts separates RLF into two components, RLF-B and RLF-M (17). RLF-M represents a hetero-hexamer complex that is conserved in all eukaryotes analyzed so far and consists of six members of the Mcm family of proteins. Mcm proteins are essential for DNA replication initiation (18) and were initially isolated as genetically defined mutants from *Saccharomyces cerevisiae* by their inability to replicate plasmids containing certain yeast replication origins (19). Mcm proteins are loaded at the origin of replication and move with the replication fork (20); they show homologies with DNA-dependent ATPases (21) and co-fractionate with a helicase activity *in vitro* (22), which taken together suggests that RLF-M is a putative candidate for a eukaryotic hexameric replication fork helicase.

In this study we show that Mcm7 interacts, in the context of the entire Mcm complex, with the HPV-18E6 onco-protein, and this discovery led us to identify Mcm7 as a novel E6-AP substrate. We describe that Mcm7 is ubiquitinated in an E6-AP-dependent manner, and we define the substrate recognition sequence for E6-AP in Mcm7. Intriguingly, the E6-AP/Mcm7 interaction is obtained through a novel homotypic motif, and this motif in turn is also used by the HPV-18E6 protein for interaction. We call this motif the L2G box.

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[1] The abbreviations used are: E2, ubiquitin carrier protein; HPV, human papillomavirus; UBE3A/E6-AP, E3-ubiquitin ligase E6-AP, in short E6-AP for E6-associated protein; Mcm, multicopy maintenance protein; RLF-M, replication licensing factor-M; GST, glutathione *S*-transferase; hect domain, homologous to the E6-AP carboxyl terminus; DTT, dithiothreitol; NEM, *N*-ethylmaleimide; HA, hemagglutinin; His_6, 6 histidines; LLnL, calpain I inhibitor, *N*-acetyl-Leu-Leu-norleucinal; aa, amino acid(s); NTA, nitrilotriacetic acid; NXT, nuclear extract.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Screen—2×10^7 yeast transformants from a human lymphocyte library in the vector pACT were screened with selection at 30 μg/ml 3-aminotriazole using pAS1-GAL4 fused in frame to full-length HPV-18E6 as a bait. Positive cells were analyzed for β-galactosidase activity. For confirmation of the screen the plasmids from 3-aminotriazole-resistant and β-galactosidase-positive colonies were rescued in *Escherichia coli* DH5α and then retransformed into *S. cerevisiae*.

HeLa Cell Nuclear Extracts and Superose 12 Gel Filtration—HeLa cell nuclear extracts were prepared essentially as described previously (23). HeLa cells were grown in suspension culture and harvested at mid–logarithmic phase at a cell density of $4–5 \times 10^5$ cells/ml. Prior to harvesting the cells were grown in the presence of the peptide aldehyde LLnL (25 μM, Sigma) for 2 h (the addition of the proteasome inhibitors proved to be essential for the isolation of 18E6 as a high molecular component associating with the RLF-M complex). Lysis buffer A contained 20 mM Hepes, pH 7.5, 3.5 mM $MgCl_2$, 25 mM KCl, 0.2 mM EDTA, 0.2 mM EGTA, 10% glycerol, 20 μM L-1-tosylamido-2-phenylethyl chloromethyl ketone, 20 μM 1-chloro-3-tosylamido-7-amino-2-heptanone, 50 μM LLnL, 5 mM *N*-ethylmaleimide (NEM, Sigma), 0.2 mM NaF, and 30 mM 4-nitrophenyl phosphate. Nuclei were isolated and separated at $100,000 \times g$, and the supernatant was designated cytoplasmic extract. For the peptide assays shown in Fig. 2*B*, nuclei were first extracted in buffer B (as for A but with 20% glycerol and 100 mM KCl), and after centrifugation at $100,000 \times g$, the supernatants obtained were designated soluble nuclear extract. The pellet was then extracted in buffer A containing 0.4 M KCl. After centrifugation at $100,000 \times g$, the supernatant was designated high salt nuclear extract. The remaining pellet contained virtually no Mcm7 nor Mcm3 protein as judged by Western blotting of aliquot fractions. For gel filtration experiments as shown in Fig. 1, *A* and *B*, the nuclei were extracted with buffer B containing 0.4 M KCl (NXT), diluted after centrifugation 1:3 in buffer A, and stepwise precipitated with polyethylene glycol employing a 3 and 9% step as described (17). The 9% fraction was immediately loaded onto a Superose 12 column of a fast protein liquid chromatography-Pharmacia system and equilibrated with buffer A containing 150 mM KCl. Loading volume was 100 μl. 1-ml fractions were collected, and the column was standardized with marker proteins as indicated. Fractions obtained were concentrated by 7.5% trichloroacetic acid precipitation in the presence of 0.01% sodium deoxycholate prior to analysis on reducing SDS-polyacrylamide gel electrophoresis.

Constructs—MCM7 (fragment 175–720) cDNA was obtained by polymerase chain reaction amplification from an oligo(dT)-primed cDNA from HeLa cells with primers designed according to sequences in GenBank™ accession number D26091. Constructs for deletion analysis were done in the vector bluescribe SK (Stratagene) with the coordinates as indicated in Fig. 1*D*. GST constructs have been described previously (24). His_6-HPV-18E6- and His_6-MCM7-containing vectors were constructed by in-frame ligation of the full-length HPV-18E6 or an Mcm7 fragment (fragment 554–720) into the vector pET2A-(Stratagene) containing the 6-histidine-tag $(His)_6$. The His_6-Mcm7 reporter construct for the ubiquitination assays was cloned by ligating a *Bam*HI/*Sal*I fragment from MCM7 (fragment 175–720) in-frame into a His_6-containing pcDNA3 vector (Invitrogen). The Mcm7 (Δ640–646) deletion mutation was generated by oligonucleotide-directed mutagenesis using the polymerase chain reaction and ligated into pcDNA3 (pcDNA3-His_6-Mcm7ΔL2G). All constructs were verified by dideoxy sequencing. The E6-AP-containing plasmids were done in pcDNA3-E6-AP and pcDNA3-E6-APΔE from human E6-AP DNA isoform 1, originally obtained from M. Scheffner (see Ref. 10). The HA-ubiquitin-containing plasmid pCMV-HA-Ubi was obtained from D. Bohmann (see Ref. 25).

Peptide Affinity Assays—Immobilization of cysteine-containing peptides onto SulfoLink (Pierce) resins and the estimation of the coupling efficiency, as measured with the Ellman's Reagent (Pierce) reaction, were done according to the supplier's instructions. Coupling efficiency was comparable among the peptides used, and resins contained approximately 400 μg of peptide/ml of 50% gel slurry. For the assay, 100 μg of extract in 250 μl of buffer A and 30 μl of 50% peptide containing gel slurry were incubated at 4 °C for 20 min and then intensively washed with buffer A containing 0.4 M KCl. Proteins were eluted at 94 °C in sample solution containing 2% SDS, and aliquots were analyzed in Western blots as indicated.

Immunoprecipitations and Western Blots—HeLa cell nuclear extracts or Superose 12 fractions were precleared in a mixture of protein A- and protein G-Sepharose prior to incubation with specific antibodies (2 μg/ml) on ice and subsequent purification with a mixture of protein A and protein G-Sepharose (preincubated in 10 mg/ml bovine serum albumin). Precipitations from extracts shown in Fig. 1*C* and Fig. 2*E* were done from 150 μg (NXT) diluted 1:1 in buffer A in the presence of 0.05% SDS and 0.05% sodium deoxycholate. Samples were washed six times in buffer A containing 0.4 M KCl and twice in buffer A alone. For Western blots, the resins were boiled in loading buffer containing 2% SDS and 0.1 M DTT. Western blots were developed using peroxidase-conjugated secondary antibodies and the enhanced chemiluminescence substrates (Amersham Pharmacia Biotech).

In Vitro Binding Assays—Assays were performed with either *in vitro* translated ^{35}S-labeled proteins produced with the TNT kit (Promega) or *E. coli* expressed proteins at concentrations indicated. Binding reactions were done for 2 h at either 4 °C for the assays containing TNT proteins or at room temperature in buffer A containing 0.9 M $(NH4)_2SO_4$ and 0.2 μg/μl bovine serum albumin, and then washed as indicated in Fig. 2*C* with the same buffer containing Tween 20 at concentrations indicated. Binding with ^{35}S-labeled proteins was detected by exposure to x-ray films and in Western blots for binding reactions with the recombinant *E. coli* expressed proteins.

Recombinant Proteins, Antibody Production, and Purification—*E. coli* proteins were purified from the strain BL21(DE3) by glutathione-Sepharose affinity chromatography for the GST fusion proteins and Ni^{2+}-NTA-agarose (Quiagen) chromatography for the $(His)_6$-tagged proteins. Antibodies were raised and purified essentially as described (26). Briefly, polyclonal antibodies to His_6-18E6 and His_6-Mcm7 (554–720) were raised in rabbits. Antibodies used were affinity purified against His_6-18E6 or His_6-Mcm7 proteins covalently linked to BrCN-activated Sepharose (Amersham Pharmacia Biotech). The antibodies to GST were obtained and affinity purified in a similar way. Polyclonal peptide antibodies were raised against the peptide CATLGVGSSGRGTTYQSRPA for Mcm3 and against SAYLENSKGAPNNSC for E6-AP (peptide-E6-AP antibody) coupled to keyhole limpet hemocyanin. These antibodies were affinity purified against the peptide coupled to SulfoLink resins (Pierce). Antibodies to GST-E6-AP were a gift from M. Scheffner and purified in batch against a GST-E6-AP protein that was bound to GST-Sepharose and immobilized by cross-linking with dimethyl suberimidate (GST-E6-AP antibody).

Ubiquitination Assays—For the Mcm7 *in vivo* ubiquitination assay, we used the protocol essentially as described (25) with minor modifications as follows. The pH 5.8 washing step of the Ni^{2+}-NTA-agarose was omitted, and proteins were eluted with 1.8 ml of 200 mM imidazole. For detection of the HA-ubiquitin we used the 16B12 (Babco) monoclonal anti HA antibody; Mcm7 was detected on parallel blots. HEK 293 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and transiently transfected at a density of 0.8×10^5/10-cm dish, using the calcium phosphate precipitation method. Cells were collected 24 h after transfection. LLnL, if used, was added 2 h prior to harvesting of the cells. For all the experiments shown, supernatants were included in the protein purifications.

RESULTS

HPV-18E6 Interacts with a Subpopulation of the Mcm Holocomplex in Vivo—We used the HPV-18E6 oncoprotein fused to the Gal4 DNA binding domain as a bait in a yeast two-hybrid interaction screen (27). A cDNA identical to the C-terminal part of the p85 Mcm sequence (28), now referred to as the human Mcm7 protein, was isolated in the screen and represented approximately 15% of the positive colonies obtained. Control strains carrying Gal4-p53, Gal4-HPV-16E5, or Gal4-HPV-16E7 baits did not show interaction with Mcm7 in a yeast two-hybrid assay, thus proving specificity for the selection procedure used (not shown). Six different types of Mcm proteins have been described, and together they form a hetero-hexamer complex which is the functional unit (29–31) of the replication licensing factor M (RLF M) (17, 32, 33). We established procedures to analyze the entire Mcm hexamer complex from HeLa cells a natural source of HPV-18E6 protein (29–31) (see "Experimental Procedures") in order to ask whether E6 is present within this complex. From the 9% polyethylene glycol fraction (a fraction that was previously shown to contain RLF-M (17)) from high salt nuclear extracts (NXT), HPV-18E6 and Mcm7 co-elute in overlapping fractions on a Superose 12 column with an apparent molecular mass of 400–600 kDa (Fig. 1*A*), which

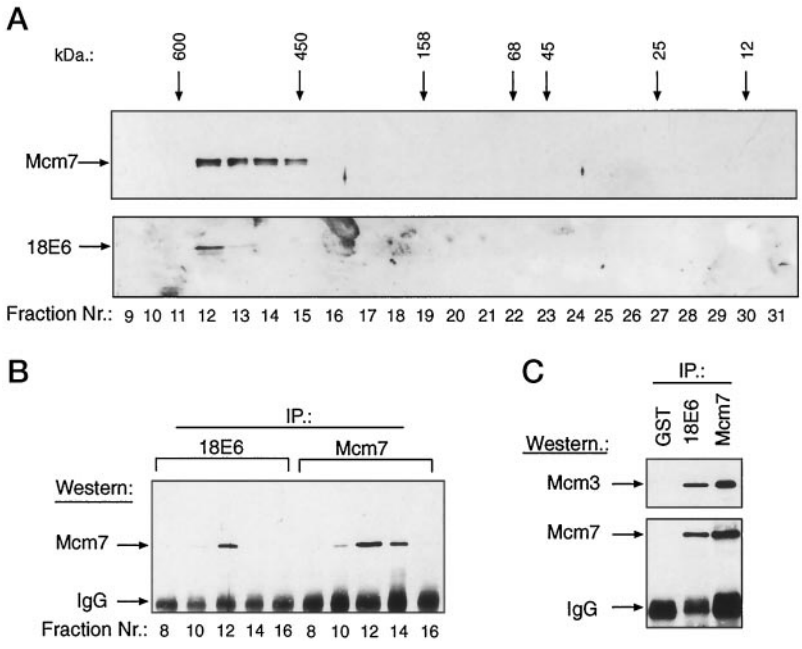


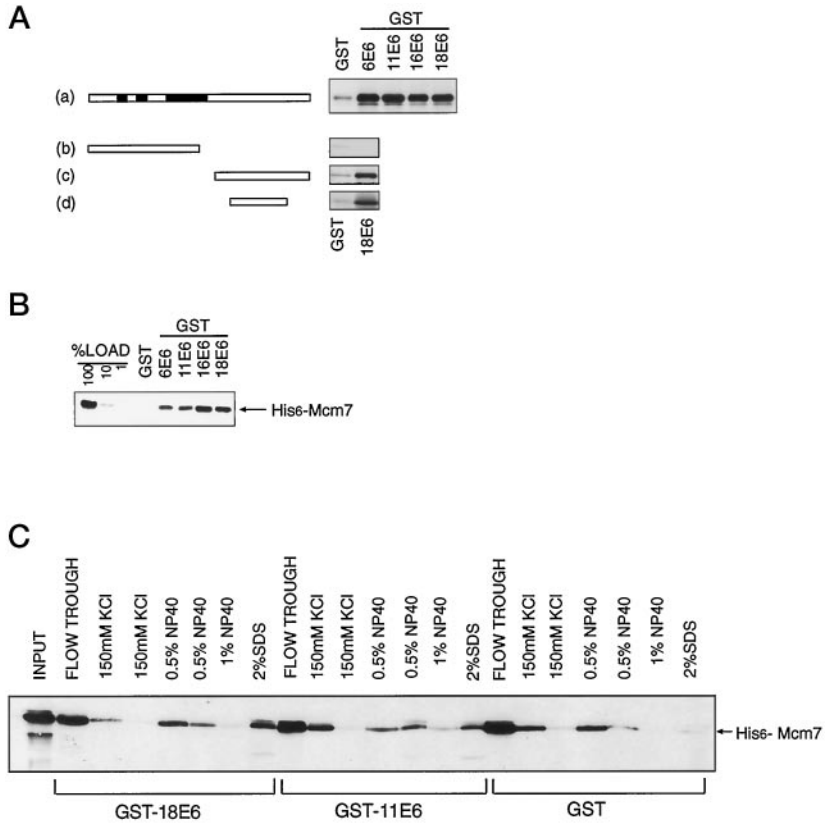
FIG. 1. **Mcm7 interacts with HPV-18E6 *in vivo*.** *A*, Western blot analysis of various fractions obtained by size fractionation on Superose 12. Blots were probed for Mcm7 and HPV-18E6 as indicated. *B*, HPV-18E6- and Mcm7-containing fractions were immunoprecipitated with affinity purified antibodies specific for HPV-18E6 (18E6) or Mcm7. Immunoprecipitates were analyzed for Mcm7 by Western blotting. *C*, co-immunoprecipitation from NXT of Mcm7 and HPV-18E6 with *GST, 18E6,* and *Mcm7* antibodies. Co-precipitations of Mcm3 and Mcm7 were detected by Western blot analysis.

is within the range reported previously for an active RLF-M fraction (29–31). Immunoprecipitations of the relevant peak fractions, using either HPV-18E6 or Mcm7 monospecific antibodies, show precipitation of the Mcm7 protein from these Superose fractions (Fig. 1*B*). HPV-18E6 co-precipitates Mcm7 only from the early Mcm7 peak fractions around 600 kDa, whereas immunoprecipitations of Mcm7 show the presence of Mcm7 proteins in fractions ranging from 600 to 400 kDa, thus indicating that HPV-18E6 interacts with a subpopulation of the Mcm complex. In addition, Mcm3 co-precipitates from these extracts with antibodies against both HPV-18E6 and Mcm7 (Fig. 1*C*) demonstrating the integrity of the functional Mcm holocomplex. Mcm3 was previously shown to be less tightly associated with the RLF-M hexamer complex and was therefore used as an indicator for the integrity of the entire complex (31).

Mcm7 Is a Direct Target for HPV-E6 Proteins in Vitro—By having shown that the HPV-18E6/Mcm7 interaction takes place *in vivo,* we were next interested in determining whether E6 proteins from other HPV types could interact with Mcm7. To do this, the E6 proteins from the low risk types HPV-6 and 11 as well as from the high risk types HPV-16 and 18 were expressed as glutathione *S*-transferase-E6 fusion proteins (GST), and binding to *in vitro* translated ^{35}S-labeled Mcm7 protein was assessed. As can be seen from Fig. 2*A*, Mcm7 interacts with GST-18E6, GST-16E6, GST-11E6, and GST-6E6 indicating a strong conservation of binding to Mcm7 among both high and low risk HPV types. In order to define the region of Mcm7 bound by the E6 proteins, a deletion analysis of the Mcm7 protein was performed. A region of 78 amino acids was defined that is required for binding to HPV18-E6 (Fig. 2*A*), and furthermore, a binding assay using purified recombinant His_6-Mcm7 (fragment 577–719) and GST-E6 proteins verified that the Mcm7/E6 association is direct (Fig. 2*B*). As Mcm7 interactions showed a high background with GST protein alone, the nature of specific binding conditions was determined in more detail (Fig. 2*C*). This interaction was stabilized in the presence of 0.9 M $(NH_4)_2SO_4$ or 2 M KCl, whereas for the elution of specific bound proteins the ionic detergent SDS was necessary, conditions that indicate that the interactions observed are specific and hydrophobic in nature (Fig. 2*C* and data not shown).

Homotypic Interaction of UBE3A/E6-AP and Mcm7 Defines a Novel Motif, the L2G Box—Sequence analysis of the Mcm7 region essential for its interaction with HPV-18E6 (Fig. 2*A*) defines a stretch of amino acids that has significant homology with a region of the ubiquitin ligase, E6-AP, that was previously shown to be sufficient for interacting with HPV-16E6 *in vitro* (34) (Fig. 2*A*). In addition, this region shows similarity to a sequence recently described as an E6 consensus binding site that was derived from mapping data obtained for the E6-binding protein (E6BP) (35) and from a random peptide library screen (36). This region is not conserved between the different Mcm family paralogues but is nearly identical among the Mcm7 vertebrate orthologues. The corresponding region in E6-AP is not conserved in any of the recently discovered E6-AP/hect domain (37, 38) containing members of the ubiquitin ligase family. To evaluate the significance of this homology between Mcm7 and E6-AP for the E6 interactions, we used peptides spanning the core of the relevant regions (Fig. 3*A*). These peptides were covalently coupled by an additional C-terminal cysteine via iodoacetyl groups to a non-ionic chromatographic "SulfoLink"-agarose matrix and used to test for specific precipitation of the Mcm7 and HPV-18E6 proteins, respectively, from HeLa cell extracts. Cytoplasmic extracts, low salt nuclear fractions, or high salt nuclear fractions (chromatin-bound proteins) were passed through these peptide columns, and after several washes with 0.4 M KCl containing buffers, specifically bound proteins were eluted with 2% SDS and analyzed by Western blot for HPV 18E6 and Mcm7. Peptides of the homologous region in the E6-AP protein were able to specifically retain the HPV-18E6 protein from cytoplasmic and nuclear high salt fractions (Fig. 3*B*). More strikingly, the E6-AP homology motif-containing peptides are also able to enrich for Mcm7 from cytoplasmic as well as from low and high salt nuclear fractions. Peptides that contain a Thr substitution for the conserved Leu^{402} show a reduction in both the HPV-18E6 and Mcm7 binding. The Mcm7 peptide columns enrich for HPV-18E6 protein in the chromatin-bound fractions only but do not form homodimers with the Mcm7 protein. In contrast to the data observed for the E6-AP resins, Mcm7 peptides carrying a Thr substitution for the corresponding Leu^{644} still show binding with HPV-18E6, probably reflecting a different binding affinity for either binding site to HPV-18E6.

To investigate the E6-AP/Mcm7 interaction further, an *in*



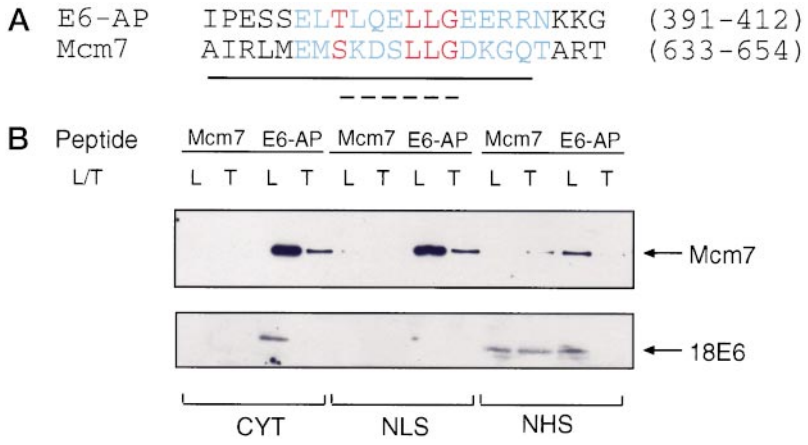


FIG. 2. **Analysis of the HPV-E6/Mcm7 interaction *in vitro*.** *A*, interaction studies were performed with E6 proteins from both high risk (16E6 and 18E6) and low risk HPV types (*6E6* and *11E6*). The GST-E6 fusion proteins (0.5 μg) or GST (5 μg) were incubated with *in vitro* translated, ^{35}S-labeled Mcm7 (aa 175–720) protein (*a*). Conserved regions previously mapped for all six members of the Mcm proteins (30) are indicated schematically with *black bars*. Specifically retained proteins were separated on an SDS-polyacrylamide gel electrophoresis and analyzed by autoradiography and are shown on the *right* of the figure. The region important for the HPV-18E6/Mcm7 interaction was mapped with a series of Mcm7 deletion mutants. *b,* residues aa 176–367; *c,* residues aa 554–720; *d,* residues aa 576–655. Protein retained by GST and GST-HPV18E6 is shown. *B*, the E6/Mcm7 interaction is direct. Purified $(His)_6$-Mcm7 (aa 554–720) (0.2 μg) was incubated with the different purified GST-E6 proteins (0.5 μg) or GST (5 μg), and specifically bound Mcm7 protein was detected by Western blot analysis. *C*, binding requirement for a HPV-E6/Mcm7 interaction. Binding assays were performed as in *B*, and aliquots of supernatants from various washing conditions, as indicated, were analyzed for Mcm7 in Western blots.

FIG. 3. **Binding of Mcm7 by HPV-18E6 and E6-AP requires a novel structural motif, the L2G box.** *A*, a stretch of amino acids that is conserved between Mcm7 and E6-AP was identified in a region that was previously shown to be necessary for the HPV-18E6 interaction with E6-AP. Alignment of the conserved region is shown. *Unbroken line,* smallest region in E6-AP previously mapped for HPV-18E6 interaction (34); *red,* conserved residues; *blue,* region spanning the peptide sequences used in *B*; *dashed line,* deletion introduced in Mcm7 used in Fig. 4*B* and Fig. 6. *B,* affinity purification of Mcm7 and HPV-18E6 from HeLa cell extracts with peptides spanning the conserved regions indicated in *A*. HeLa cell extracts were incubated with peptides immobilized on SulfoLink resins, and specifically retained proteins were analyzed by Western blotting. *L,* represents peptide with wild-type sequence; *T,* represents peptide with Leu^{644} to Thr substitution for Mcm7 peptide and Leu^{402} to Thr substitution for E6-AP peptide. Assays were performed with cytoplasmic (*CYT*), nuclear low salt (*NLS*), and nuclear high salt extract (*NHS*).

vitro binding assay was performed using purified GST-E6-AP and purified His-Mcm7 proteins. The results demonstrate that the E6-AP/Mcm7 interaction observed in the peptide assays is direct (Fig. 4*A*) and argue that no additional bridging or auxiliary factors are required for the E6-AP/Mcm7 interaction. Moreover, *in vitro* translated Mcm7 proteins deleted for the core E6-AP homology region (Δ640–646) failed to bind GST-E6-AP (Fig. 4*B*). To determine whether E6-AP interacts with Mcm7 in mammalian cells, HeLa cell nuclear extracts (Fig. 4*C*) were immunoprecipitated with specific antibodies to both of the proteins (Mcm7 and E6-AP) and a control antibody (GST) and analyzed in Western blots for cross-immunoprecipitation. A subpopulation of E6-AP as well as of Mcm7 specifically co-precipitate with each other (*lanes 2* and *3*), whereas no co-immunoprecipitation is seen with the control antibodies (*lane 1*). Again, as was seen for the HPV18-E6/Mcm7 interaction, these complexes were detectable only from log phase nuclear extracts prepared after incubation and in the presence of isopeptidase and proteasome inhibitors (see "Experimental Procedures", negative results with no inhibitors not shown). Collectively, these data show that the E6-AP and Mcm7 proteins are able to interact *in vitro* and *in vivo* and that this interaction is mediated by homotypic motifs which we call the L2G box. In addition, the small region of Mcm7 found to be essential for the Mcm7/E6-AP interaction also represents a specific contact site for HPV-18E6 (Fig. 3*B*). In contrast to the

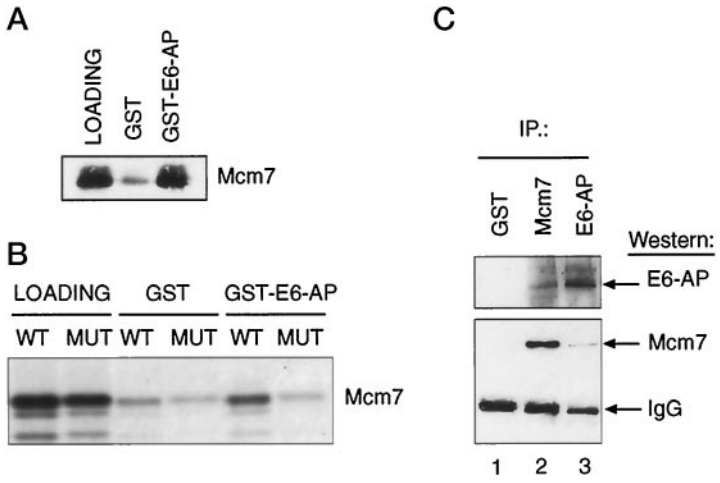


FIG. 4. **E6-AP and Mcm7 interact *in vitro* and *in vivo*.** *A*, direct interaction between E6-AP and Mcm7. Purified $(His)_6$-Mcm7-(554–720) (0.2 μg) was incubated with purified GST-E6-AP (0.5 μg) or GST (5 μg) proteins, and specifically retained Mcm7 protein was detected by Western blot analysis. The loading control was $(His)_6$-Mcm7-(554–720) protein (0.05 μg). *B*, deletion of the L2G box in Mcm7 abolishes E6-AP binding *in vitro*. GST-E6AP (0.5 μg) or GST (5 μg) was incubated with ^{35}S-labeled Mcm7-(175–720) (*WT*) or Mcm7-(175–720, Δ640–646) (*MUT*) and bound proteins determined by polyacrylamide gel electrophoresis and autoradiography. *C*, co-immunoprecipitation of E6-AP and Mcm7 from HeLa-cell extracts. HeLa cell nuclear extracts (*NXT*) were immunoprecipitated (*IP*) with Mcm7, peptide-E6-AP, and GST-specific antibodies and presence of E6-AP and Mcm7 assessed by Western blot analysis with Mcm7-and GST-E6-AP-specific antibodies.

E6-AP/Mcm7 interaction, this site would not appear to be the only site on Mcm7 of interaction with HPV-18E6, since the Mcm7 (Δ640–646) deletion mutant still shows HPV-E6 binding *in vitro* (data not shown).

Mcm7 Is Ubiquitinated in Vivo—We find Mcm7 associated with both the E6 and the E6-AP proteins, both of which appear to use the same stretch of amino acids in Mcm7 as a platform for direct interaction. A prediction from the E6-AP/Mcm7 interaction would be that Mcm7 is a substrate for ubiquitination, and the L2G box in Mcm7 serves as an E6-AP specific substrate recognition site. Indeed, Western blot analysis of the endogenous Mcm7 protein purified from HeLa cells with the E6-AP peptide columns identified several slower migrating Mcm7 species when cellular isopeptidases (39) were inhibited (*+NEM* Fig. 5*A*), indicative of ubiquitination. To confirm that Mcm7 is a substrate for polyubiquitination, we performed an *in vivo* ubiquitination assay (25). A hexa-histidine-tagged Mcm7 C-terminal reporter fragment (His_6-Mcm7) was used, together with a plasmid encoding a multimeric precursor molecule composed of eight hemagglutinin-tagged ubiquitin units (HA-ubiquitin), in transient transfection assays. The use of the His_6-Mcm7 construct allows analysis under denaturing conditions, which should minimize possible artifacts during extract preparation resulting from de-ubiquitinating activities or unspecific proteases capable of degrading ubiquitin-marked proteins *in vitro*. HEK 293 cells transfected with His_6-Mcm7 plus HA-ubiquitin, but not with either plasmid alone, showed polyubiquitination of the His_6-Mcm7 protein (Fig. 5*B*). Comparable results were obtained with either human Saos-2 osteosarcoma cells or with mouse NIH3T3 cells (not shown). Parallel blots stained for Mcm7 detected the His_6-Mcm7 as a 36-kDa protein mainly in the non-ubiquitinated form, Mcm7 specific ladders being visible only after long exposures (not shown). The appearance of the ubiquitinated form of Mcm7 as a subpopulation of the total Mcm7 pool is similar to that described for the c-Jun protein (25) and probably reflects a high turnover of the ubiquitinated *versus* the non-ubiquitinated protein fraction, consistent with the ubiquitination step being rate-limiting.

The L2G Box in Mcm7 Is Functional in Vivo and Is the Substrate Recognition Site for the Ubiquitin Ligase E6-AP—To ask if the ubiquitination observed for Mcm7 is directly linked to the E6-AP ubiquitin ligase system *in vivo*, co-transfection experiments with either HPV-18E6 or E6-AP-encoding plasmids were performed in the assay system described above. The presence of HPV-18E6 (Fig. 6*A*, *lane 2*) or additional E6-AP (Fig. 6*B*, *lane 3*) results in a sharp decrease in the levels of both the ubiquitinated and non-ubiquitinated His_6-Mcm7 fraction. This decrease was not observed when specific peptide aldehyde proteasome inhibitors were added 2 h prior to the protein extraction (Fig. 6, *A*, *lane 3,* and *B*, *lane 4*), suggesting E6-AP and HPV-18E6 targeted degradation of the Mcm7 protein by the proteasome. The fact that the addition of proteasome inhibitors for as short as 2 h caused a stabilization of the Mcm7-specific ubiquitin ladders proved to be a valuable and essential test for the specificity of the E6-AP or HPV-E6-dependent degradation of Mcm7. Noteworthy, His_6-c-Jun protein used in a similar assay was not affected by E6-AP or HPV-18E6 (not shown). The decrease of the Mcm7 product observed in the presence of additional E6-AP indicates that E6-AP can target Mcm7 in the absence of E6. An E6-AP mutant protein deleted for the HPV-16E6/Mcm7 interaction domain (E6-APΔE) did not reduce the Mcm7 product in the same assay (Fig. 6*B, lanes 5* and *9*), consistent with the interpretation that this site is also essential for an HPV-E6-independent function of the E6-AP protein.

We then further analyzed an Mcm7 protein with a seven-amino acid deletion in the E6-AP interaction site (Fig. 6, *A*, *lanes 4–6,* and *B*, *lanes 5–8*). Interestingly, His_6-Mcm7ΔL2G, which is no longer capable of binding E6-AP (see Fig. 3*B*), is still polyubiquitinated *in vivo* (see "Discussion"), but no degradation is observed in response to HPV-18E6 or E6-AP (Fig. 6, *A, lane 5,* and *B*, *lane 7*) confirming that the L2G box is a substrate recognition site for the E3 ubiquitin-protein ligase E6-AP *in vivo*. Similar results were obtained by using either Leu/Thr^{644} or Leu/Thr^{645} or Glu/Ala^{646} substitution mutations from the Mcm7 L2G box.[2] We did not map the binding site for non-oncogenic E6 proteins further and do not yet know if these proteins interact with the Mcm7-L2G box or through other regions of the Mcm7 protein. It has been shown that low risk E6 proteins do not interact with the homotypic region now defined as the L2G box in E6-AP (34) and that only oncogenic associated HPV-E6 types can efficiently target p53 for ubiq-

[2] C. Kühne, unpublished observations.

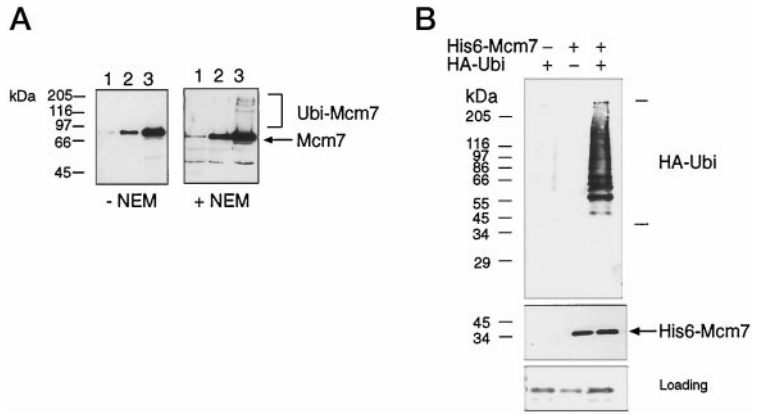


FIG. 5. **Mcm7 is ubiquitinated *in vivo*.** *A*, high molecular weight species of Mcm7 indicative of ubiquitin conjugates. HeLa cell nuclear extracts (*NLS*) were incubated with peptides coupled to resins as described in Fig. 3*B*. Specifically retained proteins were analyzed in Western blots with Mcm7-specific antibodies from samples prepared in the presence of 100 mM DTT and absence of NEM (*−NEM*) or absence of DTT and presence of 5 mM NEM (*+NEM*); *lane 1,* Mcm7 wild-type peptide; *lane 2,* E6-AP peptide with Leu^{402} to Thr substitution; *lane 3,* E6-AP wild-type peptide. *B,* Mcm7 is ubiquitinated *in vivo*. HEK 293 cells were transiently transfected with the plasmids pcDNA3-His6-Mcm7 (3 μg) and pCMV-(HA-ubiquitin) (2 μg) as indicated. The His_6-Mcm7 reporter protein was isolated by Ni^{2+}-NTA-agarose affinity chromatography, and proteins were analyzed in Western blots using anti-HA or Mcm7 antibodies as indicated. The protein stain of a prominent protein retained on the Ni^{2+}-NTA-agarose served as *Loading* control.

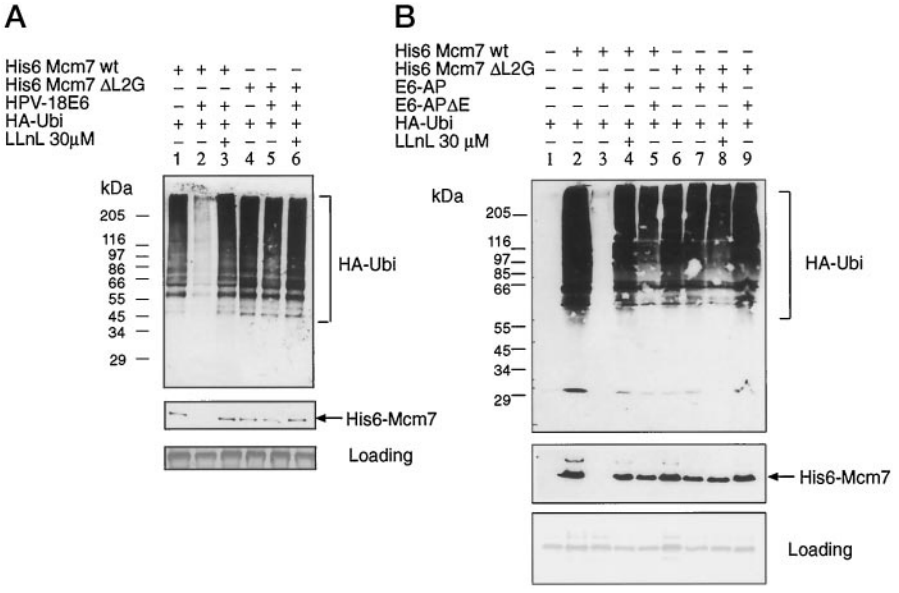


FIG. 6. **Mcm7 is ubiquitinated *in vivo* by an E6-AP-dependent and -independent pathway.** *A*, HPV-18E6 induces degradation of Mcm7 *in vivo*. Transient transfection assays with the plasmids pCMV-HA-Ubi (2 μg), pcDNA3-His6-Mcm7 (3 μg), pcDNA3-His_6-Mcm7-ΔL2G (3 μg), and pcDNA3-HPV-18E6 (5 μg) in the combinations indicated; analysis was done as in Fig. 5. *B*, E6-AP induces degradation of Mcm7 *in vivo*. Transient transfection assays with the plasmids pCMV-(HA-ubiquitin) (2 μg), pcDNA3-His6-Mcm7 (3 μg), pcDNA3-His_6-Mcm7ΔL2G (3 μg), pcDNA3-E6-AP (6 μg), and pcDNA3-E6-APΔE (6 μg), in the combinations as indicated. Detected as for Fig. 5*B*.

uitin-mediated degradation via E6-AP (5, 7, 40, 41). However, recent studies have shown that low as well as high risk E6 proteins can interact with p53 (40). Our preliminary studies suggest that Mcm7 is not degraded by HPV-11E6.[2]

Additional L2G Box Candidates, Involvement in a Common Pathway of Regulation—The region of p53 involved in the interaction with HPV-18E6 and E6-AP is currently not mapped to the extent presented here for Mcm7 (40). However, the L2G box consensus (S/T)*XXX*LLG can also be found in the p53 core region that spans the putative interaction site (Fig. 7). Interestingly, this L2G consensus is located between the two DNA-contacting residues Arg^{248} and Arg^{273}, the region of p53 most frequently mutated in human tumors (42). This site is not conserved in the more recently discovered homologue p73 (43). A data base search for additional (S/T)*XXX*LLG-containing proteins revealed a small cluster of proteins that function as regulators of DNA replication initiation and/or progression (Fig. 7). Noteworthy, cyclin D that has been previously shown to be ubiquitinated (44) and a member of the c-Abl tyrosine kinase family (c-Abl2) (45) contains an L2G consensus motif. Further matches were seen with essential DNA-modifying enzymes such as DNA polymerase-α, DNA polymerase-ϵ, the telomerase catalytic subunit (EST2) (45–49), and the proto-oncogene BLM, a DNA-helicase previously identified as the Bloom's syndrome gene product (50). Strikingly, the translation initiation factor EIF3β (51, 52), a prime candidate for an effector protein for the regulation of the general protein translation turnover and thus of cell growth, contains the L2G motif. Although interaction of these proteins with the homotypic L2G motif in E6-AP is speculative at present, this cluster of L2G box-containing proteins may also be similarly recognized by E6-AP as is Mcm7.

DISCUSSION

Proteins from small DNA tumor viruses interfere with central cellular control proteins such as p53 or the retinoblastoma protein pRB (53), and this results in a loss of tumor suppression, a hallmark of tumor development (54, 55). Based on this, viral proteins serve as valuable tools for screening for new candidate proteins that are involved in cellular regulatory pathways. In recent years, interaction screens with various viral oncogenes have become a "classical" tool in molecular biology for the discovery not only of particular interaction partners but also for finding cellular components which in turn

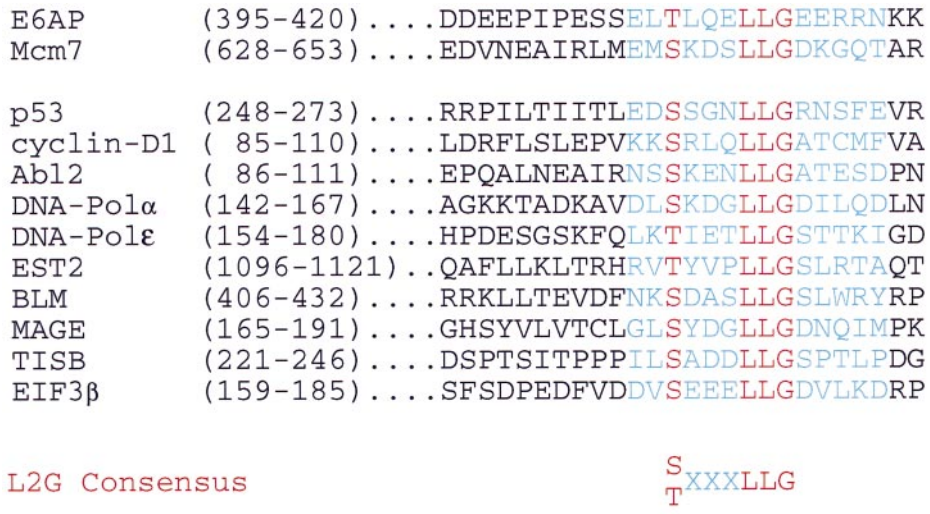


FIG. 7. **The L2G domain is conserved in proteins that function as regulators of DNA replication initiation and/or progression.** Entries were retrieved from the Swiss Prot data base by a search with the Prosite package and (S/T)*XXX*LLG as a query. Alignment is shown only of the human L2G box containing proteins. *Letters* in *blue* and *red* mark region mapped for Mcm7 and E6-AP to be sufficient and essential for contact between E6-AP and HPV-18E6. *Red,* conserved residues. The amino acid coordinates are indicated in *parentheses*. Although only the human sequences are shown, this motif is conserved for all the respective vertebrate orthologues isolated so far. Abbreviations and data base accession numbers for the proteins obtained are as follows: E6-AP (L07557); Mcm7 (P33993); p53 (M60950); cyclin-D1 (P24385), c-Abl2 (P42684), c-Abl1 shows an inversion (LL*AG*) in the L2G region (not shown); DNA polymerase-α (DNA-Pol α, P09884); DNA polymerase-ϵ subunit B (DNA-Pol ϵ, P56282); telomerase catalytic subunit (hEST2, AF018167); Bloom's syndrome gene product (BLM, P54132); MAGE (P43355) (61); nuclear early response gene TISB (Q07352) (62); eukaryotic translation initiation factor 3β (p116) (EIF3β, P55884); cyclin-D2 is identical in the boxed region indicated for cyclin D1 (not shown). Only MAGE-1 is shown, the family members 1, 2, 3, 6 and 8 are identical for the region indicated.

could be linked to pathways that are affected in virus pathology. We reasoned that the oncogenic HPV-18E6 protein, a viral component previously shown to be involved in E6-AP-dependent degradation of p53, should help to identify new E6-AP substrates. As a result of an interaction screen, we demonstrate an HPV-E6 association with the RLF-M component Mcm7. The characterization of this interaction in turn led to the discovery that Mcm7 is a substrate for both E6-AP-dependent and -independent ubiquitination and is specifically targeted for degradation by the 26 S proteasome. Subsequent detailed mapping of the HPV-18E6/Mcm7 binding requirements revealed two features as follows: first the interaction domain used by the virus and by the enzyme E6-AP are contained within 14 amino acids, suggesting an overlapping binding site, and second, the E6-AP interaction is mediated by a homotypic motif present in the substrate and the enzyme which we call the L2G box. The fact that Mcm7 is still polyubiquitinated in the absence of a specific binding site in-*cis* for E6-AP argues for an additional, E6-AP-independent process involved in the regulation of the polyubiquitin-mediated turnover of the Mcm7 protein. This probably regulates the basic turnover of the Mcm7 protein. We propose that this turnover is modulated by the L2G-binding proteins such as HPV-18E6 and E6-AP in response to as yet unidentified regulators, placing the L2G box as a highly entropically structured module.

It is striking that substrate and enzyme use the L2G box for interaction, and this interaction is sufficient for ubiquitin-mediated degradation by E6-AP; however, the E6 protein does not contain an L2G motif yet interacts with and thus functions via the L2G motif. This suggests that HPV E6 proteins have evolved to interfere with a regulatory pathway in total, such that they interfere with the substrate recognition site (L2G box) of the ubiquitin ligase E6-AP. This observation gives important information concerning tumor virus/host interactions and has exciting evolutionary implications for a virus/host adaptation. Data base searches for this L2G motif identify a small cluster of proteins that are likely candidates for a similar regulation and suggest that E6-AP has more *in vivo* substrates than was previously anticipated. We would speculate that, at least for some of these proteins, the L2G box was adopted by HPV-E6 proteins for host protein recognition. The E6-binding site motif has been proposed as a basis for an "anti-HPV drug" design (35, 36), although the knowledge of this cellular (evolutionary) context presented above will now have to be considered.

Implications for other Hect Domain Containing E3-Ubiquitin Ligases—A family of structurally and functionally related E3-ubiquitin protein ligases was recently identified which have a C-terminal homology motif with the E6-AP ubiquitin ligase catalytic domain (hect domain) in common (37). The hect domain spans approximately 350 amino acids within the C-terminal regions of the proteins, but the N terminus of every individual member shows distinct features. The human genome encodes at least 20 different hect domain proteins (38). We find in the case of E6-AP that the substrate recognition site is in the nonconserved N terminus of the protein, and we show that substrate recognition is facilitated by a homotypic interaction of the enzyme with its substrate. This might well be a precedent for a general mode of specificity selection for hect domain E3 ubiquitin ligases. Arguing that specificity of a particular ubiquitin-dependent degradation pathway is provided mainly by its E3 enzymes, the assumption of substrate selection by homotypes should help to define individual ubiquitination pathways for other hect E3 type enzymes.

E6-AP and Licensing for DNA Replication—Our findings link the E3-ubiquitin ligase E6-AP, originally discovered as the E3 ligase for HPV E6-dependent degradation of p53, to a key mediator of the "once-per-cell-cycle control" of DNA replication. Loss of cell cycle control is one of the hallmarks of cancer (54, 56), and DNA tumor viruses have been invaluable in dissecting these controls in higher eukaryotes. Mcm7 could represent an E6-AP-regulated checkpoint control element for DNA licensing, consistent with a licensing model for DNA replication, in which the activated and thus functional RLF-M hexamer for initiation would be irreversibly destroyed by proteolysis after successful initiation (note that this might well be an RLF-M subpopulation). Moreover, we speculate that the outcome of a malfunction of E6-AP should be rather a loss of checkpoint

function than *a priori* over-replication. The binding of the Mcm complex to chromatin is strongly inhibited by the recently identified, anaphase-promoting complex-regulated geminin proteins (57). These proteins were proposed to sequester the Mcm complex after DNA replication initiation from its chromatin loading and thus are thought to resemble part of a "licensing surveillance" mechanism. It will be interesting to test if the E6-AP control of Mcm7 represents either a parallel or a linear succession of these proposed mechanisms and thus if geminin links E6-AP to the cell cycle clock. We favor the idea that shortly before or after initiation of replication the Mcm7 protein in the RLF-M complex becomes a repressor of progression which is resolved by degradation. These speculative predictions of an involvement of Mcm7 in a "licensing checkpoint" are further supported by a more recent discovery of a possible inhibition of *Xenopus* DNA replication initiation due to an interaction of Mcm7 with the retinoblastoma tumor supressor proteins (58).

An early G_1-phase arrest point was identified, called the origin decision point, that ensures specific recognition of the dihydrofolate reductase origin locus by *Xenopus* egg extracts (59). Intriguingly, transformation by SV40 can override this arrest point (60). With the knowledge presented above, it should be possible to test if the modulation of Mcm7 abundance plays a direct role in this early G_1 decision point and if the HPV oncogene E6 can bypass the origin decision point, as shown for the SV40 proteins.

E3 enzymes are a central issue because they are potential regulators of ubiquitination timing and substrate selection. We analyzed substrate selection for Mcm7, and we define a substrate recognition motif for the E3-ubiquitin ligase UBE3/E6-AP *in vivo* which we call the L2G box. Substrate recognition defined to the extent presented above now allows analysis of the timing of the E6-AP function in the cell, and it is this aspect that we expect to be by-passed, not only by the viral but also by cellular oncogenes in cancer. Identification of the L2G box as a specific homotypic protein-protein interface for E6-AP and its substrates implies the existence of a common regulation for both enzyme and substrate and might serve as the basis for a regulation of an E6-AP-dependent pathway.

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E3-Ubiquitin Ligase/E6-AP Links Multicopy Maintenance Protein 7 to the Ubiquitination Pathway by a Novel Motif, the L2G Box

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