The E6 oncprotein of human papillomaviruses associated with cervical cancer targets the tumor suppressor p53 and several other cellular proteins including the human homologs of Dlg and Scribble for degradation via the ubiquitin-proteasome system. Similar to p53 degradation, E6-induced degradation of Scribble is mediated by the ubiquitin ligase E6-AP. In contrast, degradation of Dlg \textit{in vitro} and within cells has been reported to be independent of E6-AP suggesting that the E6 oncprotein has the ability to interact with ubiquitin ligases other than E6-AP. Furthermore, the ability of the E6 oncprotein to interact with these yet unidentified ubiquitin ligases may be shared by the E6 protein of so-called low risk human papillomaviruses that are not associated with cervical cancer. In this manuscript, we used the RNA interference technology and mouse embryo fibroblasts derived from E6-AP deficient mice to obtain information about the identity of the ubiquitin ligase(s) involved in E6-mediated degradation of Dlg. We report that, within cells, E6-mediated degradation of Dlg depends on the presence of functional E6-AP and provide evidence that the E6 protein of low risk human papillomaviruses functionally interacts with E6-AP. Based on these data, we propose that, in general, the proteolytic properties of human papillomavirus E6 proteins are mediated by interaction with E6-AP.

The E3 ubiquitin ligase E6-AP was originally discovered as a cellular protein that interacts with the E6 oncprotein of so-called high risk human papillomaviruses (HPVs1) and, in complex with E6, targets the tumor suppressor p53 for ubiquitination and degradation (1-4). High risk HPV types including HPV16 and HPV18 preferentially infect mucosal epithelia and have been etiologically associated with the development of cervical cancer (5). It is commonly assumed that, in the absence of E6, E6-AP is not involved in p53 degradation and that E6/E6-AP-mediated degradation of p53 contributes to HPV-induced cellular transformation. Indeed, interference with E6-AP activity in HPV-positive cells but not in HPV-negative cells results in accumulation and activation of p53 (6-9). Furthermore, E6 proteins derived from so-called low risk HPVs that preferentially infect mucosal epithelia but are not associated with malignant lesions only weakly interact with p53 and do not target p53 for degradation (4,10,11). The inability to target p53 for degradation may be explained by the notion that bacterially expressed low risk HPV E6 proteins do not form complexes with E6-AP that are detectable under the conditions of coprecipitation experiments (1,2).

Although the ability of high risk HPV E6 proteins to target p53 for degradation contributes to their oncogenic potential, it is also clear that high risk HPV E6 proteins have oncogenic activities that are independent of p53 (summarized in 4,11). Numerous cellular proteins have been reported to interact with high risk E6 proteins (e.g. Bak, c-Myc, E6BP/ERC55, E6TP1, IRF-3, Mcm7, Paxillin, Dlg, MAGI-1, MUPP-1, Scrib) and some of these have been reported to be targeted for degradation in an E6-AP-dependent (e.g. Bak, c-Myc, Mcm7, Scrib) or E6-AP-independent (Dlg, MAGI-1, MUPP-1) manner (4,11). In addition, some of these proteins (Mcm7, Bak) have also been reported to be targeted by E6 proteins derived from so-called low risk HPVs including HPV6 and HPV11 that have not been associated with malignant lesions (12-14). However, the mechanism by which low risk HPV E6 proteins...
target these proteins for degradation is not known at present. Dlg, MAGI-1, MUPP-1, and Scrib are PDZ domain containing proteins (11). In a simplified view, PDZ domains can be considered as protein-protein recognition modules that are part of molecular scaffolds that hold multisubunit protein complexes together. The high risk HPV E6 proteins, but not the low risk HPV E6 proteins, contain at their very C terminus a PDZ-binding motif, a rather short motif (xT/SxV) generally found in proteins that specifically interact with PDZ domains (15,16). Indeed, binding of high risk HPV E6 proteins to PDZ domain containing proteins is dependent on the presence of an intact C terminus (11). Significantly with respect to oncogenesis, the very C-terminal region of E6 is not required for interaction with E6-AP and p53 but C-terminal deletion mutants of E6 are impaired in their transforming capacity (11,17-19). This indicates that the interaction with and, possibly, degradation of PDZ domain containing proteins contribute to the oncogenic potential of E6. However, although PDZ domain containing proteins are targeted for degradation by the high risk HPV E6 proteins, the mechanisms involved in the degradation of these proteins were reported to be different. Unlike binding to p53, binding of E6 to PDZ domain containing proteins including Dlg and Scrib is independent of E6-AP (4,11). However, E6-induced degradation of Scrib proceeds via E6-AP-dependent ubiquitination (20), whereas E6-induced degradation of Dlg or MAGI-1 has been proposed to involve E3 ligases other than E6-AP (21-23). The latter notion is mainly based on the observations that, in vitro, high risk HPV E6 proteins can target Dlg for degradation in the absence of E6-AP and that low risk HPV E6 proteins, when fused to a C-terminal PDZ-binding motif, can target Dlg for proteasome-mediated degradation within cells (21-23).

In order to confirm that E6-induced degradation of Dlg is independent of E6-AP, we set out to characterize E6-mediated degradation of Dlg and p53 in cells that are deficient for E6-AP. Unexpectedly, we observed that high risk HPV E6-mediated degradation of Dlg requires the presence of catalytically active E6-AP. Furthermore, degradation of Dlg mediated by a chimeric protein consisting of the C-terminal PDZ-binding motif of high risk HPV E6 proteins fused to a low risk HPV E6 protein was also dependent on the presence of E6-AP. This indicates that low risk HPV E6 proteins can functionally interact with E6-AP. Finally, we provide evidence that low risk HPV E6 proteins and E6-AP can form complexes, when both proteins are coexpressed within cells. Thus, the ability to interact with E6-AP appears to be more conserved among HPV E6 proteins than previously assumed.

Experimental Procedures

Cell lines and Plasmids - Mouse embryo fibroblasts (MEFs) prepared from E6-AP knock-out mice (kindly provided by A. Beaudet, Houston, Texas) (24), HeLa cells, HEK293 cells, and H1299 cells were grown in DMEM supplemented with 10% (vol/vol) FBS. Bacterial expression constructs for glutathione S-transferase (GST) fusion proteins of HPV11 E6 and HPV18 E6 were described previously (1). Bacterial expression constructs for GST fusion proteins of various HPV E6 chimeric proteins (18C11, 11C18, 5C18, 6C18, 16C18) (see Fig. 1A and Supplemental Information Fig. 2) were generated by PCR based approaches (further details will be provided upon request). For transient transfection experiments, the various E6 proteins were expressed with an N-terminal HA-tag or N-terminal GFP-tag from the eukaryotic expression vector pcDNA3 (Invitrogen). GFP was expressed from pcDNA3. (Invitrogen). The expression construct for wild-type p53 was described previously (25). The expression constructs for rat Dlg (kindly provided by L. Banks, ICGEB, Trieste, Italy), HA-tagged hDlg (kindly provided by C. Pique, Paris, France), and Flag-tagged MAGI-1 (kindly provided by S. Butz, University of Muenster, Germany) were described previously (21,26,27). Rat Dlg, wild-type E6-AP (isoform 1) (28), the catalytically inactive mutant E6-AP-C820A, and the internal deletion mutant E6-APΔE6bs (2) (deletion of amino acid residues 378-395; numbering according to E6-AP isoform 1) were expressed as N-terminally HA-tagged or Flag-tagged forms from pcDNA3. Transfection and Antibodies - In transient expression experiments, cells were transfected with the respective constructs in the presence of a reporter construct encoding β-galactosidase by lipofection
(Lipofectamine 2000; Invitrogen) according to the manufacturer's instructions. Protein extracts were prepared 24 h after transfection as described (25), and transfection efficiency determined by measuring β-galactosidase activity. Levels of p53, HA-tagged Dlg (rat or human; note that similar results were obtained for rat and human Dlg in all the experiments performed), Flag-tagged MAGI-1, E6-AP and its mutant forms, the various HA-tagged E6 proteins, and GFP were determined by Western blot analysis by using transfection efficiency adjusted protein amounts. The antibodies used for detection were, the mouse monoclonal HA.11 (Hiss Diagnostics, Freiburg, Germany) for HA-tagged proteins, the mouse monoclonal M2 (SIGMA) for Flag-tagged proteins, the mouse monoclonal DO1 (Calbiochem) for p53, a mixture of the mouse monoclonal antibodies 7.1 and 13.1 (Roche) for GFP, a mouse monoclonal antibody for tubulin (kindly provided by A.A. Noegel, Koeln, Germany), and a mouse monoclonal antibody (9) for E6-AP. Endogenous Dlg and E6-AP present in protein extracts prepared from HeLa cells were detected by mouse monoclonal antibody 2D11 (SANTA CRUZ, Santa Cruz, California) and a mouse monoclonal antibody (9), respectively.

To generate cell lines, in which E6-AP expression is stably suppressed by RNA interference, H1299 cells were transfected with pRetroSUPER-E6-AP expressing an siRNA directed against nucleotides 300-318 of the E6-AP mRNA (numbering according to E6-AP isoform 1, with nucleotide 1 referring to A of the start codon) by lipofection (Lipofectamine 2000; Invitrogen). Cells stably containing the expression construct were selected by resistance to puromycin (Sigma). Cells stably containing the expression construct were selected by resistance to puromycin (Sigma). After establishing single cell clones, protein extracts were prepared (25) and E6-AP levels determined by Western blot using a mouse monoclonal antibody (9).

siRNAs against mRNAs encoding E6-AP, HPV-18 E6, or renilla luciferase were obtained from MWG (Ebersbach, Germany). The respective sequences used were described previously (9,29). For siRNA-mediated knockdown of the respective mRNAs, exponentially growing cells were transfected with 0.6 nmol of the respective siRNA per 6 cm plate by Oligofectamine according to the manufacturer's instructions (Invitrogen). Protein extracts were prepared 72 h after transfection and the levels of endogenous E6-AP and Dlg detected by Western blot analysis.

Ubiquitination and coprecipitation assays - For in vitro ubiquitination experiments, the various E6 proteins were expressed as glutathione S-transferase fusion proteins in Escherichia coli DH5α. The ubiquitin-activating enzyme E1, E6-AP, and the ubiquitin-conjugating enzyme UbcH5 were expressed in the baculovirus system or in Escherichia coli BL21 by using the pET expression system as described (30). For in vitro ubiquitination, 1 µl of wheat germ extract-translated 35S-labeled substrate (p53, rat Dlg or human Dlg) was incubated with 50 ng E1, 50 ng UbcH5, and 10 µg ubiquitin (SIGMA) in the absence or in the presence of bacterially expressed E6 (50 ng) and baculovirus expressed E6-AP (50 ng) in 50 µl volumes. In addition, reactions contained 25 mM Tris-HCl (pH 7.5), 60 mM NaCl, 3 mM DTT, 2-4 mM ATP, and 4-8 mM MgCl2. After incubation at 30°C for 2 h, total reaction mixtures were electrophoresed in 10% SDS-polyacrylamide gels and the 35S-labeled substrates detected by fluorography.

For coprecipitation experiments, two 10 cm plates of HEK293 cells were transfected with the respective expression constructs for HA-tagged HPV E6 proteins and the various forms of E6-AP (see Fig. 6). Protein extracts were prepared 24 h after transfection and precleared with Protein A Sepharose. Then, 4 µg of the mouse monoclonal antibody HA.11 were added, the extracts incubated for 4 h at 4°C, and bound proteins precipitated by Protein A Sepharose. 10 percent of the precipitate was used for Western blot analysis to detect the respective E6 protein and potentially coprecipitated E6-AP. With the remaining 90 percent of the precipitate, in vitro ubiquitination assays in the presence of 50 ng E1, 50 ng UbcH5, and 10 µg ubiquitin were performed under standard reaction conditions (see above).

RESULTS

E6-AP mediates high risk HPV E6-induced ubiquitination of Dlg in vitro - It was previously reported that high risk HPV E6 proteins induce degradation of wheat germ extract translated Dlg in vitro (23). Since wheat germ extract does not contain E6-AP (1,2), this indicated that E6-induced
degradation of Dlg does not require the presence of E6-AP. However, no experimental evidence was provided to show that E6-induced degradation of Dlg \textit{in vitro} requires ubiquitination of Dlg. In an effort to set up an E6-dependent \textit{in vitro} ubiquitination system for Dlg, we observed that, in the presence of E6-AP, HPV18 E6 (Fig. 1) or HPV16 E6 (data not shown) are capable of inducing the ubiquitination of in wheat germ extract translated radioactively labeled Dlg (note that in this and all subsequent experiments similar results were obtained for rat and human Dlg). Thus, no distinction will be made between rat and human Dlg. Furthermore, E6-mediated ubiquitination of Dlg was dependent on the presence of the C-terminal PDZ binding motif of E6 indicating the specificity of this reaction. Thus, at least \textit{in vitro}, E6-AP can support E6-induced ubiquitination of Dlg. These data are consistent with results recently published by Matsumoto et al. (31).

A fusion protein consisting of the C-terminal PDZ binding motif of HPV18 E6 fused to HPV11 E6 was reported to induce degradation of Dlg \textit{in vitro} and within cells (22). Thus, we generated bacterial and mammalian expression constructs encoding a similar fusion protein ("11C18", Fig. 1A). However, while the 11C18 E6 protein was able to bind to Dlg \textit{in vitro} (data not shown) and to target Dlg for degradation within cells (see below), bacterially expressed 11C18 was not able to induce ubiquitination of Dlg \textit{in vitro} under the conditions used, neither in the absence (not shown) nor in the presence of E6-AP (Fig. 1).

In the following, data will be presented that were obtained with HPV18 E6 and HPV11 E6. However, we will refer to "high risk" (HPV18) and "low risk" (HPV11) HPV E6 proteins rather than to HPV18 E6 and HPV11 E6, since similar data were obtained for the E6 proteins derived from HPV16 ("high risk") and HPV6 ("low risk").

\textit{E6-AP is required for E6-mediated degradation of Dlg in murine cells} - To address the role of E6-AP in E6-facilitated degradation of Dlg within cells, mouse embryo fibroblasts (MEFs) were prepared from genetically engineered E6-AP null mice (24) and the ability of E6 to target Dlg and p53, respectively, for degradation was determined in cotransfection assays. As expected, high risk HPV E6 proteins but not low risk HPV E6 proteins induced degradation of p53 in the presence of ectopically expressed wild-type E6-AP (Fig. 2A). Furthermore, disappearance of p53 was not observed in the presence of an E6-AP mutant devoid of the E6 binding site (E6-AP-ΔE6bs) (2) or of a catalytically inactive E6-AP mutant (E6-AP-C820A) (32) indicating that E6-AP null MEFs are suited to address the role of E6-AP in the degradation of a given protein.

Similar to the results obtained in the \textit{in vitro} ubiquitination system, high risk HPV E6-induced degradation of Dlg was dependent on the presence of wild-type E6-AP and the presence of the C-terminal PDZ binding motif of E6 ("18C11") (Fig. 2B). Furthermore, the 11C18 protein was capable of inducing degradation of Dlg but surprisingly, only in the presence of wild-type E6-AP. Although it is not possible to conclude from these data that E6-AP is directly involved in low risk HPV E6-induced degradation of Dlg, the finding that degradation of Dlg is not observed in the presence of E6-AP-ΔE6bs is consistent with this possibility.

\textit{E6-AP is required for E6-mediated degradation of Dlg in human cells} - Although the experiments with E6-AP null MEFs showed that E6-AP is required for E6-induced degradation of Dlg within murine cells, it remained unclear if this result can be extrapolated to human cells. For example, it could be envisioned that human cells, but not murine cells, express an E3 ligase that interacts with both low risk and high risk HPV E6 proteins and that this E3 ligase can substitute for E6-AP in the degradation of Dlg. Human cell lines deficient in E6-AP expression have not yet been described. To generate cells that express no or at least significantly decreased levels of E6-AP, the H1299 cell line was used, since this cell line is readily transfectable and proficient in E6-induced degradation of both p53 and Dlg (Fig. 3A). H1299 cells were transfected with pRetroSUPER-E6-AP, which should allow the stable suppression of E6-AP expression by RNA interference (33). Upon selection, single cell clones were established and the individual clones checked for E6-AP expression by Western blot analysis. As shown in Fig. 3B, some of the selected clones had E6-AP levels similar to the parental H1299 cells, while in other clones (i.e. K2 and K3) E6-AP expression was significantly decreased suggesting that E6-AP-specific siRNAs are expressed at different levels in the individual cell
clones. Furthermore, upon cotransfection, neither high risk HPV E6 proteins nor 11C18 were able to induce degradation of p53 and Dlg in the K3 or the K2 clone (Fig. 3C, and data not shown).

The above data indicate that, with respect to E6-induced degradation of p53 and Dlg, the clones K2 and K3 can be considered as "E6-AP null" cells. To unambiguously demonstrate that the inability of K2 and K3 cells to support E6-mediated degradation processes is indeed due to insufficient expression levels of E6-AP, rescue experiments were performed. To do so, expression constructs for E6-AP and its mutants were generated that are resistant to the siRNA used to abrogate expression of endogenous E6-AP by introducing silent mutations in the region of the E6-AP mRNA targeted by the siRNA (Supplemental Data Fig. 1). Indeed, coexpression of the respective wild-type E6-AP expression construct resulted in efficient E6-facilitated degradation of p53 and Dlg, respectively (Fig. 4A). Furthermore, the requirements for E6-AP to support degradation of Dlg were similar for both high risk HPV E6 proteins and 11C18 insofar as the region of E6-AP that mediates the interaction with high risk HPV E6 proteins is also required for 11C18-induced degradation of Dlg.

Similar to Dlg, E6-induced degradation of MAGI-1 has been reported to be independent of E6-AP (23). Thus, we determined the ability of E6 to induce degradation of MAGI-1 in parental H1299 cells (data not shown) and K3 cells (Fig. 4B). As for Dlg, E6-induced degradation of MAGI-1 was observed only upon coexpression of wild-type E6-AP indicating that, in general, E6-induced degradation of PDZ domain containing proteins is mediated by E6-AP.

**Downregulation of E6-AP expression induces Dlg accumulation in HeLa cells** - We have recently reported that downregulation of E6-AP expression by RNA interference results in accumulation of p53 and induction of apoptosis in HPV18-positive HeLa cells and other HPV-positive cell lines (9). In contrast, the cytotoxic effect of downregulation of E6-AP expression was significantly diminished in HeLa cells, in which p53 expression was stably suppressed by RNA interference ("p53 null" HeLa cells). If E6-AP is involved in E6-facilitated degradation of Dlg in HPV-positive cell lines, abrogation of the ubiquitin ligase activity of E6-AP should result in accumulation of endogenous Dlg. Indeed, transient downregulation of E6-AP by RNA interference resulted in a moderate but significant increase in Dlg levels in "p53 null" HeLa cells (Fig. 5). Furthermore, the relative increase in Dlg levels was similar to the one observed upon downregulation of expression of the endogenous E6 protein, while Dlg levels were not affected by downregulation of E6-AP expression in HPV-negative H1299 cells (see Fig. 3B). Thus, E6-AP appears to be intrinsically involved in E6-mediated degradation of Dlg in HeLa cells but does not play a role in the degradation of Dlg in HPV-negative cells, consistent with the observation that E6-AP does not bind to Dlg in the absence of E6.

**Low risk HPV E6 proteins can bind to E6-AP** - The results presented above indicate that, within cells, low risk HPV E6 proteins have the ability to functionally interact with E6-AP. To obtain evidence that E6-AP functions as ubiquitin ligase in low risk HPV E6-mediated degradation of Dlg, we revisited the possibility that low risk HPV E6 proteins form complexes with E6-AP. As reported previously (1,2) and in line with the in vitro ubiquitination data presented above, an interaction between bacterially expressed low risk HPV E6 proteins and in vitro translated E6-AP was not observed (data not shown). However, when both proteins were coexpressed in mammalian cells, E6-AP was coprecipitated with low risk HPV E6 (Fig. 6A). Importantly, binding of 11C18 E6 to E6-AP required the region of E6-AP that was previously shown to be necessary and sufficient for interaction with high risk HPV E6 proteins (2). Furthermore, the precipitated complex was able to induce in vitro ubiquitination of Dlg but not of p53 indicating the specificity of the in vitro reaction (Fig. 6B). Similar to previous reports (22), however, a chimeric protein consisting of the C-terminal PDZ binding motif of HPV18 E6 fused to the E6 protein of a cutaneous HPV type (HPV5, has not been associated with mucosal lesions) did not coprecipitate with E6-AP and did neither induce ubiquitination of Dlg in vitro (Fig. 6) nor degradation of Dlg within cells (Supplemental Data Fig. 2). This indicates that the ability to interact with E6-AP is conserved among the E6 proteins derived from mucosal HPVs but not among HPV E6 proteins in general.

**DISCUSSION**
Using *in vitro* ubiquitination assays and cellular degradation assays, we provide evidence that the ability of high risk HPV E6 proteins to utilize the cellular ubiquitin ligase E6-AP to target associated proteins for ubiquitination and degradation is shared by the low risk HPV E6 proteins. Furthermore, based on the results obtained with E6-AP null MEFs and H1299 cells, in which expression of endogenous E6-AP was ablated by RNA interference, we conclude that, in contrast to previous assumptions (21-23), E6-AP is required for E6-mediated degradation of the PDZ domain containing proteins Dlg and MAGI-1.

It was previously reported that, *in vitro*, E6 targets Dlg for proteasome-mediated degradation in an E6-AP-independent manner (23). However, no evidence was provided to indicate that this E6-mediated degradation process requires prior ubiquitination of Dlg. Since our results show that, within cells, E6-mediated degradation of both Dlg and MAGI-1 is dependent on the presence of E6-AP, we do not favor the hypothesis that another ligase may substitute for E6-AP in the *in vitro* system. Several proteins including c-Jun and c-Fos have been reported to be degraded by the proteasome in a ubiquitin-dependent and ubiquitin-independent manner (34,35). Thus, an interesting but purely speculative possibility is that, *in vitro*, E6 can target PDZ domain containing proteins to the proteasome in a ubiquitin-independent and, thus, E6-AP-independent manner.

Reconstitution experiments in H1299 cells, in which expression of endogenous E6-AP was stably ablated by RNA interference, showed that wild-type E6-AP is required for E6-induced degradation of p53 and Dlg (Fig. 4). Accordingly, expression of mutant forms of E6-AP that either are not capable of binding to E6 or are catalytically inactive did not rescue E6-mediated proteolytic processes. These results demonstrate that the RNA interference technology can be used to perform structure/function analyses of a given protein within cells, thereby providing an excellent tool to correlate known biochemical properties of a protein with physiological functions. Furthermore, the results indicate that E6-induced degradation of Dlg involves ubiquitination of Dlg. It should be noted, however, that attempts to accumulate ubiquitinated forms of Dlg by the addition of proteasome inhibitors to transfected cells failed. This is probably explained by the observation that proteasome inhibitors only marginally interfered with E6-induced degradation of Dlg under the conditions used (data not shown) (the possibility that the disappearance of Dlg in the presence of E6 is due to non-proteolytic events was excluded by the use of the ubiquitin fusion protein system developed by the Varshavsky lab (36); see Supplemental Data Fig. 3). In comparison to non-transfected cells, transfected cells appear to be more sensitive to cytotoxic effects of proteasome inhibitors. Thus, to exclude possible artifacts, transfected cells were treated with proteasome inhibitors only for a limited amount of time (4 hours). Since only marginal increases of Dlg were observed under these conditions, we propose that, even in the presence of E6, Dlg has a half-life of more than 4-6 hours. In agreement with this hypothesis, HeLa cells have to be treated for 8-10 hours with proteasome inhibitors to observe a 2-fold increase in endogenous Dlg levels (data not shown), although Dlg is at least in part degraded via the E6/E6-AP complex in these cells (Fig. 5).

Although our data clearly show that low risk HPV E6 proteins have the ability to interact with E6-AP, the physiological significance of this interaction remains to be established. To do so, it will be important to identify interaction partners of low risk HPV E6 proteins and to determine if their stability is affected by the E6 proteins in an E6-AP-dependent manner. Previous studies have shown that the E6 proteins of both low risk and high risk HPVs have the ability to target Bak for degradation (13,14). For unknown reasons, we have not been able to observe E6-mediated degradation of ectopically expressed Bak in cotransfection experiments. In this context, it should be noted that our results do not exclude the possibility that the E6 proteins of low risk and/or high risk HPVs have the ability to functionally interact with other yet unidentified ubiquitin ligases in the degradation of proteins other than p53 and PDZ domain containing proteins.

In agreement with previous reports, we were not able to observe complex formation between bacterially expressed low risk HPV E6 proteins and *in vitro* translated E6-AP or E6-AP present in mammalian cell extracts and vice versa (1,2). However, coexpression of both proteins within cells resulted in the formation of a complex containing low risk HPV E6 proteins and E6-AP. Importantly,
complex formation required the region of E6-AP that also mediates the interaction of E6-AP with high risk HPV E6 proteins. Furthermore, catalytically active E6-AP is required for 11C18 E6 to target Dlg for ubiquitination in vitro and degradation within cells. Taken together, these data indicate that low risk HPV E6 proteins directly interact with E6-AP and that the low risk HPV E6/E6-AP complex functions as ubiquitin ligase. However, we cannot exclude the possibility that an additional yet unknown ubiquitin ligase contributes to E6/E6-AP-mediated ubiquitination/degradation of Dlg. Similarly, it is possible that additional yet unknown proteins are required to facilitate the interaction of E6-AP with low risk HPV E6 proteins (e.g. by posttranslational modification of E6 or E6-AP or both or by affecting the structure of E6 or E6-AP). Mass spectrometric analyses of purified low risk HPV E6/E6-AP complexes should provide insight into the composition of this complex and provide first hints to answer these questions.

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FOOTNOTES

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1The abbreviations used are: HPV, human papillomavirus; MEFs, mouse embryo fibroblasts, GST, glutathione-S-transferase; siRNA, small interfering RNA.

FIGURE LEGENDS

**Fig. 1.** E6-AP facilitates E6-mediated ubiquitination of Dlg *in vitro*. *A*, Schematic representation of the E6 proteins used and their ability to bind and to degrade p53 and Dlg, respectively. 18C11, chimeric protein consisting of the 10 C-terminal amino acid residues of HPV11 E6 fused to amino acid residues 1-141 of HV18 E6 (consists of 158 residues); 11C18, chimeric protein consisting of the 17 C-terminal amino acid residues of HPV18 E6 fused to amino acid residues 1-140 of HV11 E6 (consists of 150 residues). +, binds to p53 and Dlg, respectively; -, does not bind to p53 and Dlg, respectively. *B*, Bacterially expressed GST fusion proteins of the E6 proteins indicated were incubated with *in vitro* translated radiolabeled Dlg under standard ubiquitination conditions for 2 h in the presence or absence of baculovirus-expressed E6-AP and ubiquitin, respectively, as indicated. The reaction products were analyzed by SDS-polyacrylamide gel electrophoresis followed by fluorography. Running positions of the nonmodified form and of the ubiquitinated forms of Dlg are indicated by an arrowhead and an asterisk, respectively.

**Fig. 2.** E6-AP is required for E6-induced degradation of Dlg in MEFs derived from E6-AP null mice. *A-C*, Mouse embryo fibroblasts derived from E6-AP null mice (24) were cotransfected with expression constructs for p53 and HA-tagged Dlg in the absence and presence of expression constructs encoding various E6 proteins and different forms of E6-AP isoform 1 (28) as indicated. Protein extracts were prepared 24 hours after transfection and levels of p53, Dlg, and E6-AP determined by Western blot analysis as indicated. Note that the anti-p53 antibody used (DO1) recognizes ectopically expressed human p53 but not endogenous murine p53. wt, wild-type E6-AP isoform 1; C820A, catalytically inactive E6-AP with a substitution of the catalytic site cysteine residue at position 820 (numbering according to E6-AP isoform 1) by alanine; ∆E6bs, E6-AP isoform 1 with a deletion of the E6 binding region (amino acid residues 378-395). *, denotes a cross-reactive band serving as loading control.

**Fig. 3.** Stable knockdown of E6-AP expression in H1299 cells by RNA interference. *A*, H1299 cells were cotransfected with expression constructs for p53, HA-tagged Dlg, and GFP in the absence and presence of expression constructs encoding various HA-tagged E6 proteins as indicated. Protein extracts were prepared 24 hours after transfection and levels of p53, Dlg, GFP, and E6 determined by Western blot analysis as indicated. *B*, To generate H1299 cells, in which E6-AP expression is stably suppressed by
RNA interference, H1299 cells were transfected with pRetroSUPER-E6-AP that, in addition to an siRNA specific for E6-AP mRNA, expresses the puromycin resistance gene. Cells stably containing the expression constructs were selected by resistance to puromycin and single cell clones established. Protein extracts were prepared from several single cell clones (K1-K7) and E6-AP levels determined by Western blot analysis using a mouse monoclonal anti-E6-AP antibody. par., extract prepared from parental H1299 cells. C, The K3 clone was cotransfected with expression constructs for p53, HA-tagged Dlg, and GFP in the absence and presence of expression constructs encoding various HA-tagged E6 proteins as indicated. Protein extracts were prepared 24 hours after transfection and levels of p53, Dlg, GFP, and E6 determined by Western blot analysis as indicated.

Fig. 4. Reconstitution of E6-induced degradation of Dlg and MAGI-1 in "E6-AP null" H1299 cells. A, The "E6-AP null" H1299 clone K3 was cotransfected with expression constructs for p53 and HA-tagged Dlg in the absence and presence of expression constructs encoding HPV18 E6 and 11C18, respectively, and different forms of E6-AP isoform 1 (28) as indicated. Protein extracts were prepared 24 hours after transfection and levels of p53, Dlg, and E6-AP determined by Western blot analysis as indicated. B, K3 cells were cotransfected with expression constructs for Flag-tagged MAGI-1 and GFP in the absence and presence of expression constructs encoding HPV18 E6 and 11C18, respectively, and different forms of E6-AP isoform 1 (ref) as indicated. Protein extracts were prepared 24 hours after transfection and levels of MAGI-1, GFP, and E6-AP determined by Western blot analysis as indicated. wt, wild-type E6-AP isoform 1; C820A, catalytically inactive E6-AP with a substitution of the catalytic site cysteine residue at position 820 (numbering according to E6-AP isoform 1) by alanine; ΔE6bs, E6-AP isoform 1 with a deletion of the E6 binding region (amino acid residues 378-395).

Fig. 5. Downregulation of E6-AP expression results in accumulation of Dlg in "p53 null" HeLa cells. The previously described "p53 null" HeLa clones HA1 and HC2 (9) were transfected with chemically synthesized siRNAs specifically targeting HPV18 E6 (E6) or E6-AP (E6-AP). As control, HA1 and HC2 cells were transfected with siRNA directed against renilla luciferase (con). Protein extracts were prepared 72 hours after transfection and levels of Dlg, E6-AP, and tubulin (tub; loading control) determined by Western blot analysis as indicated.

Fig. 6. Functional interaction of low risk HPV E6 proteins with E6-AP. HEK293 cells were transfected with expression constructs encoding various forms of HA-tagged E6 and Flag-tagged E6-AP, respectively, as indicated. Protein extracts were prepared 24 hours after transfection. 5 percent of the respective extracts were directly used for determination of the expression levels of the various forms of E6 and E6-AP, respectively (Input). The remaining extracts were used for immunoprecipitation of E6 with an anti-HA antibody. A, 10 percent of the precipitates were analyzed by Western blot analysis to determine the levels of the respective immunoprecipitated E6 and of potentially coprecipitating E6-AP. B, The remaining precipitates were used for in vitro ubiquitination assays using in vitro translated radiolabeled p53 or Dlg as substrate. The running position of ubiquitinated forms of p53 and Dlg, respectively, are indicated by an asterisk. 11, HPV11 E6; 18, HPV18 E6; 11C18, chimeric protein consisting of the 17 C-terminal amino acid residues of HPV18 E6 fused to amino acid residues 1-140 of HPV11 E6; 5C18, chimeric protein consisting of the 17 C-terminal amino acid residues of HPV18 E6 fused to amino acid residues 1-150 of HPV5 E6; wt, wild-type E6-AP isoform 1; C820A, catalytically inactive E6-AP with a substitution of the catalytic site cysteine residue at position 820 (numbering according to E6-AP isoform 1) by alanine; ΔE6bs, E6-AP isoform 1 with a deletion of the E6 binding region (amino acid residues 378-395).
**Fig. 1** Kuballa et al.

**A**

|       | p53 | Dlg |
|-------|-----|-----|
| 18 E6 | +   | +   |
| 11 E6 | -   | -   |
| 18C11 | +   | -   |
| 11C18 | -   | +   |

**B**

|       | E6 | E6-AP | ubi |
|-------|----|-------|-----|
| 18    | -  | -     | +   |
| 18    | +  | +     | +   |
| 11C11 | +  | +     | +   |
| 18C11 | +  | +     | +   |

*Figures showing results of experiments.*
**Fig. 2** Kuballa et al.

A

| Dlg | p53 | E6 | E6-AP |
|-----|-----|----|-------|
| -   | -   | -  | -     |
| +   | +   | 18 | wt    |
|   - |   - | -  | wt    |

B

| E6  | E6-AP |
|-----|-------|
| -   | -     |
| 11  | wt    |
| 18  | wt    |
| 18C11| wt    |
| 11C18| wt    |

C

| E6  | E6-AP |
|-----|-------|
| -   | wt    |
| 11C18| wt    |
|     | wt    |
| 18  | C820A | ΔE6bs |
|     | C820A | ΔE6bs |

*Dlg* and *p53* levels are shown in the Western blots.
Fig. 3  Kuballa et al.

A  

**H1299**

|   | E6 | -  | 11 | 18 | 18C11 | 11C18 |
|---|----|----|----|----|-------|-------|
| Dlg | ![Dlg lane](image) |
| p53 | ![p53 lane](image) |
| E6 | ![E6 lane](image) |
| GFP | ![GFP lane](image) |

B  

**H1299 par.**

|   | K1 | K2 | K3 | K4 | K5 | K6 | K7 |
|---|----|----|----|----|----|----|----|
| E6-AP | ![E6-AP lane](image) |
| Dlg | ![Dlg lane](image) |
| tub. | ![tub lane](image) |

C  

**K3**

|   | E6 | -  | 11 | 18 | 18C11 | 11C18 |
|---|----|----|----|----|-------|-------|
| Dlg | ![Dlg lane](image) |
| p53 | ![p53 lane](image) |
| E6 | ![E6 lane](image) |
| GFP | ![GFP lane](image) |
Fig. 4 Kuballa et al.

A

E6  -  -  18  11C18
E6-AP  -  wt  -  wt  \(\Delta E6bs\) C820A  wt  \(\Delta E6bs\) C820A

Dlx

p53

E6-AP

B

E6  -  -  18  11C18
E6-AP  -  wt  -  wt  \(\Delta E6bs\) C820A  -  wt  \(\Delta E6bs\) C820A

MAGI

E6-AP

GFP
Fig. 5  Kuballa et al.

| siRNA | con | E6  | E6-AP | con | E6  | E6-AP |
|-------|-----|-----|-------|-----|-----|-------|
| HA1   |     |     |       |     |     |       |
|       |     |     |       |     |     |       |
|       |     |     |       |     |     |       |
| HC2   |     |     |       |     |     |       |
|       |     |     |       |     |     |       |
|       |     |     |       |     |     |       |

- Dlg
- E6-AP
- tub.
Fig. 6  Kuballa et al.

| A       | Anti-HA-IP |               |               |               |               |
|---------|------------|---------------|---------------|---------------|---------------|
| E6      | 11         | 18            | 11C18         | 5C18          |               |
| E6-AP   | wt         | wt            | ΔE6bs C820A   | ΔE6bs C820A   | wt            |
| E6AP    |            |               |               |               |               |
| HA-E6   |            |               |               |               |               |

| B       |               |               |               |               |               |
|---------|---------------|---------------|---------------|---------------|---------------|
| E6      |               | 11            | 18            | 11C18         | 5C18          |
| E6-AP   |              | wt            | wt            | ΔE6bs C820A   | ΔE6bs C820A   |
| *       | p53          |               |               |               |               |

* Dlg
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