Identification of HHR23A as a Substrate for E6-associated Protein-mediated Ubiquitination*

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The human papilloma virus E6-associated protein (E6AP) functions as a ubiquitin protein ligase (E3) in the E6-mediated ubiquitination of p53. E6AP is also an E3 in the absence of E6, but its normal cellular substrates have not yet been identified. Here we report the identification of HHR23A, one of the human homologues of the yeast DNA repair protein Rad23, as an E6-independent target of E6AP. HHR23A binds E6AP and is ubiquitinated in vitro in an E6AP-dependent manner. Ubiquitinated forms of endogenous HHR23A are detectable in mammalian cells. Overexpression of wild-type E6AP in vivo enhances the ubiquitination of HHR23A, whereas a dominant negative E6AP mutant inhibits HHR23A ubiquitination. Although HHR23A is a stable protein in nonsynchronized cells, its levels are regulated in a cell cycle-dependent manner, with specific degradation occurring during S phase. The S phase degradation of HHR23A could be blocked in vivo by dominant negative E6AP, providing direct evidence for the involvement of E6AP in the regulation of HHR23A. Consistent with a role of the HHR23 proteins in DNA repair, UV-induced DNA damage inhibited HHR23A degradation. Although the precise role of HHR23 proteins in DNA repair and cell cycle progression remains to be elucidated, our data suggest that E6AP-mediated ubiquitination of HHR23A may have important implications in DNA repair and cell cycle progression.

Protein ubiquitination is implicated in a variety of cellular processes, including DNA repair, cell cycle control, chromosomal organization, intracellular translocation of proteins, and apoptosis (1–3). Ubiquitin-dependent proteolysis is the best known aspect of the ubiquitin pathway. The covalent conjugation of multiple ubiquitin molecules to lysine residues of a target protein serves to signal its recognition and rapid degradation by the 26 S proteasome (3, 5, 6). Ubiquitination of protein substrates is a multi-step process that involves the concerted action of at least three classes of enzymes as follows: ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzymes (E2a), and ubiquitin protein ligases (E3s) (3). Although the biochemical mechanisms of ubiquitin transfer within the enzymatic components of the pathway and its subsequent conjugation to target proteins is now understood in considerable detail, it is still unclear how specific proteins are recognized by the ubiquitin system as substrates. E1 first activates ubiquitin in an ATP-dependent reaction through the formation of ubiquitin adenylate, followed by a thiol ester bond between the carboxyl terminus of ubiquitin and thiol group of a specific cysteine residue in E1. Ubiquitin is then transferred either directly to a substrate or to E3 enzymes that finally catalyze the formation of an isopeptide bond between the carboxyl terminus of ubiquitin and the ε-amino group of lysine residues on a target protein (3, 7, 8). A substrate may be multiply ubiquitinated by sequential linkage of additional ubiquitin molecules to each other through specific lysine residues (Lys-48 or Lys-63). Multi-ubiquitination of a protein leads to its recognition and consequent degradation by the 26 S proteasome (3, 5, 6).

The mechanisms involved in the recognition of specific proteins as substrates of the ubiquitin system are not fully understood. However, it is likely that E3 ubiquitin protein ligases are the key components that provide specificity to the ubiquitin system by direct interaction with specific substrates. Although two E3 activities had previously been identified from rabbit reticulocytes (E3a and E3b) (9–11), it was the cloning and characterization of E6AP that revealed structural and functional features of a new class of E3 enzymes. E6AP was initially identified as a 100-kDa cellular protein that, in conjunction with the E6 oncoprotein of human papilloma virus type 16 (HPV), constituted the E3 activity in the ubiquitination of p53 (7, 12–14). E6AP was also found to promote the ubiquitination of cellular proteins in the absence of E6, indicating that E6AP could function as an E3 enzyme independent of E6 (7). Sequence analysis of E6AP revealed a region of approximately 350 amino acids in the carboxyl terminus that was highly conserved among a number of proteins from various organisms (15). This region, subsequently termed the HECT domain, contains a conserved cysteine residue that serves as the active site for thiol ester formation with ubiquitin (15). In addition to E6AP, several other Hect domain proteins have now been shown to be capable of forming thiol ester complexes, suggesting that Hect-containing proteins belong to a family of structurally related ubiquitin protein ligases (8, 15). As mentioned above, the specificity of substrate recognition by the ubiquitin system may be achieved by E3 enzymes capable of direct interaction with specific substrates. In the case of Hect proteins for instance, their divergent amino-terminal sequences may provide unique recognition sites for the cell cycle progression of human papilloma virus; HA, hemagglutinin; mAb, monoclonal antibody; XP, Xeroderma pigmentosum; XPC, xeroderma pigmentosum group C.

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† The abbreviations used are: E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzymes; E3, ubiquitin protein ligase; E6AP, E6-associated protein; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; NER, nucleotide excision repair; WGE, wheat germ extracts; CMV, cytomegalovirus; WT, wild type; HPV,
vide the necessary diversity required for substrate recognition, whereas their conserved carboxyl terminus (Hect domain) can interact with specific E2 enzymes and catalyze the ubiquitination of bound substrates (16–18).

To date, only a small number of proteins have been identified as substrates of Hect E3 enzymes. The general amino acid permease Gap1 and uracil permease Fur4 have been reported to be ubiquitinated by RSP5 (NP11), a Hect protein of Saccharomyces cerevisiae (19, 20). The large subunit of RNA polymerase II (Rpb1) was recently identified also as an RSP5 substrate (17). In addition, the Schizosaccharomyces pombe homologue of RSP5, Pub1, has been shown to target the CDC25 phosphatase for ubiquitin-dependent degradation (21). In the case of E6AP, p53 is the only known substrate; however, the ubiquitination of p53 by E6AP is dependent upon the presence of oncogenic E6 proteins of HPV. To date no other substrate for E6AP-mediated ubiquitination has been isolated. Interestingly, E6AP was recently identified as the gene affected in Angelman syndrome, a genetic neurological disorder. A majority of the mutations in E6AP is predicted to abolish the catalytic activity of E6AP, raising the possibility that deregulation of E6AP substrates may contribute to the pathogenesis of Angelman syndrome (22, 23).

In an attempt to identify E6-independent substrates of E6AP, we isolated one of the human homologues of yeast Rad23 (HHR23A) as an E6AP-interacting protein using the yeast two-hybrid system (18, 24). Rad23 is involved in excision repair of UV-damaged DNA and has also been implicated in spindle pole body duplication and cell cycle progression in S. cerevisiae (25–27). Two homologues of Rad23 exist in humans, HHR23A and HHR23B (24). Both of these proteins have been reported to bind and function with the xeroderma pigmentosum group C (XPC) protein in nucleotide excision repair (NER) (28, 29). Our results identify HHR23A as a novel, E6-independent substrate of E6AP. We demonstrate that HHR23A interacts with E6AP and is efficiently ubiquitinated in an E6AP-dependent manner in vitro. By using anti-HHR23A and anti-ubiquitin antibodies, a small fraction of endogenous HHR23A was found conjugated to ubiquitin. Transient expression of wild-type E6AP enhanced the ubiquitinated fraction of HHR23A, whereas a dominant negative E6AP mutant inhibited HHR23A ubiquitination in vivo. Although HHR23A appears to be a stable protein in asynchronously growing cells, we have found that HHR23A protein levels are regulated during cell cycle progression. The level of HHR23A was found to be the highest in M phase and early G1, with a consistent 3–5-fold decrease occurring during late G1 and early S phase, indicating targeted degradation of the protein at specific stages of the cell cycle. The decrease in HHR23A protein levels was completely blocked by transient expression of dominant negative E6AP, providing direct evidence for the involvement of E6AP in the cell cycle-dependent degradation of HHR23A. Significantly, treatment of cells with UV radiation also abolished HHR23A degradation, suggesting that DNA damage may regulate HHR23A stability. At present, the precise roles of HHR23 proteins in repair of UV-damaged DNA is not clear. Nevertheless, our data suggest that E6AP-mediated ubiquitination of HHR23A may be important in regulating its function in DNA repair and cell cycle progression.

EXPERIMENTAL PROCEDURES

Isolation of HHR23A cDNA—A modified version of the yeast two-hybrid screen used to identify E6AP-interacting proteins has been described previously (18). Interacting clones were isolated, and their DNA sequence was determined by dideoxynucleotide sequencing. The BLAST algorithm was used to search GenBank™ data bases, and eight independent inserts of varying lengths were identified as HHR23A. Clone 15-2, containing the entire open reading frame of HHR23A, was used for further analysis.
were collected and replated for indicated amounts of time, and cell extracts were prepared at the end of each time point. In experiments involving UV radiation (Fig. 6), 2 h after plating (post-mitotic shake), cells were rinsed with phosphate-buffered saline and irradiated with an 8-J UV dose using UV Stratalinker 1800 (Stratagene). The cells were immediately refed with fresh media and transferred back to 37°C, 5% CO₂ incubator.

**Immunoprecipitation and Immunoblot Analysis**—Rabbit polyclonal sera against GST-HHR23A were generated at Babco. To detect ubiquitinated HHR23A species in mammalian cells (untransfected or transfected with E6AP constructs), whole cell extracts of COS-7 and U2OS cells were prepared in RIPA buffer (20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 150 mM NaCl, 0.25% SDS, 1% Nonidet P-40, 1% deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 1 µg/µl aprotinin/leupeptin mix). Cells from three confluent 10-cm plates were lysed, and HHR23A was immunoprecipitated from 2 mg of total cell extract in RIPA buffer at 4°C with anti-HHR23A antibodies. 40 µl of protein A-Sepharose (v/v) was added after 4 h, and samples were washed extensively in RIPA buffer to avoid co-precipitation of proteins other than HHR23A. As control, equal amounts of cell extracts (2 mg) were subjected to precipitation with preimmune sera. Immunoprecipitates were boiled for 5 min in SDS sample buffer and resolved by SDS-PAGE. Proteins were transferred to polyvinylidene difluoride membranes (Nen) using standard procedures, and immunoblot analysis was carried out with anti-HHR23A and anti-ubiquitin antibodies. Detection of proteins was performed with ECL reagents (Nen). HHR23A ubiquitination after transfection of COS-7 cells with CMV-HA-E6AP constructs was detected similarly by immunoprecipitation (in RIPA buffer) and immunoblotting of 2 mg of whole cell lysates with anti-HHR23A antibodies. Expression of E6AP (WT and CS33A) in U2OS cells was detected by immunoblotting with anti-HA MAb (12CA5, Babco).

For the time course analysis, GM6419, U2OS, or XPC cell extracts were prepared in lysis buffer by gentle sonication, and 100 µg of whole cell lysates were resolved by SDS-PAGE. HHR23A and cyclin A were detected by immunoblotting with anti-HHR23A or anti-cyclin A antibodies (BF683, Santa Cruz Biotechnology). Quantitation of relative protein amounts was performed by using the Gel Plotting Macros feature of NIH-Image software.

**RESULTS**

**Isolation of HHR23A cDNA**—In an attempt to identify potential substrates and regulators of E6AP, we used a modified version of the yeast two-hybrid system to isolate cDNA clones that encode E6AP-interacting proteins. A catalytically inactive form of E6AP in which the active site cysteine residue is substituted with alanine was used as bait to avoid potential degradation of interacting proteins (18). E6AP (CS33A) was fused in frame with the Ga4 DNA-binding domain and introduced into the yeast reporter strain MaV103. Expression of the fusion protein was confirmed by immunoblot analysis with anti-E6AP and anti-Ga4 DNA-binding domain antibodies. The prey cDNA library, fused to the Ga4 activation domain, was derived from activated human T cells. Interacting clones were isolated by plating transformants on histidine drop-out plates containing 25 mM 3-aminotriazole (18). Interaction positive cDNAs were rescued, and the DNA sequence of isolated clones was determined. Using the BLAST algorithm, eight independent clones consisting of cDNA inserts of varying lengths were identified as HHR23A, one of the human homologues of the yeast DNA repair protein Rad23 (24, 26, 27). Clone 15-2, containing the entire open reading frame of HHR23A, was subcloned into appropriate vectors and used in further analysis.

**Interaction of HHR23 Proteins with E6AP**—To confirm the interaction between HHR23A and E6AP, the HHR23A cDNA was cloned into pGEX-4T-2 vector and expressed as a GST fusion protein in bacteria. We also wanted to ascertain whether the second human homologue of yeast Rad23, HHR23B, which shares over 70% sequence homology with HHR23A, would interact with E6AP (24). Consequently, we obtained the HHR23B cDNA and expressed it as a fusion protein with GST in bacteria. Both HHR23 proteins were purified using glutathione-Sepharose beads and assayed for their ability to bind radiolabeled E6AP synthesized in vitro using wheat germ extract (which does not contain endogenous E6AP). Fig. 1A (lanes 2 and 3) shows that GST-HHR23A and GST-HHR23B are both capable of efficient interaction with E6AP. The GST portion alone failed to bind E6AP, serving as negative control (lane 1). One interesting feature of the HHR23 proteins is the presence of a ubiquitin-like region in their amino terminus (24, 34). This region is most likely an integral part of these proteins as it lacks the Gly-Gly sequence present in the carboxyl terminus of ubiquitin required for cleavage of ubiquitin peptides from linear molecules (3, 35). Hence, it is unlikely that the ubiquitin-like region of HHR23 proteins is cleaved or conjugated to other proteins in a manner similar to authentic ubiquitin. Since both of the HHR23 proteins can interact with E6AP and share the conserved ubiquitin-like region, we next determined whether this region was required for binding E6AP. HHR23A lacking the amino-terminal ubiquitin-like sequences could interact with E6AP with similar efficiency as WT-HHR23A (denoted by ++). The catalytic Hect domain of E6AP (E6AP-Hect) fails to interact with HHR23A (denoted by –). The unique amino-terminal region (E6APDHect) is sufficient for interaction with HHR23A, although its binding efficiency is weaker than WT-E6AP (denoted by +). Binding assays were performed as described in A. ND, not done.

![Fig. 1. A, interaction of HHR23 proteins with E6AP. E6AP was synthesized in WGE in the presence of [35S]methionine and incubated with glutathione-Sepharose-coupled GST or GST-HHR23 proteins for 3 h at 4°C. Samples were washed four times in binding buffer and resolved by SDS-PAGE. Both HHR23A and HHR23B efficiently bound in vitro synthesized E6AP (lanes 2 and 3). The GST moiety alone failed to show any interaction (lane 1), serving as negative control. The amount of E6AP bound to the GST-HHR23 proteins varied from 10 to 20% between experiments. B, schematic summary of HHR23A and E6AP interactions. HHR23A deleted of UbL (HHR23AΔUbL) can bind E6AP with similar efficiency as WT-HHR23A (denoted by ++). The catalytic Hect domain of E6AP (E6AP-Hect) fails to interact with HHR23A (denoted by –). The unique amino-terminal region (E6APDHect) is sufficient for interaction with HHR23A, although its binding efficiency is weaker than WT-E6AP (denoted by +). Binding assays were performed as described in A. ND, not done.](image-url)
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**Fig. 2.** E6AP-mediated ubiquitination and degradation of HHR23A and HHR23B. HHR23 proteins were synthesized in the presence of [35S]methionine using WGE. Lanes 1 and 5 show the total input amounts (5 μl) of HHR23A and HHR23B used in each reaction, respectively. Aliquots of translated products were incubated with ATP, ubiquitin, E1, and E2 (UbcH7 or UbcH8) (lanes 2–4 and 6–8) for 1.5 h at 30 °C. Addition of WT-E6AP (lanes 3 and 7) resulted in significant ubiquitination (evidenced by higher molecular weight bands) and degradation of both HHR23A and HHR23B. Addition of catalytically inactive E6AP mutant (lanes 4 and 8) had no such effect. The position of unmodified HHR23A and HHR23B is indicated by arrows.

| E1 | HHR23A | HHR23B |
|----|--------|--------|
|    | − + + + | − + + + |
| E2 (UbcH7) | − + + + | − + + + |
| E6AP (WT) | − − − + | − − − + |
| E6AP (C833A) | − − − + | − − − + |

E6AP-mediated Ubiquitination and Degradation of HHR23A and HHR23B—The interaction of HHR23 with E6AP raised the possibility that these proteins may be substrates for E6AP-mediated ubiquitination. We therefore examined the ability of E6AP to ubiquitinate HHR23A and HHR23B. The HHR23 proteins were synthesized in WGE in the presence of radiolabeled methionine and incubated with E1, E2 (UbcH7 or UbcH8) (18, 36), and ubiquitin, either in the absence of any E6AP or in the presence of the wild-type or the catalytically inactive mutant (C833A) of E6AP. As shown in Fig. 2 (lanes 2 and 6), incubation of HHR23A and HHR23B with E1, E2, and ubiquitin did not result in any ubiquitination. Upon the addition of wild-type E6AP, both proteins were significantly ubiquitinated as seen by the appearance of higher molecular weight bands (lanes 3 and 7). Degradation of the HHR23 proteins due to the presence of proteasome activity in WGE is also evident in these reactions. Addition of mutant E6AP (C833A), which retains efficient interaction with the HHR23 proteins, failed to promote their ubiquitination or degradation (lanes 4 and 8) indicating that the transfer of ubiquitin to these proteins requires catalytically active E6AP. These results clearly demonstrate that E6AP can induce the ubiquitination and consequent degradation of HHR23 proteins, and unlike p53, the presence of E6 is not required for this process. As such, HHR23 proteins are the first E6-independent substrates identified for E6AP.

Although the precise role of the ubiquitin-like region in the amino terminus of HHR23 proteins is not known, it has been reported for the yeast Rad23 protein that this region is required for its function in DNA repair (37). Our binding assays between E6AP and HHR23A revealed that the ubiquitin-like region of HHR23A is dispensable for interaction with E6AP (Fig. 1B). We therefore examined whether this region in HHR23A contributed to its ubiquitination by E6AP. In vitro synthesized HHR23A lacking the amino-terminal ubiquitin-like sequences showed greatly reduced ubiquitination in comparison to the wild-type protein (data not shown), indicating that the presence of the ubiquitin-like sequence is required for efficient ubiquitination. These data suggest that ubiquitination of HHR23 proteins in vivo may be important for their function in DNA repair.

**HHR23A Is Ubiquitinated in Vivo**—The finding that HHR23A could be efficiently ubiquitinated by E6AP in vitro prompted us to look for evidence of HHR23A ubiquitination in vivo. For this purpose, we raised polyclonal antibodies against HHR23A and confirmed reactivity of the sera against endogenous HHR23A from mammalian cells by immunoprecipitation and immunoblot analysis. Despite the high degree of sequence conservation between the two HHR23 proteins, anti-HHR23A antibodies did not cross-react with the HHR23B protein (data not shown) (24). Immunoprecipitation of HHR23A from mammalian cell extracts followed by immunoblot analysis with anti-ubiquitin monoclonal antibodies revealed a set of higher migrating bands that potentially represent ubiquitinated HHR23A species (Fig. 3A, right panel, lane 2). These bands (marked by asterisks) are undetectable in immunoprecipitates of preimmune serum (right panel, lane 1). In the left panel of Fig. 3A, the same immunoprecipitates were immunoblotted with anti-HHR23A antibodies (left panel, lanes 2 and 3) and, in addition to unmodified HHR23A, revealed an identical set of bands seen in the anti-ubiquitin blot (left panel, lane 3). Immunoprecipitation with preimmune serum failed to bring down either the unmodified HHR23A or the higher migrating bands (left panel, lane 2). Lane 1 of the left panel shows an immunoblot with anti-HHR23A antibodies without prior immunoprecipitation to indicate the position of endogenous HHR23A. Although the mobility of immunoglobulin heavy chains is similar to that of HHR23A (as seen in lanes 2 and 3 of the left panel), it is possible to detect unmodified HHR23A co-migrating with heavy chain (lane 3). Taken together, these results demonstrate that ubiquitinated species of endogenous HHR23A can be detected in vivo. Our results are consistent with the finding that the yeast Rad23 protein is also ubiquitinated in vivo (53). In both cases, however, only a minor fraction of the total HHR23A/Rad23 proteins could be detected in the modified form.

Effect of Wild-type and Mutant E6AP on HHR23A Ubiquitination in Vivo—In order to ascertain whether E6AP is involved in the in vivo ubiquitination of HHR23A, wild-type and mutant forms of E6AP were transfected into mammalian cells, and their expression was confirmed by immunoblot analysis with anti-E6AP antisera (32). HHR23A ubiquitination was detected by immunoprecipitation and immunoblot analysis with anti-HHR23A antibodies (Fig. 3B). Transient expression of wild-type E6AP resulted in an enhancement of HHR23A ubiquitination (Fig. 3B, compare lanes 1 and 2). In contrast, expression of the E6AP (C833A) catalytically inactive mutant, which retains efficient interaction with HHR23A, behaved in a dominant negative fashion resulting in decreased overall ubiquitination of HHR23A (compare lanes 1 and 2 with lane 3). The inhibitory effect of E6AP (C833A) mutant on HHR23A ubiquitination is consistent with its dominant negative effect on p53 stabilization when overexpressed in HPV-positive cells (32). Taken together, the enhancement of HHR23A ubiquitination by wild-type E6AP and the dominant negative effect of the
Ubiquitination of HHR23A by E6AP

Ubiquitination of HHR23A

A.

**Fig. 3.** A detection of ubiquitinated HHR23A in mammalian cells. Approximately 2 mg of whole cell extract from COS.7 cells was immunoprecipitated (IP) with preimmune sera (left panel, lane 2; right panel, lane 1) or anti-HHR23A antibodies (left panel, lane 3; right panel, lane 2) and immunoblotted with anti-ubiquitin monoclonal antibodies (right panel, lanes 1 and 2) or anti-HHR23A antibodies (left panel, lanes 2 and 3). Several higher molecular weight bands (marked by asterisks) potentially representing HHR23A-ubiquitin conjugates are visible in the anti-ubiquitin immunoblot (right panel, lane 2). The same bands are also detected by anti-HHR23A antibodies (left panel, lane 3). Immunoprecipitation with preimmune sera failed to bring down either HHR23A (left panel, lane 2) or HHR23A-ubiquitin conjugates (right panel, lane 1). The apparent molecular mass of unmodified HHR23A is approximately 55 kDa, and the protein co-migrates with immunoglobulin heavy chain (marked by heavy arrow on left). Unmodified HHR23A protein, absent in the anti-ubiquitin blot (right panel, lane 2), can be seen in the anti-HHR23A blot (marked by arrow on right, left panel, lane 3). Lane 1 of the left panel is an immunoblot of 100 µg of cell extract with anti-HHR23A antibodies (without prior immunoprecipitation) to show the position of endogenous HHR23A (marked by arrow on left). B. Effect of wild-type and dominant negative E6AP mutant on HHR23A ubiquitination. COS.7 cells were mock-transfected (lane 1) or transfected with 10 µg each of either WT-E6AP (lane 2) or the catalytically inactive mutant (C833A) E6AP mutant (lane 3). Cell lysates were prepared 60 h post-transfection. Expression of E6AP constructs was confirmed by immunoblotting with anti-E6AP antibodies (not shown). To detect HHR23A-ubiquitin conjugates, 2 mg of each whole cell extract was immunoprecipitated in RIPA buffer and immunoblotted with anti-HHR23A antibodies. Two predominant bands representing ubiquitinated HHR23A are visible in lane 1 (marked by asterisks on left). Expression of WT-E6AP results in enhancement of HHR23A ubiquitination as seen by the increased intensity of the same ubiquitinated bands and the appearance of additional higher molecular weight bands (lane 2). Expression of dominant negative E6AP (C833A) inhibits HHR23A ubiquitination as evidenced by the overall decreased intensity of bands in lane 3 (compare lane 3 with lanes 1 and 2). The blot was cut off at the position of Ig heavy chain to facilitate detection of HHR23A-ubiquitin conjugates. WB, Western blot.

**Fig. 4.** HHR23A protein level during cell cycle progression. A. U2OS cells were synchronized in M phase by nocodazole treatment, and mitotic cells were collected by shake-off and centrifugation. An aliquot representing cells in M phase was removed (lane 1), and equal numbers of remaining cells were replated for the indicated amounts of time (lanes 2–7). At the end of each time point, cells were rinsed once in phosphate-buffered saline and whole cell lysates prepared. 100 µg of extracts from each sample were subjected to SDS-PAGE, and HHR23A was detected by immunoblot analysis. Cell cycle stages indicated below are derived from cyclin A profile by immunoblotting the same samples with anti-cyclin A mAb (not shown). B. HHR23A and cyclin A levels were quantitated using Gel Plotting Macros feature of NIH-Image software. HHR23A protein levels were highest during M and early G1 (see Fig. 5A, lanes 1 and 2). A 4–5-fold decrease in HHR23A is seen 16 h post-mitotic shake (early S, lane 3) and at later time points (20 and 24 h; lanes 6 and 7) the protein levels show a gradual increase.

HHR23A Protein Levels Are Regulated during Cell Cycle Progression—Ubiquitination of HHR23A in vivo implies that HHR23A may be subject to degradation by the proteasome. However, since only a small fraction of total HHR23A can be detected in the modified form in mammalian cells, it is possible that either only a subset of HHR23A is degraded and/or that its proteolysis occurs at specific times during the cell cycle. Since the half-life of HHR23A in asynchronously growing cells exceeded 4–5 h (data not shown), we next investigated whether HHR23A degradation occurred only at specific stages of the cell cycle. Indeed, studies with a functional allele of yeast Rad23 have shown it to be a stable protein in stationary cultures and very unstable (with a 30-fold decrease in half-life) in actively growing cultures (38). Human U2OS cells were synchronized in M phase by nocodazole treatment (33), and after a shake-off step equal numbers of mitotic cells were plated for time course analysis. Cells were harvested every 4 h for 24 h, and equal amounts of cell extracts were immunoblotted for HHR23A. As shown in Fig. 4A, the protein level of HHR23A was found to be the highest during M phase and early G1 (4 h after release; lanes 1 and 2). A 4–5-fold decrease in HHR23A protein level was seen in late G1 and early S (12 and 16 h after release; lanes 4 and 5). At later time points after release HHR23A protein levels showed a gradual increase (20 and 24 h; lanes 6 and 7).

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3. C. Schauber, W. Potts, R. D. Kirkpatrick, R. D. Gietz, L. Chen, and K. Madura, submitted for publication.
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These results were quantitated using the NIH-IMAGE software and are represented graphically in Fig. 4B. Similar sets of results were obtained from GM6419 primary human fibroblasts. Three different cell synchronization methods in GM6419 cells, namely nocodazole treatment, contact inhibition, or serum starvation, all yielded identical profiles for HHR23A protein levels during the cell cycle (data not shown). Furthermore, we were able to exclude any effects of fluctuations in protein synthesis on the HHR23A protein profile shown in Fig. 4A by pulse-labeling cells for 20 min at each of the time points analyzed. Immunoprecipitation of HHR23A after pulse labeling did not show any difference in protein synthesis indicating that the decrease in HHR23A during late G1/early S phases of the cell cycle is attributable to protein degradation. These results are consistent with the detection of ubiquitinated forms of yeast Rad23 during G1/S transition.3

In order to establish a cell cycle profile for the time course analysis, cell extracts from each time point were immunoblotted with anti-cyclin A antibodies (40, 41). Cyclin A protein was undetectable in M phase (nocodazole-treated population) and in the 4- and 8-h time points after release. Twelve hours after release, low levels of cyclin A were detected, and the levels reached a peak at 16 and 20 h. At the 24-h time point cyclin A levels began to diminish (Fig. 4B). The cyclin A profile indicates that the 4- and 8-h time points correspond to G1, whereas the 12- and 16-h time points correspond to S phase. Lower cyclin A levels at the 24-h time point suggest that the cells had exited S and were in the G2/M phase of the cell cycle (40, 41).

Effect of Dominant Negative E6AP on the Cell Cycle-dependent Degradation of HHR23A—In order to investigate whether the observed degradation of HHR23A at G1/S transition involved E6AP, U2OS cells were transfected with wild-type or dominant negative (C833A) E6AP using FuGene transfection reagent. Twenty four hours post-transfection, cells were synchronized by nocodazole treatment (for 24 h). Cell extracts were prepared at the indicated times after mitotic shake and replating. HHR23A (above) and E6AP (below) were detected by immunoblot analysis with anti-HHR23A antibodies and anti-HA mAb, respectively. Expression of dominant negative E6AP resulted in complete inhibition of HHR23A degradation at the 16 h (G1/S) time point (compare lane 3 and 7). A faster-migrating band that cross-reacts with anti-HHR23A antibodies in immunoblots is denoted by an asterisk.

The decrease in HHR23A seen at 16 h after release (lane 3) was completely blocked by expression of dominant negative E6AP (lane 7). Transfection of wild-type E6AP resulted in a slight enhancement of HHR23A degradation (compare lanes 3 and 11). These results are consistent with the effects of wild-type and dominant negative E6AP on the ubiquitination of endogenous HHR23A (Fig. 3B) and provide direct evidence for the involvement of E6AP in the cell cycle-dependent regulation of HHR23A. Furthermore, the effects of wild-type and dominant negative E6AP on HHR23A protein levels do not result from altered cell cycle since expression of E6AP (wild-type or dominant negative) in U2OS cells does not affect the cell cycle profile (32). The faster migrating band seen below HHR23A (denoted by asterisk), cross-reacts with anti-HHR23A antibodies in immunoblots but not in immunoprecipitations.

Effect of UV Radiation on HHR23A Levels—Since HHR23A proteins have been implicated in the repair of UV-damaged DNA, we next examined the effect of DNA damage induced by UV radiation on HHR23A levels during cell cycle progression. After nocodazole shake-off and plating, U2OS cells were UV-irradiated (8 J) at 2 h after release. In contrast to the 3-fold decrease seen in HHR23A protein level at 16 h after release (Fig. 6, lanes 1–3; see graph), UV treatment completely blocked HHR23A degradation during the late G1/early S phases (Fig. 6, compare lanes 2 and 5). Since UV irradiation at a dose of 8 J does not cause a significant delay in cell cycle progression of the U2OS osteosarcoma cells, these results indicate that UV-induced DNA damage may stabilize the fraction of HHR23A that undergoes degradation at the G1/S boundary.

DISCUSSION

Xeroderma pigmentosum (XP) is a rare autosomal recessive disorder associated with a high incidence of sunlight (UV)-induced skin cancers (42). At least seven complementation groups (XP-A to XP-G) have been reported in humans (43). The primary defect in XP lies in the nucleotide excision repair (NER) subpathway responsible for eliminating various lesions in DNA, including UV-induced cyclobutane pyrimidine dimers and photoproducts (44–46). Based on sequence homology with yeast Rad23, the HHR23A proteins are believed to function in...
the nucleotide excision repair pathway (24, 27, 29). Yeast lacking the rad23 gene are UV-sensitive and exhibit a partial NER defect (37). Consistent with a role of HHR23 proteins in NER, HHR23B has been found to exist in a tight complex with the XPC protein in vivo (24). XPC-p125 has been identified as the affected gene in complementation group C of XP (43, 47, 48). Recently Sugasawa et al. (29) reported that both of the HHR23 proteins can stimulate XPC repair activity and are functionally interchangeable in complex formation with XPC-p125. Their results demonstrate a direct involvement of HHR23 proteins in NER reactions through interaction with XPC-p125.

We isolated HHR23A in a yeast two-hybrid screen with E6AP as bait. By using in vitro binding assays, we demonstrate that HHR23A, as well as the second human homologue of yeast Rad23, HHR23B, interact with E6AP. The conserved Hect domain of E6AP, which is required and sufficient for interaction with specific E2s (18), is dispensable for binding HHR23A. The amino terminus of E6AP lacking the Hect domain could interact with HHR23A suggesting that the unique amino-terminal sequences in E6AP confer substrate specific binding properties. We also examined the role of the ubiquitin-like region conserved in both HHR23 proteins in E6AP binding. Deletion of the ubiquitin-like region of HHR23A had no effect upon its ability to interact with E6AP suggesting that other conserved sequences in HHR23 proteins may be involved in this interaction.

The interaction of HHR23A with the amino terminus of E6AP led us to examine whether HHR23A is a substrate for E6AP-mediated ubiquitination. By using partially reconstituted in vivo ubiquitination assays, we demonstrate that both HHR23 proteins are efficiently ubiquitinated and degraded in the presence of wild-type E6AP but not in the presence of a catalytically inactive mutant. Since HHR23A lacking the ubiquitin-like region could still interact with E6AP, we investigated the role of this region in ubiquitination. HHR23A deleted of its ubiquitin-like region was very poorly ubiquitinated compared with full-length HHR23A indicating a requirement for this ubiquitin-like region was very poorly ubiquitinated compared with full-length HHR23A indicating a requirement for this region in the efficient ubiquitination and degradation of HHR23A in vitro. The ubiquitin-like region present in yeast Rad23 has been shown to be required for its function in DNA repair (37). Replacement of the wild-type rad23 gene with a mutant version missing the ubiquitin-like region results in UV sensitivity (37). Taken together, our data suggest that ubiquitination of HHR23 proteins may play a role, either directly or indirectly, in their NER function.

Yeast Rad23 has been reported previously to undergo ubiquitination in vivo (37). By using anti-HHR23A and anti-ubiquitin antibodies, we demonstrate that a small fraction of endogenous HHR23A can be detected as ubiquitinated species in cells. Transient expression of wild-type E6AP enhanced HHR23A ubiquitination, whereas a dominant negative mutant inhibited this process. These results demonstrate the direct involvement of E6AP in HHR23A ubiquitination in vivo. Unlike p53, the E6 oncoprotein of HPV is not required for E6AP-mediated ubiquitination of HHR23A. However, it is not clear at this stage whether the presence of E6 may affect HHR23A ubiquitination in cells, either positively or negatively.

Although HHR23A ubiquitinated forms can be found in vivo, by pulse-chase analysis the protein appears to be stable in asynchronously growing cells. As proteolysis is a common consequence of ubiquitin conjugation, this observation suggests that perhaps only a subset of HHR23A undergoes degradation in cells, and/or its degradation occurs at specific stages of the cell cycle. The detection of only a fraction of endogenous HHR23A as ubiquitinated species is consistent with the above possibilities. In addition, the finding that yeast Rad23 exhibits a greatly reduced half-life in actively growing cultures compared with stationary cultures further supports this hypothesis (38).

Our time course analysis of HHR23A protein level in synchronized cells revealed that only a fraction of HHR23A is degraded at the G1/S boundary. In agreement with this observation, a small fraction of yeast Rad23 has been shown to undergo ubiquitination at the beginning of S phase. At present, the reason for HHR23A degradation at G1/S transition is unclear. It is possible that HHR23A degradation may serve in part as a signal that indicates completion of repair such that cells can exit G1 and enter S phase. Furthermore, proteolysis of HHR23A at the G1/S boundary may result in disassembly of repair complexes, thereby allowing access to DNA by proteins involved in DNA synthesis. This is consistent with the observation that yeast Rad23 facilitates complex formation between at least two components of the repair machinery, multisubunit transcription/repair factor TFIH and Rad14, a UV-specific DNA damage recognition protein (49, 50). Disassembly of repair complexes at the beginning of S phase may also increase the availability of factors common to NER and replication (such as replication protein A and proliferating cell nuclear antigen) for DNA synthesis.

The existence of HHR23B poses additional questions with regard to the regulation of HHR23 proteins. Although HHR23A and HHR23B are expressed in the same cells (24), it is not yet known whether the two proteins have a redundant function and are regulated similarly in vivo. The only other example of duplication of genes involved in the DNA repair and ubiquitin pathways comes from the existence of two mammalian homologues of yeast Rad6. Gene inactivation experiments in mice have indicated functional redundancy between the two proteins (51). Ubiquitination of HHR23B by E6AP in vitro suggests that HHR23B may also undergo ubiquitination in cells; however, our studies have not yet addressed this possibility. In additional, Sugasawa et al. (29) have recently demonstrated that the two HHR23 proteins behave similarly in stimulating XPC repair activity in vitro. Taken together, these studies suggest that at least certain aspects of HHR23 function may be redundant and subject to similar regulatory controls.

It has been reported previously that only a small fraction of HHR23B protein can be found in a complex with the XPC protein p125 in cells (24, 52, 53). This observation has led to the hypothesis that HHR23 proteins may have other functions in addition to a role in DNA repair (52, 53). This is further supported by genetic evidence in yeast demonstrating the involvement of Rad23 in spindle pole body duplication and cell cycle progression (25). Our results show that not all of the HHR23A undergoes degradation during cell cycle progression, suggesting perhaps that only the fraction of HHR23A that is involved in NER (29, 54) may be targeted for degradation. Preliminary experiments aimed at investigating this possibility suggest that HHR23A remains stable throughout the cell cycle in repair-deficient XPC cells that lack the functional p125/XPC protein (43, 47, 48). Since the overall level of HHR23 proteins in cells is much higher than XPC-p125 and only a fraction of HHR23B is found in complex with XPC-p125 (24), these results would be consistent with a model in which only the pool of HHR23 proteins involved in NER may be targeted for proteolysis. As noted earlier, complex formation between XPC and HHR23B proteins has been reported to stimulate XPC repair activity (29). As such, the degradation of HHR23A at specific stages of the cell cycle may result in the regulation of NER activity during cell cycle progression.

Treatment of cells with UV radiation is known to induce a delay in the onset of DNA synthesis in order to allow time for
the repair of DNA lesions (55). It therefore seems reasonable to postulate that, in the presence of DNA damage, HHR23 proteins may be stabilized to ensure uninterrupted repair. Indeed, UV-induced DNA damage inhibited HHR23A proteolysis, indicating that the presence of DNA lesions results in stabilization of HHR23A.

Additional evidence for the involvement of HHR23A in the regulation of cell cycle progression and a link between DNA repair and cell cycle comes from studies on the Vpr protein of HIV-1. Expression of Vpr in cells has been reported to cause an arrest at the G2/M phase of the cell cycle (56). It has been proposed that Vpr may interfere with signaling events involved in DNA repair in order to induce cell cycle arrest. Recently, Withers-Ward et al. (39) have shown that Vpr co-localizes and physically interacts with HHR23A and that Vpr-mediated cell cycle arrest can be alleviated by overexpression of HHR23A. Although the precise mechanism by which HHR23A may function in cell cycle control is unclear at present, our results suggest that HHR23A degradation at the G2/S boundary may be an important signal that provides a link between DNA repair and the initiation of DNA synthesis. Future studies aimed at understanding the regulation of HHR23A degradation should be useful in elucidating its potential role in coordinating DNA repair and cell cycle progression.

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