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Reconstitution of p53–ubiquitinylation reactions from purified components: The role of human ubiquitin-conjugating enzyme UBC4 and E6-associated protein (E6AP)

(human papilloma virus/cervical cancer/degradation)

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ABSTRACT **The E6 protein of the high-risk human papillomaviruses inactivates the tumor suppressor protein p53 by stimulating its ubiquitinylation and subsequent degradation. Ubiquitinylation is a multistep process involving a ubiquitin-activating enzyme, one of many distinct ubiquitin-conjugating enzymes, and in certain cases, a ubiquitin ligase. In human papillomavirus-infected cells, E6 and the E6-associated protein are thought to act as a ubiquitin–protein ligase in the ubiquitinylation of p53. Here we describe the cloning of a human ubiquitin-conjugating enzyme that specifically ubiquitinylates E6-associated protein. Furthermore, we define the biochemical pathway of p53 ubiquitinylation and demonstrate that *in vivo* inhibition of various components in the pathway leads to an inhibition of E6-stimulated p53 degradation.**

The p53 protein is a key regulator of mammalian cell growth and its gene is frequently mutated in a wide range of human tumors (1). Furthermore, many DNA tumor viruses encode viral antigens that inactivate p53 (2). The high-risk human papillomaviruses (HPVs) 16 and 18 are strongly implicated in the pathogenesis of cervical carcinoma (3). These viruses encode two transforming proteins, E6 and E7, that target the cellular growth regulators p53 and pRb, respectively (4, 5). The mode of inactivation of p53 by E6 has been determined (6). Viral E6 and a cellular E6-associated protein (E6AP) combine to stimulate the ubiquitinylation of p53 and, thus, target p53 for degradation. In this reaction, E6 and E6AP are thought to be providing a ubiquitin ligase or E3-like activity (7).

The human ubiquitin-conjugating enzyme (UBC) (E2) involved in p53 ubiquitinylation has not been molecularly characterized, although a biochemical fraction from rabbit reticulocytes has been shown to contain a UBC capable of supporting E6-stimulated p53 turnover (7, 8). We have cloned a human UBC* and here define its biochemical role in the ubiquitinylation of p53. Surprisingly, the enzyme specifically ubiquitinylates the E6AP. We demonstrate that ubiquitinylated E6AP is the ultimate donor of ubiquitin to p53 in an E6-stimulated reaction. We also show that specific *in vivo* inhibition of the biochemical components involved in p53 ubiquitinylation leads to an inhibition of E6-mediated p53 turnover.

MATERIALS AND METHODS

Cloning of hUBC4. Two pairs of PCR primers were used in a reverse transcription–PCR approach to clone UBCs from HeLa poly(A)$^+$ RNA. Pair 1 was GCGCGCAAGCTTTAYGARGGWGGWGTYTTYTT and GCGCGCGAATTCACNGCRTAYTTYTTNGTCCCAYTC, where Y is T or C, R is A or G, W is A or T, and N is A, C, G, or T (see ref. 9). Pair 2 was GCGCGCAAGCTTCCNGTNGGNGAYGAYTTRTTYCAYTGGCA and GCGCGCGAATTCATNGTNARNGCNGGCGACCA. Pair 1 primers gave rise to a 310-bp fragment and pair 2 primers produced a 230-bp fragment. The fragments were cloned, sequenced, and used to screen a HeLa cDNA library for isolation of the full-length cDNA.

Protein Expression and Purification. Native UBC4 was expressed from *Escherichia coli* BL21(DE3) using an isopropyl β-D-thiogalactoside-inducible T7 promoter plasmid and purified. UBC4-containing *E. coli* cell pellets were suspended in PBS containing 1 mM EDTA, 1 mM dithiothreitol (DTT), leupeptin (1 μg/ml), aprotinin (1 μg/ml), soybean trypsin inhibitor (10 μg/ml), 0.2 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride, and lysozyme (0.1 mg/ml). The suspension was incubated at 4°C for 1 h and then sonicated in three 45-sec bursts. The lysate was centrifuged at 15,000 rpm in a JA20 rotor for 30 min. The supernatant was gel-filtered over Superdex 200 26/60 that had been preequilibrated in PBS containing 1 mM DTT and 1 mM EDTA. The cleanest 17-kDa fractions (as judged by SDS/gel electrophoresis) were pooled, diluted 1:3 with 20 mM Tris·HCl (pH 8.5), and applied to a Mono Q column preequilibrated with the same buffer. UBC4 passes through Mono Q column while the major contaminant is retained.

Native p53 was expressed from the baculoviral vector pVL1392 in Sf9 insect cells by following the manufacturer's instructions (PharMingen) and purified on a p53 affinity column. Insect-cell pellets were suspended in lysis buffer containing 50 mM Tris·HCl (pH 7.5), 150 mM NaCl, 10% (vol/vol) glycerol, 1 mM DTT, 1 mM Na_3VO_4, leupeptin (1 μg/ml), aprotinin (1 μg/ml), soybean trypsin inhibitor (10 μg/ml), and 0.1 mM phenylmethylsulfonyl fluoride. Cells were sonicated for 30 sec to achieve lysis. The lysate was centrifuged at 15,000 rpm in a JA20 rotor for 30 min. A p53-specific monoclonal antibody (DO-1) was cross-linked to protein A-Sepharose beads. Such beads were packed into a column that was subsequently blocked with 10% (wt/vol) bovine serum albumin in PBS and equilibrated with PBS containing 10% glycerol. The p53-containing supernatant was applied to the column. The column was washed extensively with PBS/10% glycerol. p53 was eluted with 50 mM glycine hydrochloride, pH 3.0/150 mM NaCl/10% glycerol. Samples were immediately neutralized with 100 mM Tris·HCl (pH 7.5). HPV-18 E6 was expressed in *E. coli* BL21 as a glutathione *S*-transferase (GST) fusion protein and purified on glutathione (GSH)-Sepharose. Human E1 was cloned by PCR from the published cDNA

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Abbreviations: E6AP, E6-associated protein; DTT, dithiothreitol; GST, glutathione *S*-transferase; GSH, glutathione; HPV, human papillomavirus; UBC, ubiquitin-conjugating enzyme.

*The sequence reported in this paper has been deposited in the GenBank data base (accession no. L40146).

sequence (10) and native protein was expressed and purified from baculoviral-infected cells. E6AP was expressed in *E. coli* JM109 as a GST fusion protein and purified on GSH-Sepharose (pGEX.E6AP was a generous gift from Peter Howley, Harvard Medical School, Boston).

Ubiquitinylation Reactions. Ubiquitinylation reaction mixtures contained 50–200 ng of the indicated proteins in 50 mM Tris·HCl, pH 7.5/5 mM $MgCl_2$, 2 mM adenosine 5′-[γ-thio]triphosphate/0.1 mM DTT/5 μM ubiquitin. Total reactions (30 μl) were incubated at 25°C for 3 h and then reaction products were loaded on an 8% SDS gel for analysis of p53 ubiquitinylation or a 4–20% gradient gel for analysis of ubiquitinylation of the UBCs and E6AP. The gels were electrophoresed and proteins were electrophoretically transferred to nitrocellulose. p53 proteins were detected with the monoclonal antibody DO-1 (Oncogene Science) and the ECL system from NEN. Ubiquitinylated proteins were visualized using Extravidin–horseradish peroxidase from Sigma and the ECL system from NEN.

Microinjection. MDA-MB-468 cells growing on glass coverslips (at ≈60% density) were microinjected with an automated microinjection system (AIS, Zeiss; ref. 11). All microinjection experiments were carried out in 3.5-cm Petri dishes containing 3 ml of carbonate-free Dulbecco's modified Eagle's medium (DMEM) to avoid a decrease in pH of the medium during the injection. The computer settings for injection were as follows: angle, 45; speed, 10; time of injection, 0.0 sec. Each cell was injected with the indicated vectors at a pressure between 50 and 150 hPa (for more details on the microinjection procedure, see ref. 12). The cytomegalovirus expression vectors were obtained by inserting the indicated cDNAs, in a sense or antisense orientation, in the pX plasmid (13). Plasmids were purified with a Promega Wizard maxiprep kit and injected at 100 μg/ml in the presence of a microinjection marker (affinity-purified normal rabbit antibody at 3 mg/ml in PBS). Twenty-four hours after microinjection, coverslips were rinsed in PBS, fixed for 10 min at −20°C in cold methanol/acetone, 1:1 (vol/vol), and washed again three times with PBS. Cells were incubated for 1 h with a monoclonal antibody to p53 (14), purchased from Oncogene Science and diluted 1:1000. After three washes in PBS, the coverslips were incubated for 30 min with biotinylated horse anti-mouse secondary antibody (Vector Laboratories, dilution 1:50), washed three more times with PBS, and incubated with Texas red-conjugated streptavidin (Vector Laboratories, dilution 1:100) and fluorescein isothiocyanate-conjugated goat anti-rabbit antibody (Vector Laboratories, dilution 1:50). All antibody reactions were carried out in a humidified chamber at room temperature and dilutions were made in DMEM containing 10% (vol/vol) fetal calf serum. Counterstaining for DNA was performed by adding bisbenzimide (Hoechst Pharmaceuticals 33258) at 1 μg/ml into the final PBS wash. Immunofluorescence samples were directly mounted in Crystal/mount medium (Biomed). Photographs were taken by using a Plan Neofluar ×40 lens mounted on a Zeiss Axiophot photomicroscope and printed with a Mavigraph color video printer on Sony UPC-3010 print paper.

RESULTS

Cloning of hUBC4. By using a PCR-based approach, we isolated a HeLa-cell cDNA fragment encoding a protein with high sequence homology to UBCs (E2) (Fig. 1). This human E2 appears to be a member of the UBC4 class of enzymes that is responsible for the turnover of short-lived cellular proteins (15). The predicted human protein is most similar to the UBC4 proteins from *Drosophila melanogaster* (9) and *Caenorhabditis elegans* (16), sharing ≈94% identity. It is also similar to the *Saccharomyces cerevisiae* (17) UBC4 protein, sharing 78% sequence identity.

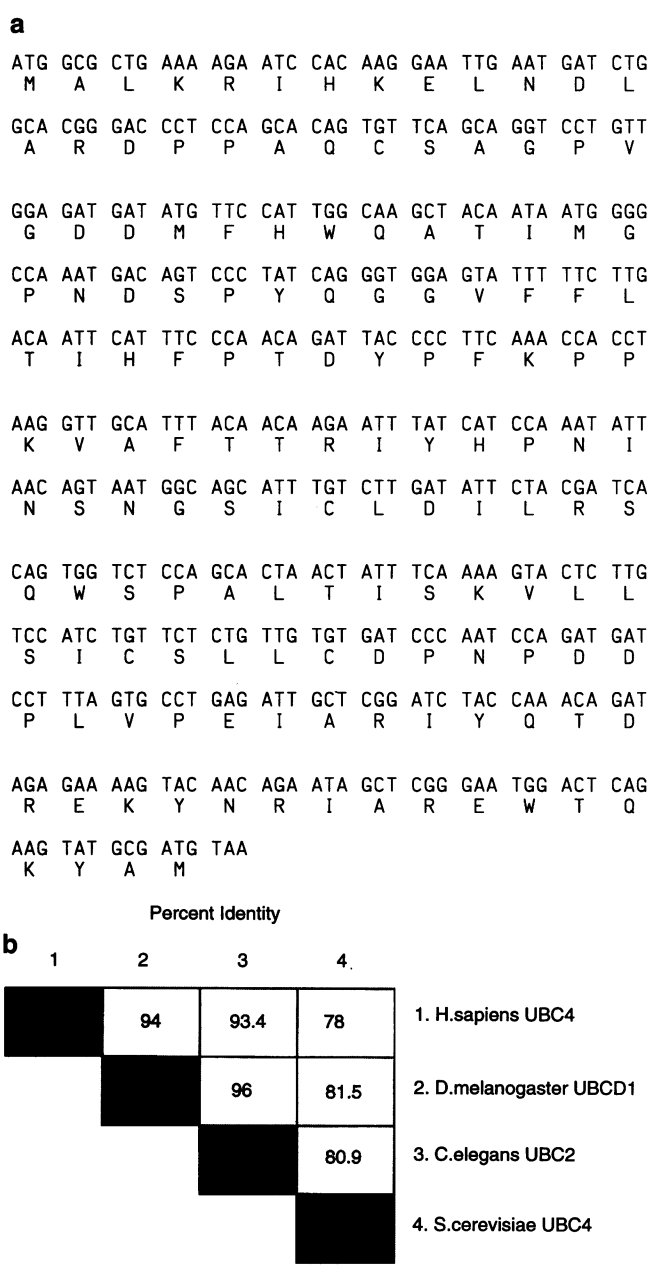

FIG. 1. (*a*) Nucleotide and predicted amino acid sequence of human UBC4. (*b*) Homology matrix of human UBC4 and "UBC4-like" genes from *D. melanogaster, C. elegans*, and *S. cerevisiae.*

***In Vitro* Ubiquitinylation of p53.** A full-length hUBC4 cDNA was isolated from a λZAP library and the entire open reading frame was cloned into a bacterial expression vector for production of the full-length native protein. Soluble protein was purified from *E. coli* and used in an *in vitro* ubiquitinylation reaction (Fig. 2). The appearance of specific p53–ubiquitin conjugates was demonstrated to require this E2, HPV-18 E6, E6AP, ubiquitin, and E1, the ubiquitin-activating enzyme.

A cDNA encoding a second human UBC, UBC2, the human homolog of the *S. cerevisiae* DNA repair gene Rad6 (18), was also cloned and the protein was expressed and purified from *E. coli.* This protein was active in a minimal conjugation reaction containing E1, ATP, and ubiquitin, in that E1 could activate ubiquitin and transfer it onto UBC2 (see below). However, UBC2 could not substitute for UBC4 in the p53 conjugation reaction (Fig. 2). We made an active-site Cys → Ser mutation in UBC4. Such active-site E2 mutants should accept activated ubiquitin from E1 but should not ubiquitiny-

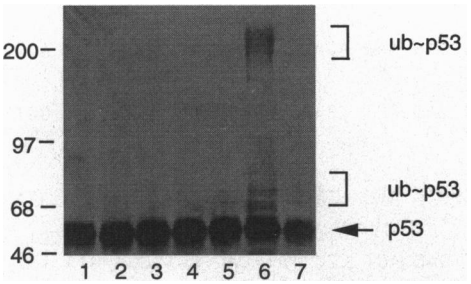

FIG. 2. UBC4-dependent ubiquitinylation of p53. A complete ubiquitinylation reaction mixture shown in lane 6 contained E1, UBC4, E6, E6AP, p53, and ubiquitin. The following changes were made. Lanes: 1, no E6; 2, no E6AP; 3, UBC2 replaces UBC4; 4, no E1; 5, no ubiquitin; 7, mutant UBC4 replaces wild-type UBC4. ub~p53, Ubiquitinylated p53.

late their downstream substrates due to the high stability of the ester linkage formed between the active-site serine and the C terminus of ubiquitin. This mutant was inactive in the p53 conjugation reaction (Fig. 2, lane 7). These results demonstrate that a catalytically active UBC4 is absolutely required for generation of ubiquitinylated p53 in this *in vitro* system.

The Pathway of Ubiquitin Transfer to p53. We wished to dissect the pathway of ubiquitin transfer in the ubiquitinylation of p53. Scheffner *et al.* (7) had proposed that E6–E6AP complex acted as a ubiquitin protein ligase in the p53 ubiquitinylation pathway. We wanted to investigate the possibility that UBC4 could directly ubiquitinylate E6AP since we had failed to detect a direct interaction between UBC4 and p53 *in vitro* (data not shown). We carried out ubiquitinylation reactions using the purified recombinant proteins described in Fig. 2. We used biotinylated-ubiquitin and Extravidin-horseradish peroxidase to allow the detection of all ubiquitinylated intermediates generated in these reactions.

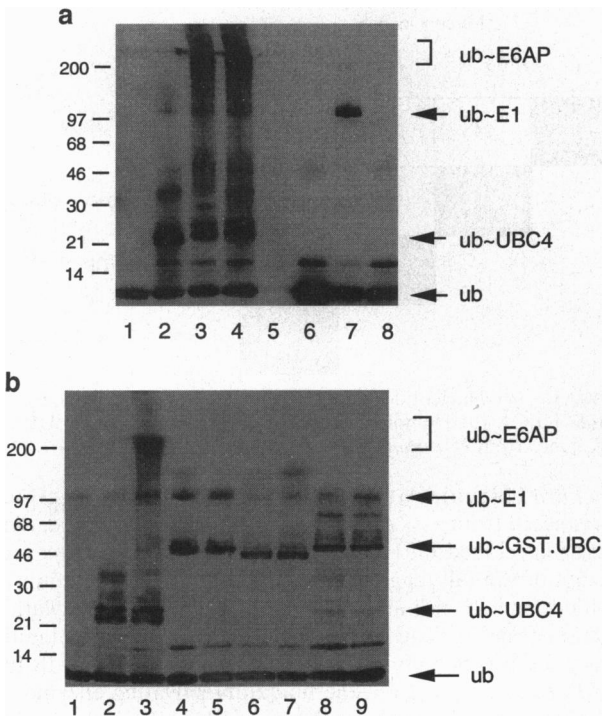

FIG. 3. (*a*) Ubiquitinylation of E6AP. Purified proteins were used in ubiquitinylation reactions containing biotinylated ubiquitin. Lanes: 1, ubiquitin; 2, E1, ubiquitin, and UBC4; 3, E1, ubiquitin, UBC4, and E6AP; 4, E1, ubiquitin, UBC4, E6AP, and E6; 5, E1, UBC4, E6AP, and E6; 6, ubiquitin, UBC4, and E6AP; 7, E1, ubiquitin, and E6AP; 8, ubiquitin and E6AP. (*b*) UBC4-specific ubiquitinylation of E6AP. All lanes contained E1 and ubiquitin with the following additions: Lanes: 1, nothing; 2, UBC4; 3, UBC4 and E6AP; 4, GST–UBC8; 5, GST–UBC8 and E6AP; 6, GST–UBC2; 7, GST–UBC2 and E6AP; 8, GST–epiUBC; 9, GST–epiUBC and E6AP. ub, Ubiquitin; ub~, ubiquitinylated.

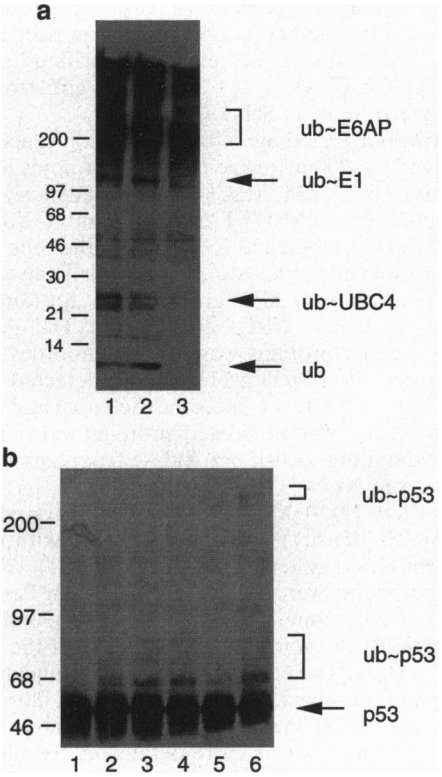

FIG. 4. (*a*) Purification of ubiquitinylated E6AP. Lanes: 1, E1, ubiquitin, UBC4, and E6AP; 2, as lane 1 and unbound by GSH-Sepharose; 3, as lane 1 and bound to GSH-Sepharose (10 μl loaded). (*b*) Purified ubiquitinylated E6AP can donate ubiquitin to p53 in an E6-dependent reaction. All lanes contained 50 ng of p53. Lanes: 1, 10 μl of ub~E6AP and no E6; 2, 1 μl of ub~E6AP and 50 ng of E6; 3, 5 μl of ub~E6AP and 50 ng of E6; 4, 10 μl of ub~E6AP and 50 ng of E6; 5, 20 μl of ub~E6AP and 50 ng of E6; 6, complete *in vitro* reaction mixture (as in Fig. 2, lane 6). ub, Ubiquitin; ub~, ubiquitinylated.

In Fig. 3*a*, we show that ubiquitinylated E1 could transfer ubiquitin efficiently to UBC4 but not directly to E6AP and that ubiquitinylated UBC4 transferred ubiquitin to E6AP in a reaction that was not further stimulated by E6. All of these ubiquitinylation reactions required the presence of the ubiquitin-activating enzyme E1 and ubiquitin.

To address the issue of the specificity of UBC4-mediated ubiquitinylation of E6AP, we performed ubiquitinylation reactions with purified recombinant UBC4, GST–UBC2, GST–UBC8 (19), and a GST fusion of the so-called epidermal UBC (20). Each of these recombinant proteins could accept activated ubiquitin from E1, but only UBC4 could donate ubiquitin to E6AP (Fig. 3*b*). We also confirmed that native UBC2 could accept ubiquitin from E1 but could not donate ubiquitin to E6AP (data not shown).

We then purified the ubiquitinylated GST–E6AP by affinity chromatography on GSH-Sepharose and demonstrated that it did not contain appreciable amounts of ubiquitinylated E1, ubiquitinylated UBC4, or free ubiquitin (Fig. 4*a*). Furthermore, upon reprobing of the gel shown in Fig. 4*a* with antisera specific for UBC4 and E1, no immunoreactivity was detectable in lane 3 (data not shown). A band with slightly faster mobility than band ub~E1 is evident in lane 3 but is missing from lanes 1 and 2. This is monoubiquitinylated GST–E6AP and becomes apparent in lane 3 as a result of the substantial enrichment achieved during the GSH-Sepharose purification step. The

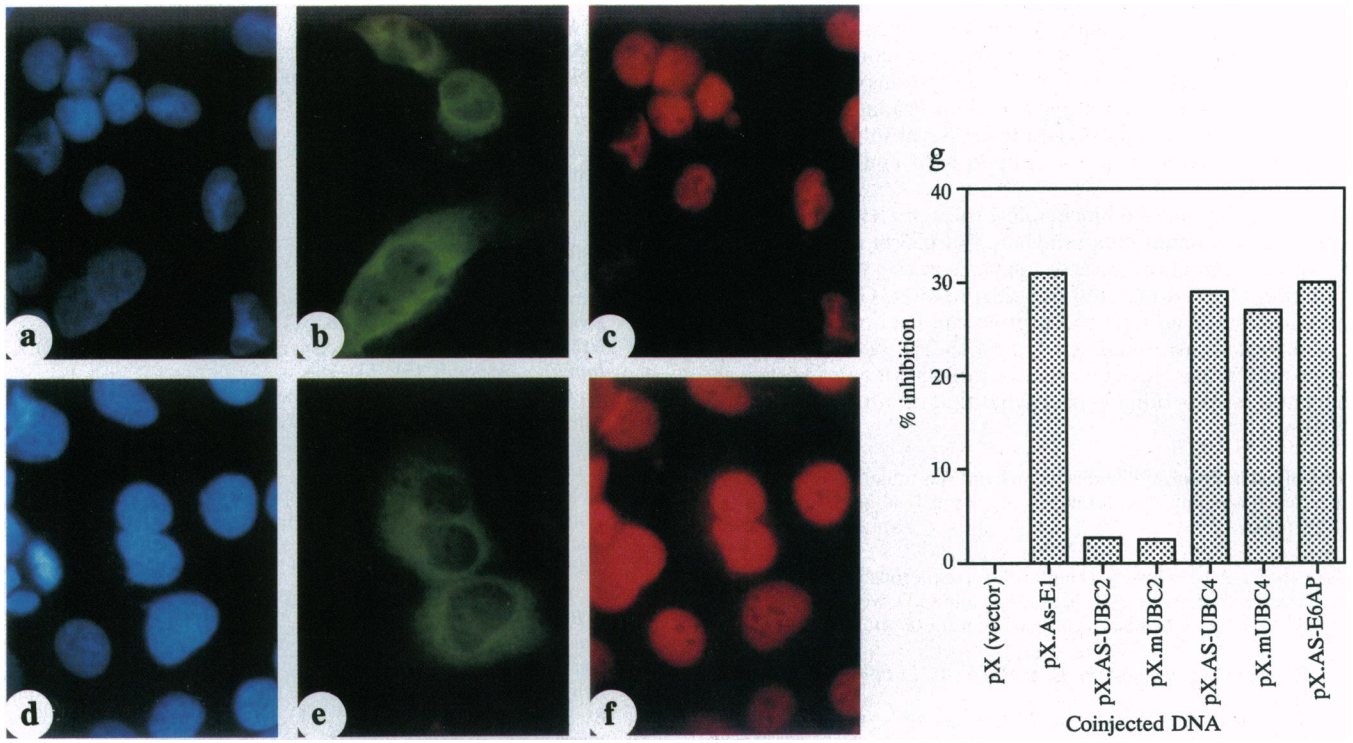

FIG. 5. (*a–f*) Inhibition of E6-stimulated p53 degradation by mUBC4. Immunofluorescence analysis of microinjected cells. (*a–c*) Cells were injected with pX.E6. (*d–f*) Cells were injected with pX.E6 and pX.mUBC4. (*a* and *d*) Stained with Hoechst. (*b* and *e*) Stained with an anti-rabbit IgG to reveal injected cells. (*c* and *f*) Stained with the p53-specific monoclonal antibody DO-1. pX.E6 injection led to a disappearance of p53 staining in 80–90% of the injected cells. (*g*) Inhibition of E6-stimulated p53 degradation in coinjection experiments. The indicated DNAs were coinjected with pX.E6. The levels of inhibition of the E6-stimulated p53 degradation are derived from an analysis of ≈150 injected cells per experimental point in at least two experiments. To calculate a percent inhibition, we carefully counted the number of injected cells and then counted those injected cells that stained positively for p53. Thus if 150 cells were injected with pX and pX.E6, typically only 15–30 could be stained for p53 24 h later. Coinjection with antisense UBC2 had a minimal effect on the pX.E6-stimulated p53 turnover with 20–35 injected cells staining for p53. Coinjection with pX.ASUBC4, pX.ASE1, pX.ASE6AP, and pX.mUBC4 had a substantial effect on the E6-stimulated p53 turnover with 60–70 cells staining for p53. The percent inhibition is calculated as follows: 100 − {[(no. cells injected with pX.E6 and 2nd plasmid − no. p53-positive cells)/(no. cells injected with pX.E6 − no. p53-positive cells)] × 100}.

purified ubiquitinylated GST–E6AP could donate ubiquitin to p53 in an E6-dependent reaction, albeit inefficiently (Fig. 4*b* and see *Discussion*).

Inhibition of p53 Turnover *in Vivo*. To investigate the consequences of interfering with UBC4 and E6AP function *in vivo*, DNA microinjection experiments were carried out. To facilitate the detection of p53 by indirect immunofluorescence, the experiments were performed in the human tumor cell line MDA-MB-468, which contains high levels of mutant p53(Arg273His). In this line, the degradation of p53 could be stimulated by microinjection of an HPV-18 E6 expression plasmid (Fig. 5).

To determine whether UBC4 and E6AP mediate the E6-dependent ubiquitinylation and degradation of p53 *in vivo*, coinjection experiments were performed. When either an antisense or mutant UBC4 (Fig. 5*g*) expression plasmid or an expression plasmid encoding antisense E6AP was coinjected with the E6 expression plasmid, the E6-stimulated degradation of p53 was inhibited (Fig. 5*g*). Similar results were obtained when polyclonal antibodies generated against human UBC4 or an expression plasmid encoding a mutant form of E6AP (21) were microinjected (data not shown). Coinjection of an E6 expression plasmid with an expression plasmid encoding antisense E1 also inhibited the E6-stimulated degradation of p53. Coinjection of antisense or mutant UBC2 expression plasmids had a negligible effect on the E6-stimulated degradation of p53 (Fig. 5*g*).

DISCUSSION

We have described the cloning of a human UBC that specifically ubiquitinylates E6AP and is involved in the turnover of p53 *in vivo*. We have defined several discrete biochemical steps in the activation and transfer of ubiquitin onto p53. These biochemical reactions provide two levels of specificity in the ubiquitinylation of p53, the UBC4-dependent ubiquitinylation of E6AP, and the E6-dependent transfer of ubiquitin from ubiquitinylated E6AP to p53. These results confirm and extend those of Scheffner *et al.* (7) who demonstrated an E3-like function for E6 and E6AP in the ubiquitinylation of p53. The only other molecularly cloned E3 is the *UBR1* gene in *S. cerevisiae*, whose protein product is involved in recognition of substrates that are degraded via the N-end rule pathway (22). There is no evidence that the Ubr1 protein forms a thioester with ubiquitin. Our findings propose an enzymatic activity for an E3 protein, but the generality of this observation must await characterization of other members of this family of proteins.

We could only generate low levels of mono-, di-, and tri-ubiquitinylated p53 when we used purified ubiquitinylated E6AP and E6 in the p53 conjugation reactions (Fig. 4*b*, lanes 2–5). Despite the fact that the reactions carried out with purified components are somewhat inefficient (Figs. 2, lane 6, and 4*b*, lane 6), transfer from the purified ubiquitinylated E6AP to p53 to generate these low molecular weight p53 conjugates did indeed occur and was as efficient as the generation of the low molecular weight conjugates in a total *in vitro* reaction.

Inhibition of UBC4 and E6AP enzyme function *in vivo* causes an inhibition of E6-stimulated p53 degradation. The level of inhibition achieved in the microinjection experiments was 25–30%. This may be a consequence of not every injected cell achieving high-level expression of the injected construct,

a phenomenon we have noted before in many different systems (A.T. and M.P., unpublished). It may also suggest that there is some redundancy in the cellular ubiquitin conjugation machinery or that the intracellular concentrations of E1, UBC4, and E6AP are not rate-limiting for p53 degradation in the cell line used. All of our data suggest that E6 is absolutely required for ubiquitinylation of p53 in our *in vitro* and *in vivo* assay systems.

We have defined the biochemical roles of UBC4 and E6AP in the E6-stimulated ubiquitinylation of p53 *in vitro* and have shown that inhibition of these enzymes *in vivo* can lead to an inhibition of E6-stimulated p53 degradation. Given that elevated levels of wild-type p53 protein can lead to apoptosis in a variety of transformed cell types (23–25), both UBC4 and E6AP may be attractive therapeutic targets not only in cervical cancer but also in other cancer types that retain wild-type p53 protein.

Note Added in Proof. While this manuscript was under review, a paper appeared describing the cloning of a gene that is highly related to UBC4 (26).

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