A novel member of the ubiquitin carrier protein family, designated E2EF, has been cloned by our laboratory and expressed in a bacterial system in an active form. Ubiquitin carrier proteins, or E2s, catalyze one step in a multistep process that leads to the covalent conjugation of ubiquitin to substrate proteins. In this paper, we show that recombinant E2EF catalyzes auto/multiubiquitination, the conjugation of multiple ubiquitin molecules to itself. Multiubiquitination has been shown previously to be required for targeting of a substrate protein for rapid degradation. Using a rabbit reticuloocyte lysate system, E2EF was shown to support the degradation of a model substrate in an ATP- and ubiquitin-dependent fashion. In contrast to a previous study which showed that selective protein degradation in one system is dependent upon multiubiquitination via the lysine 48 residue of ubiquitin, multiubiquitination, and proteolytic targeting by E2EF was shown here to be independent of the lysine 48 multiubiquitin linkage. This functional characterization of E2EF revealed a combination of features that distinguishes this enzyme from all previously characterized members of the ubiquitin carrier protein family. These results also suggest several possible autoregulatory models for E2EF involving auto- and multiubiquitination.

Ubiquitin, a highly conserved 76-amino acid polypeptide, is present in all eukaryotic cells either in a free state or covalently conjugated to various cellular proteins (1, 2). Ub-protein conjugates are formed through an isopeptide linkage between the carboxy-terminal glycine residue of ubiquitin and ε-amino groups of substrate proteins (3, 4). Ub-protein conjugates typically account for about 50% of total cellular ubiquitin (5) and have been localized to various cellular compartments including the cytosol (6), nucleus (7), cell surface (8), and mitochondrion (6). Ubiquitin conjugation has been implicated in a variety of cellular functions. The best understood of these is the targeting of the substrate protein for selective degradation via a nonlysosomal pathway (9). Other cellular processes mediated by the ubiquitin conjugation system include DNA repair (10), cell cycle progression (11), regulation of chromatin structure (12), cell surface recognition (13), and regulation of transcription factors, e.g. NF-κB (14).

The formation of ubiquitin-protein conjugates involves a three-step process (15, 16). The first step is the ATP-dependent activation of ubiquitin by the 105-kDa ubiquitin activating (E1)3 enzyme (15, 17) involving the formation of a thiol ester linkage between the ubiquitin carboxyl terminus and a thiol group of E1. In the second step, ubiquitin is transferred to the active site cysteine residue of a ubiquitin carrier protein (or E2). In the last step, an isopeptide bond is formed between the carboxyl terminus of ubiquitin and a lysyl ε-amino group within a substrate protein, a reaction catalyzed either directly by the E2 enzyme or via a third enzyme designated isopeptide ligase (E3). Enzymes designated ubiquitin isopeptidases have also been described which deubiquitinate conjugates (18), consistent with evidence for the dynamic balance governing ubiquitin adduct pools (5).

E2s exist as a family of isozymes that exhibit variability in terms of molecular weight, physiological function, substrate specificity, and dependence on E3 in vitro systems (2, 19, 20). For example, in the yeast Saccharomyces cerevisiae, eight genes (designated UBC1–8) encoding distinct E2s have been described (2, 21). The RAD6 (UBC2) protein functions in DNA repair, sporulation, and induced mutagenesis (10). CDC34 (UBC3) is an E2 of 24 kDa that is essential for G1/S transition during mitosis (11). The basic structure of E2s consists of a 153-amino acid core domain containing an active site cysteine within a highly conserved random coil segment. Additional carboxyl-terminal extension domains present on many isozymes are often acidic and generally show a high degree of sequence divergence, suggesting that they may play a role in substrate specificity during E3-independent conjugation or may contribute to the specificity of binding to cognate E3 isoforms.

A subset of E2s has been shown to support multiubiquitination, a process in which successive ubiquitin molecules are linked by an isopeptide bond between a side chain amino group of one ubiquitin and the terminal carboxyl group of a second ubiquitin molecule. In vitro studies have demonstrated that E3-independent multiubiquitination by several E2 isozymes is sufficient for degradative targeting by the 26 S multicatalytic protease complex (22). In contrast, polyubiquitination refers to the conjugation of multiple ubiquitin molecules directly to different lysine residues within a single substrate molecule. Studies show that RAD6, CDC34, and the rabbit reticuloocyte E232K catalyze E3-independent multiubiquitination and support E3-dependent ubiquitin conjugation; however, these enzymes differ in their linkage specificity for multiubiquitination, with CDC34 and E232K using Lys-48 (20) and RAD6 utilizing Lys-6.

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The abbreviations used are: E1, ubiquitin activating enzyme; E2, ubiquitin carrier protein; E3, ubiquitin-protein isopeptide ligase; EPF, endemic pemphigus foliaceus; GST, glutathione S-transferase; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; FPLC, fast protein liquid chromatography; Ub, ubiquitin; BSA, bovine serum albumin; rmBSA, reduced, carboxymethylated form of BSA; rmUb, radiolabeled methylated form of Ub.
Multiubiquitination via lysine 48 of ubiquitin has also been demonstrated in the E2_{29K} of wheat germ (23) and E2_{25K} from calf thymus (24). Formation of branched, multiubiquitin adducts have been shown to be involved in targeting of the substrate protein for selective degradation by an ATP-dependent protease complex (25). Chau and co-workers (26, 27) have shown that proteolytic targeting can be inhibited by preventing multiubiquitination via the lysine 48 residue of ubiquitin.

A novel member of the E2 protein family, E2_{EPF}, has recently been cloned from human keratinocytes (28). E2_{EPF} is unique among E2s in that it contains a highly basic carboxy-terminal extension domain. The E2_{EPF} transcript was also shown to encode an antigenic polypeptide recognized by antialoantibodies from pemphigus foliaceus patients. E2_{EPF} is therefore the first member of the E2 enzyme family to be implicated in a disease process. The present paper reports the detailed enzymatic characterization of recombinant E2_{EPF}, revealing a set of functional properties that distinguishes this isozyme from all other characterized E2s. E2_{EPF} is shown to exhibit auto- and multiubiquitination activities. We further show that this E2 supports the ubiquitin-dependent protein degradation pathway in the absence of Lys-48 multiubiquitination. The latter observation indicates that multiubiquitination by linkage other than Lys-48 are competent degradative intermediates, supporting a role for subpopulations of ubiquitin having different linkage specificities within the overall pathway of ATP, ubiquitin-dependent protein degradation.

**Materials and Methods**

Ubiquitin, rcmbSA (reduced, carboxymethylated form of BSA), UbK48R (site-directed mutant of Ub in which the lysine residue at position 48 has been replaced by arginine), and rmUb (reductively methylated form of ubiquitin in which all free amino groups are blocked by methyl groups) were prepared as described previously (20). Rabbit reticulocyte E1 and E2_{4K} were purified to homogeneity by a combination of affinity and high performance chromatography and then quantitated by stoichiometric activity assays (29). Recombinant yeast RAD6 was expressed and purified as described previously (20). Protein concentrations were determined by the Bio-Rad dye binding assay using BSA as a standard. All other proteins and reagents were purchased from Sigma unless otherwise indicated.

Preparation of E1/E2-depleted Reticulocyte Fraction II—The recombinant ubiquitin, E1, and various E2 isozymes from rabbit reticulocyte lysate was accomplished using previously described procedures (29, 30). Rabbit reticulocyte lysate was depleted of ubiquitin according to a modified procedure for fraction II (29, 30) followed by covalent affinity depletion of E1 and E2s as described previously (29). The resulting unadsorbed fraction from the ubiquitin affinity column (depleted fraction I) was determined to be quantitatively depleted of ubiquitin, E1, and E2 isozymes by its inability to support 125I-ubiquitin conjugation without supplementation by exogenous E1 and E2{sub}4K and inability to support 125I-rcmBSA degradation without further addition of ubiquitin.

Expression and Purification of Cloned E2_{EPF}—Cloned E2_{EPF} was expressed in Escherichia coli DH5a harboring the pGEXEPF5S1F2B expression construct (28). Cells were grown at 37°C in LB medium containing 50 μg/ml ampicillin. When the culture reached A{sub}600 of 0.5, isopropyl-1-thio-β-D-galactopyranoside was added to a final concentration of 0.5 mM. After an additional 3-h incubation, the cells were harvested by centrifugation and resuspended in 0.04 volume of solution TD (50 mM Tris-Cl (pH 7.5), 1 mM EDTA, and 1 mM DTT). The cells were lysed by passing the suspension twice through a French press at 1000 p.s.i. The cell lysate containing the glutathione S-transferase (GST)-E2_{EPF} fusion protein was clarified by centrifugation at 10,000 × g for 60 min at 4°C. The fusion protein was purified by gluthatione-agarose affinity chromatography as described previously (31). Ten ml of the clarified cell extract was mixed with 10 ml of 50% (v/v) glutathione-agarose (Sigma) suspension in solution TD and rocked at 4°C for 30 min. The agarose beads were washed 5 times with 30 ml of solution TD. The washed beads were then poured into a column and washed again with 5 bed volumes of solution TD. The bound fusion protein was eluted with 10 mM glutathione (Sigma) in solution TD. The capacity of the glutathione-agarose affinity column was calculated to be approximately 1 mg of fusion protein per mg of the glutathione-agarose matrix under the conditions described.

Isolation of E2_{EPF} (CDNA-encoded polyepitope without the amino-terminal GST moiety) was accomplished as follows. The immobilized fusion protein was incubated with a highly purified preparation of thrombin (4000 NIH units/mg of protein; Sigma) at a concentration of 30 NIH units/ml in TD buffer resulting in elution of the E2_{EPF} while the GST moiety of the fusion protein remained bound to the column. Optimal digestion conditions were 1 unit of thrombin per 5 μg of fusion protein at 25°C for 1–2 h. Longer incubations resulted in partial degradation of E2_{EPF} (data not shown). To remove contaminants including thrombin, undigested fusion protein, and GST, the eluted fraction was subjected to further purification by anion exchange chromatography (29). Prior to chromatography, the sample was filtered through a 0.2-μm membrane. The filtrate was chromatographed at 4°C on a Mono Q HR 5/10 anion exchange column equilibrated with solution TD using a Pharmacia Biotech Inc. fast protein liquid chromatography system (29). Flow rate was maintained at 1 ml/min during sample loading and gradient elution. After sample injection, the column was washed with solution TD and eluted with a linear 0–0.5 M NaCl gradient having a slope of 12.5 mM/min. Eluted fractions were assayed by both SDS-PAGE and ubiquitin thiol ester formation (29). E2_{EPF} consistently eluted as a single peak at 125 mM NaCl. A typical yield of E2_{EPF} based on the thiol ester formation assay was approximately 10 nmol/liter bacterial culture. Purified E2_{EPF} was stored at −80°C. Under these conditions, E2_{EPF} retained full activity for over 8 months and after two cycles of freeze thawing.

Assay of Thiol Ester Formation—Purified E2_{EPF} was assayed for the ability to form a thiol ester linkage with ubiquitin, according to the thiol ester formation assay. Thiol ester bands were cut from the gel and quantified by γ counting on an automatic γ counter (Micromedic 4/600 Plus, ICM Micromedic Systems Inc.). The absolute content of thiol ester was calculated based on the specific activity of 125I-ubiquitin (32).

Assay of Ubiquitin Conjugation—Conjugation assays for measuring steady state levels of conjugates were performed at 37°C for 60 min as described previously (29). The assay mixtures contained 50 mM Tris-Cl (pH 7.5), 2 mM ATP, 0.5 mM DTT, 10 mM MgCl₂, 20 IU/ml inorganic pyrophosphatase, various concentrations of 125I-ubiquitin (native, UbK48R, or rmUb), purified rabbit reticulocyte E1, and purified E2 (E2{sub}4K, E2_{EPF}, or CDC34). The reaction products were resolved by SDS-PAGE (12% acrylamide) and visualized by autoradiography.

A similar approach was used to assay the conjugation of ubiquitin to endogenous reticulocyte conjugates. Assays contained 50 mM Tris-Cl (pH 7.5), 2 mM ATP, 10 mM MgCl₂, 0.5 mM DTT, 20 IU/ml inorganic pyrophosphatase, various concentrations of 125I-ubiquitin (4–8 × 10⁶ cpm/mmol), 10 mM E1, indicated concentrations of E2s, and the depleted fraction II (158 μg) as a source of both E3 and substrates.

Degradation of Protein Degradation of reduced, carboxymethylated BSA (rcmbBSA) was quantitated by generation of trichloroacetic acid-soluble radioactivity similar to that described previously (9). Briefly, the reactions of 50 μl total volume containing 50 mM Tris-Cl (pH 7.5), 0.5 mM DTT, 10 mM MgCl₂, 2 mM ATP, 20 IU/ml creatine phosphokinase, 5 μg ubiquitin, 4 μg 125I-rcmbBSA (4 × 10⁶ cpm/mmol), and the indicated concentrations of E2s, as described. The reaction products were resolved by SDS-PAGE (12% acrylamide) and visualized by autoradiography.
described by Smith and Johnson (31). As shown in Fig. 1, GST. The recombinant protein was purified using the glutathione-epoxycarboxyl terminus of 26-kDa segment of 1EPF5-ORF2B as described previously (5). The fusion protein vector, pGEX-2T (Pharmacia), to generate construct pGEX-EPF5-ORF2B. Expression and Purification of E2EPF—The coding region of the E2EPF cDNA was subcloned into the plasmid expression vector, pGEX-2T (Pharmacia), to generate construct pGEX-EPF5-ORF2B as described previously (5). The fusion protein encoded by this construct consists of the full-length 24-kDa E1 ternary complex to either free E2EPF or the GST fusion is complete within the 1.5-min incubation time employed, as is typical of the analogous loading reaction with other members of the E2 family (20). On longer incubation with free E2EPF, a series of higher molecular weight 125I-ubiquitin adducts was consistently observed on nonreducing thiolester gels (Fig. 3A). Unlike the 125I-ubiquitin-E2EPF thiol ester formed following short incubation (Fig. 2), the higher molecular weight bands formed during prolonged incubation were stable to reducing conditions, Fig. 3B, indicating that they represented peptide conjugates to radiolabeled ubiquitin. In addition, a small fraction of the monoubiquitin adduct was also stable to reducing conditions and showed a time-dependent accumulation on prolonged incubation. Therefore, the E2-Ub1 band in Fig. 3 represents a mixture of 125I-ubiquitin thiol ester and conjugate adducts at long incubation times but principally only thiol esters at short times. The relative molecular weights of the ladder of ubiquitin conjugates corresponded to integer multiples of the molecular weight for ubiquitin ligated to free E2EPF. The bands present under reducing conditions in Fig. 3B were confirmed as E2EPF-ubiquitin conjugates by comigration of identical bands when similar incubations containing unlabeled ubiquitin were analyzed on immunoblots probed with either anti-E2EPF or anti-ubiquitin antibodies (not shown). Thus, E2EPF catalyzes an E3-independent autoubiquitination reaction resulting in the formation of mult ubiquitin homopolymer chains.

E2EPF Catalyzes the Formation of Multiubiquitin Chains with Linkage Specificity Distinct from Lysine 48—Accumulation of autoubiquitinated E2EPF adducts of molecular weights greater than that predicted for monoubiquitination of all 17 lysines within E2EPF (Fig. 3) indicates that some or all of the attached ubiquitin moieties are present as multiubiquitin chains. To demonstrate this conclusively, E2EPF autoubiquitination was carried out in the presence of 125I-rmUb which is incapable of supporting chain elongation due to the absence of primary lysyl ε-aminos (20, 33). Inability to form adducts above E2EPF-Ub6 (Fig. 4, lane 2), under conditions for which there is accumulation of these products with wild-type ubiquitin (lane 1), demonstrates that ubiquitin moieties ligated to E2EPF can increase on longer incubation and thus did not result from a lower rate of transthiolation from the E1 ternary complex (not shown). The lower level of GST-E2EPF thiol ester also did not result from inactivation since the predicted amount of free E2EPF thiol ester, based on protein determination, was observed if the fusion protein was first incubated with thiophilic anion exchange chromatography as described under “Materials and Methods.” The purity of the resulting E2EPF polypeptide was demonstrated by electrophoretic analysis (Fig. 1, lane 3). The apparent molecular weight of the E2EPF polypeptide agreed with the size predicted by sequence analysis. The Mono Q chromatography step resolved intact E2 from minor fragmentation products generated during thrombin processing (not shown). Note that E2EPF exhibits a more intense level of staining by the silver technique relative to that of an equal mass of GST-E2EPF fusion protein (Fig. 1, lanes 2 and 3). This is attributable to differences in mole numbers between the two proteins when normalized for equal mass and a greater mole fraction of lysine for E2EPF compared to GST, since lysine serves as the site of silver deposition.

The GST-E2EPF fusion protein is catalytically active in forming the corresponding 125I-ubiquitin thiol ester in the presence of E1 (28). Fig. 2 shows that recombinant E2EPF processed from GST by thrombin and subsequently purified by Mono Q FPLC is also active in ubiquitin thiol ester formation (lane 5). The amount of thiol ester formed to free E2EPF, determined by quantification of 125I radioactivity associated with the corresponding band in lane 5 of Fig. 2 (20), agreed with that predicted from the mass of E2EPF protein determined as described under “Materials and Methods.” The 125I-ubiquitin thiol ester adducts of both E2EPF and GST-E2EPF formed in incubations parallel to those of Fig. 2 were quantitatively labile to reducing conditions in the presence of 2-mercaptoethanol (not shown), confirming that the associated 125I-ubiquitin was in thiol ester linkage (20). We consistently noted that, at equimolar concentrations, free E2EPF formed more ubiquitin thiol ester than did the GST-E2EPF fusion protein (Fig. 2, lanes 4 and 5). The amount of thiol ester formed to the fusion protein did not.
results in the loss of monoubiquitinated E2EPF. During multiubiquitination with 125I-rmUb (Fig. 4, lane 5), molecular weight CDC34-ubiquitin conjugates when autoubiquitinated with 125I-UbK48R show similar conjugate patterns (Fig. 4, lanes 1 and 3). This is in contrast to CDC34 which utilizes lysine 48 (20), confirmed by the absence of higher molecular weight CDC34-ubiquitin adducts (Fig. 4, lanes 4 and 6).

E2EPF supports E3-dependent ubiquitin conjugation—Those E2 isoforms capable of catalyzing E3-independent conjugation are bifunctional and support significant rates of E3-dependent ubiquitin ligation (20, 33). The ability of E2EPF to also support E3-dependent 125I-ubiquitin conjugation was tested using reticulocyte fraction II depleted of endogenous E1 and E2 isoforms by prior passage through a ubiquitin affinity column (see “Materials and Methods”). Initial rates of conjugation were measured in 5-min incubations to preclude significant accumulation of the E2EPF auto-mult ubiquitination products observed in Fig. 3B. Fig. 5 shows the electrophoretic pattern of the products of these conjugation reactions. No conjugation of radioiodinated ubiquitin is observed in the presence of only pure E1 (lane 2) or E214K (lane 3), confirming that the fraction II is quantitatively depleted of both activating enzyme and the major cognate E3-dependent E2 isoform (20). Comparison of lanes 1–3 of Fig. 5 also demonstrate that the purified E1 and E214K proteins do not contain catalytically significant concentrations of other components required for ubiquitin ligation. Supplementation of the depleted fraction II with both E1 and E214K results in a significant accumulation of 125I-ubiquitin conjugates (Fig. 5, lane 4). Like results were in E214K, E2EPF supported conjugation of ubiquitin to endogenous proteins present in the depleted fraction II in the presence of added E1 (Fig. 5, lane 6), but not in its absence (lane 5). The size distribution of the resulting adducts indicated that they consist primarily of conjugates between ubiquitin and endogenous reticulocyte proteins, although certain of the lower molecular weight bands may correspond to E2EPF-Ub1 and E2EPF-Ub2 (compare lane 6 of Fig. 5 to the lane corresponding to the 5-min time point in Fig. 3B). It was also noted that the ubiquitin conjugate patterns for E2EPF and E214K are qualitatively similar when incubated at similar concentrations and resolved by SDS-PAGE (Fig. 5, lanes 4 and 6). Densitometric measurements revealed that the conjugation rate of E2EPF is approximately 67% of that exhibited by an equivalent concentration of E214K.

E2EPF supports Ubiquitin-dependent Degradation of 125I-rmBSA—Since E2EPF supports E3-dependent conjugation in depleted fraction II extracts, the ability of E2EPF also to support ubiquitin-dependent proteolysis of 125I-rmBSA was assayed in parallel incubations (34). Initial rates of 125I-rmBSA degradation were measured by the formation of trichloroacetic acid-soluble radioactivity (Table I). Degradation of 125I-rm-
BSA was not stimulated above the basal rate of depleted fraction II alone when supplemented with saturating concentrations of ATP, ubiquitin, or both (Table I, Experiment A). Also, degradation was not stimulated by addition of either E1 or E214K alone (Table I, Experiment B); however, when the depleted fraction II was supplemented with ubiquitin, ATP, purified E1, and recombinant E214K, a significant stimulation in degradation above the basal rate was observed (Table I, Experiment B). The results of Experiments A and B confirm the conclusions from Fig. 5 that the fraction II extract is depleted of E1 and E2 isoforms.

When similar experiments were conducted using recombinant E2EPF, this isoform also was shown to stimulate 125I-rcmBSA degradation by E2EPF in the depleted fraction II alone when supplemented with pure E1 (Table I, Experiment C). Thus, in this in vitro system, E2EPF can function in targeting a substrate protein for selective degradation in an energy- and ubiquitin-dependent fashion. This targeting appears to be dependent on E3 activity since in the absence of a reticulocyte extract BSA does not function as an E2EPF substrate (not shown). Table I also shows that the efficiency of E2EPF in supporting degradation approaches that of the cognate E214K at equimolar concentrations. The degradation rate of E2EPF is approximately 75% of that exhibited by E214K, which agrees favorably with the difference in initial rates of E3-dependent conjugation observed between E2EPF and E214K in Fig. 5 (lanes 4 and 6).

Ubiquitin-dependent Proteolysis Supported by E2EPF Does Not Exclusively Require a Lys-48 Multiubiquitin Linkage—As described above, we have shown that E2EPF catalyzes auto- and multienzyme ubiquitination (Fig. 3) which is not restricted to the Lys-48 residue of ubiquitin (Fig. 4) and that E2EPF supports ubiquitin-dependent protein degradation (Table I). These results led to the question of whether multiubiquitination involving linkages other than Lys-48 of ubiquitin was sufficient to target substrate proteins for degradation. To address this issue, we replaced native ubiquitin with either UbK48R or rmUb in proteolytic assays otherwise identical with those of Table I. Data summarized in Table II demonstrate that initial rates of degradation supported by E2EPF are almost exclusively dependent on Lys-48 ubiquitination since substitution with either UbK48R or rmUb yielded rates less than or equal to 2% of that observed with native ubiquitin (Experiment A). In contrast, UbK48R did not similarly block E2EPF-supported 125I-rcmBSA degradation but resulted in a consistent 50% reduction compared to rates in the presence of native ubiquitin (Experiment B). The remaining stimulation of 125I-rcmBSA degradation by E2EPF supported by UbK48R must be due to multiubiquitin conjugate intermediates containing linkages other than those to Lys-48 since substitution with rmUb completely blocked proteolysis (Table II, Experiment B).

**DISCUSSION**

In this paper, we report the functional characterization of a new member of the E2 enzyme family, E2EPF, from human epidermis. Sequence comparison with all previously characterized E2s revealed that E2EPF exhibited the highest degree of homology with yeast UBC4, with a 60% similarity and a 38% identity (28). UBC4 and UBC5 are central components of the ubiquitin-mediated proteolytic pathway in yeast (35) and are categorized as class I E2s (2) defined as those members of the E2 protein family that lack adjacent sequences extending from the E2 core structure. In contrast, class II E2s contain carboxy-terminal extensions that are highly divergent and are thought to play a role in substrate specificity (12, 36). For example, RAD6 and CDC34, which have polyacidic carboxy-terminal tails, catalyze ubiquitination of the highly basic histone proteins (20). E2EPF has a polybasic carboxy tail with a primary structure that is unique among the E2 family. This keratinocyte enzyme may therefore have a highly restricted substrate specificity, possibly limited to certain acidic proteins.

A variety of model proteins (such as histones, lysozyme, cytochrome c, myoglobin, hemoglobin, and BSA) have been assayed with E2EPF in the in vitro E3-independent ubiquitin conjugation assay, but none has been found to function as a substrate in this system. The identification of E2EPF-specific substrate(s) will be an important step in characterizing the physiological role of this enzyme.

It has been demonstrated that reticulocyte E220K, E228K, RAD6, and CDC34 are bifunctional enzymes capable of catalyzing both E3-independent and E3-dependent ubiquitin ligation (20, 29, 33). A recent study has shown that ubiquitination and selective degradation of some proteins is dependent upon specific association with E3 (20, 33, 37). The results presented in this report show that E2EPF is also bifunctional. Figs. 3 and 4 demonstrate that E2EPF can catalyze ubiquitination in the absence of an E3. Evidence that E2EPF also conjugates ubiquitin to substrate proteins in an E3-dependent manner came from the results of the in vitro degradation assay (Table I). Radiolabeled rcmBSA, which is not a substrate for E2EPF in
the absence of E3, was shown to be targeted for degradation by E2EPF in a ubiquitin-dependent manner using a reticulocyte lysate containing E3. We speculate that E2EPF may normally conjugate ubiquitin to a restricted set of specific substrate proteins in the absence of E3 and may function in an E3-dependent pathway with a more general range of substrate proteins.

Several E2s, including RAD6, CDC34, rabbit E23K, and bovine E23K5 have been shown to support the formation of multiubiquitin chains (20, 25). In the present study, we demonstrate that human E2EPF supports both auto- and multiubiquitination (Fig. 3) via sequential addition of ubiquitin to the growing multiubiquitin chain. Although rigorous kinetic studies have not yet been done, it appears that the rates of mono- and diubiquitinations are much slower than those of the subsequent elongation of ubiquitin chains to form higher order conjugates. Auto-multiubiquitination has also been documented in yeast CDC34 (20). Unlike CDC34, multiubiquitination by E2EPF does not appear to be restricted to the ubiquitin lysine 48 linkage since substitution of ubiquitin with UbK48R did not significantly alter the resulting conjugation pattern (Fig. 4). Parallel work with Lys to Arg ubiquitin mutants has not significantly altered the resulting conjugation pattern lysine 48 linkage since substitution of ubiquitin with UbK48R did result in a 50% decrease in E2EPF-dependent conjugation. This site may be involved in the recognition of specific substrate proteins (E3-independent pathway) and in the interaction with specific E3(s) which, in turn, determine substrate specificity (E3-dependent pathway).

The demonstration that E2EPF catalyzes auto- and multiubiquitination and targets substrate proteins for selective degradation suggests several possible autoregulatory models for E2EPF. In one model, E2EPF could down-regulate its own activity by targeting itself for degradation using the ubiquitin-dependent pathway. Alternatively, the ubiquitin conjugation activity of E2EPF may be determined by the conjugation state of the enzyme (native, mono-, or multiubiquitinated). But, in either case, the possibility that autoubiquitination of E2EPF in vivo may be coupled to an additional signal, e.g. phosphorylation or interaction with other cellular proteins, cannot be ruled out. The enzymatic properties of E2EPF documented in this report provide a useful model system to address these important issues regarding autoregulation of this keratinocyte E2.

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