Identification of the Ubiquitin Carrier Proteins, E2s, Involved in Signal-induced Conjugation and Subsequent Degradation of IκBα*

(Received for publication, January 21, 1999, and in revised form, March 16, 1999)

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The last step in the activation of the transcription factor NF-κB is signal-induced, ubiquitin- and proteasome-mediated degradation of the inhibitor IκBα. Although most of the components involved in the activation and degradation pathways have been identified, the ubiquitin carrier proteins (E2) have remained elusive. Here we show that the two highly homologous members of the UBC5 family, UBC5b and UBC5c, and CDC34/UBC3, the mammalian homolog of yeast Cdc34/Ubc3, are the E2 enzymes involved in the process. The conjugation reaction they catalyze in vitro is specific, as they do not recognize the S32A,S36A mutant species of IκBα that cannot be phosphorylated and conjugated following an extracellular signal. Furthermore, the reaction is specifically inhibited by a doubly phosphorylated peptide that spans the ubiquitin ligase recognition domain of the inhibitor. Cys-to-Ala mutant species of the enzymes that cannot bind ubiquitin inhibit tumor necrosis factor α-induced degradation of the inhibitor in vivo. Not surprisingly, they have a similar effect in a cell-free system as well. Although it is clear that the E2 enzymes are not entirely specific to IκBα, they are also not involved in the conjugation and degradation of the bulk of cellular proteins, thus exhibiting some degree of specificity that is mediated probably via their association with a defined subset of ubiquitin-protein ligases. The mechanisms that underlie the involvement of two different E2 species in IκBα conjugation are not clear at present. It is possible that different conjugating machineries operate under different physiological conditions or in different cells.

Generation and activation of the transcription factor NF-κB involve two successive ubiquitin- and proteasome-mediated proteolytic steps: (i) processing of the precursor protein p105 to the active subunit p50 and (ii) signal-induced degradation of the inhibitor IκBα. Degradation of IκBα is triggered by a broad array of stimuli, such as binding of cytokines or viral products to their appropriate receptors. The receptors then recruit adaptors such as TRADD and TRAF. Consequently, activated NF-κB-inducing kinase (1) is released and sequestered into a large complex that contains, among other proteins, IκB kinases α and β (see, for example, Ref. 2), NEMO (NF-κB essential modulator) (3) or IκB kinase γ (4), and IκB kinase complex-associated protein (5). Complex formation leads, most probably, to activation of the IκB kinases that phosphorylate NF-κB-complexed IκBα on serines 32 and 36. This modification leads to its targeting and rapid degradation by the ubiquitin-proteasome pathway (6–10). Most of the upstream components of the signaling pathway have been identified. Also, recent studies have identified an SCF complex that contains Skp1p, Cullin1, and the F-box protein β-TrCP as the ubiquitin-IκBα ligase complex (see for example Refs. 11–13). However, all these studies have not identified the ubiquitin carrier protein(s) involved in targeting of phosphorylated IκBα (pIkBα), and the identity of the enzyme(s) has remained elusive.

The ubiquitin system targets a wide array of short-lived regulatory proteins such as transcriptional activators, tumor suppressors and growth modulators, cell cycle regulators, and signal transduction pathway components. Consequently, it is involved in the regulation of many basic cellular processes. Among these are cell cycle and division, differentiation, and development; response to stress and extracellular stimuli; modulation of cell-surface receptors; DNA repair; regulation of the immune and inflammatory responses; biogenesis of organelles; and apoptosis. Degradation of a protein by the ubiquitin system involves two distinct and successive steps: (i) covalent attachment of multiple ubiquitin molecules to the target protein and (ii) degradation of the tagged substrate by the 26S proteasome. Conjugation proceeds via a three-step mechanism involving three enzymes. Initially, ubiquitin is activated by the ubiquitin-activating enzyme (E1). One of several E2 enzymes (ubiquitin carrier proteins or ubiquitin-conjugating enzymes (designations abbreviated as pIkBα, phosphorylated IκBα; E1, ubiquitin-activating enzyme; E2, ubiquitin carrier protein; E3, ubiquitin-protein ligase; TNF-α, tumor necrosis factor α; PAGe, polyacrylamide gel electrophoresis).
nated UBCs) transfers ubiquitin from E1 to the substrate, either directly or via a member of the ubiquitin-protein ligase family of enzymes (E3) to which the substrate protein is specifically bound. The first ubiquitin moiety typically binds via its C-terminal Gly and generates an isopeptide with an ε-NH2 group of a Lys residue of the protein substrate. In successive reactions, a polyubiquitin chain is synthesized by transfer of additional activated ubiquitin moieties to Lys\(^{48}\) of the previously conjugated molecule. The structure of the ubiquitin system appears to be hierarchical: a single E1 carries out activation of ubiquitin required for all modifications. It can transfer ubiquitin to several species of E2 enzymes. Each E2 acts in concert with either one or several E3 enzymes. Following conjugation, the protein moiety of the adduct is recognized, most probably via its polyubiquitin chain, and degraded by the 26 S proteasome complex. Free and reutilizable ubiquitin is released via the activity of isopeptidases (for recent reviews and a monograph on the ubiquitin system, see, for example, Refs. 14–17).

A few E2 enzymes, such as E2-C, which is involved in the targeting cyclins (18), appear to be E3- and substrate group-specific. Other E2 enzymes are involved in transfer of ubiquitin to several E3 enzymes and in the targeting of different groups of substrates. Three homologous human enzymes (UBCH5a, UBCH5b, and UBCH5c) have been described (19) that are closely related to Saccharomyces cerevisiae Ubc4 and Ubc5, which are involved in targeting many cellular proteins, particularly under stress. They are also homologous to Drosophila melanogaster UBCD1 and to Arabidopsis thaliana UBC8–12. UBCH5b and UBCH5c are ~98% identical, whereas UBCH5a is ~90% similar to the b and c species. Members of the family have been shown to be involved in cell-free conjugation of several proteins (e.g. p53 (20) and p105 (21)) and to interact with certain E3 enzymes such as members of the HECT domain family of ligases (22). The cellular substrates of these enzymes have, however, remained obscure. S. cerevisiae cdc34/ubc3 and E2-14K were purified from rabbit reticulocyte lysates as described (31). Recombinant E2-14K was as described (18). cDNA coding for E2-14K was as described (32). Following induction of cell disruption, the bacterial cell extract was resolved on DEAE-cellulose, and the enzyme that was contained in Fraction I was further purified via gel filtration chromatography on a Superdex 75 HiLoad column (16 × 600 mm; Amersham Pharmacia Biotech). Recombinant E2-8A (33) was expressed in bacteria, and the protein was partially purified as described above for UBCH7. cDNAs coding for UBCH5a (20) and UBCH5b and UBCH5c (19) were obtained from Dr. Allan Weissman (National Institutes of Health). cDNAs coding for human CDC34/UBC3 (24), E2-25K (34), and E2-20K (35) were cloned by reverse transcription-polymerase chain reaction from 293 cells. Active-site, dominant-negative mutants of the different E2 enzymes (C85A UBCH5a, C85A UBCH5b, C85A UBCH5c, C93A CDC34/UBC3, C92A E2-25K, and C87A E2-20K) were generated by two-step polymerase chain reaction as described (36). For bacterial expression, the cDNAs coding for wild-type and mutant UBCH5a, UBCH5b, and UBCH5c, for CDC34/UBC3, and for E2-25K and E2-20K were subcloned into the pT7-7 vector (37) following their modification by polymerase chain reaction to contain an N-terminal His\(^{6}\) tag. For transient expression in mammalian cells, the cDNAs were subcloned into the pCAGGS vector (38), whereas for tetracycline-inducible expression, they were cloned into the pHS111-3 vector (39). Sequences of all the constructs were verified using the ABI 310 or ABI 377 autosequencers (Perkin-Elmer). Identification of the transfected cDNAs was carried out by reverse transcription-polymerase chain reaction of cellular RNA following induction by doxycycline and primers from noncoding regions in the vectors used for transfection. Amplified cDNAs were diagnosed by specific restriction analysis. For purification of the His\(^{6}\)-tagged E2 enzymes, BL21 (DE3) cells, transformed with the appropriate expression constructs, were cultured in 2× YT medium, and isopropyl-β-D-thiogalactopyranoside (400 μM) was added when A\(_{600}\) nm attained 0.6. The induced proteins were purified over Ni\(^{2+}\)-nitritotratic acid-agarose according to the manufacturer’s instructions. For transient expression of the various E2 enzymes, 293 cells were transfected with the various E2 constructs using the DEAE-dextran method (40). Transfection efficiency was always ~75% as determined by parallel transfection with a gene encoding green fluorescent protein. Experiments were carried out 40–48 h following transfection. Stable transfectants of HeLa Tet-on cells (CLONTECH) for inducible expression of mutant E2 enzymes were established by calcium phosphate transfection as described (41). Protein expression was induced by the addition of 1 μg/ml doxycycline for 48 h. Induction of the proteins was monitored by Western blot analysis using anti-His tag antibody.

**Conjugation of IxBo and Cellular Proteins in Vitro**–[\(^{35}\)S]Methionine-labeled IxBo complexed to HeLa p50/p65 was generated as described (9, 10). Briefly, cDNAs coding for wild-type or S32A,S63A mutant IxBo were translated/transcribed in vitro in wheat germ extract in the presence of \(^{35}\)S-labeled methionine. The translation mixture was added to endogenous cellular NF-kB complex, the labeled protein was incubated in HeLa cell extract in the presence of okadaic acid. Following incubation, anti-p65 antibody was added, and the immune complex was immunoprecipitated with protein A-Sepharose beads. The washed immobilized complex was used as a substrate in the different conjugation assays. Conjugation of IxBo to ubiquitin was monitored essentially as described.
(9, 10, 30, 31). Briefly, the reaction mixture contained (in final volume of 25 μl) 4 μl of packed and washed protein A-Sepharose beads containing the labeled IκBα protein (20,000 cpm), 40 μl Tris-HCl (pH 7.6), 5 mM MgCl$_2$, 2 mM dithiothreitol, 5 μg of ubiquitin, and 0.5 μg of the isopeptidase inhibitor ubiquitin aldehyde (42). Crude HeLa cell extract (60 μg), Fraction II (45 μg of protein), Fraction I (15 μg; as a source of E2), and Fraction IIA (25 μg; as a source of E3); E1 (0.75 μg); E2-14K (0.75 μg); E2-C (0.5 μg); E2-8A (0.75 μg); UBCH7 (0.75 μg); and E2-25K (1.0 μg) were added as indicated. UBCH5a, UBCH5b, and UBCH5c were added at 0.75 μg or as indicated, whereas CDC34/UBC3 was added at 1.25 μg or as indicated. The different Cys-to-Ala mutant species of UBCH5a, UBCH5b, and UBCH5c and of CDC34/UBC3 were also added as indicated. 0.2-20K was added at 0.8 μg or as indicated. The biological activity of the different E2 enzymes was monitored using formation of E2-specific ubiquitin thiol ester in the presence of E1 as described (29). The IκBα phosphopeptidase and S32A,S36A peptide were added at 40 μg. In mixtures containing the peptides, bestatin was added at 20 μg/ml and was incubated for 15 min at room temperature with all the components of the reaction except for the peptides and the labeled substrate. Following addition of the peptides, the reaction mixture was further incubated for 5 min at 30 °C prior to the addition of the labeled substrate. All mixtures containing the peptides contained also 2 μg/μl okad acid. When complete HeLa cell extract (25 μg) was used as a source of endogenous substrates, endogenous E1 and E2 and E3 enzymes were purified by N-ethylmaleimide (10 mM; 10 min at room temperature) followed by neutralization with dithiothreitol (6 mM; 1 min at room temperature). E1, the different E2 enzymes, Fraction IIA (as a source of E3 enzymes), and 125I-labeled ubiquitin (0.1 μg; ~100,000 cpm) were added as described above and in the figure legends. The complete reaction mixtures were incubated for 30 min at 37 °C in the presence of ATP (0.5 mM ATP and ATP-regenerating system) (30,31). Reactions were terminated by the addition of 12.5 μl of 3-fold concentrated sample buffer and, following boiling, were resolved via SDS-PAGE (10%). Gels were dried, and 125I-labeled proteins were visualized using a PhosphorImager (Fuji, Japan). 125I-Labeled proteins were visualized following exposure to Kodak XAR-5 film.

Degradation of IκBα in Vitro—The fate of IκBα was monitored in cells that were stably or transiently transfected with the different species of E2 enzymes. Following incubation in the presence of TNF-α (10 ng/ml), cells were harvested at the indicated time points, lysed in sample buffer, and resolved via SDS-PAGE (10%). The resolved proteins were blotted onto nitrocellulose paper, and the inhibitor was visualized by Western blot analysis using a specific antibody, a secondary horseradish peroxidase-conjugated antibody, and ECL reaction as described (31).

Degradation of MyoD—Degradation of bacterially expressed MyoD was followed in crude HeLa cell Fraction II by Western blot analysis as described (31).

Degradation of the General Population of Short-lived Proteins—HeLa Tet-on cells stably transfected with C85A UBCH5b and C85A UBCH5c were labeled with [38S]methionine (100 μg/ml) for 5 min (pulse). Following removal of the labeling amino acid, the cells were incubated for the indicated time periods in the presence of excess unlabeled methionine (chase), and degradation was monitored by measuring release of trichloroacetic acid-soluble radioactivity into the medium as described (43).

Determination of Ubiquitin Conjugates in Cells—Cell extracts derived from uninduced or doxycycline-induced HeLa cells stably transfected with Cys-to-Ala mutant UBCH5b and UBCH5c or from 293 cells transiently transfected with Cys-to-Ala mutant CDC34/UBC3 were resolved via SDS-PAGE (10%). Resolved proteins were blotted onto nitrocellulose paper and probed with anti-ubiquitin antibody as described (31).

Protein Concentration—Protein concentration was determined according to Bradford (44) using bovine serum albumin as a standard.

Indication of Ubiquitin—Ubiquitin was iodinated, using the chloramine-T method, as described (29).

RESULTS

UBCH5b, UBCH5c, and CDC34/UBC3 Are the Ubiquitin Carrier Proteins Involved in Conjugation of NF-κB-complexed and Phosphorylated IκBα in Vitro—To identify the ubiquitin carrier proteins involved in conjugation of pIκBα in the context of the heterotrimeric pIκBα:p50:p65 complex, a cell-free system was reconstituted. The system contained E1, Fraction IIA (as a source of E3), and different species of purified E2 enzymes. As shown in Fig. 1A, only UBCH5b and UBCH5c conjugated the inhibitor in a specific manner. The adducts are of high molecular mass, but more important, they are specific to the phosphorylated species of the inhibitor: they are not generated when the S32A,S36A mutant species of IκBα is used as a substrate. UBCH5a, UBCH7, and E2-8A also conjugated the inhibitor; however, the conjugates are mostly of the low molecular mass type and do not appear to be specific: S32A,S36A IκBα is also targeted. E2-20K, E2-25K, E2-C, and E2-14K did not conjugate the inhibitor at all. To further establish the specificity of these two E2 enzymes, we tested the effect of a specific phosphopeptide that spans the phosphorylation domain of IκBα. This peptide blocks specifically the conjugation reaction by interfering with the recognition by E3 (10). As shown in Fig. 1B, the phosphopeptide inhibited specifically the UBCH5b- and UBCH5c-mediated conjugation, but not UBCH5a-, UBCH7-, and E2-8A-mediated conjugation. The S32A,S36A peptide had no effect on the specific conjugation. As noted, the conjugates generated by UBCH5a, UBCH7, and E2-8A are of low molecular mass. UBCH5b and UBCH5c also catalyzed formation of such conjugates (see Fig. 1, A and B).

However, they were also generated when S32A,S36A mutant IκBα was used as a substrate, and the process was not sensitive to the phosphopeptide. Thus, they do not appear to be specific to the complexed and signal-induced phosphorylated inhibitor and/or do not lead to its degradation (for the possible significance of these conjugates, see “Discussion”).

Recent evidence suggests that human β-TrCP is the receptor subunit of the IκBα ligase (11–13). β-TrCP is an F-box-containing WD protein that, unlike the initial finding report (11), does not act alone. It associates with Skp1p and Cullin1 to generate an SCF complex that serves as the IκBα E3 (12, 13). As mentioned above, these complexes are involved also in cell cycle control via their ability to target certain phosphorylated cell cycle regulators. They act in concert with CDC34/UBC3 that serves as the E2 enzyme. Therefore, it was important to study the role of this E2 in pIκBα conjugation. Our initial results indicated that CDC34/UBC3 is not the E2 enzyme involved in targeting the inhibitor. As shown in Fig. 1C, the enzyme did not conjugate the inhibitor (compare lanes 4 and 5). Furthermore, the enzyme was clearly confined to Fraction II (Fig. 1D), and one would expect that if it plays a role in the conjugation of the inhibitor, ubiquitin-supplemented Fraction II alone would be sufficient to support conjugation. However, this was not the case (Fig. 1C, lane 2), and the process required the addition of both Fraction I as a source of a different E2 (Fig. 1C, lane 3) or an E2 enzyme such as UBCH5c that is contained in Fraction I (lane 5) (all members of the UBCH5 family are contained in Fraction I (19, 20, 30, 32)). The confinement of CDC34/UBC3 to Fraction II implies that potential CDC34/UBC3-mediated conjugation of pIκBα should not require the addition of Fraction I or of an E2 contained in this fraction. Other studies have yielded similar results and have shown that pIκBα conjugation requires an E2 enzyme contained in Fraction I. Alkalay et al. (9) and Winston et al. (12) used crude Fraction I as a source of E2 in the reconstituted cell-free conjugation reaction. They could not demonstrate any conjugating activity in Fraction II. Similarly, Yaron et al. (11) and Spencer et al. (13) used, for the reconstitution assays, UBCH5c and UBCH5, respectively, (the species of the UBCH5 enzyme is not indicated), which are contained in Fraction I (both groups, however, have not shown the specificity of the enzyme, the role of other members of the family or other E2 enzymes in the process, and the involvement of the enzyme in IκBα proteolysis in vivo; see below). Taken together, these findings strongly suggested that CDC34/UBC3 is not involved in IκBα conjugation. However, more careful
reconstitution assays have shown a specific role for CDC34/UBC3 in conjugation of the inhibitor in vitro (Fig. 1E) and in vivo (see below). As shown clearly in Fig. 1E (lanes 2 and 7), the phosphorylated pIkBaR50/p65 substrate recruited into the complex the E3 enzyme, and conjugation of the inhibitor required only E1 and an E2 (similar results as for the presence of endogenous E3 in the signal-induced phosphorylated substrate complex have been reported also by Yaron et al. (11)). In the absence of added exogenous E3, CDC34/UBC3 supported conjugation of the inhibitor (Fig. 1E, lane 2). Conjugation was specific, as it was inhibited by the IkBa phosphatepeptide (Fig. 1E, lane 5), but not by the S32A,S36A mutant peptide (lane 4).

Also, the S32A,S36A mutant species of IkBa was not conjugated by CDC34/UBC3 (Fig. 1E, lane 6). Interestingly, however, the addition of crude Fraction II as a source of E3 strongly inhibited the CDC34/UBC3-catalyzed conjugation, although additional endogenous CDC34/UBC3 was contained in this fraction (Fig. 1, C, lanes 2 and 4; and E, lane 5). Similar results were obtained using Fraction IIA as a source of E3 (data not shown). In striking contrast, the addition of Fraction II did not inhibit the UBC5c-catalyzed reaction. On the contrary, this reaction was further stimulated by Fraction II, a stimulation that was probably due to the addition of exogenous E3 complex (Fig. 1E, compare lanes 7 and 8; compare also the amount of conjugates in Fig. 1, C, lane 5; and E, lane 7). The inhibition of CDC34/UBC3-mediated conjugation by Fractions II and IIA is probably the reason for the conclusion in other studies (9, 11–13) that the IkBa E2 is contained in Fraction I. Although the reason for the inhibitory effect is not clear (see “Discussion”), it appears that CDC34/UBC3 is also involved in signal-induced targeting of the inhibitor in vivo (see below), thus suggesting a physiological role for the enzyme in the turnover of the inhibitor in the intact cell (see “Discussion”).

To dissect further the mechanism of action of the E2 enzymes and to enable the development of tools with which it will be possible to analyze the function of these enzymes in vivo, we tested the effect of catalytic site mutant species of the different E2 enzymes on the conjugation of the inhibitor in vitro. As shown in Fig. 2A, a mutant species of UBC5c in which the ubiquitin-binding residue Cys85 was substituted with Ala, strongly inhibited the wild-type enzyme-catalyzed reaction. C85A mutant UBC5b had a similar effect (Fig. 2B). Interestingly, C85A mutant UBC5a also inhibited the reaction, although to a lesser extent (Fig. 2B). It is possible that this enzyme, which is 90% homologous to UBC5b and UBC5c, binds to the IkBa E3, but cannot catalyze the ubiquitin transfer reaction (see also below).

Not surprisingly, E2-20K, which cannot catalyze conjugation, did not inhibit it as well (Fig. 2B). Similarly, wild-type E2-25K and the Cys-to-Ala mutant species of E2-20K and E2-25K did not have any effect (data not shown). Thus, it appears that these enzymes do not interact with any component of the IkBa-conjugating machinery (see also below).

**Fig. 1. Specificity of different species of E2 enzymes in conjugating IkBa in vitro.** A, effect of different species of E2 enzymes on the conjugation of wild-type and S32A,S36A mutant IkBa proteins. Conjugation reactions were carried out as described under “Experimental Procedures” and contained labeled, NF-kB-complexed wild-type and phosphorylated (pWT) or S32A,S36A (S/A) mutant IkBa proteins, E1, HeLa cell Fraction IIA (as a source of E3 enzymes), and the indicated species of E2 enzymes. E2 enzymes were added as described under “Experimental Procedures.” Following incubation, reactions were resolved via SDS-PAGE, and proteins were visualized by a PhosphorImager. B, effect of the IkBa phosphopeptide and S32A,S36A peptide on the conjugation of IkBa by different E2 enzymes. Conjugation reactions were carried out, and proteins were visualized as described for A and under “Experimental Procedures.” E2 and Peptide denote the different species of the E2 enzymes and peptides added to the mixtures, respectively. PP denotes the IkBa phosphopeptide, whereas NR denotes the S32A,S36A IkBa peptide. Conj. denote conjugates. IkBa and pIkBa denote unmodified and phosphorylated IkBa, respectively. C, effect of Fraction I, CDC34/UBC3, and UBC5c on the conjugation of pIkBa. Conjugation reactions were carried out essentially as described under “Experimental Procedures” and contained labeled wild-type pIkBa, ubiquitin, and, when indicated, HeLa cell Fraction I (FrI; 15 μg), Fraction II (FrII; 45 μg), extract (Ext.; 60 μg), CDC34/UBC3 (0.75 μg), and UBC5c (0.75 μg). D, distribution of CDC34/UBC3 between Fractions I and II. HeLa cell extract (60 μg), Fraction I (15 μg; derived from ~60 μg of HeLa cell extract), and Fraction II (15 and 45 μg; 45 μg was derived from ~60 μg of crude HeLa cell extract) were resolved via SDS-PAGE (12.5%). Following transfer to nitrocellulose paper, CDC34/UBC3 was detected via Western blot analysis as described under “Experimental Procedures.” E, effect of the IkBa phosphopeptide, the S32A,S36A peptide, and Fraction II on conjugation of pIkBa by CDC34/UBC3. Conjugation reactions were carried out essentially as described under “Experimental Procedures” and for A–C. All E2 enzymes employed were added at a concentration that catalyzes the transfer of ~2 pmol of [125I]-labeled ubiquitin to the E2 enzyme under the conditions employed (29, 30).
substrates, the pIκBα conjugation reaction is relatively specific, and pIκBα is conjugated only by UBCH5b, UBCH5c, and CDC34/UBC3. This is due, most probably, to the specific interaction of these E2 enzymes with the specific SCF E3 complex that recognizes only the post-translationally modified and complexed inhibitor. To further demonstrate the limited, although not entirely specific, scope of these enzymes and to corroborate the notion that they are not involved in targeting the bulk of cellular proteins in vitro, we have shown that their corresponding mutant species do not inhibit conjugation of labeled ubiquitin to the general population of cellular proteins (Fig. 4A). In addition, they do not inhibit ubiquitin-mediated degradation of MyoD (Fig. 4B), a bona fide substrate of the ubiquitin system (31). For this substrate, it has been reported that it is targeted by E2-14K (31), but interestingly, also by CDC34/UBC3 (45).

UBCH5b, UBCH5c, and CDC34/UBC3 Are the Ubiquitin Carrier Proteins Involved in Signal-induced Degradation of IκBα in Vivo—To study the role of UBCH5b, UBCH5c, and CDC34/UBC3 in signal-induced degradation of IκBα in vivo, we tested the effect of catalytic site mutant species of the enzymes on TNF-α-induced degradation of IκBα in cells. As shown in Fig. 5, transient expression of mutant UBCH5b and UBCH5c (panel B) as well as of CDC34/UBC3 (panel C) significantly inhibited the degradation of the inhibitor. Expression of any one of the two UBCH5 mutant enzymes alone had a similar effect monitored with both of them (data not shown; see below, however). Not surprisingly, mutant E2-20K and E2-25K did not have any effect in the transiently transfected cells (Fig. 5D). A similar effect was observed also in stably transfected cells (Fig. 6). UBCH5b and UBCH5c, expressed either singly or together, strongly inhibited the degradation of the inhibitor (Fig. 6, A–C). Like its effect in vitro (see Fig. 2B), UBCH5a also inhibited the degradation of IκBα, although the effect was weaker compared with that of UBCH5b and UBCH5c (Fig. 6D).

To test for the specificity of the inhibitory effect of the mutant E2 enzymes on the degradation of IκBα in vivo, we monitored the effect of expression of these enzymes on the degradation of the general bulk of short-lived proteins that are known to be targeted by the ubiquitin system (46). As shown in Fig. 7A, expression of mutant UBCH5b and UBCH5c did not affect the stability of the general population of short-lived proteins. Furthermore, it did not decrease the steady-state level of ubiquitin conjugates in the cell (Fig. 7, panel B1). Similarly, transient expression of C93A CDC34/UBC3 did not decrease the steady-state level of cellular ubiquitin conjugates either (Fig. 7, panel B2). Although it is clear that UBCH5b and UBCH5c, but also CDC34/UBC3, are not entirely specific to IκBα, it is obvious that they are also not involved in the deg-
radiation of the bulk of short-lived cellular proteins, but rather in targeting of a more limited set of unstable proteins.

**DISCUSSION**

We have shown that UBCH5b, UBCH5c, and CDC34/UBC3 are the ubiquitin carrier proteins involved in the conjugation of signal-induced phosphorylated and NF-κB-complexed IκBa. In vitro, conjugation is specific to the post-translationally modified protein: the S32A,S36A mutant is not conjugated, and the reaction is inhibited specifically by a doubly phosphorylated peptide that spans the IκBa recognition domain. Catalytic site mutant species of the UBCH5 enzymes and of CDC34/UBC3 inhibit conjugation of IκBa in vitro and degradation of the protein in vivo. They probably act via binding to the E3 enzyme and render it inaccessible to association with wild-type endog-

![Fig. 4. Specificity of the conjugating activity of UBCH5b and UBCH5c. A, lack of effect of C85A (C/A) UBCH5a, C85A UBCH5b, and C85A UBCH5c on the conjugation of ubiquitin to endogenous HeLa cell proteins. The C85A species of UBCH5c, UBCH5b, and UBCH5a were added at the indicated concentrations to a reaction mixture containing [125I]-labeled ubiquitin (125I-Ub), crude HeLa cell extract, and ATP. Reactions were carried out as described under "Experimental Procedures," and conjugates (Conj.) were visualized by autoradiography. B, lack of effect of the C85A mutant species of UBCH5a, UBCH5b, and UBCH5c on the degradation of MyoD. Degradation of MyoD was carried out in ubiquitin-supplemented crude HeLa cell Fraction II and ATP as described under "Experimental Procedures." Mixtures also contained the indicated amounts of the C85A mutant species of UBCH5a, UBCH5b, and UBCH5c.

![Fig. 5. Effect of transiently expressed catalytic site mutant species of UBCH5b and UBCH5c, CDC34/UBC3, E2-20K, and E2-25K on TNF-α-induced degradation of IκBa in 293 cells. Empty pCAGGS vector (A) or vectors containing C85A UBCH5b and UBCH5c (B), C93A CDC34/UBC3 (C), and C87A E2-20K or C92A E2-25K (D) were transiently transfected into 293 cells. The effect of expression of the different E2 enzymes on TNF-α-induced degradation of IκBa was monitored 48 h following transfection as described under "Experimental Procedures" using Western blot analysis and ECL.

![Fig. 6. Effect of mutant species of UBCH5a, UBCH5b, and UBCH5c on TNF-α-induced degradation of IκBa in Tet-on HeLa cells. C85A UBCH5b (A), C85A UBCH5c (B), C85A UBCH5b and C85A UBCH5c (C), and C85A UBCH5a (D) were stably transfected into Tet-on HeLa cells as described under "Experimental Procedures." Stability of endogenous IκBa was monitored via Western blot analysis and ECL in uninduced and doxycycline-induced cells following the addition of TNF-α. Numbers indicate time (minutes) following addition of the cytokine. E demonstrates the effect of doxycycline on expression of UBCH5c. Here, UBCH5c was detected using an anti-His antibody as described under "Experimental Procedures."
process in other cell types. Why does the degradation of a single protein employ different species of E2 enzymes? As for UBCH5b and UBCH5c, because the two enzymes are almost identical (19), they are probably functionally redundant, act via the same E3 enzyme, and can substitute for each other. This assumption is supported by the findings that mutant UBCH5b can inhibit UBCH5c conjugation in a cell-free system (Fig. 2B) and that there is no difference between the effect of the two enzymes in vivo (Fig. 6): expression of either one or two of the UBCH5 species displays a similar inhibitory effect on IκBα degradation. As for the involvement of CDC34/UBC3 in the process, it is possible that this enzyme acts via an E3 that is different from the one with which the UBCH5 enzymes catalyze their reaction. Alternatively, the two enzymes can catalyze conjugation of pIκBα via the same E3, but carry it out in different cell types or in the same cell, but under different pathophysiological conditions. As noted, the activity of CDC34/UBC3 appears to be different from that of its UBCH5 counterparts as it is strongly inhibited by Fraction II or IIA (Fig. 1, C, lanes 2 and 4; and E, lane 5; for possible mechanism of this inhibition, see below). In contrast, the activity of UBCH5 is further stimulated following the addition of Fraction II (Fig. 1, E, compare lanes 7 and 8). Experimentally, the distinct E3 enzyme hypothesis can be tested, at least initially, using cross-inhibition by catalytic site, mutant species of one class of E2 along with the wild-type species of the other class. If distinct E3 enzymes are involved, C93A CDC34/UBC3 will not be able to inhibit wild-type UBCH5b- and UBCH5c-mediated conjugation, and similarly, C85A UBCH5b and C85A UBCH5c will not be able to inhibit the CDC34/UBC3 activity. However, because the reaction requires Fraction II, which may provide one or more E3 enzymes, and because Fraction II is inhibitory for CDC34/UBC3 activity, experimental examination of this theory may be difficult at present. Also, experiments with inhibitory inactive mutant species are not conclusive at times. The inhibitory effects of UBCH5a demonstrated in this study illustrate the limit of such probes as faithful experimental tools. It appears therefore that elucidation of the underlying mechanisms will have to await reconstitution of the system from the different purified components. Of note is that a requirement for two distinct E2 enzymes has been demonstrated previously for different substrates. We have shown that lysosome can be targeted by E2-14K and E2-F1 (UBCH7) and that each acts with a distinct E3 enzyme that recognizes a different structural motif in the protein (47). Chen et al. (48) have shown that the yeast transcriptional activator Mato2 is also targeted by two distinct E2 enzymes, Ube6 and Ubc7; however, the mechanistic basis for their differential activity has not been resolved.

The reason for the inhibitory effect of Fractions II and IIA on the activity of CDC34/UBC3 is not known. It is possible that the enzyme binds to an E3 in Fraction II and is not available for ubiquitin transfer to the substrate-bound E3. Initial experiments in which we increased the concentration of CDC34/UBC3 in the reaction mixture failed to corroborate this hypothesis (Fig. 1C, lane 4), although did not exclude it altogether. Another possibility is that the conjugates generated by CDC34/UBC3 are different from those generated by the UBCH5 enzymes and are more sensitive to the activity of ubiquitin C-terminal hydrolases (isopeptidases). These hydrolases must be insensitive to ubiquitin aldehyde, which is included in all reactions. Interestingly, C85A UBCH5a also inhibits the conjugation of the inhibitor in vitro (Fig. 2B) and its subsequent degradation in vivo (Fig. 6D), although the inhibition is weaker than that exerted by its b and c counterparts. Wild-type UBCH5a also catalyzes pIκBα conjugation, although the reaction appears to be nonspecific (Fig. 1, A and B). UBCH5a is ~90% similar to UBCH5b and UBCH5c, which are 98–99% identical to each other (19). It is possible that UBCH5a recognizes and binds to the IκBα E3, although with a lower affinity than UBCH5b and UBCH5c, but cannot catalyze ubiquitin transfer to the inhibitor.

The nonspecific conjugation catalyzed by UBCH5a, UBCH7, and E2-8A in the cell-free system (Fig. 1, A and B) also deserves some attention. It is possible that these two E2 enzymes are involved in the conjugation and subsequent degradation of the free dissociated inhibitor. This process is clearly signal-independent and must be slower then the signal-induced proteolytic cleavage of the inhibitor. It is clear that this process does not require phosphorylation of the inhibitor, as the S32A,S36A mutant species of the inhibitor is also conjugated, and the process is not inhibited by the Ubα phosphopeptide. The protein is probably recognized by a constitutive, non-regulated motif. It is possible that during incubation, a small part of the complexed inhibitor is dissociated and targeted by different E2 enzymes. Alternatively, the complexed inhibitor may be recognized simultaneously via fast-reacting regulated and specific and slow-reacting non-regulated signals. Of note is that all the nonspecific conjugates are of relatively low molecular mass and that even UBCH5b and UBCH5c catalyze generation of such low molecular mass, nonspecific adducts (Fig. 1, A and B). Only the high molecular mass adducts appear to be regulated. Thus, it appears that two conjugation events that serve different purposes occur in parallel. The physiological significance of these processes is still obscure. Another explanation for the involvement of multiple E2 enzymes in the nonspecific conjugation of IκBα is that the cell-free system does not faithfully reproduce the cellular events, and these E2 enzymes are inactive toward IκBα in the intact cell.

In a recent study, it has been reported that UBC9/HUS5 is the E2 involved in IκBα degradation (49). However, this enzyme cannot conjugate ubiquitin and is involved in modification of substrate proteins with the ubiquitin-like protein SUMO-1. Thus, if it acts at all on IκBα degradation, it has an indirect effect. The recent report that modification by SUMO-1 renders IκBα stable and protects it from ubiquitin-mediated degradation (50) makes the involvement of UBC9 in the process, even in an indirect manner, highly unlikely.

Acknowledgments—We thank Drs. Allan Weissman for the cDNA clones of UBCH5a, UBCH5b, and UBCH5c; Simon S. Wing (McGill University) for the clone of UBCH7; Avram Hershko (Technion) for purified E2-2; Goujun Bu (Washington University School of Medicine) for the cDNA encoding green fluorescent protein; and Avraham Yaron for thoughtful advice during the initial phase of the work.

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