Identification of a Novel Family of Ubiquitin-conjugating Enzymes with Distinct Amino-terminal Extensions*

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The ubiquitin/proteasome system is the main eukaryotic nonlysosomal protein degradation system. Substrate selectivity of this pathway is thought to be mediated in part by members of a large family of ubiquitin-conjugating (E2) enzymes, which catalyze the covalent attachment of ubiquitin to proteolytic substrates. E2 enzymes have a conserved ~150-residue so-called UBC domain, which harbors the cysteine residue required for enzyme-ubiquitin thioester formation. Some E2 enzymes possess additional carboxyl-terminal extensions that are involved in substrate specificity and intracellular localization of the enzyme. Here we describe a novel family of E2 enzymes from higher eukaryotes (Drosophila, mouse, and man) that have amino-terminal extensions but lack carboxyl-terminal extensions. We have identified four different variants of these enzymes that have virtually identical UBC domains (94% identity) but differ in their amino-terminal extensions. In yeast, these enzymes can partially complement mutants deficient in the UBC4 E2 enzyme. This indicates that members of this novel E2 family may operate in UBC4-related proteolytic pathways.

In eukaryotes, selective protein degradation is largely mediated by the ubiquitin/proteasome system (for reviews, see Refs. 1–5). Degradation by this system was recently found to be instrumental in a variety of cellular functions such as DNA repair, cell cycle progression, signal transduction, transcription, and antigen presentation. Known substrates of this pathway include transcription factors (MATα2, GCN4, c-jun, p53, NF-κB), protein kinases (Mos), cyclins, inhibitors of cyclin-dependent kinases (SIC1, p27), and subunits of trimeric G proteins (for review, see Refs. 1–5). Moreover, the ubiquitin/proteasome system also eliminates abnormal proteins, e.g. misfolded, mislocalized, or misassembled proteins.

Substrate recognition by this pathway involves a specialized recognition and targeting apparatus, the ubiquitin-conjugating system, which operates spatially detached from the proteasome. Proteins recognized by this system are earmarked by the covalent attachment of ubiquitin, a small and highly stable protein. In most cases, ubiquitination involves the formation of multiubiquitin chains attached to the substrate that are subsequently recognized by a specific receptor of the (26 S) proteasome. Proteins bound to the receptor are then probably unfolded and translocated into the central cavity of the proteasome where they are degraded to small polypeptides. Ubiquitin chains are released from substrates and recycled to single ubiquitin moieties (for review, see Ref. 5).

Ubiquitin conjugation involves a reaction cascade (1, 3, 6, 7). Initially, ubiquitin-activating (E1)1 enzyme hydrolyses ATP and forms a thioester bond between itself and ubiquitin. Ubiquitin is then passed on to ubiquitin-conjugating (E2) enzymes and often subsequently to ubiquitin ligases (E3). Each step involves the formation of a thioester-linked ubiquitin-enzyme intermediate (E1, E2, or E3) intermediate (7). E2 and/or E3 enzymes finally catalyze isopeptide formation between the carboxyl terminus of ubiquitin and ε-amino groups of internal lysine residues of target proteins. Both E2 and E3 enzymes exist as protein families, and diverse combinations of E2/E3 enzyme complexes are thought to define the substrate specificity of the conjugation system.

In the yeast Saccharomyces cerevisiae, 12 different genes for ubiquitin-conjugating enzymes (UBC genes) have been detected to date (3, 6). Genetic studies revealed that the encoded enzymes mediate strikingly diverse functions such as DNA repair (8), sporulation (3, 8), cell cycle progression (9, 10), peroxisome biogenesis (11), membrane-protein degradation (12), heat shock resistance (13), and cadmium tolerance (15). One of the most prominent E2 enzymes from yeast is UBC4 (13). A principal function of UBC4 and the highly related UBC5 enzyme appears to be the degradation of abnormal proteins as indicated by the sensitivity of ubc4 ubc5 double mutants to heat shock, canavanine (an arginine analog), and cadmium (13, 15). In addition, UBC4/5-mediated proteolysis is important for some regulatory processes. One example is the UBC4/UBC5-mediated degradation of the yeast transcription factor MATα2 involved in mating type control (16). UBC4/UBC5 homologs have been described from several organisms including Drosophila (UbxD1; Ref. 17), Caenorhabditis elegans (ubc-2; Ref. 18) and man (Ubch5; Ref. 19). The function of these enzymes in these organisms is not known, but, in the cases tested, the respective genes can fully complement yeast ubc4 ubc5 mutants. Interestingly, vertebrate UBC4 homologs can mediate p53 (19) and cyclin (20) ubiquitination in vitro, suggesting that UBC4/5-mediated degradation may be of central regulatory importance.

Here we report the identification of a novel family of ubiquitin-conjugating enzymes (UBC genes) that are involved in substrate specificity and intracellular localization of the enzyme. We have identified four different variants of these enzymes that have virtually identical UBC domains (94% identity) but differ in their amino-terminal extensions. In yeast, these enzymes can partially complement mutants deficient in the UBC4 E2 enzyme. This indicates that members of this novel E2 family may operate in UBC4-related proteolytic pathways.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank1**, EMBL Data Bank with accession number(s) X92663, X92664, and X92665.

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Novel Ubiquitin-conjugating Enzyme Family

Bacterial and Yeast Strains, Media—Growth and handling of Escherichia coli were by standard techniques (21). Strain XL1 was used as a host for plasmids. Transformation was carried out using the electro-pro-secution procedure. λ phage was propagated on strain C600hfr. S. cerevisiae cultures were grown in rich (YP) or synthetic (S) media containing either 2% glucose (YPD or SD media), 2% raffinose (YPraf or SRaf media), or 2% galactose (YPgal or SGal media) as carbon sources. Yeast transformation was carried out using standard protocols (21).

**RESULTS**

Cloning of a Novel UBC Gene Family From Higher Eukaryotes—To study the function of the ubiquitin system in higher eukaryotes, we initiated a homology-based screen for UBC genes from organisms, which are amenable to genetic analysis such as Drosophila, C. elegans, and mouse (17, 18). Ubiquitin-conjugating enzymes are highly related proteins showing at least 30% amino acid sequence identity between different members of this enzyme family. In particular, sequences within the UBC domain, which harbors the cysteine residue required for ubiquitin-thioester formation, are highly similar. Primer pairs specific for conserved sequences were designed (see “Experimental Procedures”) and used for the PCR. From Drosophila and mouse genomic DNA as templates, we were able to amplify fragments of sizes equivalent to the corresponding regions within yeast UBC genes. DNA sequence analysis indicated that the amplified fragments correspond to segments of five different genes encoding novel ubiquitin-conjugating enzymes. Interestingly, the deduced amino acid sequence of one of the PCR fragments isolated from Drosophila and two fragments isolated from mice were virtually identical, suggesting that we have identified three members of a family of highly related UBC genes (see below). Using the cloned five PCR fragments as probes, we isolated the complete cDNAs for the corresponding genes from a plasmid-born Drosophila library and a mouse cDNA library in λ phage, respectively. We name the three Drosophila genes UbcD1, UbcD2, and UbcD3

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and the two mouse genes UbcM2 and UbcM3 (ubiquitin-conjugating enzymes from Drosophila or mice; the numbering reflects the order of identification). As reported previously (17), the UbcD1 enzyme is the structural and functional homolog of yeast UBC4. UbcD3 identified by our screen is identical to bendless, a gene implicated in Drosophila nervous system development characterized while this work was in progress (26, 27). We mapped UbcD1, UbcD2, and UbcD3 on the Drosophila genome to positions 88D, 32A/B, and 12D, respectively (data not shown).

A Family of Highly Related Ubiquitin-conjugating Enzymes with Distinct Amino-terminal Extensions—The DNA sequences and the deduced amino acid sequences of the three new genes, UbcD2, UbcM2, and UbcM3 are shown in Fig. 1. The initiator methionines of the three genes were assigned to the first ATG of the cDNAs preceded by stop codons. The open reading frames of UbcD2, UbcM2, and UbcM3 predicted proteins of 232 (24.5 kDa), 207 (22.9 kDa), and 193 (21.3 kDa) residues, respectively. By Northern analysis using total RNA isolated either from Drosophila or mouse, we identified with the respective probes transcripts for UbcD2, UbcM2, and UbcM3 of 1.5, 1.7, and 1.5 kb, respectively (Fig. 2). The sizes fit well to the corresponding cDNAs and indicate that the isolated cDNAs contain the complete open reading frames.

A comparison of the deduced amino acid sequences of UbcD2, UbcM2, and UbcM3 shows that they are highly related (Fig. 3). Unlike previously identified E2 enzymes (3, 6), these new enzymes possess amino-terminal extensions in addition to the UBC domain. The UBC domains of the three enzymes are almost identical in sequence (94% identity over 149 amino acid residues; between 72 and 79% at the DNA level). UbcD2 differs from the UbcD2/UbcM2/UbcM3 consensus by 6, UbcM2 by 2, and UbcM3 by 3 residues (Fig. 3). In contrast to the extreme conservation of the UBC domains, the amino-terminal extensions (designated extensions A, B, and C; Fig. 3) show little sequence similarity among each other and differ in size (Fig. 3). The weak sequence similarity of the extensions is largely restricted to clusters of serine/threonine and basic residues. No significant sequence similarities between the extensions and known sequences in the databases were found except for short consensus sequences for phosphorylation sites.

Further database searches detected (in addition to a human UbcM2 homolog designated UbcH9) a partial open reading frame from a human cDNA fragment (designated UbcH8), and this represents a probable fourth member of this enzymefamily. This partial sequence exhibits a 100% match to the corresponding sequences of the UBC domains of UbcD2, UbcM2, and UbcM3 and an amino-terminal extension (designated extension D) nonidentical, but related to, extension B of...
UbcM2 and UbcH9 (Fig. 3). This suggests that the novel E2 enzyme family described here has at least four distinct members.

UbcD2, UbcM2, and UbcM3 Are Structurally and Functionally Related to Yeast UBC4—Computer aided sequence comparisons of the UBC domains of UbcD2, UbcM2, and UbcM3 with the UBC domains of other known ubiquitin-conjugating enzymes revealed that their closest homologs are yeast UBC4/UBC5 (an enzyme pair expressed from duplicated genes; Refs. 13 and 28) and their respective homologs from higher eukaryotes, including the Drosophila UbcD1 (17), C. elegans ubc-2 (18), and human UbcH5 (19) gene products. The UBC domains of UbcD2, UbcM2 and UbcM3 share 64% sequence identity with these UBC4-like enzymes, suggesting that the
newly identified enzymes may be functionally related to UBC4 (Fig. 3). Yeast UBC4/UBC5 ubiquitin-conjugating enzymes are involved in stress-related functions and in the turnover of regulatory proteins (Ref. 13; see the Introduction). Single mutations in these genes are viable and lead to only moderate mutant phenotypes, but ubc4 ubc5 double mutants are slowly growing and inviable at elevated temperatures (13). As reported previously, UbcD1, the UBC4 homolog from Drosophila (80% identical to yeast UBC4), can rescue the deficiencies of yeast ubc4 ubc5 mutants (17). Complementation of UBC4/UBC5 functions by UbcD1 was nearly complete, even when the enzyme was expressed from a single copy in the genome (17).

To study the activity of UbcD2, UbcM2 and UbcM3 in yeast, we cloned the respective reading frames into yeast high copy number, 2 micron based expression vectors. When yeast cells were transformed with these plasmids, all three genes could express the following UBCs (in clockwise orientation). Vector as negative control, amino-terminal truncated UbcM3 (pUBcM3), UbcM2 (pUBcM2), UbcD2 (pUBcD2), and yeast UBC4 (pUBC4) as positive control. B, Western blot analysis of total yeast proteins from ubc4 ubc5 cells expressing UbcM3 and UbcM3 Δ1–47, respectively, with antibodies generated against the conserved UBC domain of UbcD2/UbcM2/UbcM3 family. Additional protein bands cross-reacting with the antisera serve as loading control. Size references are given on the right.

**FIG. 4.** Complementation of the yeast ubc4 ubc5 mutant by expression of UbcD2, UbcM2, UbcM3 and an amino-terminal truncated UbcM3. A, growth of yeast ubc4 ubc5 double mutant on YPGal plates at normal growth temperature (30 °C) and heat shock temperature (37 °C) expressing the following UBCs (in clockwise orientation). Vector as negative control, amino-terminal truncated UbcM3 (pUBcM3 Δ1–47), UbcM3 (pUBcM3), UbcM2 (pUBcM2), UbcD2 (pUBcD2), and yeast UBC4 (pUBC4) as positive control. B, Western blot analysis of total yeast proteins from ubc4 ubc5 cells expressing UbcM3 and UbcM3 Δ1–47, respectively, with antibodies generated against the conserved UBC domain of UbcD2/UbcM2/UbcM3 family. Additional protein bands cross-reacting with the antisera serve as loading control. Size references are given on the right.

**FIG. 5.** Phylogenetic tree of the UBC4-related subfamily. Relatedness was calculated by the algorithm provided by the DNA Star package and compared with distantly related yeast UBC2/UBC6. Only the UBC domains were compared. Complementation of yeast ubc4 ubc5 mutants (growth at 30 and 37 °C) is indicated; +++, full complementation; ++, partial complementation by overexpression; +, partial complementation by overexpression; −, no complementation (see text for experimental details).

**DISCUSSION**

Previously identified ubiquitin-conjugating enzymes (3, 6) are small proteins (~14–32 kDa), which either consist of the UBC domain only (class I E2 enzymes) or they possess additional carboxyl-terminal extensions (class II enzymes). Here we describe a novel family of ubiquitin-conjugating enzymes from higher eukaryotes that have amino-terminal but lack carboxyl-terminal extensions (designated class II enzymes). We have cloned three members of this class and identified a fourth in the data base. The UBC domain of these novel enzymes is virtually identical, but the amino-terminal extensions show limited sequence similarity. The recent identification of human homologs to UbcM2 (UbcH9; Fig. 3) and UbcM3 (UbcH6; Fig. 3), which are homologous to their respective murine counterparts over their entire lengths (including the extensions), and the extreme conservation of the UBC domains of different members of this family strongly suggest that a homolog of each of these novel four UBCs may be present in each of these species, i.e. Drosophila, mouse, and man. We have unsuccessfully tried to identify yeast homologs to these enzymes using different PCR strategies. Thus we assume this family probably evolved relatively late in evolution and may be unique to multicellular organisms. Intriguingly, these enzymes are among to the most highly conserved proteins of these organisms. The UBC domains of UbcD2 from Drosophila and UbcM2, and UbcM3 from mice share 94% identical amino acid residues (homologs of other UBCs are typically 70–80% identical in sequence; Refs. 17–19, 29–32; see Fig. 5). The extreme conservation of UbcD2, UbcM2, and UbcM3 is even more remarkable given the likely possibility that the true homologs, i.e. the enzymes with similar extensions are yet to be identified. Proteins of similar high conservation, e.g. histones or ubiquitin, either have multiple interacting partners or most of their amino acid residues participate in intramolecular contacts. Both types of interactions are thought to prevent evolutionary amino acid sequence drift. We thus assume that the novel UBC enzymes interact with several proteins. Candidates for binding partners are components of the ubiquitin/proteasome system or substrates. Since overexpressed UbcD2, UbcM2, and UbcM3 can partially sup-
press UBC4/UBC5 deficiency in yeast, these enzymes and UBC4/UBC5 probably have many substrates in common. However, the presence of multiple, highly conserved extensions of these enzymes suggests that they are likely to carry out specialized functions distinct from those of UBC4. What these functions are is not known at present, but the gene expression pattern of the Drosophila UBCs may provide some clues. Interestingly, UbcD1, the UBC4 homolog, is continuously expressed throughout development consistent with a "housekeeping" function of the encoded enzyme. Transcripts of UbcD3 (endless), another UBC4-related gene (which is actually unable to rescue ubc4 ubc5 mutants; Fig. 5) can also be detected at all developmental stages of Drosophila development. In contrast, UbcD2 appears to be exclusively expressed at postlarval (L3) stages, but in eggs the transcript is supplied maternally. Thus the functions of these class III enzymes may be predominantly restricted to distinct tissues in pupae or adult flies.

The significance of the amino-terminal extensions is currently unclear, but their conservation between species (e.g. the extensions B and C; Fig. 3) indicate that they are probably relevant to their cellular functions. The carboxyl-terminal extensions of class II E2 enzymes are known either to contribute to their substrate specificity (UBC2, UBC3; Refs. 9 and 33–35) or they mediate intracellular localization (UBC6; Ref. 12). The prevalence of putative phosphorylation sites within the extensions of the UbcD2, UbcM2, and UbcM3 enzymes may indicate that the enzymatic activity or a possible interaction with other proteins is possibly controlled by enzyme phosphorylation. Alternatively, these sequences rich in serine, threonine, and basic residues may represent binding sites for specific components of the ubiquitin-conjugating system or proteolytic substrates. Class I ubiquitin-conjugating enzymes have highly conserved three-dimensional structures with exposed amino termini (36). This suggests that the highly charged amino-terminal extensions of the class III enzymes described here may fold into separate domains, which are probably readily accessible to interacting partners.

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