UBE1L2, a Novel E1 Enzyme Specific for Ubiquitin

Received for publication, June 4, 2007, and in revised form, June 18, 2007.

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UBE1 is known as the human ubiquitin-activating enzyme (E1), which activates ubiquitin in an ATP-dependent manner. Here, we identified a novel human ubiquitin-activating enzyme referred to as UBE1L2, which also shows specificity for ubiquitin. The UBE1L2 sequence displays a 40% identity to UBE1 and also contains an ATP-binding domain and an active site cysteine conserved among E1 family proteins. UBE1L2 forms a covalent link with ubiquitin in vitro and in vivo, which is sensitive to reducing conditions. In an in vitro polyubiquitination assay, recombinant UBE1L2 could activate ubiquitin and transfer it onto the ubiquitin-conjugating enzyme UbcH5b. Ubiquitin activated by UBE1L2 could be used for ubiquitylation of p53 by MDM2 and supported the autoubiquitination of the E3 ubiquitin ligases HectH9 and E6-AP. The UBE1L2 mRNA is most abundantly expressed in the testis, suggesting an organ-specific regulation of ubiquitin activation.

Conjugation of ubiquitin to target proteins occurs via a well-known enzymatic pathway (1, 2). In the first step, the activating enzyme (E1)2 forms an initial ubiquitin adenylate intermediate, which is then transferred to the active site cysteine of the E1, forming a thiolester linkage between this cysteine and the C-terminal glycine of ubiquitin (3, 4). Next, ubiquitin is transferred to a conjugating enzyme (E2) and then to a protein ligase (E3), which forms an isopeptide bond between ubiquitin and a lysine within the target protein (5). The attachment of ubiquitin to substrates has several effects on cellular processes. In most cases, Lys-48-linked polyubiquitin chains are connected to target proteins, leading to degradation via the 26S proteasome. On the other hand, chains linked via Lys-63 of ubiquitin or monoubiquitylation are involved in DNA repair, endocytosis, or activation of kinases (6).

The ubiquitin-activating enzyme is highly conserved in yeast (7), plants (8), and humans (9). So far, it has been assumed that only a single activating enzyme for ubiquitin exists. The E1 enzyme plays an essential role in yeast since the deletion of the yeast E1 enzyme called UBA1 is lethal (7). Moreover, a hypomorphic allele of UBA1 was identified, which impairs ubiquitin conjugation to substrate proteins (10). Several mammalian cell lines with mutations that render the Ube1 gene temperature-sensitive showed a severe defect in ubiquitin conjugation when shifted to a non-permissive temperature (11–13). However, since the defect was not absolute, it could not be excluded that other ways of activating ubiquitin may exist (14).

So far, several members of the family of ubiquitin-like modifiers, including ubiquitin, SUMO, NEDD8, ISG15, Apg12, and Ufm1, have been shown to possess their private E1-activating enzymes, which specifically bind and activate their cognate modifier (15). While searching for an E1 enzyme specific for the ubiquitin-like modifier FAT10, we investigated a so far barely studied putative E1 enzyme named UBE1L2. Surprisingly, this enzyme, although failing to activate any of the tested ubiquitin-like modifiers, was readily able to activate ubiquitin in vitro and in vivo. Since UBE1L2 is highly expressed in testis, this second ubiquitin-specific E1 type enzyme may serve an organ-specific function.

EXPERIMENTAL PROCEDURES

Vector Constructs—For generation of HA-UBE1L2, cDNA of total RNA from SW620 colon carcinoma cells was used to PCR-amplify human UBE1L2 using 5′-CGA AGA GTG ACC TAT GGA AGG ATC CGA GCC TGT-3′ as forward and 5′-GCC GGA AGG ATC CGA GCC TGT-3′ as reverse primers. The PCR product was cloned with an N-terminal HA tag into the vector pcDNA3.1 using Asp-718 and Xhol restriction sites. UBE1L2 was also cloned in pGEX2TK using 5′-AGA GCT AGC TTA ATC AGT GTC ACT GA-3′ as reverse primer and human Urm1 using sense 5′-GCC TGC GCC GCT GG-3′ and antisense 5′-GCC GAA TTT GCC GGC GTG CAG ACT G-3′ primers. Both PCR products were cloned into pGEX2TK using BamH and EcoRI for Ufm1ΔC2 and BamHI and Xmal sites for hUrm1. Bacterial expression constructs (pGE-2TK) encoding glutathione S-transferase (GST) fusion proteins of ubiquitin, Nedd8, SUMO-1, and ISG15, respectively, and a mammalian expression vector (pcDNA3) for HA-tagged human UBE1 were generated by PCR-based approaches (further details will be provided upon request).
Expression and Purification of GST Fusion Proteins—The plasmids pGEX, pGEX-ubiquitin (16), pGEX-IG515, pGEX-Nedd8, pGEX-Sumo-1, pGEX-FAT10 (17), pGEX-Uml1ΔC2, and pGEX-hUrm1 were used for expression of GST and GST fusion proteins. Expression vectors were transformed into BL21(DE3)pLysS bacteria, and expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside. pGEX-UBE1L2 was transformed into competent bacteria of Escherichia coli host strain BL21-CodonPlus (DE3)-RIPL (Stratagene), and expression was induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside. Purification was carried out using glutathione-Sepharose™ 4B (Amersham Biosciences) according to the manufacturer’s protocol.

GST Pulldown Assay—HA-UBE1L2 and HA-UBE1 were in vitro transcribed and translated with the TNT T7-coupled reticulocyte lysate system (Promega) as recommended by the manufacturer. 10 μl of the lysate were incubated for 30 min at room temperature with GST or GST fusion proteins bound to 40 μl of glutathione-Sepharose™ 4B in a total volume of 50 μl containing 20 mM Tris/HCl, pH 7.6, 50 mM NaCl, 10 mM MgCl₂, 4 mM ATP, 0.1 mM dithiothreitol, and 5 units/ml inorganic pyrophosphatase. After washing five times with phosphate-buffered saline, SDS sample buffer was added to the beads either with or without β-mercaptoethanol before boiling. The samples were analyzed on 10% SDS-PAGE and detected by autoradiography. The expression of the GST fusion proteins was visualized by Coomassie Blue staining.

Immunoprecipitation Assay—HEK293T cells were transfected with pcDNA3.1-HA-UBE1L2 or pcDNA3.1-HA-UBE1 either with or without p3×FLAG-CMV-ubiquitin (18) using FuGENE 6 (Roche Applied Science). 16 h after transfection, cells were lysed in 20 mM Tris, pH 7.6, 50 mM NaCl, 10 mM MgCl₂, 4 mM ATP, 0.1 mM dithiothreitol, and 5 units/ml inorganic pyrophosphatase. After washing five times with phosphate-buffered saline, SDS sample buffer was added to the beads either with or without β-mercaptoethanol before boiling. The samples were analyzed on 10% SDS-PAGE and detected by autoradiography. The expression of the GST fusion proteins was visualized by Coomassie Blue staining.

FIGURE 1. UBE1L2, a novel E1 enzyme. The program ClustalW was used to align the amino acid sequences of human UBE1 (GenBank™ accession number NP_695012) and UBE1L2 (GenBank accession number NP_060697). The putative ATP-binding site (residues 467–474) and the active site (residues 623–631) are underlined. Asterisks indicate identical amino acids; single and double dots indicate weakly and strongly similar amino acids, respectively.
phosphatase, 20 mM creatine phosphate, and 4 μg/ml creatine phosphokinase. Immunoprecipitation was performed with 40 μl of HA-affinity matrix (Sigma) for 2 h at 4°C. Beads were washed twice with NET-TON buffer (650 mM NaCl, 5 mM EDTA, 50 mM Tris/HCl, pH 8.0, 0.5% Triton X-100, 0.05% NaN₃, 1 mg/ml ovalbumin) and three times with NET-T buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris/HCl, pH 8.0, 0.5% Triton X-100, 0.05% NaN₃) prior to boiling in SDS sample buffer either with or without 10% β-mercaptoethanol. Samples were separated on 10 or 15% SDS-PAGE and analyzed by Western blot probed with anti-HA Peroxidase conjugate (Sigma) and monoclonal and polyclonal anti-FLAG antibodies (Sigma).

Ubiquitylation Assays—For in vitro ubiquitylation experiments, E6-AP, UBE1, and Ubch5b were expressed in the baculovirus system or in E. coli BL21 as described (19). HectH9 and Mdm2 were expressed as GST fusion proteins in E. coli BL21 (20, 21). For in vitro autoubiquitylation, 10 μg of E6-AP or GST-HectH9 were incubated in the absence or in the presence of 2 μg of UBE1 or 200 ng of GST-UBE1L2, 50 ng of Ubch5b, and 20 μg of ubiquitin (Sigma) in 120-μl volumes. For in vitro ubiquitylation of p53, 1 μl of rabbit reticulocyte lysate-translated 35S-labeled p53 was incubated with 50 ng of UBE1 or GST-UBE1L2, 50 ng of Ubch5b, and 20 μg of ubiquitin in the absence or in the presence of 200 ng of GST-Mdm2 in 40-μl volumes (21). In addition, reactions contained 25 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM dithiothreitol, 2 mM ATP, and 4 mM MgCl₂. After incubation at 30°C for 2 h, total reaction mixtures were electrophoresed in 8% SDS-polyacrylamide gels, and proteins were stained with Coomassie Blue.

Quantitative Real-time RT-PCR—Real-time RT-PCR was used to quantify expression levels of the ube1l2 gene in different mouse organs. Total RNA was extracted from indicated organs of 1-month-old C57BL/6 mice using a NucleoSpin RNA II extraction kit (Macherey-Nagel). 1 μg of total RNA was reverse-transcribed using oligo(dT) primers and the reverse transcription system (Promega). Quantitative PCR was performed with the LightCycler® instrument (Roche Applied Science) using the LightCycler® TaqMan® Master reaction mix with ube1l2-specific forward (5’-GTT CTC GCC CTT GTG TCA-3’) and reverse primers (5’-GAT TGC CTA GGG ATT CCA CA-3’) and probe number 84 of the Universal Probe Library (Roche Applied Science). Mouse hprt (hypoxanthine-guanine phosphoribosyl transferase) was used as reference gene with the following primers: 5’-GCT GGT GAA AAG GAC CAC-3’ (forward) and 5’-CAC AGG ACT AGA ACA CCT-3’ (reverse). This reaction was performed with SybrGreen®.

RESULTS

Identification of a Novel Human E1-like Enzyme, UBE1L2—We originally tried to identify a possible E1 enzyme for the ubiquitin-like modifier FAT10 by performing NCBI BLAST searches looking for proteins with homology to UBE1. Throughout the search, a protein sequence with 40% identity to the UBE1, named UBE1L2 (ubiquitin-activating enzyme E1-like protein 2) or MOP-4 (monocyte protein 4), was found comprising 1052 amino acids (Fig. 1). The theoretical molecular mass of UBE1L2 is 118 kDa, which corresponds well to the apparent molecular mass on SDS-PAGE. The ube1l2 gene is only found in vertebrates (human, mouse, chicken, fish) but neither in invertebrates (Drosophila, Caenorhabditis elegans) nor in plants or yeast. Recently, a splice variant of this gene named nUBE1L1 has been described as being strongly expressed in adult human testis (22). The sequence of UBE1L2 also contains two important consensus motifs conserved among E1 enzymes: the highly conserved ATP-binding motif (GXGXXGCE; amino acids 467–474) and the putative active site with the consensus sequence PXCTXXXP (amino acids 623–631).
surrounding Cys-625, which may form a thiolester with ubiquitin.

**UBE1L2 Specifically Activates Ubiquitin in Vitro**—Initially, the ability of UBE1L2 to activate ubiquitin or ubiquitin-like proteins by thiolester formation was investigated in vitro. GST-tagged ubiquitin and the ubiquitin-like proteins FAT10, ISG15, NEDD8, SUMO-1, UFM1, and URM1 were used to investigate an interaction with in vitro transcribed and translated HA-UBE1L2 in the presence of ATP in GST pulldown experiments. Samples were subjected to SDS-PAGE under reducing or non-reducing conditions. From Fig. 2A, it is evident that HA-UBE1L2 was unable to activate GST-FAT10 or other GST-tagged ubiquitin-like modifiers, although each of the used recombinant GST modifier proteins was able to interact specifically with their respective cognate E1 enzyme in vitro (data not show). Intriguingly, we found that UBE1L2 could form a thiolester linkage specifically with GST-ubiquitin, as seen in a higher molecular weight band when samples were applied to the gel in the absence of reducing agent (Fig. 2A). The covalent linkage between UBE1L2 and ubiquitin could be broken in the presence of β-mercaptoethanol, suggesting that the two proteins were thiolester-linked (Fig. 2B).

**Co-immunoprecipitation of UBE1L2 and Ubiquitin under Non-reducing Conditions**—To determine whether the interaction between UBE1L2 and ubiquitin also occurs in intact cells, HEK293T (human embryonic kidney) cells were transfected with expression constructs for either HA-UBE1L2 or HA-UBE1 alone or together with a FLAG-ubiquitin construct to carry out co-immunoprecipitation experiments. UBE1L2 was immunoprecipitated with anti-HA monoclonal antibody, and samples were analyzed by immunoblotting with anti-FLAG and anti-HA antibody. Fig. 2, C and D, illustrates that FLAG-ubiquitin coprecipitated with HA-UBE1L2 in cells coexpressing the two proteins. FLAG-ubiquitin and HA-UBE1L2 were found together in a high molecular weight band under non-reducing conditions (Fig. 2C). A reduction by boiling in 10% β-mercaptoethanol strongly reduced this band (Fig. 2D), but complete separation of UBE1L2 and ubiquitin could not be achieved, which may indicate that UBE1L2 is also isopeptide-linked to ubiquitin to a low extent or that the reduction was incomplete. This phenomenon was also observed in a similar experiment with HA-UBE1 and FLAG-ubiquitin, where under reducing conditions, some adduct remains (Fig. 2, E and F). Taken together, it appears that human UBE1L2 also activates ubiquitin in intact cells.

**UBE1L2 Transfers Activated Ubiquitin onto Ubch5b and Supports E3-mediated Polyubiquitylation**—Many E3 ligases including HectH9 and E6-AP serve as their own ubiquitylation substrates in vitro (autoubiquitylation) (20). Thus, autoubiquitylation assays are ideally suited to determine whether UBE1L2 can indeed function as an E1 ubiquitin-activating enzyme in protein ubiquitylation. To test whether UBE1L2 has E1 activity, HectH9 and E6-AP were expressed in bacteria (HectH9) or in the baculovirus system (E6-AP) and incubated in the presence of UBE1 or UBE1L2 under standard ubiquitylation conditions (19, 20). As E2, bacterially expressed Ubch5b was used, which is known to interact with the E3s tested. As expected, incubation of HectH9 or E6-AP in the presence of UBE1, Ubch5b, and ubiquitin resulted in efficient autoubiquitylation of the respective E3 (Fig. 3, A and B). Interestingly, bacterially expressed UBE1L2 facilitated ubiquitylation of HectH9 (Fig. 3A) as well as E6-AP (Fig. 3B) with an efficiency similar to UBE1. In addition, UBE1L2 supported Mdm2-mediated ubiquitylation of p53 in vitro (Fig. 3C) (21). Taken together, these data clearly show that UBE1L2 can substitute for UBE1 in the ubiquitylation reactions tested and, thus, functions as an ubiquitin-activating enzyme.
Expression Analysis of ube1l2 in Several Mouse Organs—Next, we determined the expression profile of UBE1L2 mRNA in different mouse organs. The mouse has a bona fide UBE1L2 protein that is well conserved with an identity of 88% to its human homologue. Quantitative real-time RT-PCR was conducted for lung, liver, kidney, intestine, brain, thymus, spleen, lymph nodes, uterus, ovaries, and testis. The UBE1L2 mRNA expression was normalized to HPRT expression in each organ, and the relative UBE1L2 expression level was calculated with the value for the kidney being arbitrarily set to one. As shown in Fig. 4, it is striking that UBE1L2 expression in the testis is about 5-fold higher than in any of the other organs tested, which could point at a testis-specific function of UBE1L2.

DISCUSSION

In the present study, human UBE1L2 was identified as a second ubiquitin-activating enzyme. Among the known E1 enzymes, UBE1L2 has the highest identity to the classical ubiquitin-activating enzyme UBE1 (40%) followed by the ISG15-activating enzyme UBE1L (35%) and the bipartite SUMO-activating enzyme UBA2 (25%)/AOS1 (16%). The similar length of activating enzyme UBE1L (35%) and the bipartite SUMO-activating enzyme UBA2 (23, 24) and APP-BP1/Uba3 (25). Clear evidence that the UBE1L2 protein by itself is capable of forming a thioester ubiquitin-activating enzyme UBE1 and not as a heterodimeric E1 complex, as formed by AOS1/Uba2 (23, 24) and APP-BP1/Uba3 (25). Clear evidence that the UBE1L2 protein by itself is capable of forming a thiolester with ubiquitin was then provided by our in vitro assay and by the transfection experiments showing that UBE1L2 could also activate ubiquitin in vivo (Fig. 2). That UBE1L2 indeed functions as an E1 enzyme was then shown by its ability to transfer the activated ubiquitin to the broad spectrum E2 enzyme UbcH5b and to support the autopolyubiquitylation of two E3 enzymes (Fig. 3, A and B) and Mdm2-mediated ubiquitylation of p53 (Fig. 3C). It will remain to be investigated whether UBE1L2 displays some degree of selectivity with respect to the E2 enzymes, which can be supplied with activated ubiquitin.

All known E1 enzymes are conserved from yeast to human, except for UBA5, which is found in several multicellular organisms but not in yeast (26). UBE1L2 is even more restricted in distribution because it is only found in vertebrates but not in lower organisms. This indicates that UBE1L2 might have a specialized role in tissues of higher organisms, and in particular, in the testis, where it is expressed by far most abundantly (Fig. 4). Gene targeting experiments will be required to determine this particular function of UBE1L2 and to elucidate the interesting question why nature has invented a second ubiquitin-activating enzyme that has the potential to selectively supply activated ubiquitin to a subset of E2 enzymes, E3 enzymes, and substrates.

ACKNOWLEDGMENTS—We thank Kay Hofmann, Eva Schlosser, and Sebastian Lukasiak for scientific advice and Adriano Marchese for the contribution of plasmids.

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doi: 10.1074/jbc.C700111200 originally published online June 19, 2007

Access the most updated version of this article at doi: 10.1074/jbc.C700111200

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