Human Homologs of Ubc6p Ubiquitin-conjugating Enzyme and Phosphorylation of HsUbc6e in Response to Endoplasmic Reticulum Stress*

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Ubiquitin-conjugating enzyme Ubc6p is a tail-anchored protein that is localized to the cytoplasmic face of the endoplasmic reticulum (ER) membrane and has been implicated in the degradation of many misfolded membrane proteins in yeast. We have undertaken characterization studies of two human homologs, hsUbc6c and hsUbc6e. Both possess tail-anchored protein motifs, display high conservation in their catalytic domains, and are functional ubiquitin-conjugating enzymes as determined by in vitro thiol-ester assay. Both also display induction by the unfolded protein response, a feature of many ER-associated degradation (ERAD) components. Post-translational modification involving phosphorylation of hsUbc6e was observed to be ER-stress-related and dependent on signaling of the PRK-like ER kinase (PERK). The phosphorylation site was mapped to Ser-184, which resides within the uncharacterized region linking the highly conserved catalytic core and the C-terminal transmembrane domain. Phosphorylation of hsUbc6e also did not alter stability, subcellular localization, or interaction with a partner ubiquitin-protein isopeptide ligase. Assays of hsUbc6es184D and hsUbc6es184E, which mimic the phosphorylated state, suggest that phosphorylation may reduce capacity for forming ubiquitin-enzyme thiol-esters. The occurrence of two distinct Ubc6p homologs in vertebrates, including one with phosphorylation modification in response to ER stress, emphasizes diversity in function between these Ub-conjugating enzymes during ERAD processes.

Targeted protein degradation via the proteasome requires substrate modification with covalent attachment of ubiquitin (Ub) through the sequential action of three classes of enzymes (reviewed in Ref. 1). First, Ub-activating enzyme (E1) in an ATP-dependent step catalyzes the formation of a high energy thioester bond between its active site cysteine and the C terminus (Gly-76) of Ub. The activated Ub is then transferred to the active site cysteine of a Ub-conjugating enzyme (E2) in a thiolesterification reaction. Target protein ubiquitination can be achieved usually in conjunction with E3 ubiquitin-protein ligases, to regulate substrate specificity. After the initial ubiquitination is attached to an internal Lys residue of the substrate, additional ubiquitins are added sequentially forming a Lys-48-linked polyubiquitin chain, which then targets the substrate for degradation by the 26S proteasome.

Ubiquitin-mediated protein degradation is involved in many cellular processes, including ER quality control of membrane proteins. In ER quality control, terminally misfolded or misassembled membrane proteins are disposed of by a multifaceted process termed ER-associated degradation (ERAD) (reviewed in Refs. 2 and 3). To become accessible to proteasomes, targeted proteins are retrotranslocated to the cytosol with the involvement of chaperones and components of the protein translocon machinery (4–6). Ubiquitination machinery specifically localized at the cytosolic face of the ER membrane then targets ERAD substrates for proteasomal degradation (7–9). Accumulation of misfolded proteins in the ER, which can be induced in numerous physiological and pathological conditions, activates the unfolded protein response (UPR) signaling pathway (reviewed in Ref. 10). Consequences of UPR activation in mammalian cells include transcriptional induction of UPR target genes, global translational repression, and cell-cycle arrest (reviewed in Ref. 11).

Genetic and biochemical studies in yeast have implicated the Ub-conjugating enzyme Ubc6p in the degradation of numerous ERAD target proteins. These include mutant forms of the translocon pore protein Sec61p (7), vacuolar protein Cpy (12), plasma membrane protein uracil permease (13), as well as wild-type or mutant ABC transporters Ste6p (14) and Pdr5* (15). Ubc6p is a tail-anchored ER-membrane protein with a single transmembrane domain at its extreme C terminus and an N-terminal catalytic domain oriented toward the cytoplasm (16). Location of Ubc6p to the ER membrane is a requirement for the proper turnover of its ERAD substrates (7, 17).

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3 The abbreviations used are: Ub, ubiquitin; ER, endoplasmic reticulum; UPR, unfolded protein response; ERAD, endoplasmic reticulum associated degradation; DTT, dithiothreitol; EST, expressed sequence tag; CMV, cytomegalovirus; PBS, phosphate-buffered saline; BSA, bovine serum albumin;
In mammals, as in yeast, ERAD target proteins are generally degraded by components of the ubiquitin-proteasome pathway. Mammalian ERAD substrates known to be ubiquitinated prior to degradation include ApoB (18), 3-hydroxy-3-methylglutaryl coenzyme A reductase mutants (19), unassembled T-cell receptors (20), and the cystic fibrosis transmembrane conductance regulator (21, 22). Recently, two highly conserved families of Ubc6p-related proteins in vertebrates have been identified by sequence alignment (23). Mammalian members of both subfamilies are bound to the cytosolic face of the ER membrane and are involved in mammalian ERAD (24–29). Their participation in the quality control of non-membrane proteins has also been suggested (30, 31). However, these homologs have yet to be characterized in detail, and it is unknown how they are distinguished functionally. We investigated these homologs and have identified a phosphorylation modification of hsUbc6e that occurs in response to ER stress.

**EXPERIMENTAL PROCEDURES**

**Construction of hsUbc6 and hsUbc6e Mammalian Expression Plasmids**—The amino acid sequence of yeast Ubc6p (NP_011026) was used to identify human orthologs from the expressed sequence tag (EST) database division of GenBank™ using TBLASTN (32). cDNA clones of the two human homologs, hsUbc6 (Image: 754549, AA411279, refseq NM_058167) and hsUbc6e (Image: 2387149, AA798116, refseq NM_016336), were used as PCR templates for construction of pCMV expression plasmids using standard procedures (33).

For hsUbc6, the N-terminal Myc epitope tag (EQKLISEEDL) was incorporated in-frame immediately following the natural start codon with a single round of PCR using forward (5'-ATA AGA ATG CGG CCC ACC ACC ATG GAA CAA AAA CTC ATC TCA GAA GAG GAT CTG AGC AGC ACC AGC AGT AAG) and reverse (5'-ATA GTT TAG CGG CCC ACC TGT GCT GGG CAG TGT) oligonucleotides. The N-terminal Myc-tagged version of hsUbc6 lacking the transmembrane domain (hsUbc6eATMD) was generated with the forward sequence primer above and the reverse oligonucleotide (5'-ATA GTT TAG CGG CCC TTT ACC CAC CAT GAT CAG TGT G), leading to the introduction of the TAA stop codon immediately following amino acid G286 (Fig. 1A). For hsUbc6e, the N-terminal Myc epitope was incorporated using the forward (5'-ATA AGA ATG CGG CCC ACC ACC ATG GAA CAA AAA CTC ATC ATC TCA GAA GAG GAT CTG AGC AGC ACC AGC AGT AAG) and reverse (5'-ATA GTT TAG CGG CCC TTT ACC CAC CAT GAT CAG TGT G) oligonucleotides.

The Myc-tagged mutants of hsUbc6e S184A, S184D, S184E, T195A, S251A, and S266A plasmid versions were generated using the QuikChange site-directed mutagenesis kit (Stratagene) with the following oligonucleotides: S184A (5'-GGC TAG GCA AAT AGC CCT TTA GGC AGT CAA TTC ATC TGG), S184D (5'-GAA CTG CCT AGG CAA ATA GAC TTT AAG GCA GAA GTC AAT TC), S184E (5'-GAA CTG CCT AGG CAA ATA GAC TTT AAG GCA GAA GTC AAT TC), T195A (5'-CAA TTC ATC TGG AAA GGC TAT CTC TGA GTC AGA CTT AAA CC), S251A (5'-GCT AAG AAT ACC TCC ATG GCC CCT CCA CAG CGC), and S266A (5'-GCA GAG TCA GAG AAT GTC TAA GGC TAC TCC ACC AGA TGT AAT CC). All PCR-generated and coding segments of expression vectors were verified by DNA sequencing.

**Cell Cultures and Transfections**—HEK293, Panc-1, and SH-SY5Y cells were grown at 37 °C with 5% CO₂ in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and antibiotics. Subconfluent HEK293 and Panc-1 cells were transfected according to the ExGen 500 in vitro transfection reagent protocol provided by the manufacturer (MBI Fermentas), using 10 μg of expression plasmid per 100-mm plate. Cells were harvested 24 h later, or as indicated for individual experiments. perk⁻⁻ mutant cells (34) were cultured as previously described (35). SH-SY5Y cells were transfected with Lipofectamine 2000 (Invitrogen), using 5 μg of expression plasmid per 60-mm plate.

**Protein Extract Preparation, Electrophoresis, and Immunoblotting**—Transfected cells were washed with ice-cold PBS and solubilized in radioimmune precipitation assay (RIPA) buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% deoxycholate, protease inhibitor mixture (Roche Applied Science)) supplemented with phosphatase inhibitors (10 mM NaF, 10 mM β-glycerophosphate, 10 mM sodium orthovanadate, 10 mM glucose 1-phosphate, 10 mM para-nitrophenol phosphate), for 5 min on ice. Cell debris was removed by centrifugation at 15,000 × g for 15 min at 4 °C, and the supernatant protein extracts were used for immunoblot analysis or immunoprecipitation. Protein samples were separated by SDS-PAGE on 11.5% gels and transferred to Hybond-C super membranes (Amersham Biosciences). Immunoblot detection of Myc fusion proteins was performed using a 1:5000 dilution of monoclonal anti-Myc antibody (clone 9E10, Oncogene). To detect endogenous hsUbc6e, rabbit polyclonal antibodies were raised against the peptide antigen AKNTSMPQRRQQAQQQS and affinity-purified (Washington Biotechnology, Inc.). β-Tubulin, glyceraldehyde-3-phosphate dehydrogenase, and calnexin antibodies (Santa Cruz Biotechnology, Inc.) and phospho-eIF-2α (Ser51) (Cell Signaling) were used according to manufacturers’ protocols. Immunoreactive protein was detected using enhanced chemiluminescence of horseradish peroxidase-conjugated anti-mouse secondary antibody (Amer sham Biosciences) or anti-rabbit secondary antibody (Bio-Rad) with Hyperfilm™ ECL film (Amersham Biosciences).

**Phosphatase Assay**—Protein extracts from 3 × 100-mm plates transfected with hsUbc6e were collected and pooled. Immunoprecipitation of Myc-tagged hsUbc6e was performed by incubating the lysates with a 1:100 dilution of anti-Myc antibody (clone 9E10, Oncogene). To detect endogenous hsUbc6e, rabbit polyclonal antibodies were raised against the peptide antigen AKNTSMPQRRQQAQQQS and affinity-purified (Washington Biotechnology, Inc.). β-Tubulin, glyceraldehyde-3-phosphate dehydrogenase, and calnexin antibodies (Santa Cruz Biotechnology, Inc.) and phospho-eIF-2α (Ser51) (Cell Signaling) were used according to manufacturers’ protocols. Immunoreactive protein was detected using enhanced chemiluminescence of horseradish peroxidase-conjugated anti-mouse secondary antibody (Amer sham Biosciences) or anti-rabbit secondary antibody (Bio-Rad) with Hyperfilm™ ECL film (Amersham Biosciences).

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Ubc6p Homologs and ER Stress-related Phosphorylation

UPR Induction, RNA, and Protein Analysis—Cultured Panc-1 cells were incubated with 10 μg/ml tunicamycin (Calbiochem) in Me₂SO or Me₂SO alone. RNA was extracted using a QiAmp TMM Homogenizer and RNeasy® mini kit (Qiagen) according to the supplier’s protocols. 10 μg of total RNA was resolved on agarose gels containing 1% formaldehyde, followed by blotting to nylon membranes (HybTm, N, Amersham Biosciences) and UV cross-linking. Following hybridization with 32P-labeled hsUbc6e cDNA or hsUbc6 cDNA fragments, membranes were washed and exposed to film. Membranes were then deproteinized and sequentially rehybridized with Grp78 (Image 878587) and glyceraldehyde-3-phosphate dehydrogenase cDNA probes (33).

To examine ER stress effects on phosphorylation, cells were incubated with 10 μg/ml tunicamycin (Calbiochem), 1.0 μM thapsigargin (Sigma-Aldrich), or 2.0 mM DTT (Sigma-Aldrich). For UPR induction experiments with overexpressed hsUbc6e, Panc-1 cells were first transfected using 0.5 μg of Myc-hsUbc6e plasmid (mixed with pCMV vector to a total of 3 μg of DNA) per 60-mm dish, grown overnight, and then treated. Cell lysates were prepared, and protein samples were separated by SDS-PAGE for detection of endogenous or Myc-tagged hsUbc6e by immunoblot analysis, as above.

PERK in Vitro Kinase Assay—First strand cDNA from fetal liver total RNA (Stratagene) was synthesized by reverse transcription with oligo(dT)12–18 primer. Using total first strand cDNA as template, the cDNA of PERK kinase domain (amino acid residues 589–1114) was generated by standard PCR procedures with the forward (5'-AAC AGA ATG CGG CCC TCA CAG GCA AAG GAA G) and reverse (5'-CCG CTC GAG CTA ATT GCT TGG CAA AGG) oligonucleotides. The PCR-generated open reading frame segment was inserted into the pET-28a bacterial expression vector (Novagen) and verified by sequencing. Bacterial transformation, induction of protein expression, and protein purification were carried out as described below. For the in vitro kinase assay, purified PERK kinase domain (2.5 μM) or mock (vector control), was preincubated with kinase buffer (50 mM Heps, 10 mM MgCl₂, 5 mM EDTA, 100 mM NaCl, 30 μM ATP) for 10 min at 30 °C. Kinase reactions were initiated with addition of substrates, purified eIF2α (100 μM) (a gift from F. Sicheri) or His-Ubc6eΔTMD (50 μM) (described below), and 2.0 μCi of [γ-32P]ATP, with incubation for 20 min at 30 °C. Reactions were terminated with addition of Laemmli sample buffer, and products were resolved by SDS-PAGE, followed by autoradiography. Purified PERK and Ubc6e proteins were detected by immunoblot analysis with 1:7500 dilution of monoclonal anti-T7 antibody (Novagen) and enhanced chemiluminescence using Hyperfilm™ ECL film (Amersham Biosciences) as above.

Metabolic Labeling and Immunoprecipitation—Transfected cells from 100-mm plates were split 24 h post-transfection onto 60-mm plates and grown for an additional 24 h. For [32P]phosphate labeling, cells were incubated in phosphate-free Dulbecco’s modified Eagle’s medium (Cellgro) supplemented with 10% dialyzed fetal bovine serum (Invitrogen) for 2 h at 37 °C. The cells were then pulse-labeled by addition of 0.3 mCi/ml [32P]phosphate for 2 h at 37 °C, followed by a chase period of 2 h with addition of complete Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. For metabolic labeling with [35S]methionine and [35S]cysteine, cells were incubated in methionine- and cysteine-free α-MEM for 30 min at 37 °C. The cells were then pulse-labeled by addition of 140 μCi/ml [35S]methionine and [35S]cysteine (Pro-mix I-35S) in vitro cell labeling mix, Amersham Biosciences), 7 min, followed by chase periods with addition of complete Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum.

Cells were collected, and lysate volumes for immunoprecipitation were normalized for total labeled protein, as determined by scintillation counting of trichloroacetic acid-precipitated material. Immunoprecipitation of labeled extracts was performed as described above, and immunoreactive products were separated by SDS-PAGE. Gels were then fixed in 10% acetic acid and 40% ethanol, soaked for 30 min in Amplify (Amersham Biosciences), dried, and exposed to film. Labeled proteins were visualized by exposure to BioMax MR film (Eastman Kodak) for 1–3 days.

Membrane Isolation—Transfected HEK293 cells were washed with PBS and scraped into ice-cold suspension buffer (0.25 M sucrose, 10 mM triethanolamine, 1 mM EDTA, pH 7.4) supplemented with protease inhibitor mixture and phosphatase inhibitors. The cell suspension was passed 15 times through a 27-gauge needle and then centrifuged at 1000 x g for 5 min at 4 °C. The clarified extract was then centrifuged at 100,000 x g for 1 h at 4 °C. The supernatant was collected and subjected to trichloroacetic acid precipitation. Precipitated material and the 100,000 x g pellet were resuspended in an equal volume of RIPA buffer and then analyzed by immunoblot.

Immunofluorescence—COS-7 cells transfected with expression vectors were split 24 h post-transfection and cultured for an additional 24 h in 4-chamber slides (BD Falcon). The cells were fixed and permeabilized in 4% paraformaldehyde and 0.1% Triton X-100 treatments for 30-min durations at room temperature. Following blocking with 1% BSA in PBS (PBS-BSA) for 30 min, cells were incubated with anti-Grp94 antibody (Santa Cruz Biotechnology, Inc.), diluted 1:1000 in PBS-BSA, for 1 h at room temperature. Following antibody treatments, cells were washed twice with PBS-BSA and incubated with 1:1000 anti-goat rhodamine-conjugated secondary antibody (Jackson ImmunoResearch Laboratory, Inc.) for 1 h at room temperature. Cells were again washed and incubated with anti-Myc antibody, diluted 1:1000, for 1 h, then washed again and incubated with 1:300 anti-mouse fluorescein isothiocyanate-conjugated secondary antibody (Jackson ImmunoResearch Laboratory, Inc.) for 1 h at room temperature. Following antibody treatments, cells were washed twice with PBS-BSA, once with PBS, and mounted with Vectashield mounting medium (Vector Laboratories). Indirect immunofluorescence was examined with a Nikon E1000 fluorescence microscope. Images were captured using a charge-coupled device camera and OpenLab Spectrum Software (Scanalytics).

Cloning of Parkin and Pael-R cDNAs—First strand cDNA from human fetal brain total RNA (Clontech) was synthesized by reverse transcription with oligo(dT)12–18 primer. Using total first strand cDNA as template, open reading frame cDNA of Parkin was generated by standard PCR procedures with the forward (5'-ATA GTG TTT GTC AGG TTC) and reverse (5'-
CTA CAC GTC GAA CCA GTG) oligonucleotides. Open reading frame cDNA of Pael-receptor (Pael-R) was generated with two overlapping PCR products using the forward (5′-GCA GCA GTT GTC GAT CCT A-3′) and reverse (5′-TGA TGA GAA ATG CCC ACC AGA AGG-3′), and the forward (5′-GGA TTC TTG GTT GAA GGA AT-3′) and reverse (5′-CAA GTA CTG-3′) oligonucleotides. The full-length open reading frame of Pael-R was then generated by overlap extension of the two PCR fragments.

Open reading frame cDNAs were used as PCR templates for construction of pcMV expression vectors. N-terminal FLAG-tagged Parkin with start site Kozak consensus sequence was generated with the forward (5′-ATA AGA ATG CCG CCC ACC ATG TAC AAG GAT GAC GAC GAT AAG GCT ATA GTG TTT GTC AGG TTG-3′) and reverse (5′-ATA GTT TAG CGG CCG CCT ACA CGT CGA ACC AGT G-3′) oligonucleotides. C-terminal hemagglutinin (HA)-tagged Pael-R with start site Kozak consensus sequence was generated with the forward (5′-ATA AGA ATG CGG CCG CCC ACC ATG CGA GCC CCG GGC GCG CTT-3′) and reverse (5′-CAA GTA CTG-3′) oligonucleotides. All PCR-generated segments and reading frames of expression vectors were verified by DNA sequencing.

Co-immunoprecipitation of Parkin and hsUbc6e—Protein extracts were prepared from transfected HEK293 in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, 1 mM DTT, protease inhibitor mixture (Roche Applied Science)) supplemented with phosphatase inhibitors. Immunoprecipitation of FLAG-tagged Parkin was performed by incubating the lysates with a 1:1000 dilution of anti-FLAG antibody (Sigma) for 1 h at 4 °C, followed by incubation with 40 μl of 50% Protein-G-Sepharose slurry (Roche Applied Science) for 4 h at 4 °C. The beads were washed three times with ice-cold lysis buffer (1 ml). Immunoprecipitated products were separated by SDS-PAGE for immunoblot analysis.

RESULTS

HsUbc6 and hsUbc6e Sequence Analysis and Confirmation of E2 Activity by Thiol-Ester Assay—Human homologs display high sequence conservation to yeast Ubc6 in their catalytic cores, with 64 and 46% identity for hsUbc6 and hsUbc6e, respectively, and are invariant at their catalytic cysteine positions (Fig. 1A). As well, both homologs possess hydrophobic C-terminal transmembrane domains and lack N-terminal signal sequences indicating that they are tail-anchored proteins. However, with the exception of the transmembrane domain, the sequences in regions C-terminal to the catalytic core have been poorly conserved. Both Ubc6 and Ubc6e subfamilies are represented in all eukaryotic kingdoms, including fungi, protists, plant, and metazoa with Ubc6e subfamily members exhibiting divergent and longer C-terminal segments, as illustrated with hsUbc6e.

To demonstrate that the homologs are bona fide E2s, their activity was assessed by in vitro thiol-ester assay. As recombinant proteins, hsUbc6e and hsUbc6 accept ubiquitin from E1 in an ATP-dependent manner by their catalytic cysteines (Fig. 1A). To generate S184D and S184E variants, the corresponding full-length pcMV expression vectors were used as templates in PCR. The transmembrane coding regions were excluded to promote solubility of the fusion proteins. PCR-generated open reading frame segments were verified by sequencing after insertion into the pET-28a expression vector (Novagen). Escherichia coli cells (BL21(DE3)) transformed with expression plasmids were induced with 0.2 mM isopropyl-β-thiogalactopyranoside for 4 h at 37 °C and collected and lysed with B-PER® Bacterial Protein Extraction Reagent according to the manufacturer’s protocol (Pierce). Expressed proteins were purified by affinity chromatography using nickel-nitrilotriacetic acid His-Bind® resin (Novagen) according to manufacturer’s protocol.

Thiol-Ester Assay—Thiol-ester E2 complex formation with ubiquitin was measured for hsUbc6e and the S184D and S184E variants in a two-step assay. Ubiquitin-activating enzyme, E1, was preincubated as follows: 0.3 μM rabbit E1 (Boston Biochem), 1 μM ubiquitin (Sigma, U6253), 2 mM ATP, 20 mM Tris, pH 7.5, 50 mM NaCl, 15 mM MgCl2 at 37 °C for 10 min. After cooling to 7 °C, hsUbc6e was added to a final concentration of 0.2 μM, and incubation was continued as indicated. Reactions were stopped with Laemmli sample buffer, and products were resolved by SDS-PAGE, followed by immunodetection with a 1:7500 dilution of monoclonal anti-T7 antibody (Novagen) and enhanced chemiluminescence using Hyperfilm™ ECL film (Amersham Biosciences). Purified hsUbc6e was used as a standard for densitometry measurement (FluorChem 9900, Alpha Innotech).

Recombinant Expression and Purification of hsUbc6e, hsUbc6eS184D, and hsUbc6eS184E—Bacterial expression vectors of His6 tag-fused hsUbc6eΔTMD and variants were generated by PCR with forward (5′-CGC GGA TCC ATG GAG ACC CGC TAC AAC CTG) and reverse (5′-CCG CTC GAG TTA CCC ACC ATG ATC AGT GTG) oligonucleotides, leading to the introduction of the TAA stop codon following amino acid Gly-286 (Fig. 1A). To generate S184D and S184E variants, the corresponding full-length pcMV expression vectors were used as templates in PCR. The transmembrane coding regions were excluded to promote solubility of the fusion proteins. PCR-generated open reading frame segments were verified by sequencing after insertion into the pET-28a expression vector (Novagen).
regulated by the UPR in Panc-1 secretory cells. Cells were treated with tunicamycin, a potent inhibitor of N-linked glycosylation, and then subjected to mRNA analysis (Fig. 2). The ER chaperone Grp78 showed sustained increased expression confirming that UPR had been invoked. HsUbc6e showed strong induction with a 7-fold increase in expression following 24 h of tunicamycin treatment (average of two trials). HsUbc6 was also induced significantly; both /H110111.3-kb and /H110112.3-kb messages showed 2.5-fold increases following 8 h of treatment (average of two trials). Peak inductions were distinct; hsUbc6e expression increased slowly and was sustained at 24 h, very much like Grp78, whereas hsUbc6 expression increased rapidly, peaking at /H110118 h and then dissipated even with continued drug treatment. Similar distinct response patterns of specific UPR target genes have been shown to occur for other ERAD components (36).

HsUbc6e Is Phosphorylated in Response to ER Stress—To characterize the human Ubc6 homologs, N-terminal c-myc epitope fusion plasmids were constructed for transient expression in HEK293 cells. Immunoblotting of whole cell lysates using anti-Myc antibody identified protein products consistent with calculated sizes (Fig. 3A). Interestingly, a prominent doublet was observed for hsUbc6e. Sensitivity to phosphatase treatment indicated that the upper band (b-form) likely represented a phosphorylated form of the lower band (a-form) (Fig. 3B). To confirm that the endogenous hsUbc6e is also susceptible to phosphorylation, we raised polyclonal antibodies against a peptide sequence in the less conserved C-terminal region. Specificity of the antibody was confirmed by the detection of a band in whole cell extracts that co-migrated with the overexpressed hsUbc6e (Fig. 3C), and the absence of immunoreactivity with the pre-immune sera (data not shown). Endogenous hsUbc6e

FIGURE 1. Human Ubc6 and Ubc6e are active E2 enzymes. A, comparison of scUbc6, hsUbc6, and hsUbc6e amino acid sequences by ClustalW alignment. The conserved catalytic cores are boxed, and the invariant active site cysteine positions are indicated with an asterisk. C-terminal transmembrane domains are underlined. Putative hsUbc6e phosphorylation sites, predicted by NetPhos (54) and Scansite (55), are indicated by arrowheads. sc, Saccharomyces cerevisiae; hs, Homo sapiens. B, HsUbc6eTMD and hsUbc6TMD are active E2s by their ability to accept ubiquitin from E1 in non-reducing conditions. The in vitro thiol-ester assay demonstrated dependence on ATP and involvement of the catalytic cysteines as predicted by alignment. C, Alignment of sequence surrounding Ser-184 in Ubc6e subfamily members. Ser-184 and flanking predicted calmodulin kinase II and protein kinase C consensus sequences are conserved in Homo sapiens (hs), Mus musculus (mm), Gallus gallus (gg), and Xenopus tropicalis (xt) but not in Danio rerio (dd) or Caenorhabditis elegans (ce).

FIGURE 2. HsUbc6 and hsUbc6e are induced by UPR. Panc-1 cells were treated with tunicamycin (10 μg/ml in Me2SO (DMSO)) or Me2SO for the times indicated leading to increased hsUbc6 and hsUbc6e mRNA expression. Total RNA blots were probed with hsUbc6e and hsUbc6 cDNAs. Representative results are shown. The two messages observed for hsUbc6 are consistent with different polyadenylation signals as evident from two major classes of clones in dbEST of GenBankTM. Grp78 was used as a control for UPR induction and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the RNA quantitation control.
in unstressed Panc-1 cells was detected as a single band; however, when protein expression was induced by tunicamycin treatment, the upper band appeared. This upper form was confirmed to be phosphorylated by phosphatase treatment (Fig. 3D). With short durations of drug treatment, it was evident tunicamycin induced the phosphorylation of endogenous hsUbc6e, indicating that this modification is ER stress-regulated (Fig. 3E). This stress-induced modification was also observed in Caco-2, HEK293, and COS-7 cell lines (data not shown). Phosphorylation was also induced by other known activators of ER stress, thapsigargin, which promotes release of intracellular Ca\(^{2+}\) stores and DTT, a reducing agent that causes protein unfolding. Tunicamycin elicited a modest and slow increase in phosphorylated hsUbc6e, whereas thapsigargin and DTT induced stronger and more rapid responses. In tunicamycin- and thapsigargin-treated cells, phosphorylated hsUbc6e was still present after 8 h of incubation, accounting for \(\sim15\) and \(\sim25\)% of total protein, respectively, as measured by densitometry. A known responder to ER stress is translation initiation factor eIF-2\(\alpha\) whose phosphorylation during UPR results in translation attenuation (37). By comparison, strong eIF-2\(\alpha\) phosphorylation occurs only during early UPR (38); hsUbc6e phosphorylation was temporarily different, being maintained throughout tunicamycin- and thapsigargin-induced ER stress (Fig. 3E). Interestingly, DTT treatment elicited a distinct response with a peak amount of phosphorylated hsUbc6e at 1–2 h, accounting for up to \(\sim25\)% of total protein, with subsequent decrease. The same response was also observed at lower DTT levels (0.5 mM, data not shown). The decrease in phosphorylated level was not a result of DTT clearance, because the protein levels of hsUbc6e and ER chaperone Grp94 continue to increase up to at least 24 h confirming continued ER stress (data not shown). The observed differences in phosphorylation induced by tunicamycin, thapsigargin, and DTT likely reflect the distinct nature of each applied stress and the cellular responses elicited. Together, these data demonstrate that hsUbc6e is phosphorylated during the ER stress response in a manner dependent on the nature of the stressor.

**ER Stress-induced Phosphorylation of mmUbc6e Requires PERK Signaling**—It was considered that hsUbc6e may be a target of PERK, an ER transmembrane serine/threonine protein kinase that is activated during ER stress to inhibit global protein synthesis via eIF-2\(\alpha\) phosphorylation (39, 40). To examine the involvement of PERK in murine Ubc6e phosphorylation, wild-type and perk\(-/-\) mouse embryonic fibroblasts (MEFs) were stressed with thapsigargin (1.0 \(\mu\)M) and DTT (2.0 mM) for up to 8 h.

FIGURE 3. **hsUbc6e is phosphorylated during ER stress.** A, expression of Myc-tagged hsUbc6e and hsUbc6 in transiently transfected HEK293 cells. The anti-myc antibody detected two bands for hsUbc6e, referred to as forms a and b. B, Myc-tagged hsUbc6e immunoprecipitated from HEK293 transfection lysates were treated with alkaline phosphatase (5 or 10 units) for the indicated times. The b-form decreased with incubation but could be sustained in presence of phosphatase inhibitors (pi). C, detection of endogenous hsUbc6e in tunicamycin-treated Panc-1 cells using polyclonal antibodies. Panc-1 cells were treated for the times indicated with either tunicamycin (10 \(\mu\)M or Me\( \text{SO} \) (DMSO); Control lane, Panc-1 cell transfected with Myc-tagged hsUbc6e. D, endogenous hsUbc6e is phosphorylated. Lysates of tunicamycin-treated Panc-1 cells were treated with alkaline phosphatase (10 units) for 30 min. The b-form was sustained in the presence of phosphatase inhibitors. Control lane, untreated. E, endogenous hsUbc6e is phosphorylated during ER stress (upper panel). The phospho-specific antibody to eIF-2\(\alpha\) confirms the stress response was elicited (middle panel). \(\beta\)-Tubulin was used as loading control (lower panel). Panc-1 cells were treated with tunicamycin (10 \(\mu\)g/ml), thapsigargin (1.0 \(\mu\)M), and DTT (2.0 mM) for up to 8 h.
Ubc6p Homologs and ER Stress-related Phosphorylation

...toward hsUbc6e (Fig. 4C) under the conditions used. This, together with the observation of low amounts of phosphorylated mmUbc6e in perk−/− cells (Fig. 4B), suggests that PERK may not be the direct responsible kinase but that Ubc6e phosphorylation requires PERK signaling during ER stress.

**HsUbc6e Is Phosphorylated at Ser-184—**HsUbc6e protein sequence analysis identified several putative phosphorylation sites (Fig. 1A), of which Ser-184, Thr-195, Ser-251, and Ser-266 were selected as candidate sites based on sequence conservation with the mouse ortholog. Expressed versions with individual mutagenized sites then revealed that the S184A mutant showed complete absence of the b-form, whereas the other mutants retained their doublet patterns (Fig. 5A). To confirm that the b-form results from phosphorylation of Ser-184 alone, metabolically [35S]phosphate labeling of wild-type and S184A hsUbc6e was performed in transiently transfected HEK293 cells (Fig. 5B). By immunoprecipitation and SDS-PAGE, the [35S]phosphate-labeled b-form of wild-type hsUbc6e was readily detected. A corresponding band was not detected with the S184A mutant, and only very minor alternate labeled species were observed. Thus, the b-form of hsUbc6e, migrating as a major discreet band as detected by immunoblot (Fig. 5B, lower panel), is the Ser-184 phosphorylated form of hsUbc6e. To establish that the UPR-regulated phosphorylation of hsUbc6e involved Ser-184, Panc-1 cells were transfected with wild-type and S184A hsUbc6e, and then treated with tunicamycin, thapsigargin, or DTT. Immunoblots show that the amount of b-form increased during treatment with various ER stress inducers while the S184A mutant remained unchanged.

Residue Ser-184 is located between the catalytic core domain and the transmembrane domain, within a highly charged segment. The serine and flanking predicted kinase consensus sequences are completely conserved within mammalian members of the Ubc6e family, including *M. musculus* and *G. gallus* as well as with amphibian *X. tropicalis*, but not with lower vertebrates, *D. rerio*, or invertebrates, *C. elegans* (Fig. 1C), or with Ubc6 subfamily members.

**Phosphorylated hsUbc6e Is Stable and Localized to the ER Membrane—**We investigated the stability of hsUbc6e by metabolic [35S]methionine and [35S]cysteine pulse-chase labeling in HEK293 cells transfected with wild-type hsUbc6e (Fig. 6A). Following a short pulse, the phosphorylated form appeared early, being readily evident at the earliest chase periods (30 min or less), and by ~12 h accounted for as much as ~40% of the labeled protein (phosphorimaging analysis). The observed constitutive phosphorylation of Ubc6e did not appear to be related to ER stress, because Grp78 expression and phosphorylated eIF-2α protein levels remained unchanged with Ubc6e overexpression (data not shown). Overall, both forms were remarkably stable with the b-form persisting with a half-life equal to the a-form (t1/2 ~ 20 h), indicating that phosphorylation did not significantly affect the stability of hsUbc6e.
We also examined whether phosphorylation regulated or modified the ER membrane localization of hsUbc6e. Like the yeast ortholog, hsUbc6e has no known ER-retention/retrieval signal, and a basis for ER retention is not completely understood, although the hydrophobic tail segment of the yeast ortholog is thought to contain some targeting information (42). Isolation of crude membrane fractions revealed that both full-length phosphorylated and unphosphorylated forms of hsUbc6e are membrane-associated (Fig. 6B). Consistent with the involvement of stress signaling at the ER membrane, it was also evident that phosphorylation requires hsUbc6e membrane localization, as a truncated version lacking the transmembrane domain (ΔTMD, deletion of amino acid residues 287–318) was not modified, as detected by immunoblot (Fig. 6C). Immunofluorescence was then used to evaluate whether phosphorylation of hsUbc6e functioned as a targeting determinant. By indirect immunofluorescence of transiently transfected COS-7 cells, myc-tagged hsUbc6e with an intact carboxyl TMD displayed dense perinuclear and extended lace-patterned staining characteristic of ER membrane localization and strongly colocalized with the ER marker protein, Grp94 (Fig. 7B). In contrast, hsUbc6eΔTMD exhibited diffuse staining throughout the entire cell (Fig. 7A). Single site mutants S184D and S184E, which possess negatively charged amino acid side chains at position 184, were generated to assess the effect of phosphorylation on hsUbc6e localization. These phosphorylation-mimicking variants localized similarly to wild type, also displaying an ER-patterned staining (center and right panels, Fig. 7C). Moreover, S184A, which cannot be phosphorylated, also displayed similar ER-patterned staining (left panel, Fig. 7C), further demonstrating that phosphorylation has no direct role in determining hsUbc6e ER localization.

**Phosphorylation Affects Formation of Ubiquitin-hsUbc6e Thiol-Esters**—A thiol-ester assay was employed to examine whether phosphorylation of hsUbc6e alters enzymatic activity as measured by E2 bond formation with ubiquitin. Single endpoint trials revealed the capacity of both recombinant wild-type and phosphomimicking variants in pulse-chase experiments (Fig. 8B). However, it was apparent that hsUbc6e variants had no effect on Pael-R half-life (Fig. 8C). In fact, the unaltered half-life with the C91S variant indicated that alternate E2s play more prominent roles in vivo, despite the ability of hsUbc6e to bind Parkin.

**Phosphorylation Has No Effect on Parkin E3 Interaction and Degradation of Pael-R**—We next examined whether phosphorylation of hsUbc6e could modulate interaction with a reported partner E3 or influence turnover of an ERAD substrate. E3 ubiquitin-ligase Parkin can directly interact with hsUbc6e alone, or together with hsUbc7 has been shown to ubiquitinate Pael receptor (Pael-R), in vitro (26). This receptor is a transmembrane protein prone to aggregation causing ER stress in a neuronal cell model (26). With transiently transfected HEK293 cells, both phosphorylated and unphosphorylated forms co-immunoprecipitate with Parkin (Fig. 8A), indicating that phosphorylation does not modify this interaction. Next, we examined whether phosphorylation altered hsUbc6e E2 activity on Pael-R. In transiently transfected SH-SY5Y neuroblastoma cells, the turnover of metabolically labeled Pael-R was measured with co-expression of wild-type hsUbc6e and phosphorylation-mimicking variants in pulse-chase experiments (Fig. 8B). However, it was apparent that hsUbc6e variants had no effect on Pael-R half-life (Fig. 8C). In fact, the unaltered half-life with the C91S variant indicated that alternate E2s play more prominent roles in vivo, despite the ability of hsUbc6e to bind Parkin.
Ubc6p Homologs and ER Stress-related Phosphorylation

FIGURE 8. HsUbc6e phosphorylation has no effect on Parkin E3 interaction and degradation of Pael-R. A, phosphorylated and unphosphorylated hsUbc6e co-immunoprecipitate with Parkin. HEK293 cells were transiently transfected with FLAG-tagged Parkin and wild-type or S184A Myc-tagged hsUbc6e. Anti-FLAG immunoprecipitants from cells expressing Parkin and hsUbc6e contain both phosphorylated and unphosphorylated Ubc6e, as detected by α-hsUbc6e immunoblot. B, pulse-chase analysis of Pael-R and Myc-tagged hsUbc6e, wild-type, or S184E, or vector control, pCMV. Cells were pulse-labeled and chased as indicated. C, pulse-chase results for Pael-R half-life (minutes) with co-transfection of pCMV, Myc-tagged hsUbc6e, wild-type, C91S, S184A, or S184E, are presented as means ± S.E. for three independent trials.

DISCUSSION

All Ubc6p homologs possess the characteristic catalytic core and membrane-spanning domain, however, the joining segments within and between Ubc6 and Ubc6e subfamily members have diverged significantly. Particularly, the Ubc6e subfamily members that are found only in higher eukaryotes exhibit notably extended segments. We have determined that hsUbc6e is phosphorylated in vivo during ER stress at Ser-184, which is positioned in this poorly characterized and divergent segment. This modification distinguishes the Ubc6e and Ubc6 vertebrate subfamilies, and its absence in lower eukaryotic E1-ubiquitin thiol-ester intermediate was found to be rate-limiting under the reaction conditions (results not shown). Purified wild-type, S184D, and S184E hsUbc6e were incubated for short periods with the preformed E1-ubiquitin complexes. At early time points (1 and 2 min), reaction products of the phosphorylation-mimicking mutants S184D and S184E exhibited reduced ubiquitin-E2 formation compared with wild-type (Fig. 9). Under identical conditions, the converted fractions were significantly reduced compared with wild-type, suggesting that phosphorylation at Ser-184 inhibited acceptance of ubiquitin from E1.

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We have also investigated a putative role for phosphorylation in modulating E3 ligase interaction. The C-terminal regions of E2s are frequently involved in protein complex interactions, including E3 partners (51, 52), so another plausible role for phosphorylation of the acidic tail region of hsUbc6e may be to modulate interactions with its own cognate E3s, permitting, if not signaling, substrate ubiquitination events. Phosphorylation-mediated E2-E3-regulated interaction has been reported; UBC3/Cdc34 in its C-terminal domain induces its interaction with the F-box protein β-TrCP, the substrate recognition subunit of an Skp1/Cull1/F-box protein ubiquitin ligase, and as a result, enhances β-catenin degradation (53). Co-immunoprecipitation experiments with the E3 Parkin, however, indicate that phosphorylation of Ubc6e does not modify this particular E2-E3 interaction, because both phosphorylated and unphosphorylated forms interacted with the E3. Alternatively, phosphorylation of hsUbc6e may also modulate its specific interaction with other E2s or E2 complexes or perhaps even with specific substrates. The effect of phosphorylation was also examined in co-expression studies with the ERAD substrate Pael-R. Although participation with Parkin in Pael-R elimination has been shown (26), we found no affect of Ubc6e overexpression on Pael-R turnover rate. The activity of other E2s involved in ERAD, such as Ubc7 (24, 25, 27) or the homologue Ubc5, may be redundant to Ubc6e in vivo.

With the former yeast studies and numerous examples of ERAD, the implication of roles for mammalian Ubc6 and Ubc6e in ubiquitination and degradation are apparent. However, it is unclear as to how, in higher eukaryotes, Ubc6 and Ubc6e are distinguished in their ERAD functions, particularly substrate-targeting events. The identification of the ER stress-related mammalian Ubc6e phosphorylation site entices new studies toward elucidating these distinctions. With implication of PERK in Ubc6e phosphorylation during ER stress, studies examining the role of phosphorylated Ubc6e as an effector of PERK cell survival signaling can now also be pursued.

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