A role for mammalian Ubc6 homologues in ER-associated protein degradation

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Accepted 10 May 2002
Journal of Cell Science 115, 3007-3014 (2002) © The Company of Biologists Ltd

Summary

Integral membrane and secretory proteins which fail to fold productively are retained in the endoplasmic reticulum and targeted for degradation by cytoplasmic proteasomes. Genetic and biochemical analyses suggest that substrates of this pathway must be dislocated across the membrane of the endoplasmic reticulum (ER) by a process requiring a functional Sec61 complex and multiubiquitinylation. In yeast, the tail-anchored ubiquitin-conjugating enzyme Ubc6p, which is localized to the cytoplasmic surface of the ER, participates in ER-associated degradation (ERAD) of misfolded proteins. Here we describe the identification of two families of mammalian Ubc6p-related proteins. Members of both families are also located in the ER membrane and display a similar membrane topology as the yeast enzyme. Furthermore we show that expression of elevated levels of wild-type and dominant-negative alleles of these components affects specifically ERAD of the α subunit of the T-cell receptor and a mutant form of the CFTR protein. Similarly, we describe that the expression level of Ubc6p in yeast is also critical for ERAD, suggesting that the Ubc6p function is highly conserved from yeast to mammals.

Key words: Endoplasmic reticulum, Ubiquitin, Proteolysis

Introduction

Protein folding in the endoplasmic reticulum is monitored by an elaborate 'quality control' system, which prevents the deployment to the secretory pathway of misfolded or unassembled proteins. Endoplasmic reticulum associated protein degradation (ERAD), an important component of this quality assurance system, directs misfolded proteins for destruction by cytoplasmic proteasomes (Sommer and Wolf, 1997; Kopito, 1997; Bonifacino and Weissman, 1998; Plemper and Wolf, 1999). One established role for ERAD is the destruction of integral membrane proteins that fail to fold productively, either because of mutation or because of intrinsic inefficiency in folding. For example, 50-75% of newly synthesized cystic fibrosis transmembrane conductance regulator (CFTR) molecules do not fold productively (Ward and Kopito, 1994) and are rapidly degraded by proteasomes by a process that appears to be initiated co-translationally (Sato et al., 1998). Mutations in CFTR cause the autosomal recessive disease cystic fibrosis; the most frequent allele (ΔF508) interferes with productive CFTR folding leading to near quantitative degradation (Ward and Kopito, 1994; Ward et al., 1995; Jensen et al., 1995). ERAD is also responsible for the degradation of unassembled subunits of oligomeric complexes such as the T-cell receptor (Yu et al., 1997; Huppa and Ploegh, 1997; Yang et al., 1998). Because lumenal and integral ERAD substrates are biosynthetically sequestered from the cytosol, their degradation must be coupled to their dislocation across the ER membrane. This dislocation step requires the Sec61p translocon complex (Wiertz et al., 1996; Plemper et al., 1997; Pilon et al., 1997) and appears to be tightly coupled to ubiquitin-conjugation of the dislocated substrate (Biederer et al., 1997). In addition, a Cdc48p complex seems to be involved in mobilization of the ubiquitinated substrate from the ER membrane (Bays et al., 2001b; Ye et al., 2001; Jarosch et al., 2002; Rabinovich et al., 2002). Although many studies have confirmed that covalent multiubiquitinylation is essential for ERAD the precise role of ubiquitin in the dislocation and degradation process remains unclear.

Formation of ubiquitin conjugates requires three classes of enzymes, the ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzymes (E2s or Ubcs) and ubiquitin-protein ligases (E3s) (Hershko and Ciechanover, 1998). Yeast Ubc6p is the first Ubc demonstrated to be involved in ERAD. It is the only integral membrane member of the yeast Ubc family. Its active ubiquitin-conjugating domain (Ubc domain) is located at the N-terminus while the C-terminus comprises a charged tail containing a stretch of hydrophobic amino acids. This transmembrane domain localizes Ubc6p in the ER membrane with the active site facing the cytosol. Because of this topology and the lack of an N-terminal signal sequence Ubc6p belongs to the family of tail-anchored membrane proteins (Sommer and Jentsch, 1993). Both genetic and biochemical evidence suggests that Ubc6p is required for ERAD of mutant Sec61p, an integral component of the ER membrane, as well as for mutant carboxypeptidase Y (CPY*), an ER-lumenal protein (Biederer et al., 1996; Hiller et al., 1996). In addition, Ubc6p is required for degradation of some cytoplasmic substrates (Chen et al., 1993). Restriction of Ubc6p to the ER membrane

is essential for these proteolytic functions (Sommer and Jentsch, 1993; Chen et al., 1993). Surprisingly, Ubc6p is not only a component of the ERAD system but also a short-lived substrate of this system (Walter et al., 2001). A second ubiquitin-conjugating enzyme involved in ERAD, Ubc7p, is also localized to the ER membrane but lacks a membrane anchor. This Ubc is recruited to the ER surface through interaction with its membrane-bound receptor Cue1p (Biederer et al., 1997). Involvement of Ubc7p in ERAD has been demonstrated for several substrates (Biederer et al., 1996; Hiller et al., 1996; Hampton and Bhakta, 1997). Recently, Ubc1p has also been implicated in ERAD of CPY* (Friedlander et al., 2000; Bays et al., 2001a), and Ubc1p and Ubc4p in the turnover of unassembled Vph1p (Hill and Cooper, 2000). Although there has been considerable progress in the identification and functional characterization of Ubcs required for ERAD in yeast, little is known about ERAD-related Ubcs in mammalian cells.

Here we describe the identification and functional characterization of two distinct families of Ubc6 orthologues in mammals. Both families share significant sequence similarity with the yeast enzyme and display a similar structural organization. Although both share similar topological arrangement in the ER membrane with yeast Ubc6p, neither mammalian protein is capable of complementing deficiencies in yeast. Interestingly, overexpression of wild-type and mutant forms of members of both families of Ubc6p-related proteins appear to exert a dominant-negative phenotype on the degradation of two well-characterized mammalian ERAD substrates, ΔF508-CFTR and TCRα.

Materials and Methods

DNA techniques, cell culture and high-efficiency transfection

A TBLASTN search with the sequence of the yeast open reading frame UBC6 (accession no. X73234) identified several ESTs that showed similarity to the whole length of the query sequence. We obtained human cDNAs 417998, 429672, 345901 and the mouse cDNA 423319 from the RZPD (German Resource Center for Genome Research) and sequenced them using vector- and insert-specific primers. The human cDNAs 429672 and 345901 contained overlapping 5′ and 3′ sequences of the same 954 bp ORF encoding a 318 amino acid protein with 27% identity to yeast Ubc6p. The sequence was designated HSUBC6e. Both 5′ and 3′ sequences were PCR-amplified separately and fused by a second PCR. This fragment was subcloned into vector pSL1190 and checked by DNA sequencing. The final sequence has been deposited in GenBank (accession no. U93243).

The mouse cDNA 423319 contained a single 777 bp ORF and has also been deposited in GenBank (accession no. U93242). This sequence encodes a protein of 259 amino acids with 58% identity to yeast Ubc6p and was therefore termed MMUBC6. The human cDNA 417998 contained a 3′ sequence, which aligned to mouse cDNA 423319. The assumed ORF encodes C-terminal 197 amino acids with 93% identity to mmUbc6p.

To generate epitope-tagged versions of hsUbc6ep and mmUbc6p the corresponding DNA sequences were subcloned into pSL1190. Then a PCR fragment containing three successive *myc*-epitopes (Schneider et al., 1995) was ligated in-frame to the N-terminus of both sequences. PCR-mutagenesis was used to introduce the functional mutations C91S into HSUBC6e and myc-HSUBC6e, and C94S into MMUBC6 and myc-MMUBC6.

All generated versions of HSUBC6e and MMUBC6 (wild-type, *myc*-tagged wild-type, Cys/Ser-mutants and *myc*-tagged Cys/Ser-mutants) were subcloned into the following yeast expression vectors: pRS426 (2μ), pRS414 (ARS-CEN) and pDP83 (ARS-CEN). Expression was driven by the *CUP1* (pRS426), *GAL10* (pRS414) and the endogenous yeast *UBC6* (pDP83) promoter sequences.

For the expression in HEK 293 cells, HSUBC6e, HSUBC6e-C91S, myc-HSUBC6e, myc-HSUBC6e-C91S, MMUBC6, MMUBC6-C94S, myc-MMUBC6 and myc-MMUBC6-C94S were subcloned into pCMVPLD, a pCMV (Stratagene)-derived eukaryotic expression vector. HEK 293 cells were maintained in complete DMEM medium and were transiently transfected using calcium phosphate-DNA precipitates formed in Hepes (Ausubel et al., 1989).

The cDNA corresponding to 2B4 TCRα (Saito et al., 1987), a kind gift from J. Bonifacino (NIH, Bethesda, MD), was subcloned into pCDNA3.1 (Stratagene) for expression in HEK cells. HEK cells were grown in DME containing 10% fetal bovine serum, penicillin and streptomycin at 37°C and 5% CO_2. The cells (~2×10^6) were transiently cotransfected by calcium phosphate precipitation as described previously (Ward and Kopito, 1994) with 3 μg TCRα.

Expression analysis, localization and pulse chase analysis

Microsomes of pCMVPLDmyc-HSUBC6e- or pCMVPLDmyc-MMUBC6-transfected HEK 293 cells were prepared as follows: 24 hours post transfection cells were scraped, then washed once in PBS and once in HB-buffer (50 mM Tris/HCl pH 7.5, 250 mM saccharose, 2 mM EDTA, 150 mM KCl, 1 mM DTT). The cell suspensions were carefully dounced on ice for 5 minutes and then centrifuged at 3000 ***g*** for 10 minutes at 2°C. These crude homogenates were used for protease protection assays (see below). Membranes were prepared by centrifugation of the homogenates at 10,000 ***g*** for 15 minutes. The supernatants were then centrifuged at 75,000 ***g*** for 60 minutes and the pellets were resuspended in membrane buffer (150 mM sucrose, 50 mM Hepes pH 7.5, 2.5 mM MgOAc, 50 mM KOAc). Expression of *myc*-tagged Ubc6 homologues was checked by SDS/PAGE of membranes and supernatants followed by immunoblotting using an anti-*myc* antibody (Santa Cruz Biotechnology). Protease protection assays were done according to Sommer and Jentsch (Sommer and Jentsch, 1993). Crude HEK 293 microsomes remained untreated, treated with proteinase K (50 μg/ml) or treated with proteinase K (50 μg/ml) and 1% Triton X-100 on ice.

For pulse-chase experiments, 48 hours following transfection, HEK cells were starved in Cys/Met-free DME for 30 minutes, pulse-labeled with 500 μCi/ml [^{35}S]Met/Cys (>1,000 Ci/mmol, NEN), and chased in complete growth media with 75 μM emetine (Sigma). The cells were washed and lysed in IPB+ (10 mM Tris, pH 7.5, 5 mM EDTA, 0.15 M NaCl, 1% NP-40, 0.5% deoxycholate, 2 mg/ml BSA) supplemented with a protease inhibitor cocktail (1 mM PMSF, 100 μM TLCK, 200 μM TPCK, 10 μg/ml ALLN). The lysate was cleared of insoluble material by centrifugation at 16,000 ***g***. The resulting detergent-insoluble pellet was sonicated in 50 μl 10 mM Tris, pH 7.5 and 1% SDS then diluted in 0.95 ml IPB+. Samples were pre-cleared with 50 μl Gammabind Sepharose beads (Pharmacia) for 2 hours at 4°C. Immunoprecipitation was performed using monoclonal anti-TCRα antibody H28-7-10 (Becker et al., 1989) and Gammabind Plus Sepharose overnight. The Gammabind beads were washed sequentially with WB1 (10 mM Tris, pH 7.5, 0.1% NP-40, 0.15 M NaCl, 1 mM EDTA), WB2 (10 mM Tris, pH 7.5, 2 mM EDTA, 0.05% SDS), and WB3 (10 mM Tris, pH 7.5, 2 mM EDTA). The beads were boiled for 3 minutes and the immunoprecipitates fractionated in 11% SDS-polyacrylamide gel, and analyzed by fluorography or phosphorimager analysis (Molecular Dynamics).

FACS analysis

HEK 293 cells expressing GFP^u, grown on 10 cm dishes, were transfected with 8 μg of vector alone (mock-transfected shown as

control), wild-type hsubc6 or hsubc6e^{ser} using calcium phosphate precipitation. 48 hours after transfection, GFP fluorescence of transfected cells was measured using Coulter Epics XL-MCL Flow Cytometer and EXPO v.2 cytometer software.

Immunofluorescence microscopy

HEK 293 cells were grown adhered on coverslips in complete DMEM medium and transfected with myc-tagged expression constructs. 24 hours post-transfection, coverslips were washed in PBS and cells were fixed in methanol/acetone for 5 minutes at –20°C. Then coverslips were air dried, cells were rehydrated in PBS and primary antibodies were incubated for 90 minutes at room temperature. Coverslips were washed in PBS, incubated for 20 minutes with biotin-labeled secondary antibody, washed in PBS again and then transferred to Streptavidine-FITC labeled antibody solution for 20 minutes. After a final washing procedure with PBS, coverslips were embedded in mounting solution and prepared for fluorescence microscopy, which was performed using a Zeiss Axioplan 1 microscope equipped with a MC 100 SPOT camera (Zeiss, Oberkochen, Germany).

Yeast experiments

Yeast minimal media (SD) were prepared as described and standard transformation techniques were employed (Ausubel et al., 1989). The following yeast strains were used: YWO1 [*MATα, trp1-1(am), his3-Δ200, ura3-52, lys2-801, leu2-3,-112* (Seufert et al., 1990)], YTX150 (*MATα, sec61-2, trp1-1(am), his3-Δ200, ura3-52, lys2-801, leu2-3,-112)*, YTX140 [*MATa, prc1-1, trp1-1(am), his3-Δ200, ura3-52, lys2-801, leu2-3,-112* (Biederer et al., 1997)], and K700 [*MATα, trp1-1, can1-100, ura3, leu2-3,-112, his3-11,-15, ssd1-d* (K. Nasmyth, IMP, Vienna, Austria)]. YTX150 was constructed by cloning the *sec61-2* allele [YFP338 (Biederer et al., 1996)] into pRS406 and subsequent integration into YWO1 by the plasmid shuffle technique (Ausubel et al., 1989). The integration vector was sequenced (E.H. and T.S., unpublished) and correct integration into YWO1 was confirmed by testing for temperature sensitivity. Ubc6p and the active site mutant Ubc6^{ser}p were overexpressed from a high-copy plasmid (pSEY8) as described (Sommer and Jentsch, 1993). The expression vector pUB23P, which encodes for the N-end rule substrate Pro-β-galactosidase (Bachmair et al., 1986), was a kind gift of J. Dohmen (Cologne).

Pulse chase experiments were basically conducted as described previously (Biederer et al., 1996), except that total lysates were prepared for anti-CPY* and Ub-Pro-βGal immuno-precipitation (Friedlander et al., 2000).

Results

To identify proteins from higher eukaryotic cells with functions similar to those of yeast Ubc6p we performed a TBLASTN search against the NCBI databases using the entire open reading frame of yeast *UBC6* as the query sequence. Several genomic sequences and expressed sequence tags (ESTs) from different species were identified that could be assembled into complete putative open reading frames and that displayed significant similarities to Ubc6p (Fig. 1A). In this study, we concentrated on those data base entries that also contain a C-terminal membrane-anchor, since this is one of the major criteria to define Ubc6p.

All sequences harbored an N-terminal Ubc domain that differed significantly in its primary sequence from that of their closest homologues, the Ubc4/5 protein family. Sequence comparison between the Ubc domain of yeast and human Ubc6p revealed an identity of 55.5%, while human Ubc4p and Ubc6p showed only 23.5% identity. All Ubc6 homologues contained a tail region consisting of many charged amino acids following the Ubc domain. The extreme C-terminus contained stretches of hydrophobic amino acids, which may serve as membrane anchors. In no case was there evidence of an N-terminal signal sequence. Thus, all members of the Ubc6p family appear to belong to the class of tail-anchored (type IV) membrane proteins.

The Ubc6p family could be further divided in two different sub-families (Fig. 1A,B): the first group comprised cDNAs encoding proteins with a high degree of overall sequence identity with Ubc6p (approximately 40%) and a similar calculated molecular weight (28 kDa). The second group displayed less similarity to yeast Ubc6p (approximately 25% identical residues) and contained a longer C-terminal tail region containing the transmembrane segment. The first group was named Ubc6p and the latter sub-family, Ubc6ep. Sequence comparison of the two subtypes derived from one organism make it unlikely that the two proteins are generated by alternative splicing (data not shown). In both groups the sequence identity in the Ubc domain was higher than in the tail region: comparison of human Ubc6ep and yeast Ubc6p revealed 25% overall sequence identity and 36% identity in the Ubc domain. In contrast, 40% overall identity was found in a comparison of yeast and human Ubc6p, while in the Ubc domain the conservation was even higher (55%).

For functional analysis, cDNAs corresponding to mammalian representatives of each sub-family were obtained from the German Resource Center for Genome Research (RZPD) and their identity to the corresponding proposed open reading frame was verified by sequencing. A sequence alignment of these proteins, mmUbc6p, a member of the highly conserved class of Ubc6 homologues, and hsUbc6ep, a member of the larger class, with yeast Ubc6p is shown (Fig. 1B).

While members of the Ubc6p family seem to be present in all eukaryotic kingdoms, including fungi, plant, protists and metazoa, a representative of the Ubc6ep family was not found in yeast or *D. melanogaster*, but was in plants (including the green algae *Chlamydomonas rheinhardii*) and in many groups of the metazoa (worms, vertebrates). Several protists also contain open reading frames that display striking similarity to Ubc6ep; however, at present it is not clear whether these genes code for Ubc6ep-like membrane-anchored proteins or whether they represent another gene orthologue to Ubc6p that codes for a soluble protein. The existence of both families in many metazoa indicates that the function of the single Ubc6p found in the yeast *Saccharomyces cerevisiae* may be subsumed by two distinct membrane-bound ubiquitin-conjugating enzymes in higher eukaryotic cells.

Ubc6 homologous proteins can be divided into two families

We were interested whether members of both families of proteins exhibit the same membrane topology and are located in the same cellular membrane as yeast Ubc6p. For this purpose we fused a c-myc epitope to the N-terminus of mmUbc6p, and to hsUbc6ep, a human member of the Ubc6e-family. The tagged versions were expressed in HEK 293 cells from plasmids under control of the CMV-promotor. Signals

corresponding to myc-mmUbc6p and myc-hsUbc6ep were detectable with an anti-myc antibody in immunoblots of total membrane preparations from these transfectants but not in mock-transfected cells (Fig. 2A). This observation, together with the finding that relatively little Ubc6p immunoreactivity partitions in cytosolic fractions (data not shown), suggest that these proteins, like their yeast counterpart are membrane-associated. Immunfluorescence microscopy revealed that myc-tagged mmUbc6p and hsUbc6ep were distributed in a classical reticular pattern similar to that observed for an ER membrane protein marker, TRAPα (Fig. 2B). Taken together, these results suggest that members of both families of mammalianUbc6p-related proteins are associated with ER membranes.

To assess the nature of the interaction of Ubc6 homologues with membranes, microsomes from transient transfectants were subjected to extraction with inorganic chaotropes or detergent (Fig. 3A). Similar to the known ER membrane protein TRAPα, the mammalian Ubc6 homologues were resistant to extraction by chemical chaotropes but were extracted in non-ionic detergent. Since the membrane anchors of all the proteins investigated were at the extreme C-terminus, we performed protease protection assays using microsomal vesicles prepared from cells expressing the epitope-tagged Ubcs in order to assess the topology of the Ub-conjugation domain with respect to the bilayer (Fig. 3B). Both myc-mmUbc6p and hsUbc6ep are susceptible to digestion by proteinase K in the absence and, of course, also in the presence of detergent. At the same time, the lumenal protein GRP78 was protected from the protease, indicating that the membranes were sealed. These data suggest that, like yeast scUbc6p, both hsUbc6ep and mmUbc6p are oriented in the ER membrane with their epitope-tagged N-termini exposed to the cytosol.

The mammalian Ubc6 homologues are tail-anchored ER-membrane proteins

In order to test a possible involvement in ERAD, we expressed hsUbc6ep and mmUbc6p in yeast mutants lacking scUbc6p and bearing the *sec61-2* allele. Cells expressing the conditional lethal allele *sec61-2* are unable to form colonies at 37°C, because its gene product is degraded in a scUbc6p-dependent manner at elevated temperature (Biederer et al., 1996). Loss-of-function mutants of scUbc6p exhibit reduced ERAD capacity and thus are suppressors of the temperature sensitivity of the *sec61-2* allele (Sommer and Jentsch, 1993). HsUbc6ep and mmUbc6p are unable to replace yeast scUbc6p in this assay, since the temperature-sensitive phenotype of *sec61-2Δubc6* was not restored upon expression of the mammalian homologues. In parallel, controls with yeast scUbc6p were performed and the expression of the mammalian Ubc6p-related proteins was verified by immunoblotting (data not shown). However, the expression level of Ubc6p might be critical for normal ERAD-function, as it was reported that overexpression of wild-type scUbc6p also suppresses the temperature-sensitive growth of a *sec61-2* mutant (Sommer and Jentsch, 1993). Therefore we tested different expression levels of hsUbc6ep and mmUbc6p for complementation. Irrespective of whether we overexpressed the proteins (multi-copy vector, induced *CUP* promotor, galactose induced GAL10-promotor) or whether we expressed them at low levels from single-copy vector, neither mmUbc6p nor hsUbc6ep restored the temperature sensitivity of *sec61-2Δubc6* cells at elevated

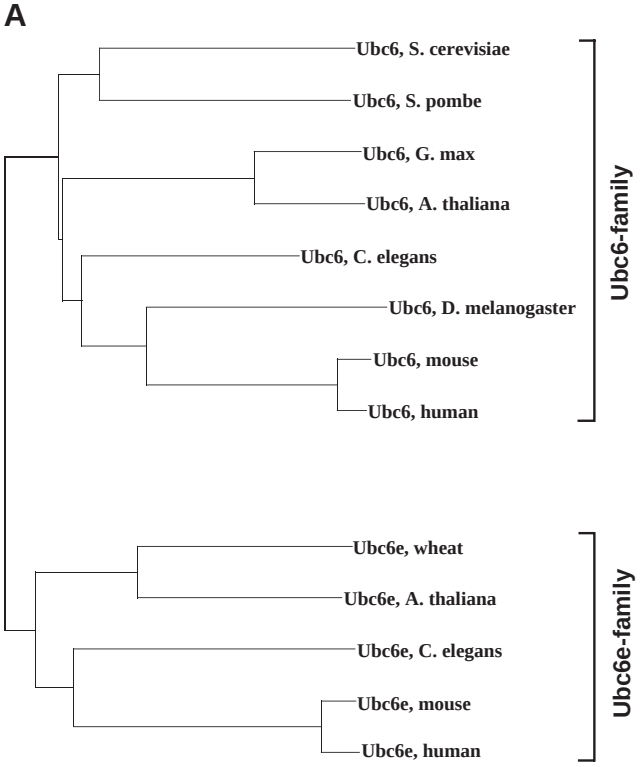

Fig. 1. (A) The CLUSTALX-analysis of the indicated amino acid sequences demonstrates the two classes of Ubc6-related proteins. (B) Comparison of the amino acid sequences of scUbc6p, mmUbc6p and hsUbc6ep. The CLUSTALW-alignment displays the well-conserved N-terminal Ubc domain (marked by a dotted line) and the less conserved tail region in both Ubc6 subfamilies. The conserved active site cysteine is indicated by an asterisk and the region of the membrane spanning domains is underlined.

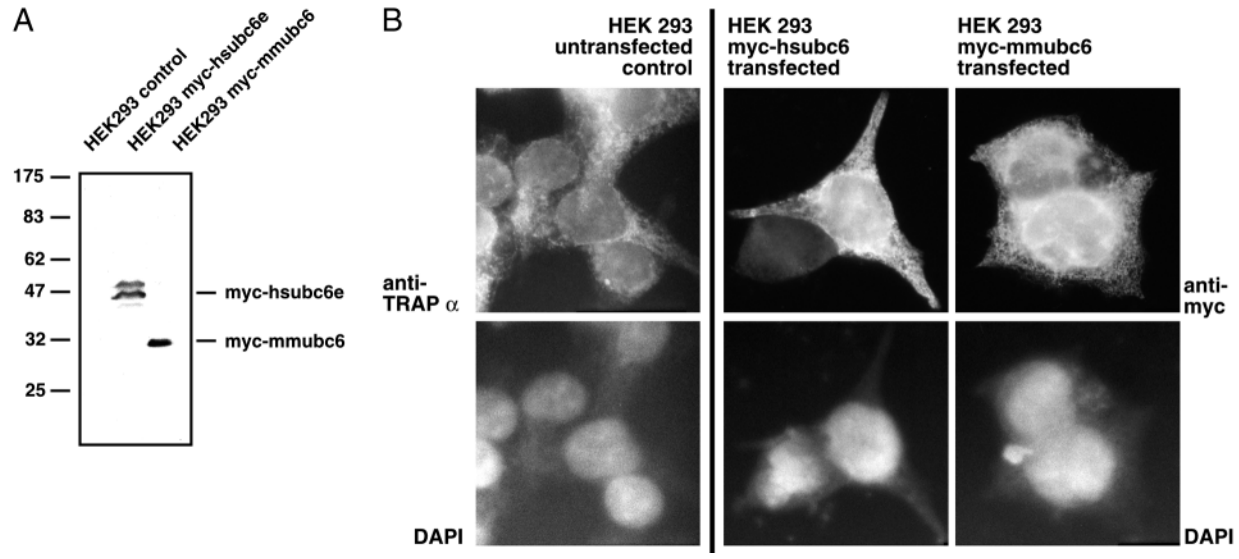

Fig. 2. (A) Western blotting of membranes prepared from HEK 293 cells transiently transfected with a control plasmid or with expression vectors containing the indicated myc-tagged Ubc6 homologues. For unknown reasons the anti-myc antibody detected three bands for the epitope-tagged human homologue of the second class. (B) Immunofluorescence microscopy of HEK 293 cells expressing epitope-tagged versions of both classes of Ubc6 homologues. In the non-transfected control, anti-TRAPα was used to stain a typical ER-membrane protein. The anti-myc antibody displays a very similar staining pattern for both Ubc6 classes.

temperature. Even when we expressed both mammalian enzymes under control of the weak yeast *UBC6* promotor, complementation could not be achieved (data not shown).

In contrast to the results with the mammalian homologues, we found that overexpression of yeast scUbc6p (from a multi-copy plasmid) exerts a dominant inhibitory effect on ERAD. Elevated levels of yeast scUbc6p restored growth of *sec61-2* cells at non-permissive temperature, indicative of stabilization of Sec61-2p. This effect was even more pronounced when a non-functional version of scUbc6p was used (Fig. 4A). In this mutant (scUbc6p^{ser}), the active-site cysteine residue was replaced by serine, thereby abolishing ubiquitin-conjugating activity. We also tested the effect of overexpresion of scUbc6p and scUbc6p^{ser} on the degradation of mutant carboxypeptidase Y (CPY*) (*prc1-1*). In agreement with the genetic experiments using *sec61-2*, we observed that the half-life of CPY* was prolonged from 17.5 minutes to 31.5 minutes in scUbc6p-overexpressing cells. Elevated levels of scUbc6p^{ser} further increased the half-life of CPY* to 60 minutes (Fig. 4B). These data suggest that overexpressed scUbc6p, and to a greater extent scUbc6p^{ser}, is a dominant inhibitor of ERAD in yeast.

To exclude that this effect is due to a general delay in ubiquitin-dependent proteolysis, we measured the turnover of an N-end rule substrate, Ub-Pro-βGal, in parallel. Turnover of this cytosolic substrate was unaffected and thus we concluded, that specifically ER-associated proteolysis was affected (Fig. 4C).

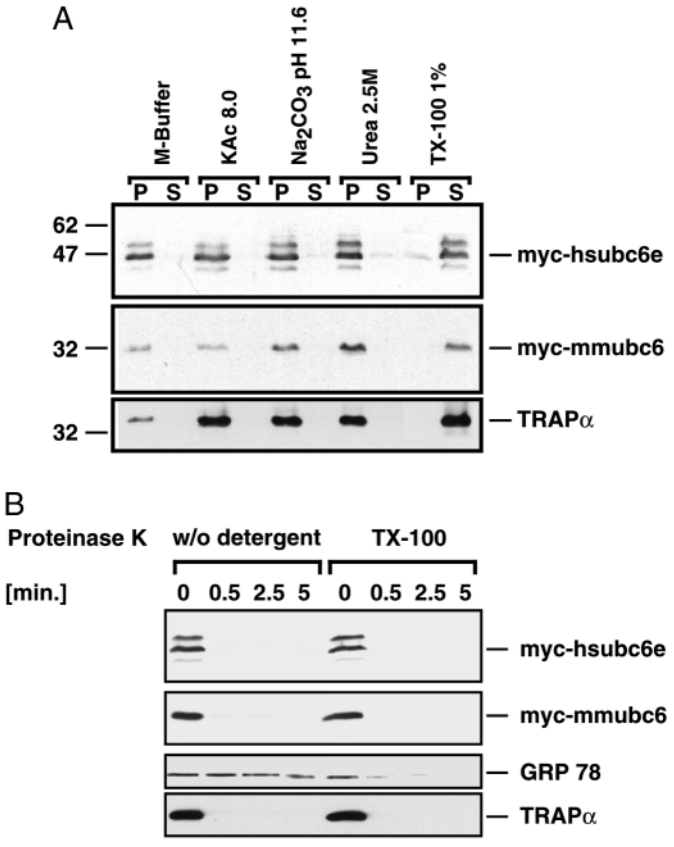

Fig. 3. (A) Epitope-tagged mmUbc6p and hsUbc6ep are integral membrane proteins, as is the control TRAPα. Membrane preparations were extracted with buffer containing 2.5 M urea or 800 mM potassium acetate, with Na_2CO_3 (pH 11.6), or with 1.0% detergent. P, pellet; S, supernatant. (B) The ubiquitin-conjugating domain of both myc-tagged mmUbc6p and hsUbc6ep is orientated to the cytosol. Protease protection assay of myc-mmUbc6 and myc-hsUbc6p and the ER-lumenal control GRP78 (Santa Cruz Biotechnology, Santa Cruz, CA) and the integral ER membrane protein TRAPα. Inside-out vesicles prepared from cells expressing the epitope-tagged Ubc6 homologues were treated with proteinase K or treated with 1% Triton X-100 and proteinase K for the indicated time. Samples were subjected to western blotting using the indicated antibodies.

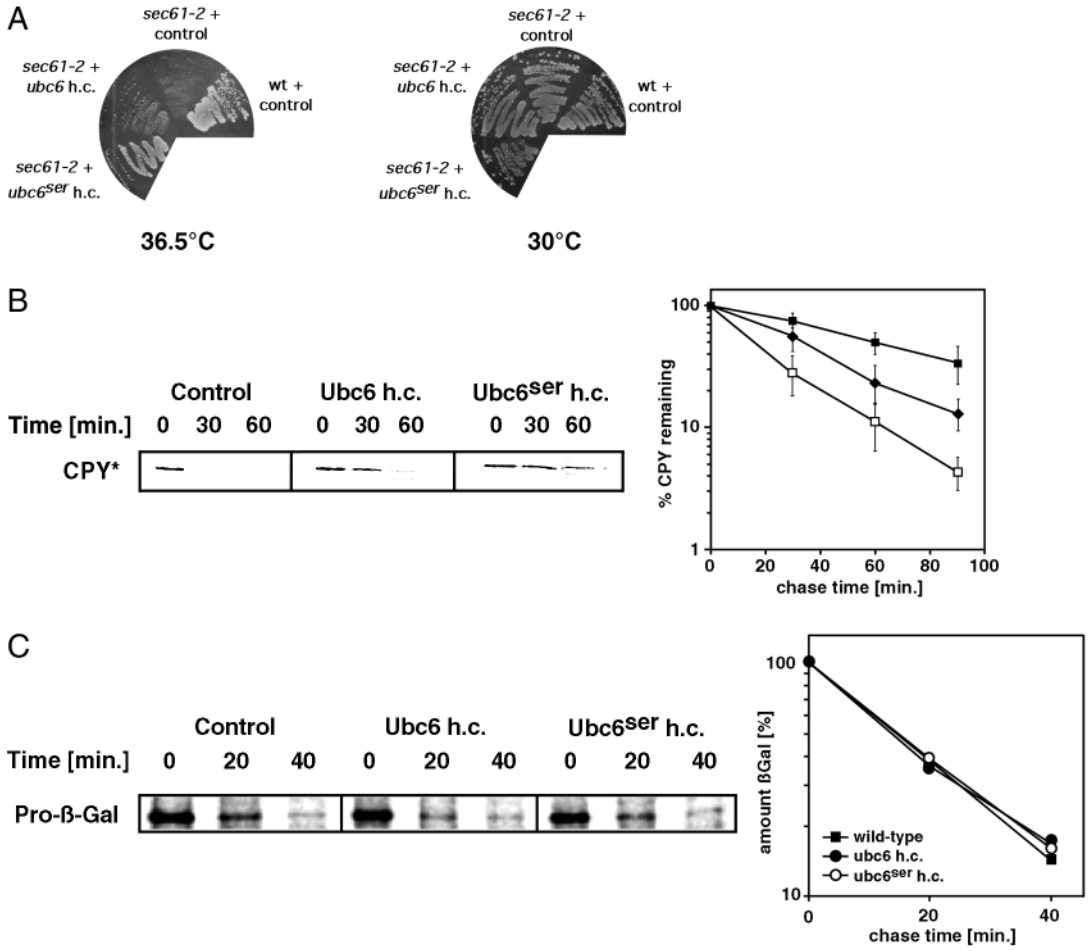

Fig. 4. (A) Wild-type and *sec61-2* yeast strains were transformed with high-copy plasmids coding for Ubc6p or Ubc6p^{ser} or with an empty vector as indicated. Plates were incubated at 30 and 36.5°C as indicated. (B) A yeast strain coding for the mutant carboxypeptidase Y (CPY*) was transformed with high-copy plasmids coding for scUbc6p (◆) or scUbc6p^{ser} (■) or with an empty (□) vector. Equal amounts of cells were labeled with ^{35}S-cysteine/methionine and chased with non-radioactive medium. Aliquots were taken after 0, 30, 60 and 90 minutes chase-time and immunoprecipitated for CPY*, subjected to SDS-PAGE and analyzed by autoradiography. A typical experiment is shown. The average and standard deviation of four (scUbc6p^{ser}) or six (empty plasmid, scUbc6p) independent experiments was calculated and is displayed as a graph. (C) Yeast strain K700 strain was co-transformed with pUB23P, which encodes for the N-end rule substrate Pro-β-Galactosidase, and with high-copy plasmids coding for scUbc6p or scUbc6p^{ser}, or with an empty vector. Equal amounts of cells were labeled with ^{35}S-cysteine/methionine and chased with non-radioactive medium. Aliquots were taken after 0, 20 and 40 minutes chase-time and immunoprecipitated for β-galactosidase, subjected to SDS-PAGE and analyzed by autoradiography. A typical experiment is shown in the upper part. The average of three independent experiments was calculated and is displayed as a graph.

Dominant-negative alleles of Ubc6 interfere with ERAD in yeast and mammals

To assess the role of Ubc6p in mammalian ERAD we overexpressed wild-type mmUbc6p, hsUbc6ep and the corresponding mutants in the active site Cys (C94S for mmUbc6p^{ser} and C91S for hsUbc6ep^{ser}) together with ERAD substrates. Pulse-chase labeling was used to assess the influence of these Ubc6 constructs on the stability of TCRα or ΔF508 transiently co-transfected into HEK cells (Fig. 5A). Similar to the results in yeast, overexpression of either mammalian wild-type Ubc6p isoform induced a small, but reproducible stabilization of both substrates. Likewise, overexpression of the mammalian active site mutants resulted in significant additional stabilization of TCRα (~50-80 minutes) and of ΔF508 (~75-140 minutes). These results do not simply reflect a nonspecific effect of protein overexpression since the Ubc6 data were compared with cells in which equal amounts of GFP plasmid were cotransfected. In addition, a short-lived cytosolic substrate, GFP^{u} (Bence et al., 2001) was investigated. Its steady state level was not altered by overexpression of wild-type hsUbc6ep and the corresponding active site mutant hsUbc6e-C91S (Fig. 5B). Since the steady state level of GFPu rises rapidly if its turnover is reduced, we concluded that the half-life of GFP^{u} was not affected by Ubc6p overexpression. In parallel experiments, an increased GFP^{u} level was observed upon overexpression of ubiquitin K48R, a dominant inhibitor of ubiquitin-dependent proteolysis (data not shown). Thus, similar to the situation in yeast, the delay in turnover is be limited to proteolytic events at the ER membrane.

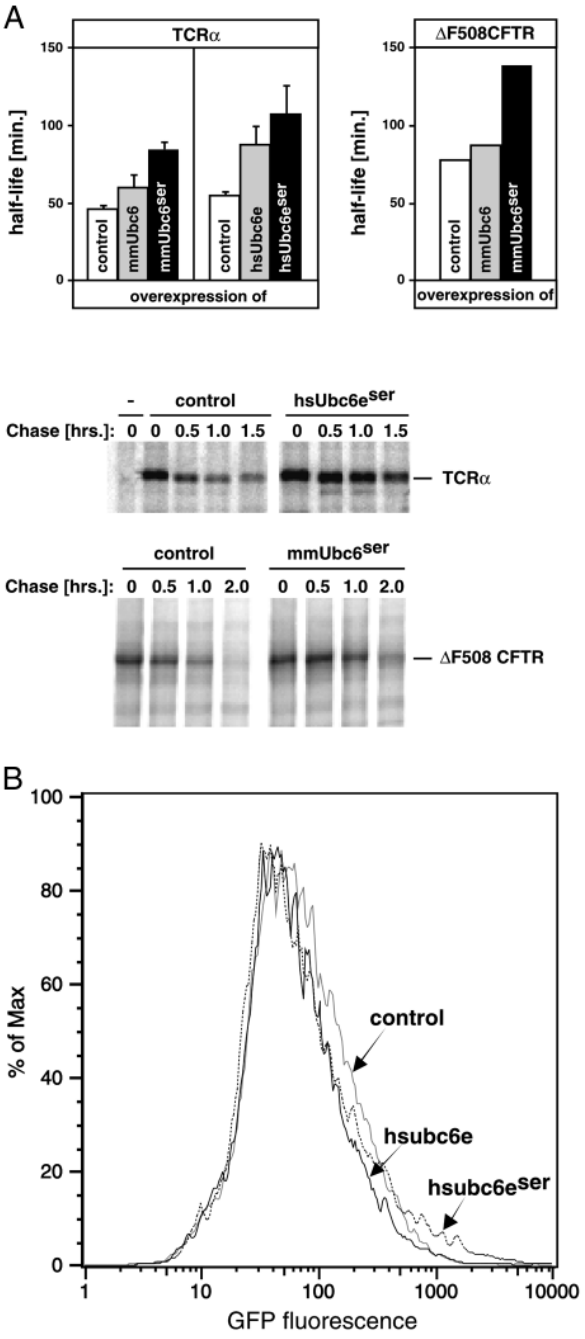

Fig. 5. (A) Effect of Ubc6p overexpression on mammalian ERAD. The turnover of TCRα and ΔF508CFTR was determined in pulse-chase experiments with or without expression of wild-type and mutant Ubc6. The calculated half-life of both substrates is summarized in the plot. The turnover was measured upon co-expression of either mmUbc6 or hsUbc6e as indicated. Of each conjugating enzyme a wild-type version (grey bars) or an active site mutant (black bars) was used. As a control, an unrelated protein (GFP; white bars) was expressed. For TCRα, data indicated are mean±s.e.m. of three experiments derived from linear regressions of semi-logarithmic transformations of decay kinetics. For ΔF508 CFTR, data are from two representative experiments. Below the plot, representative autoradiograms are shown. (B) Cells stably expressing an unstable cytosolic protein GFP^{u} were transfected with wild-type hsUbc6e or mutant hsUbc6e-C91S, and GFP fluorescence was determined by FACS analysis 2 days later.

Discussion

The data presented here demonstrate the existence of two distinct classes of tail-anchored ubiquitin-conjugating enzymes in higher eukaryotic cells that are highly similar to scUbc6p of *S. cerevisiae*. One of these, designated Ubc6p, is similar in overall length and sequence (40% identity) to the yeast protein and is thus likely to be the mammalian orthologue. The second, Ubc6ep, is 57 amino acids longer and displays lower sequence identity (25%) compared with yeast scUbc6p. While this study was in progress, a similar finding was published (Lester et al., 2000). We propose that Ubc6e represents a new subfamily of C-tail anchored ubiquitin conjugating protein localized to the endoplasmic reticulum.

We observed that overexpression of wild-type scUbc6p in *S. cerevisae* delays turnover of the soluble ERAD substrate CPY* and restores growth of *sec61-2* mutant cells. These effects were even more pronounced when a catalytically inactive version of scUbc6p is overexpressed. Similar to the observation in yeast, we noticed that overexpression of wild-type and mutant version of mmUbc6p and hsUbc6ep delay the turnover of TCRα and ΔF508CFTR in mammalian cells. However, in both yeast and mammals the slowed decay rates are limited to ERAD substrates, since the turnover short-lived cytosolic substrates remained unaffected. It is likely that this dominant effect of Ubc6p overexpression, which has not been hitherto reported, reflects titration of a limiting component in the ERAD pathway. Our results differ from previous studies (Tiwari and Weissman, 2001) reporting that overexpression of murine $Ubc6p^{ser}$ did not influence the turnover of TCRα. However, under the experimental conditions used by Tiwari et al., the half-life of TCRα – in the absence of any Ubc6p overexpression – was between 3.5 and 4.1 hours. This is significantly slower than all other previous reports of TCRα, which put the half-life between 30 and 60 minutes, independent of the particular cell line or expression level (Lippincott-Schwartz et al., 1988; Bonifacino et al., 1989; Yu et al., 1997; Huppa and Ploegh, 1997; Yang et al., 1998). Thus stabilizing effects caused by Ubc6p overexpression might have been hardly detectable under these conditions.

Our results suggest that in mammalian cells, like in yeast cells, the expression level of Ubc6p is critical for normal ERAD. The complete absence of Ubc6p in yeast leads to a slight delay in ERAD of CPY* and Sec61-2p but has no effect on HmgR degradation (Biederer et al., 1996; Hiller et al., 1996; Friedlander et al., 2000). As shown here, elevated levels of scUbc6p also lead to a decrease in the turnover of some ERAD substrates. Thus, it is feasible to speculate that Ubc6p functions as a modulator of the ERAD activity. In this context, it is interesting to note that scUbc6p is itself a short-lived protein, the degradation of which is dependent on membrane-bound Ubc7p (Walter et al., 2001). In mammalian cells, elevated levels of wild-type and active site center mutants of Ubc6p have comparable effects on the mammalian ERAD substrates TCRα and ΔF508CFTR. Therefore, not only the sequence of Ubc6p is highly conserved during evolution, but also its function seems to be preserved from yeast to mammals.

In conclusion, the data reported here identify two mammalian homologues of yeast scUbc6p that are residents of the ER membrane, and suggest that these ubiquitin conjugating enzymes may contribute to quality control ER-associated

degradation by the ubiquitin-proteasome system. We were unable to find conditions in which either of the mammalian enzymes could replace the function of yeast scUbc6p. This might be due to the fact that it is critical to adjust the expression level in a way that allows ERAD to proceed normally or that the mammalian homologues are unable to interact with other components of the yeast ERAD machinery. An alternative explanation is that the function of the single yeast Ubc6p is split between the two mammalian homologues. Future studies will be needed to discriminate among these possibilities.

For helpful discussions and critical reading of the manuscript we are grateful to K. Stade and E. Jarosch. We thank Neil Bence for providing GFPu-expressing cells. This work was partially supported by grants from the Deutsche Forschungsgemeinschaft and the Deutsch-Israelische Projektkooperation (DIP) to T.S. and by a grant (to R.R.K.) from the National Institute of Diabetes and Digestive and Kidney Diseases. M.S.G. was funded in part by a postdoctoral fellowship from the Nationl Institutes of Health. H.Y. was a recipient of a postdoctoral training fellowship from the California chapter of the American Heart Association.

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