TEB4 is a C4HC3 RING finger-containing ubiquitin ligase of the endoplasmic reticulum

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In the present study, the human TEB4 is identified as a novel ER (endoplasmic reticulum)-resident ubiquitin ligase. TEB4 has homologues in many species and has a number of remarkable properties. TEB4 contains a conserved RING (really interesting new gene) finger and 13 predicted transmembrane domains. The RING finger of TEB4 and its homologues is situated at the N-terminus and has the unconventional C4HC3 configuration. The N-terminus of TEB4 is located in the cytosol. We show that the isolated TEB4 RING domain catalyses ubiquitin ligation *in vitro* in a reaction that is ubiquitin Lys^{48}-specific and involves UBC7 (ubiquitin-conjugating enzyme 7). These properties are reminiscent of E3 enzymes, which are involved in ER-associated protein degradation. TEB4 is an ER degradation substrate itself, promoting its own degradation in a RING finger- and proteasome-dependent manner.

Key words: E3 ubiquitin protein ligase, endoplasmic reticulum-associated degradation, proteasome, RING finger, TEB4, ubiquitin.

INTRODUCTION

Ubiquitination has been shown to be involved in many cellular processes, ranging from protein quality control, protein turnover, endocytosis, protein transport, signal transduction, protein translation and DNA repair (reviewed in [1]). Many of these processes are executed by regulated conditional degradation of cytosolic and ER (endoplasmic reticulum)-associated proteins. The attachment of ubiquitin to a target protein involves a cascade of enzymatic reactions, catalysed by three classes of enzymes, including the ubiquitin-activating enzyme E1, ubiquitin-conjugating or E2 enzymes and ubiquitin-protein ligases or E3 enzymes [2,3]. The concerted action of these enzymes results in polyubiquitination of proteins. Formation of complexes of E3 ubiquitin ligases with E2 UBCs (ubiquitin-conjugating enzymes) provides substrate specificity to the ubiquitination reaction. The fate of a ubiquitinated molecule is influenced not only by the E2–E3 complex involved in the ubiquitination reaction, but also by the site of ubiquitination, the number of ubiquitin moieties attached and the lysine residues of a ubiquitin involved in the linkage.

In humans, there are three distinct classes of E3 ligases. The first is a group of proteins carrying a 350-residue C-terminal HECT (homology to E6AP C-terminal) domain. Examples are the human papilloma virus E6-associated protein, a cofactor in the degradation of the tumour suppressor gene p53 [4], and Smurf, which has a role in embryonic pattern formation [5]. The second class is formed by U-box-containing E3s such as CHIP (C-terminus of Hsc70-interacting protein) [6]. The third class harbours RING (really interesting new gene)-finger-containing E3 ligases such as c-CBL (cellular-casitas B-lineage lymphoma), which down-regulates receptor tyrosine kinases [7] and a diverse group of membrane proteins including HRD1 (hydroxymethylglutaryl reductase degradation), gp78/AMFR (autocrine motility factor receptor) and the viral K3-related MARCH (membrane-associated RING-CH) proteins [8–17].

A RING finger consists of a double-ring structure containing eight metal-binding cysteine and histidine residues that co-ordinate two zinc ions. RING fingers of E3 ligases can be formed by different configurations of histidine and cysteine residues. The most frequently found 'classical' C3HC4 RING domains are involved in many different cellular events. Examples are c-CBL, which functions in ubiquitin-dependent lysosomal trafficking [7,18,19], and BRCA1 (breast-cancer susceptibility gene 1), which affects cell-cycle progression through its ligase activity by a mechanism that is still elusive [20,21]. RING fingers with a C3H2C3 configuration are found in membrane-associated E3 ligases catalysing the ubiquitination of degradation substrates occurring in the secretory pathway, especially the ER, and endo-lysosomal compartments. So far, three ER-resident membrane E3 ligases with C3H2C3 RING fingers have been identified: yeast HRD1/Der3 [8,9], its human homologue HRD1 [15–17] and the mammalian gp78/AMFR [10]. Some proteins carry atypical forms of RING domains, such as the 'double-RING finger' found in Parkin, an E3 ligase implicated in Parkinson's disease. Parkin belongs to the group of proteins that suppress unfolded protein-induced cell death through their E3 ubiquitin-protein ligase activities [22]. A third, frequently occurring RING finger configuration is C4HC3. Several C4HC3 RING-finger proteins, including the yeast Doa10 (degradation of alpha2-10), the murine gammaherpesvirus 68 mK3, the Kaposi sarcoma herpesvirus kK3 and kK5, the myxomavirus M153R and the human MARCH

Abbreviations used: CHX, cycloheximide; Doa10, degradation of alpha2-10; ER, endoplasmic reticulum; ERK, extracellular-signal-regulated kinase; GST, glutathione S-transferase; HRD1, hydroxymethylglutaryl reductase degradation; RING, really interesting new gene; MARCH, membrane-associated RING-CH; MEKK1, MEK (mitogen-activated protein kinase/ERK kinase) kinase 1; NP40, Nonidet P40; UBC7, ubiquitin-conjugating enzyme 7; UPR, unfolded protein response; US11, unique short region 11.

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proteins, have been found to possess E3 ubiquitin ligase activity [11–14,23–30].

In the present study, we identify the C4HC3 RING-finger-containing TEB4, originally characterized as a transcript of the Cri-du-chat critical region on chromosome 5, to be a novel ER-associated E3 ligase. TEB4 appears to be well conserved, since genes with a high degree of homology to TEB4 occur in many species. *In vitro* ubiquitination reactions indicate that the N-terminal RING finger of TEB4 acts in conjunction with the E2 enzyme UBC7 and specifically catalyses the conjugation of ubiquitin through its lysine residue at position 48.

MATERIALS AND METHODS

Plasmids

The KIAA0597 cDNA, encoding the complete sequence of TEB4, was obtained from the HUGE (human unidentified gene-encoded large proteins) sequencing project [31]. A PCR fragment with the KIAA0597 sequence beginning at the first ATG codon of the open reading frame, flanked by an EcoRI site at the 5′-end and a KpnI site at the 3′-end, was cloned into pcDNA3.1 myc/His A (−) vector (Invitrogen, Leek, The Netherlands) to obtain a plasmid containing C-terminal myc and His tags. A mutation of the first cysteine residue of the RING finger into an alanine was accomplished using the Quik Change site-directed mutagenesis kit (Stratagene, Amsterdam, The Netherlands), resulting in a product designated TEB4C9A. The plasmid containing the HLA-A0102 sequence has been described in [32].

Antibodies

Anti-myc antibodies were obtained from Roche (used for immunoblotting and immunoprecipitations) or Invitrogen (used for immunoprecipitations and immunofluorescence analysis). Anti-US11 antibody (where US11 stands for unique short region 11) was produced in rabbits as described in [32]. Anti-calnexin antiserum was a gift from Dr A. Helenius (Institute of Biochemistry, ETH Zurich, Switzerland). The anti-transferrin receptor monoclonal antibody was obtained from Roche. Anti-TEB4 antiserum was produced in rabbits using a fusion protein of TEB4 (amino acids 482–971) and the GAL4 DNA-binding domain. An antibody directed against the N-terminus of TEB4 was produced as described in [32] using a peptide derived from amino acids 65–87 of TEB4. Horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit antibodies were obtained from Jackson Immunoresearch Laboratories (West Grove, PA, U.S.A.).

Immunofluorescence analysis

HeLa cells were transfected with myc-tagged TEB4 or TEB4C9A-encoding plasmids using Lipofectamine™ Plus (Invitrogen). After 24 h, cells were fixed with 3 % (w/v) paraformaldehyde in PBS for 10 min and permeabilized for 5 min using 0.2 % Triton X-100 in PBS. Cells were stained with anti-Myc monoclonal antibody or anti-calnexin rabbit serum, followed by CY3-conjugated anti-mouse and FITC-conjugated anti-rabbit serum.

In vitro transcription and translation and proteinase K digestions

TEB4 and HLA-A0201 plasmids were linearized with AflII and XhoI respectively and used for *in vitro* transcription with T7 polymerase (Promega, Leiden, The Netherlands). Transcripts were translated in the presence of L-[^{35}S]methionine in rabbit reticulocyte lysate containing canine pancreatic microsomes. Samples of 25 μl were incubated at 30 °C for 90 min. Microsomes and the supernatant were separated by centrifugation at 14 000 ***g*** at 4 °C for 15 min. The pellets were washed twice with KMH buffer (110 mM potassium acetate, 2 mM magnesium acetate and 20 mM Hepes/KOH, pH 7.2) and 1 mM $CaCl_2$ before loading on to an SDS/polyacrylamide gel. Proteins were displayed using a Personal Molecular Imager FX phosphoimager (Bio-Rad Laboratories, Veenendaal, The Netherlands).

For proteinase K digestions, microsomal membranes were centrifuged at 14 000 ***g*** for 15 min and washed with 100 μl of KMH buffer containing 110 mM potassium acetate, 2 mM magnesium acetate and 20 mM Hepes/KOH (pH 7.2). Proteinase K digestions were performed in 50 μl of KMH or NP40 (Nonidet P40) lysis mixture on ice for 30 min. Proteinase K was used at the concentrations indicated. After digestion, 1 μl of 500 mM PMSF was added to the NP40 samples, whereas 200 μl of KMH containing 4 mM PMSF was added to the KMH samples. The microsomes were centrifuged at 14 000 ***g*** for 15 min and resuspended in 60 μl of NP40 lysis mixture containing 1 μl of 500 mM PMSF. After lysis for 20 min, samples were cleared by centrifugation at 14 000 ***g*** for 15 min. The supernatant was split and used for either direct loads or immunoprecipitations.

For proteinase K digestions on whole cells, cells were infected with recombinant vaccinia virus expressing T7 [33] and, after 1 h, transfected with pcDNA3.1 TEB4 myc/His plasmid containing the T7 promoter in front of the TEB4 open reading frame. After another 3 h, cells were pulsed for 1 h, labelled with ^{35}S-Redivue Promix (Amersham Biosciences) and subsequently resuspended in 100 μl of cold permeabilization buffer [containing 25 mM Hepes, pH 7.2, 115 mM potassium acetate, 5 mM sodium acetate, 2.5 mM $MgCl_2$, 0.5 mM EGTA and 400 μg/ml digitonin (Calbiochem, La Jolla, CA, U.S.A.)] or NP40 lysis mixture. Proteinase K (Life Technologies, Breda, The Netherlands) was added at the concentrations indicated. Permeabilized cells were incubated with the digestion mixture at 4 °C for 20 min. Proteolysis was stopped by centrifugation of the cells at 14 000 ***g*** for 10 min and resuspension of the pellets in 1 ml of NP40 lysis buffer containing 1 mM PMSF. Immunoprecipitations, SDS/PAGE and phosphoimaging were performed as described in [32].

In vitro ubiquitination assays

Bacterially expressed GST–TEB4 RING or GST–TEB4 RING-finger mutant fusion proteins (where GST stands for glutathione S-transferase) were obtained using PCR fragments encoding residues 1–69 of human TEB4 or TEB4-C9A. EcoRI and XhoI sites were included in the PCR primers. The resulting PCR fragment was inserted into the pGEX-5X-3 vector (Amersham Biosciences). *In vitro* ubiquitination assays were performed at 30 °C in a reaction mixture containing 25 mM Tris (pH 7.6), 10 mM $MgCl_2$ and 1 mM ATP [17]. Where indicated, the reaction contained E1, human UBC7, GST–TEB4 RING and ubiquitin, the ubiquitin mutant Ub-K48R ($Lys^{48} \rightarrow Arg$) or modified ubiquitin. Reactions were terminated by the addition of SDS sample buffer, and protein components were separated by SDS/PAGE and visualized by Coomassie Brilliant Blue staining. For fluorescent *in vitro* ubiquitination experiments, His-tagged K48R-ubiquitin was modified with the fluorescent probe Oregon Green. K48R-ubiquitin was labelled chemically by the fusion of 2,7-difluorofluorescein (Oregon Green) iodoacetamide to the cysteine residue and purified by size-exclusion chromatography as described earlier [34].

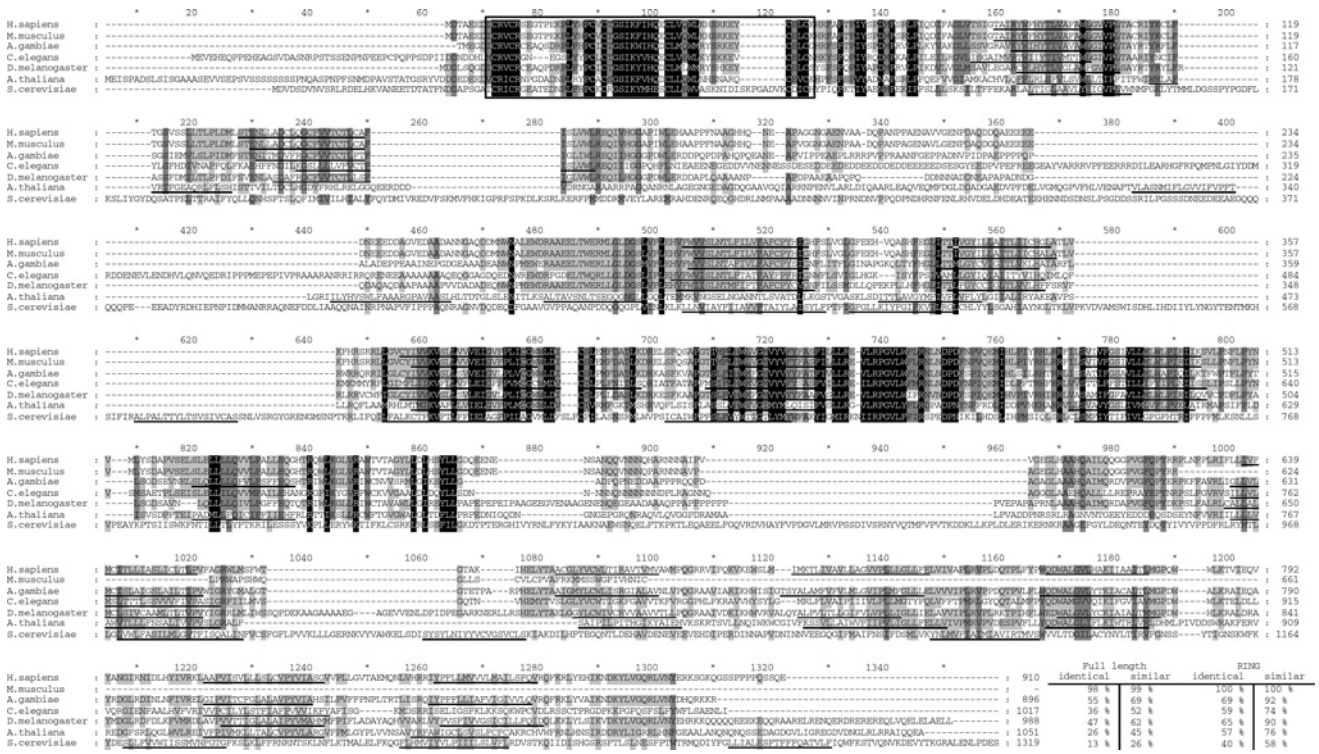

Figure 1 Amino acid sequence alignment of TEB4 homologues

TEB4s of *Homo sapiens* (T00268), *Mus musculus* (NP_766194), *Anopheles gambiae* (EAA04193), *C. elegans* (NP_492823), *Drosophila melanogaster* (NP_647715) and *Arabidopsis thaliana* (NP_195136) are putative proteins from the NCBI gene database, whereas *S. cerevisiae* Doa10 (P40318) has been cloned [31]. The RING finger is boxed and the predicted transmembrane domains are underlined. Hydropathy analysis of TEB4 was performed using www.ch.embnet.org/software/TMPRED_form.html and http://cubic.bioc.columbia.edu/predictprotein. The percentage of amino acid identity and similarity to HsTEB4 are indicated for the full-length sequences and the RING domains of the TEB4 homologues.

Peptide mass analysis

Ubiquitin or ubiquitin dimer was excised from SDS gels and digested with trypsin using an in-gel digestion procedure [35]. Mass analysis of the tryptic peptides using MALDI–TOF-MS (matrix-assisted laser desorption ionization–time-of-flight MS) was performed by a fee-for-service Core Facility at Pennsylvania State University.

RESULTS

Structural features of TEB4

An alignment of human TEB4 with related protein sequences in the mouse, *Caenorhabditis elegans*, *Anopheles*, *Arabidopsis* and *Drosophila* indicates that the TEB4 sequences are well conserved (Figure 1). The overall homology of TEB4 with the Ssm4 protein or Doa10, the proposed yeast homologue of TEB4, predominantly occurs in the N-terminal RING domains and a region spanning residues 650–800, designated the TEB4-Doa10 domain [23]. A conserved RING finger is predicted at the very N-terminus of human, mouse, *Anopheles* and *Drosophila* TEB4 (boxed in Figure 1). The homologues in *C. elegans*, *Arabidopsis* and yeast have an additional sequence that precedes the RING domain.

In vivo and *in vitro* expression of TEB4

TEB4 is expressed in many different organs, including heart, brain, placenta, lungs, liver, skeletal muscles, kidney, pancreas, spleen, thymus, prostate, testis, ovary and small intestine [31],

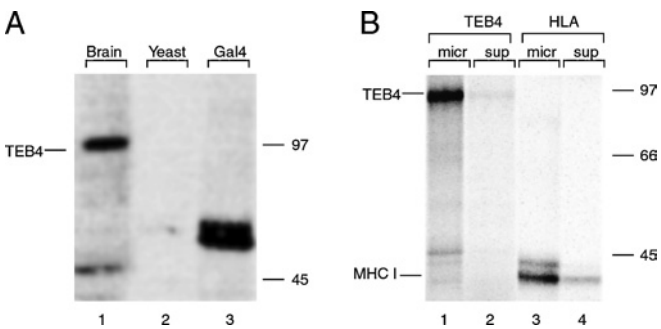

Figure 2 Expression of TEB4 *in vivo* and *in vitro*

(**A**) TEB4 expression in human brain was detected using a rabbit serum directed against amino acids 482–971 of KIAA0597 (lane 1). Yeast homogenate was used as a negative control (lane 2). The fusion protein of TEB4 (amino acids 482–971) and the GAL4 DNA-binding domain (used to generate the antibodies) served as a positive control (lane 3). Molecular masses are in kDa. (**B**) TEB4 and HLA-A2 were translated *in vitro* in the presence of dog pancreas microsomes and L-[^{35}S]methionine. One tenth of the microsomal fractions (micr) and half of the supernatant fractions (sup) were loaded on to an SDS/acrylamide gel.

suggesting that it is an important protein, involved in the general housekeeping functions of the cell. Since TEB4 is encoded in a chromosome region associated with a neurodegenerative disorder, namely the Cri-du-chat syndrome, the expression of the protein was investigated in a homogenate of human brain tissues. Detection of TEB4 by Western blotting using a TEB4-specific antiserum revealed a major polypeptide band of approx. 97 kDa (Figure 2A, lane 1). The antiserum did not cross-react with yeast Doa10

(Figure 2A, lane 2) but did recognize the TEB4 fusion protein that was used to generate the antiserum (Figure 2A, lane 3).

In vitro transcription and translation of TEB4 cDNA in the presence of microsomal membranes also yielded a translation product of 97 kDa (Figure 2B, lane 1). The polypeptide predominantly occurred in the microsomal fraction, which suggests that TEB4 is inserted into the membrane (Figure 2B, compare lanes 1 and 2). Similarly, the *in vitro*-translated MHC class I heavy-chain HLA-A2, a type I membrane protein with a single transmembrane domain, was mostly detected in the membrane fraction (lanes 3 and 4).

Membrane topology of TEB4

The hydropathy analysis of TEB4 suggests 13 transmembrane regions (Figure 1, underlined). The number and distribution of hydrophobic sequences varies somewhat among the TEB4 homologues. The putative membrane topology of human TEB4 is presented in Figure 3(A). In view of the proposed E3 ligase activity of the RING finger, this domain has been positioned in the cytosol. To confirm the cytosolic disposition of the RING domain, we used two different approaches.

First, we performed proteinase K digestions on canine pancreatic microsomes containing *in vitro*-translated TEB4. If the model shown in Figure 3(A) is correct, proteinase K digestion of the microsomes should result in the loss of an N-terminal epitope, whereas a C-terminal epitope should be protected. Treatment of the microsomes with proteinase K caused a dose-dependent loss of the translation product (Figure 3B, left panel), whereas an antibody directed against the N-terminal region of TEB4 recovered the intact protein from untreated samples, and proteinase K digestion resulted in a loss of the epitope (Figure 3B, middle panel). An antibody raised against the C-terminal myc tag was capable of precipitating a 34 kDa TEB4 fragment from proteinase K-treated microsomes, suggesting a luminal disposition of the C-terminus. At higher proteinase K concentrations, the 34 kDa fragment disappeared, presumably due to proteolysis by the enzyme.

Secondly, microsomes containing *in vitro*-translated TEB4 were incubated with antibodies directed against either the N- or C-terminus of TEB4. Subsequently, the microsomes were washed to remove excess of antibody and lysed. The lysates were incubated with Protein A–Sepharose beads to isolate the antibodies that were bound to microsome-associated TEB4. Antibodies directed against the N-terminal epitope were capable of precipitating TEB4, whereas the antibody directed against the C-terminal epitope failed to precipitate TEB4, indicating that the N-terminus but not the C-terminus of microsome-inserted TEB4 was accessible to the antibodies (Figure 3C).

To confirm the results obtained with *in vitro*-translated TEB4, the proteinase K digestion experiments were also performed on cells expressing TEB4. Figure 3(D) shows that the antibody directed against the N-terminus of TEB4 failed to bind TEB4 when the cells were permeabilized and treated with proteinase K. The antibody directed against the C-terminus of TEB4 immunoprecipitated the 34 kDa fragment that was also observed in proteinase K-treated microsomes. From the combined experiments, we conclude that the N-terminal region of TEB4 is located in the cytoplasm, whereas the C-terminus of TEB4 has a luminal deposition.

Subcellular localization of TEB4

To investigate the intracellular localization of TEB4, Myc-tagged wild-type TEB4 and a TEB4 RING-finger mutant were expressed in HeLa cells and stained using immunofluorescence. TEB4 staining patterns are reminiscent of ER localization (Figures 4a and 4d), which was confirmed by co-localization of TEB4 with the ER-resident chaperone calnexin (Figures 4c and 4f). No TEB4 was detected when non-permeabilized cells were used, indicating that TEB4 is not expressed at the cell surface (results not shown).

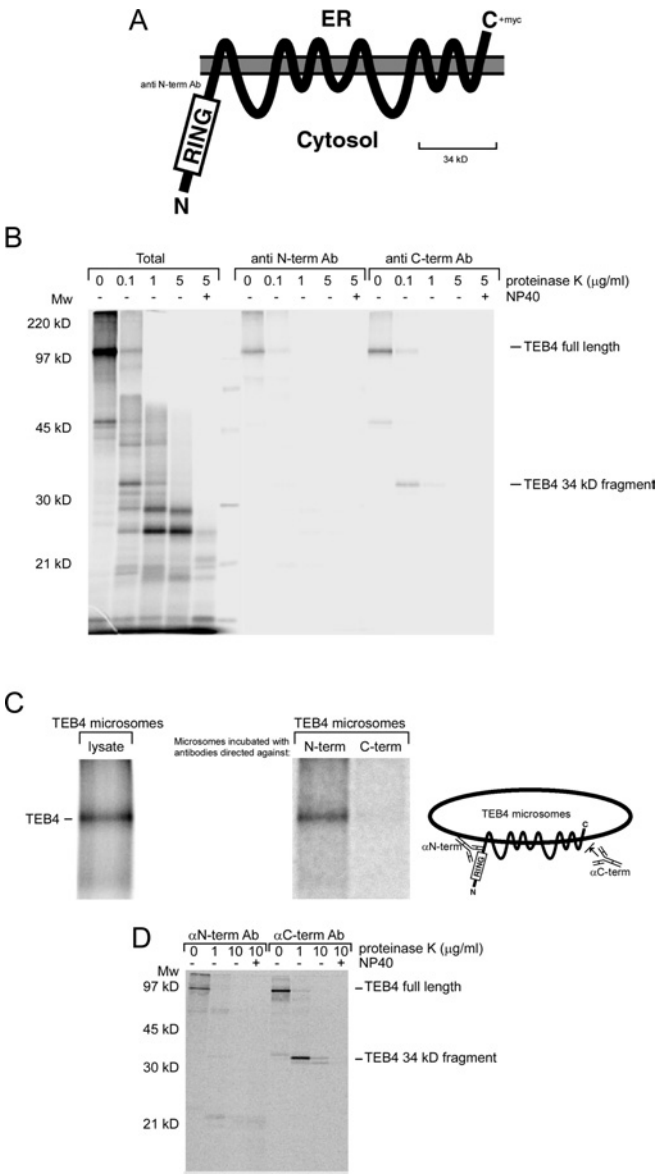

Figure 3 Membrane topology of TEB4

(**A**) Predicted membrane topology of TEB4, based on analyses with software available on www.ch.embnet.org/software/TMPRED and www.cubic.bioc.columbia.edu/predictprotein. (**B**) TEB4 was translated *in vitro* in the presence of dog pancreas microsomes. After translation, the microsomes were subjected to proteinase K digestion in KMH buffer or NP40 as indicated. One third of the microsomes was loaded directly on to a gel (total, left panel) and the rest of the microsomes were lysed and subjected to immunoprecipitation with either an antibody directed against the N-terminus of TEB4 or anti-myc antibody directed against the myc tag fused to the C-terminus of TEB4. (**C**) TEB4 was translated *in vitro* in the presence of microsomes. The total amount present in the microsomes is shown in the left panel. Intact microsomes were incubated with the same antibodies as in (**B**) for 2 h as shown pictorially on the right. The microsomes were washed to remove excess of antibodies and lysed. The clarified supernatant was incubated with Protein A–Sepharose beads to isolate TEB4–antibody complexes. (**D**) TEB4 was expressed in TS20 cells and subjected to proteinase K digestion as described in (**B**). After digestion, lysates were prepared and immunoprecipitations were performed with the antibodies indicated. Mw, molecular mass; sizes in kDa.

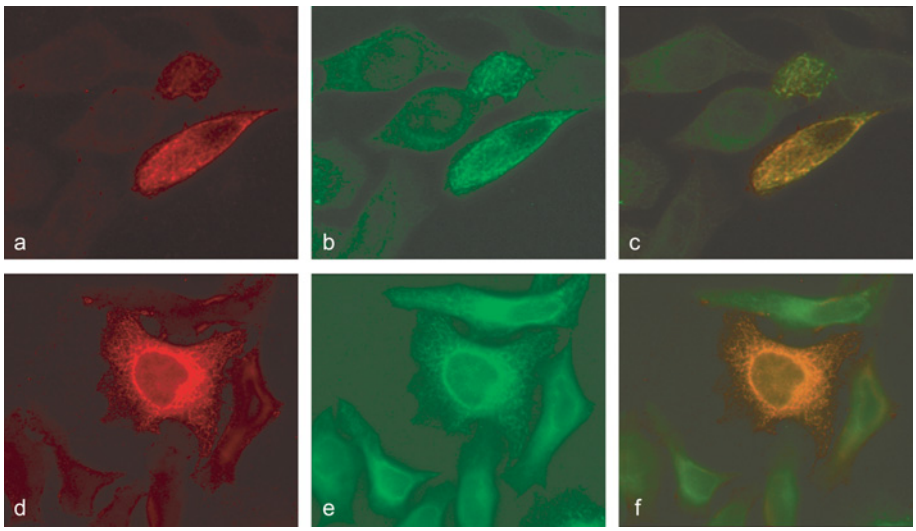

Figure 4 Subcellular localization of TEB4

TEB4 (**a**–**c**) and TEB4C9A RING mutant (**d**–**f**), both tagged with a myc epitope at their C-terminus, were transiently expressed in HeLa cells. Myc–TEB4 protein and endogenously expressed calnexin were detected by immunofluorescence using antibodies against the myc tag (**a**, **d**; red) and anti-calnexin (**b**, **e**; green) respectively. An overlay of TEB4 and calnexin staining is shown in (**c**, **f**).

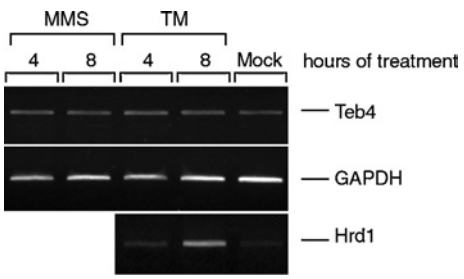

Figure 5 TEB4 expression on stress induction

HeLa cells were treated with 1 mM methyl methanesulphonate (MMS) or 10 μg/ml tunicamycin (TM) for 4 or 8 h. Total RNA was prepared and subjected to semi-quantitative reverse transcriptase–PCR using primers specific for TEB4 and HRD1.

ER localization is a conserved characteristic of human TEB4, yeast Doa10 and some of the human MARCH proteins [13,23].

TEB4 expression is not induced by ER stress

The accumulation of unfolded or misfolded proteins in the lumen of the ER leads to a stress response, UPR (unfolded protein response). Unfolded proteins can be removed by folding or degradation. During UPR, the synthesis of ER-resident protein chaperones is increased, which enhances the folding capacity in this organelle. At the same time, proteins engaged in ER-associated protein degradation, such as HRD1/Der3, are up-regulated [15,17, 36–38].

To establish whether TEB4 is up-regulated on induction of UPR, we examined the expression of TEB4 mRNA under conditions of cellular stress. Cells were treated either with the genotoxic stress inducer methyl methanesulphonate or with the N-glycosylation inhibitor tunicamycin [39]. Owing to the inhibition of N-linked glycosylation, tunicamycin interferes with proper folding of many glycoproteins, thereby inducing the ER-specific UPR. TEB4 mRNA levels were determined by semi-quantitative reverse transcriptase–PCR using specific primers. The results shown in Figure 5 indicate that the TEB4 expression does not increase in response to the various stress conditions. The mRNA level of HRD1/Der3, a known ER stress response gene product, was increased in the presence of tunicamycin, indicating induction of ER stress in the cells.

TEB4 is a RING finger-dependent ER degradation substrate

A specific characteristic of several E3 ligases analysed is the capacity to catalyse their own degradation in a RING finger-dependent manner. We therefore evaluated the stability of TEB4 and its RING-finger mutant. HeLa cells were transiently transfected with Myc-tagged wild-type and RING-finger mutant TEB4 and a chase was performed in the presence of CHX (cycloheximide). The results shown in Figure 6(A) indicate that wild-type TEB4 is completely degraded within 9 h of chase (lanes 1–4). The C9A mutation within the RING domain of TEB4 strongly impaired its degradation (lanes 5–8). Transferrin receptor remained stable throughout the chase and served as a loading control. The HCMV (human cytomegalovirus)-encoded US11 was degraded in the presence of both wild-type TEB4 and RING-finger mutant TEB4, indicating that degradation of an ER protein proceeds as normal in TEB4 mutant-transfected cells. A quantification of the TEB4 levels detected in Western blots is shown in Figure 6(B).

To investigate whether the degradation of TEB4 involves proteasomes, the pulse–chase experiment was performed in the presence of the proteasome inhibitor ZL_3H (carboxybenzyl-leucyl-leucyl-leucinal). As shown in Figure 6(C, top panel), inhibition of proteasomes results in the stabilization of TEB4 up to 9 h after the addition of CHX. These results indicate that TEB4 catalyses its own degradation in a RING finger- and proteasome-dependent manner.

The TEB4 RING domain interacts with UBC7 and promotes K48-specific ubiquitin–ubiquitin linkage

In the yeast *Saccharomyces cerevisiae*, the ER-tethered E3 ligases Hrd1 and Doa10 interact specifically with UBC7 [8,9,23]. We have shown previously that the RING domain sequence in the human and yeast ER-tethered E3 ubiquitin ligase Hrd1p interacts

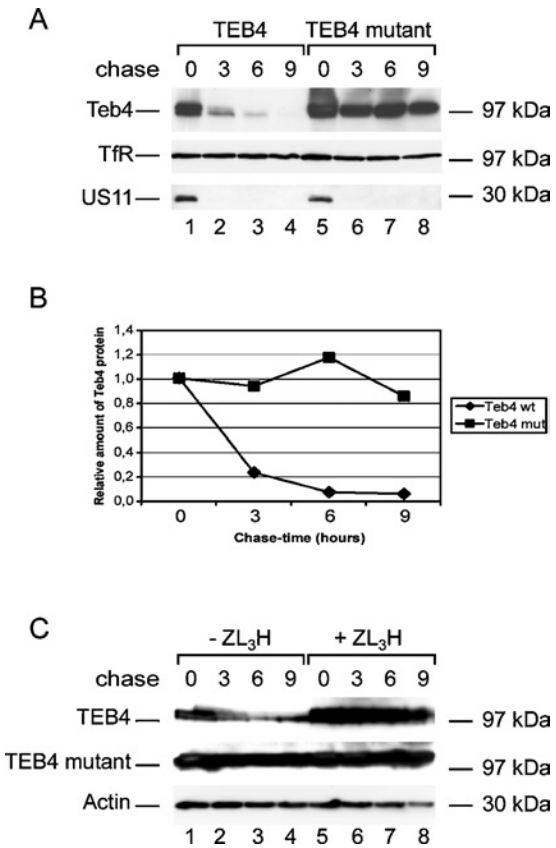

Figure 6 TEB4 is a RING finger-dependent ER degradation substrate

(**A**) HeLa cells stably transfected with HCMV-US11 were transiently transfected with myc-tagged TEB4 or TEB4C9A RING mutant; 24 h after transfection, the cells were treated with 50 μg/ml CHX and harvested at the chase times indicated. Cell lysates were subjected to SDS/PAGE and stained by Western blotting using antisera against myc tag, US11 or human transferrin receptor (TfR). (**B**) Quantification of the Western blots shown in (**A**). (**C**) Same experiment as in (**A**), but in the presence or absence of the proteasome inhibitor ZL_3H.

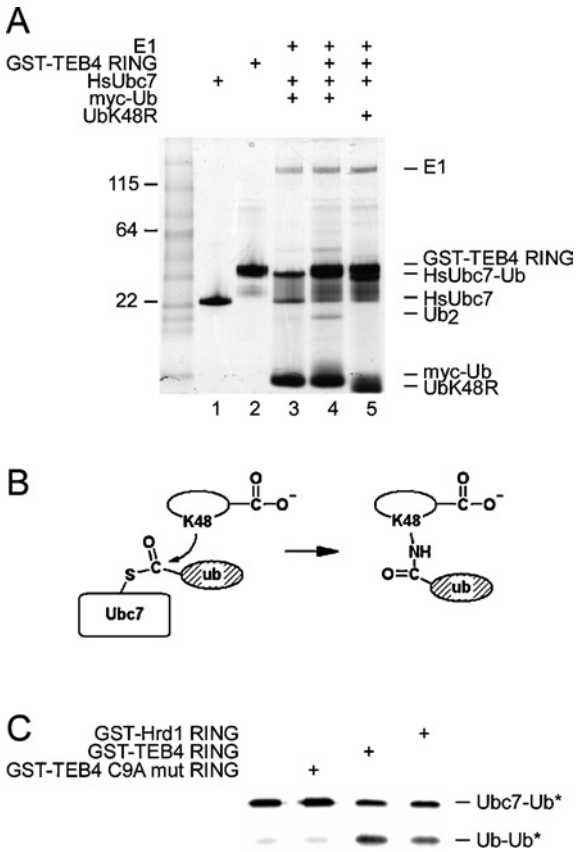

Figure 7 TEB4 facilitates a K48R-specific ubiquitination ligation reaction

(**A**) The TEB4 RING finger facilitates the *in vitro* Lys[48]-specific linkage of ubiquitin to ubiquitin. The indicated protein components were incubated at 30 °C for 10 min in a reaction buffer containing 25 mM Tris (pH 7.6), 10 mM $MgCl_2$ and 1 mM ATP. Where indicated, the reaction contains 10 nM E1, 10 μM UBC7, 10 μM GST–TEB4 RING and 50 mM poly-His-tagged ubiquitin or the ubiquitin mutant Ub-K48R. Reactions were stopped by the addition of non-reducing SDS sample buffer, and protein components were then separated by SDS/PAGE and visualized by staining with Coomassie Brilliant Blue. The migration of purified HsUbc7 and GST–TEB4 RING are shown in lanes 1 and 2 respectively. A ubiquitin dimer is formed in the reaction containing E1, GST–TEB4 RING, UBC7 and ubiquitin (lane 4) but not when ubiquitin was replaced by the mutant Ub-K48R (lane 5) or in the absence of GST–TEB4 RING (lane 3). (**B**) Schematic representation of the transfer of thioester-linked ubiquitin on UBC7 to a free ubiquitin. (**C**) A ubiquitin dimer is formed by the transfer of thioester-linked ubiquitin on UBC7 to a free ubiquitin. A reaction mixture containing 25 mM Tris (pH 7.6), 1 mM $MgCl_2$, 0.5 mM ATP, 1 nM E1, 1 μM UBC7 and 2 μM Oregon Green-labelled ubiquitin [34] was incubated at 30 °C for 1 min to generate UBC7-ubiquitin thioester (UBC7-Ub). The reaction was stopped by the addition of EDTA to a final concentration of 10 mM. The mixture was then incubated at 30 °C for 15 min with the addition of 40 μM of a truncated ubiquitin that lacks the C-terminal Gly-Gly residues and 10 μM of RING proteins as indicated. At the end of the incubation, the reaction was stopped by the addition of non-reducing SDS sample buffer. Proteins were separated by SDS/PAGE and proteins bearing the Oregon Green fluorescence (UBC7-Ub thioester and ubiquitin dimer) were visualized on a fluorescence gel scanner. Free ubiquitin migrated at the dye front and its fluorescence is masked by the presence of Bromophenol Blue in the SDS sample buffer and is not shown here.

with UBC7 and generates a robust activity for the K48-specific linkage of ubiquitin to ubiquitin [17]. We produced the TEB4 RING domain as a GST-fusion protein in bacteria and tested the purified protein for its interaction with UBC7 with a similar *in vitro* ubiquitination assay. Similar to what we observed with the GST–Hrd1 RING fusion, the inclusion of GST–TEB4 RING domain in an *in vitro* UBC7-dependent ubiquitination reaction also produced a reaction product that migrated in SDS gels with the mobility expected of a ubiquitin dimer (Figure 7A, lane 4). This activity was absent when UBC7 was replaced by UBC5, UBC2 or UBC3 (results not shown). The formation of the reaction product is dependent on the presence of Lys[48] in ubiquitin (Figure 7A, lane 5), consistent with it being a K48-linked ubiquitin dimer. To demonstrate directly the formation of a K48-linked ubiquitin dimer, we analysed the tryptic digests of the reaction product by MS. For comparison, we performed a similar analysis for ubiquitin. In a K48-linked ubiquitin dimer, K48 that is linked to the C-terminal carboxyl of another ubiquitin becomes resistant to tryptic digestion [40] and retains minimally, through an isopeptide linkage, the two C-terminal glycine residues of the other ubiquitin, when all other sites are cleaved. This isopeptide has a unique mass of 1460.75 Da that is not found in ubiquitin. In our analysis, we detected a peptide with a mass of 1460.78 Da, in addition to all the peptides that are also found in the single ubiquitin sample. Thus we conclude that the TEB4 RING sequence interacts with UBC7 and promotes the linkage of ubiquitin to ubiquitin at K48.

The UBC7-dependent ubiquitin–ubiquitin linkage reaction requires the presence of the ubiquitin-activating enzyme E1 and ATP, suggesting that the activity requires the intermediate formation of an UBC7-ubiquitin thioester. We next addressed whether the activity required one or both ubiquitin molecules to be linked by a thioester to UBC7. To test these two possibilities, we generated two distinct forms of ubiquitin, one that can form

a thioester linkage with UBC7 but lacks K48 (Ub-K48R) and another ubiquitin that lacks the C-terminal Gly–Gly residues (Ub_{74}) and cannot be activated by the ubiquitin-activating enzyme. To distinguish these two forms of ubiquitin, we extended the N-terminus of Ub-K48R with an MCHHHHHH sequence and attached a fluorescent Oregon Green label at the cysteine residue, using a previously described procedure [34]. Figure 7(C) shows that the UBC7-linked Oregon Green-labelled Ub-K48R can be transferred to Ub_{74} in a reaction that is dependent on the presence of either GST–HRD1 RING or GST–TEB4 RING. The reaction requires the presence of K48 in Ub_{74}, since a similar ubiquitin dimer was not formed with the K48R mutant of this truncated ubiquitin (results not shown). These results suggest that the ubiquitin dimer is formed by a reaction as depicted in Figure 7(B) and is reminiscent of the ubiquitin–ubiquitin linkage reaction during polyubiquitin chain elongation.

DISCUSSION

In the present study, TEB4 was characterized as a C4HC3 RING finger-containing E3 ubiquitin ligase of the ER. RING domains of E3 enzymes serve as a binding motif for UBCs (E2s). The N-terminal C4HC3 RING finger of TEB4 probably fulfils a similar function, since we have found the TEB4 RING domain to catalyse Lys^{48}-dependent ubiquitin ligation in the presence of the E2 UBC7.

C4HC3 RING fingers have been found in several viral, yeast and mammalian E3 ligases. Viral C4HC3 RING finger proteins are the murine gammaherpesvirus 68 mK3, the Kaposi sarcoma herpesvirus kK3 and kK5 and the myxomavirus M153R proteins [11,24–27,29,30,41–43]. Interestingly, these viral E3 ligases all inhibit the expression of mammalian proteins playing a role in anti-viral immunity, i.e. MHC class I complexes and the co-stimulatory molecules ICAM-1 (intercellular cell-adhesion molecule 1) and B7.2 [24–27,29,30,41–43]. Doa10 [23], also designated Ssm4 [44], was identified as a ubiquitin ligase that promotes the degradation of the soluble transcription factor Matα2, in addition to the degradation of proteins carrying a Deg1 signal (an exposed hydrophobic face of an amphipathic helix) [45]. Moreover, Doa10 and Hrd1p are together capable of suppressing growth defects due to malfunctioning of the Cdc48p–Npl4p–Ufd1p complex related to a temperature-sensitive mutation in Npl4p [46]. Recently, Doa10 and Hrd1p were shown to be involved in the ubiquitin-dependent degradation of human CFTR (cystic fibrosis transmembrane conductance regulator) ectopically expressed in yeast [47].

Human C4HC3 RING-containing proteins for which E3 ligase activity has been demonstrated are MEKK1 [MEK (mitogen-activated protein kinase/ERK kinase) kinase 1, where ERK stands for extracellular-signal-regulated kinase] [48] and the family of MARCH proteins [13]. MEKK1 is a cytosolic protein catalysing the ubiquitination of ERK-1 and -2 [48], members of the mitogen-activated protein kinase family. Except for a similar RING motif, MEKK1 does not demonstrate any obvious similarity to the MARCH proteins or TEB4 with respect to structure or subcellular localization. Interestingly, the MARCH proteins share several structural and functional properties with the viral K3 proteins [13]. Substrate specificities of the MARCH proteins and the viral E3 ligases demonstrate considerable overlap, with MHC class I molecules being one of the common substrates.

Ubiquitin can form isopeptide linkages involving its lysine residues at positions 6, 11, 29, 48 and 63. The different ubiquitin linkages are associated with various functions of ubiquitin within the cell. K63-based ubiquitin linkages have been associated with DNA repair, IκB kinase (inhibitor of nuclear factor κB kinase) activation and endocytosis (reviewed in [1,49]). The use of ubiquitin residue Lys^{48} has been linked to proteasomal degradation and dislocation of proteins from the ER into the cytosol [34,50] and binding to the AAA (ATPases associated with various cellular activities) ATPase p97–Ufd–Npl4 complex [51,52]. K11 and K29 have been associated with protein degradation by the proteasome as well.

The finding that the isolated RING domain of TEB4 catalyses an *in vitro* ubiquitination reaction involving Lys^{48} of ubiquitin points towards a role for it in proteasomal degradation. Moreover, TEB4 catalyses its own degradation in a RING finger- and proteasome-dependent manner and acts in conjunction with UBC7. These observations, combined with the association of many other C4HC3 RING-containing E3 ligases with protein degradation, led us to investigate whether TEB4 might have a similar function. Surface expression of MHC class I, Fas, TfR, CD4 and B7.2 molecules, however, was not influenced by overexpression of TEB4 or its RING finger mutant (results not shown). Previously, we have shown that dislocation and degradation of MHC class I heavy chains in the context of human cytomegalovirus US11 is ubiquitin-dependent [32,34]. The E2 and E3 enzymes involved in this process remain to be identified. Expression of either wild-type or RING-finger mutant TEB4 had no effect on US11-mediated degradation of class I heavy chains (results not shown).

The observation that TEB4 degradation involves the RING domain of TEB4 itself suggests that TEB4 catalyses its own degradation. This self-induced degradation may play a role in the regulation of TEB4 expression, as has been suggested for TRAF2 (tumour-necrosis-factor-receptor-associated factor 2) [53].

An interesting property shared by HRD1, gp78, Doa10 and TEB4 is their large number of putative transmembrane domains. HRD1, gp78, TEB4 and Doa10p are predicted to contain 6, 7, 13 and 13 membrane-spanning sequences respectively. In view of the function of HRD1 and gp78 in ER-associated degradation [10,17], one could speculate that their transmembrane domains might form part of a channel that mediates retrograde movement of substrates from the ER into the cytosol. These E3 ligases might associate with Sec61 alpha and beta translocon subunits that are also believed to be involved in dislocation of ER proteins [54–56]. These E3 ligases may also act in conjunction with Derlin-1, a protein that has recently been found to be required for US11 (but not US2)-dependent dislocation of MHC class I heavy chains [57,58]. Further studies will be required to investigate whether multimembrane-spanning E3 ligases indeed form part of a 'dislocon'.

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