Uncoupling proteasome peptidase and ATPase activities results in cytosolic release of an ER polytopic protein

Jon Oberdorf*,‡, Eric J. Carlson* and William R. Skach§

Department of Biochemistry and Molecular Biology, Oregon Health and Sciences University, 3181 SW Sam Jackson Park Road, Portland, OR 97201, USA

*These authors contributed equally to this work
‡Present address: Division of Hematology and Oncology, Portland Veterans Administration Medical Center, 3710 SW US Veterans Hospital Road, Portland, OR 97239, USA
§Author for correspondence (e-mail: skachw@ohsu.edu)

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Summary

The 26S proteasome is the primary protease responsible for degrading misfolded membrane proteins in the endoplasmic reticulum. Here we examine the specific role of β subunit function on polypeptide cleavage and membrane release of CFTR, a prototypical ER-associated degradation substrate with 12 transmembrane segments. In the presence of ATP, cytosol and fully active proteasomes, CFTR was rapidly degraded and released into the cytosol solely in the form of trichloroacetic acid (TCA)-soluble peptide fragments. Inhibition of proteasome β subunits markedly decreased CFTR degradation but surprisingly, had relatively minor effects on membrane extraction and release. As a result, large TCA-insoluble degradation intermediates derived from multiple CFTR domains accumulated in the cytosol where they remained stably bound to inhibited proteasomes. Production of TCA-insoluble fragments varied for different proteasome inhibitors and correlated inversely with the cumulative proteolytic activities of β_1, β_2 and β_5 subunits. By contrast, ATPase inhibition decreased CFTR release but had no effect on the TCA solubility of the released fragments. Our results indicate that the physiologic balance between membrane extraction and peptide cleavage is maintained by excess proteolytic capacity of the 20S subunit. Active site inhibitors reduce this capacity, uncouple ATPase and peptidase activities, and generate cytosolic degradation intermediates by allowing the rate of unfolding to exceed the rate of polypeptide cleavage.

Key words: CFTR, ER-associated degradation, Polytopic proteins, Cystic fibrosis, ER dislocation, Proteasome inhibitors, p97

Introduction

Membrane proteins in the endoplasmic reticulum (ER) that fail to acquire proper tertiary or quaternary structure are recognized by ER quality control machinery and degraded by the ubiquitin-proteasome pathway (Plemper et al., 1998; Ellgard and Helenius, 2001; Tsai et al., 2002; McCracken and Brodsky, 2003). This process, called ER-associated degradation (ERAD) (Brodsky and McCracken, 1997; Hirsch et al., 2004; Römisch, 2005), involves covalent attachment of ubiquitin by ubiquitin-conjugating (E2) and ubiquitin-ligating (E3) enzymes that either reside within (e.g. Hrd1p/Der3p, Der1p, Ubc6) or are recruited to (e.g. Ubc7, CHIP) the ER membrane (Hampton et al., 1996; Hiller et al., 1996; Biederer et al., 1997; Jiang et al., 2001; Meacham et al., 2001). Ubiquitylated substrate is then degraded by the 26S proteasome, a cytosolic protease comprised of two 19S regulatory subunits and a 20S particle containing protease activity sequestered along the interior of four stacked heptameric rings (reviewed by Voges et al., 1999).

Because proteasomes are restricted to cytosolic and nuclear compartments, degradation of membrane proteins involves multiple steps: extraction of TM segments from the lipid bilayer, retrotranslocation of lumenal domains, unfolding and insertion of polypeptide into the proteasome, and substrate cleavage by proteolytically active 20S β subunits (Wiertz et al., 1996; Pilon et al., 1997; Mayer et al., 1998; Plemper et al., 1998; Lilley and Ploegh, 2004; Ye et al., 2004). Given the potential consequences of exposing large hydrophobic domains to the cytosolic environment, one would predict that proteolytic cleavage should be tightly coordinated with unfolding and extraction. Consistent with this, several studies have indicated that degradation is coupled to retrotranslocation at the cytosolic face of the ER membrane. For example, cytosolic and lumenal epitopes of ATP-binding cassette (ABC) transporters, Pdr5 and CFTR, are degraded concurrently and require intact proteasomes as well as functional Sec61 translocation machinery (Plemper et al., 1998; Xiong et al., 1999). Intact proteasomes are also required for the membrane extraction of MHC class II proteins (Tomazin et al., 1999) and a polytopic protein containing an N-terminal degradation signal (Mayer et al., 1998). In addition, we previously showed that ubiquitylated forms of CFTR remain tightly associated with the ER membrane in vitro until they are degraded by the 26S proteasome (Xiong et al., 1999; Oberdorf et al., 2001). Other studies, however, have indicated that retro-translocation can proceed independently of degradation. CFTR and other ERAD substrates accumulate as amorphous aggregates

(aggresomes) in the cytosol of cells treated with proteasome inhibitors (Bebök et al., 1998; Johnston et al., 1998; Wigley et al., 1999). Similarly, in the presence of proteasome inhibitors, unstable connexins (VanSlyke and Musil, 2002), the T-cell receptor α subunit (Yu et al., 1997), MHC class I molecules (Wiertz et al., 1996) and other membrane proteins can be dislocated from the ER membrane and released en bloc into the cytosol. Thus, the temporal and mechanistic relationship between peptide cleavage and membrane extraction varies for different substrates and/or degradation conditions.

Eukaryotic proteasomes require that substrates are unfolded to reach the three active β1, β2 and β5 subunits within the core of the 20S particle (Voges et al., 1999). Unfolding is facilitated by a ring of AAA-ATPases located at the base of the 19S regulatory subunit that translocates polypeptide into the axial pore of cylinder (Braun et al., 1999; Köhler et al., 2001; Lee et al., 2001; Benaroudj et al., 2003; Pickart and Cohen, 2004). One appealing hypothesis has been that 19S AAA-ATPases might also provide the driving force for retro-translocation (Mayer et al., 1998; Plemper and Wolf, 1999). Indeed, the 19S subunit is sufficient to dislocate a mutant form of the soluble ERAD substrate pro-alpha factor into the cytosol (Lee et al., 2004). A second AAA-ATPase, p97(VCP/Cdc48) has also been implicated in ERAD. p97 forms a homohexameric ring, which together with adapter proteins Npl4 and Ufd1, binds ubiquitylated substrates and facilitates their presentation to the proteasome (Ye et al., 2001; Braun et al., 2002; Jarosch et al., 2002; Kobayashi et al., 2002; Rabinovich et al., 2002; Ye et al., 2004). Precisely how these two ATPase complexes function during ERAD remains unclear, but p97 inactivation or overexpression of dominant-negative p97 inhibits retrotranslocation and degradation of transmembrane substrates (Bays et al., 2001; Bays and Hampton, 2002; Kobayashi et al., 2002; Rabinovich et al., 2002; Dalal et al., 2004; Gnann et al., 2004; Huyer et al., 2004). A significant question in membrane protein degradation therefore is how the ERAD system coordinates all of the steps required for extraction and degradation while minimizing accumulation and aggregation of hydrophobic degradation intermediates.

In the current study, we examine the role of polypeptide cleavage in membrane extraction and release of a prototypical ERAD substrate, CFTR, a polytopic membrane protein with 12 transmembrane segments and three large cytosolic domains. Both in vivo and in vitro CFTR fails to efficiently mature in the ER membrane and is recognized and degraded via the ubiquitin-proteasome pathway (Jensen et al., 1995; Ward et al., 1995; Xiong et al., 1999). Using a reconstituted system that allows direct analysis of degradation products, we now show that fully functional proteasomes release CFTR into the cytosol solely in the form of TCA-soluble peptide fragments. Thus membrane extraction is normally tightly coupled to terminal cleavage events at the cytosolic face of the ER membrane. With progressive β-subunit inhibition, however, production of TCA-soluble fragments decreased, and large heterogeneous TCA-insoluble fragments were released into the cytosol in a stable complex with the proteasome. These findings support a model in which excess proteolytic capacity of the 20S proteasome is required to maintain the physiologic balance between membrane extraction and substrate cleavage. By uncoupling peptidase and ATPase activities, proteasome active-site inhibitors allow the rate of extraction to exceed the capacity for peptide cleavage and thus give rise to large cytosolic degradation intermediates.

Results

Proteasome β-subunit inhibition releases large CFTR fragments from the ER membrane

We previously developed a cell-free reticulocyte lysate-based (RRL) system that reconstitutes CFTR synthesis, core glycosylation, membrane integration and ER-associated degradation (Xiong et al., 1999). In this system, newly synthesized CFTR is selectively and specifically radiolabeled at 38 methionine residues broadly distributed throughout the molecule. This enables us to directly monitor both the rate and extent of degradation based on the generation of radiolabeled, TCA-soluble peptide fragments (Xiong et al., 1999; Oberdorf and Skach, 2002). We previously used this system to show that CFTR degradation is ATP-dependent, localized to the ER membrane and sensitive to proteasome active site (β subunit) inhibitors (Xiong et al., 1999; Oberdorf et al., 2001). Because β-subunit inhibitors block degradation but do not affect proteasome ATPase activity (Hoffman and Rechsteiner, 1996), this system provides a unique opportunity to directly examine the specific role of peptidase function during the extraction and cytosolic release of a polytopic ERAD substrate.

ER microsomal membranes containing in-vitro-synthesized CFTR were isolated, and degradation was carried out in fresh RRL in the presence and absence of proteasome inhibitors (Fig. 1). Membranes were then pelleted, and cytosolic CFTR fragments were quantified by scintillation counting before and after TCA precipitation. Under control conditions, CFTR was rapidly released from the ER membrane solely as small TCA-soluble fragments (Fig. 1A). Both degradation and membrane release were completely abolished by ATP depletion (Fig. 1G). In the presence of MG132, both the rate and extent of TCA-soluble fragment production was decreased by ~90% (Fig. 1B). Surprisingly, even though degradation was inhibited, CFTR continued to be released from the ER membrane at nearly 70% of the control rate as large TCA-insoluble fragments (Fig. 1B, arrow). A cocktail of proteasome inhibitors, clasto-lactacystin β-lactone, GPFL and leupeptin, which also inhibit all three 20S active β subunits by more than 95% (Oberdorf et al., 2001) resulted in a similar release of TCA-insoluble fragments (Fig. 1C). Clasto-lactacystin β-lactone and ALLN, which primarily inhibit chymotrypsin-like activity (β5 subunit), had a smaller effect on CFTR degradation and generated fewer TCA-insoluble fragments (Fig. 1D,E). These results support previous studies showing that CFTR degradation is proteasome mediated (Gelman et al., 2002), but can be carried out at a reduced rate by residual caspase-like (PGPH) and trypsin-like activities of β1 and β2 subunits (Oberdorf et al., 2001). In contrast to β-subunit inhibitors, hemin, an inhibitor of proteasome 19S ATPases (Hoffman and Rechsteiner, 1996; Oberdorf et al., 2001), markedly decreased CFTR release from the membrane, but the small fraction of cytosolic fragments were all TCA-soluble (Fig. 1F). Thus different mechanisms of proteasome inhibition yield very different patterns of ER fragment release.

MG132 exhibits dose-dependent effects on proteasome β-subunit activities (Bogyo et al., 1997) that directly reflect the rate of CFTR conversion into TCA-soluble fragments (Oberdorf et al., 2001). At a concentration of 10 μM, MG132

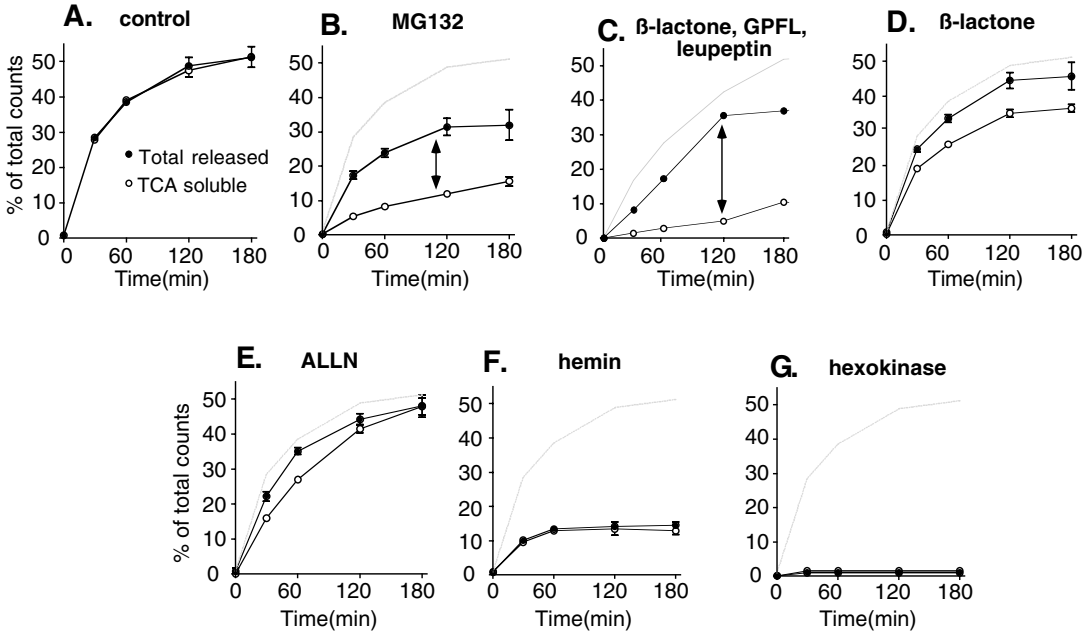


Fig. 1. Cytosolic release of TCA-insoluble CFTR fragments. CFTR degradation was carried out in the absence of inhibitors (A), or in the presence of 100 μM MG132 (B), 100 μM clasto-lactacystin β-lactone, 500 μM GPFL and 100 μM leupeptin (C), 100 μM clasto-lactacystin β-lactone (D), 100 μM ALLN (E), 40 μM hemin (F) or hexokinase and 2-deoxyglucose (G) as described in Materials and Methods. Total (●) and TCA-soluble (○) CFTR radioactive fragments recovered in the cytosolic fraction are expressed as the percentage of total CFTR counts present at *t*=0. The difference between total and TCA-soluble counts released (double arrow) indicates relative amount of CFTR released in the form of TCA-insoluble fragments. The gray line (panels B-G) shows total CFTR converted into TCA-soluble counts under control conditions.

inhibits β1, β2 and β5 proteolytic activities in RRL by 87%, 66% and >99%, respectively (Table 1), which is similar to that observed for clasto-lactacystin β-lactone (41%, 85% and >99% inhibition of β1, β2 and β5, respectively). Consistent with these findings, 10 μM MG132 resulted in total CFTR release and a relative proportion of TCA-insoluble fragments that was nearly identical to that observed for clasto-lactacystin β-lactone (compare Fig. 2B and Fig. 1D). Higher MG132 concentrations caused a dose-dependent increase in production of cytosolic TCA-insoluble fragments (Fig. 2B-E). The cumulative activities of all three proteasome β subunits therefore play a crucial role not only in the rate and extent of CFTR degradation, but also in membrane release and degradation product size.

Cytosolic degradation intermediates are heterogeneous (15-45 kDa) and derived from multiple CFTR domains

Because CFTR is the only radiolabeled substrate in cell-free reactions, we were able to examine full-length products and degradation intermediates directly by SDS-PAGE and autoradiography (Fig. 3). Cytosolic supernatants collected in the presence of MG132 revealed a significant accumulation of heterogeneous 15-45 kDa fragments as well as a much larger species that resembled poly-ubiquitylated CFTR (Fig. 3A, lanes 1-6) (Xiong et al., 1999). Fragments released in the presence of the proteasome inhibitor cocktail (clasto-lactacystin β-lactone, GPFL and leupeptin) were similar to those observed for MG132 (data not shown). ALLN and clasto-lactacystin β-lactone also generated visible cytosolic fragments of 15-30 kDa but to a lesser extent than MG132 (Fig. 3A, lanes 7-18). As expected, no cytosolic fragments were visualized in control reactions because functional proteasomes generated only TCA-soluble fragments too small to be detected (Fig. 3A, lanes 19-24). Similarly, ATP depletion stabilized full-length CFTR, and prevented cytosolic release. Finally, hemin resulted in CFTR accumulation as a high molecular mass ubiquitylated complex that remained quantitatively bound to ER membrane (Fig. 3A,B), consistent with previous membrane flotation experiments (Xiong et al., 1999).

To determine the origin of CFTR cytosolic fragments, we compared the fate of three endogenous epitopes located within N-terminal, C-terminal and internal (R) domains by counting aliquots of degradation reactions either before or following immunoprecipitation with peptide-specific antisera (Fig. 4). Because methionine residues are relatively uniformly distributed, the signal recovered by immunoprecipitation approximates the average bulk of CFTR protein attached to each epitope. Under control conditions, epitopes associated with N-, R- and C-domains were cleaved at nearly identical rates and only slightly faster than the rate of conversion into TCA soluble counts (Fig. 4, compare panels A and B). At later time points there was a minor but consistent sparing of the N-terminal epitope. MG132 markedly slowed the generation of

Table 1. Percentage residual protease activity

[MG132]	β1	β2	β5
10 μM	13±3.1	34±4.9	0.6±0.5
25 μM	5.3±1.6	16.6±2.6	n.d.
50 μM	3.3±2.3	11.1±1.8	n.d.
100 μM	1.8±0.5	6.5±0.7	n.d.

n.d., not detectable.

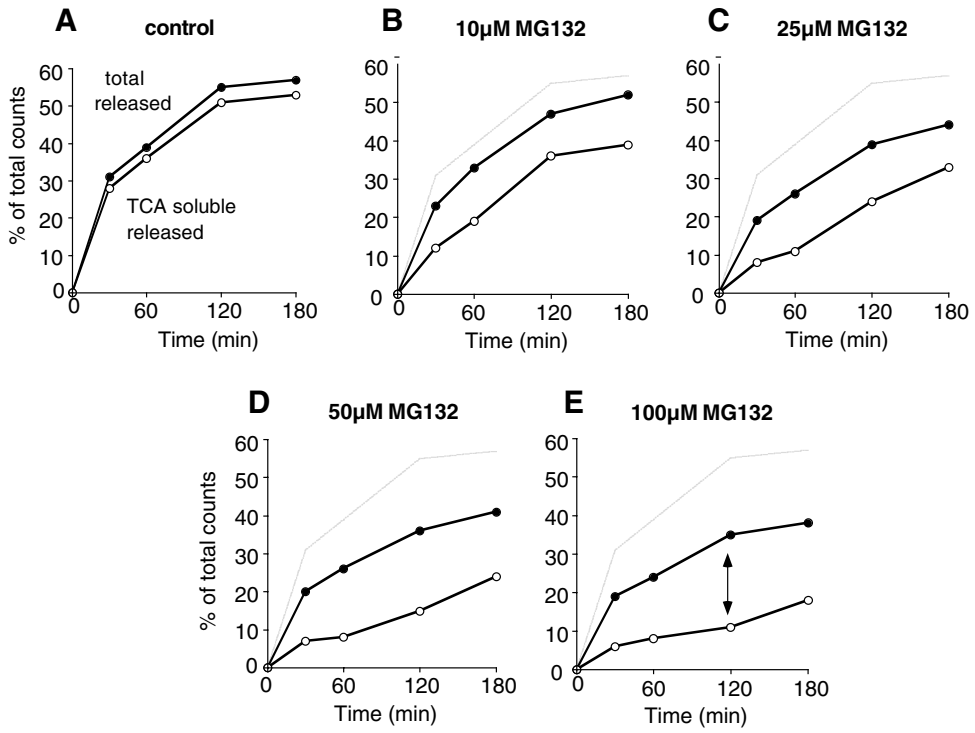


Fig. 2. Release of TCA-insoluble fragments is proportional to β-subunit inhibition. (A-E) CFTR degradation was carried out at the indicated concentrations of MG132. The amount of total (●) and TCA-soluble (○) fragments released from ER membrane was determined as in Fig. 1. TCA-insoluble fragments released are indicated by a double arrow.

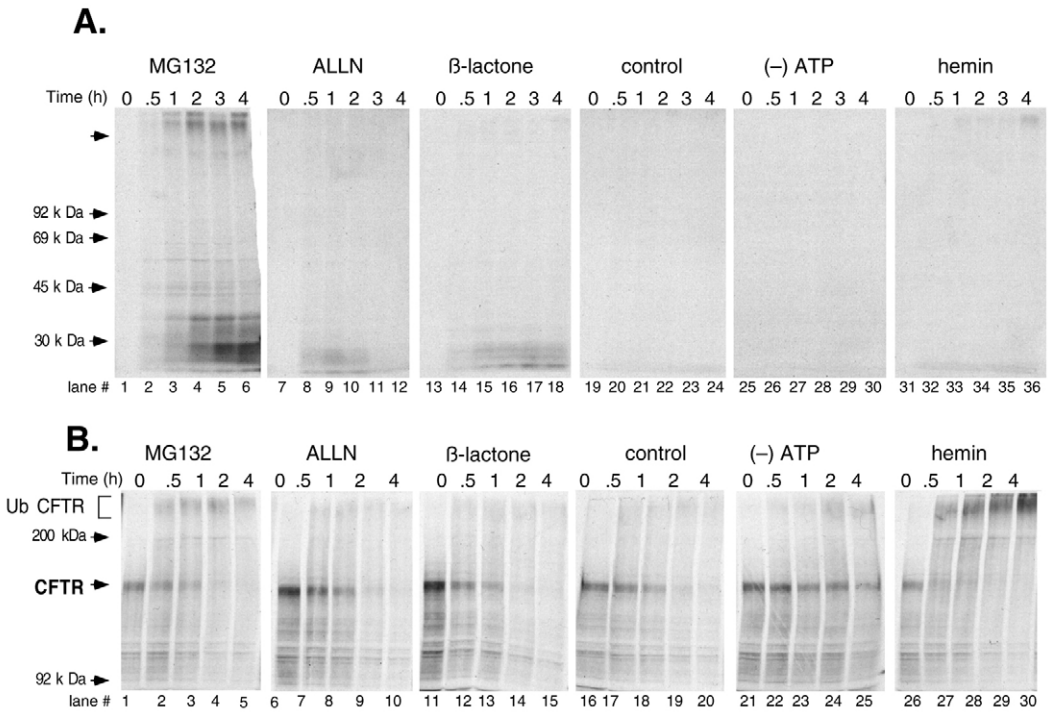
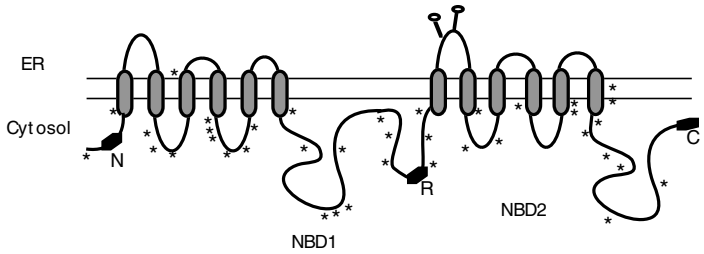
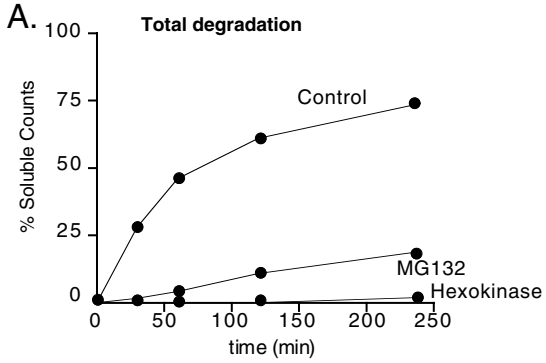
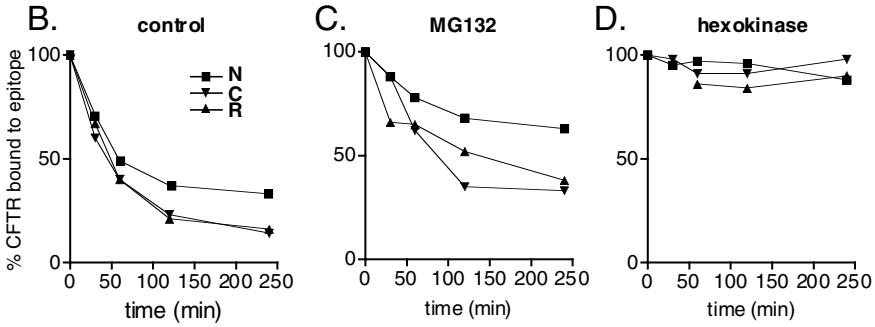


Fig. 3. Released CFTR fragments are heterogeneous. (A) Supernatants of CFTR-degradation reactions were collected at the times indicated and analyzed by SDS-PAGE (7-12% gel) and autoradiography. Inhibitor concentrations were 100 μM for MG132, ALLN, clasto-lactacystin β-lactone (β lactone) and 40 μM for hemin. Hexokinase and 2-deoxyglucose were added to deplete ATP (–ATP). (B) Total products of the degradation reaction are shown to demonstrate the rate of disappearance of CFTR protein. High M_r material in lanes 26-30 (hemin treatment) represents ubiquitylated CFTR products (Xiong et al., 1999). Exposure of the top panels was longer (5×) than the bottom panels.







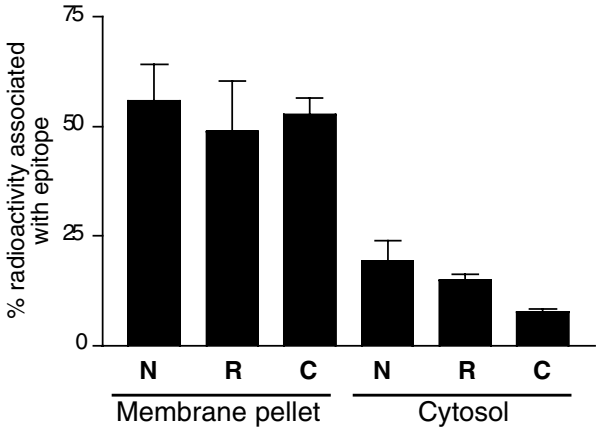


Fig. 4. CFTR degradation involves simultaneous loss of multiple epitopes. The schematic shows the topological structure of CFTR and relative locations of N-terminal (N), C-terminal (C) and regulatory (R) domain epitopes. NDB, nucleotide-binding domain; *, location of methionine residues; open circles, N-linked glycosylation sites. (A) Control CFTR degradation assay performed as in Fig. 1. (B-D) Degradation products from panel A were immunoprecipitated with peptide-specific antisera raised against the N-terminus (■), C-terminus (▼) or R-domain (▲) epitopes. Fragments recovered with each antibody were quantified by scintillation counting and expressed as the percentage of counts recovered at *t*=0.

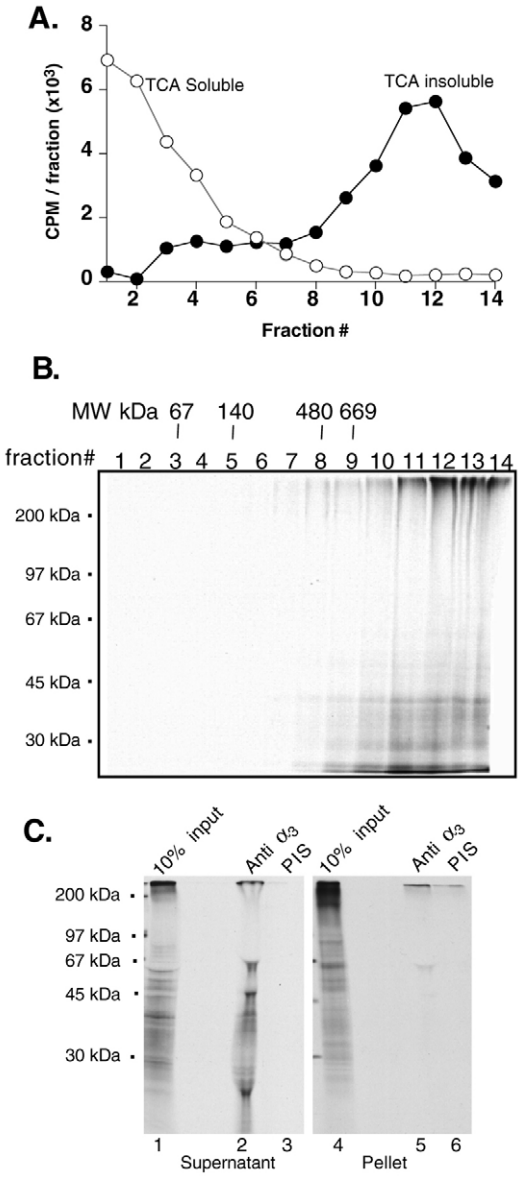
TCA-soluble counts and correspondingly decreased the rate at which epitopes were cleaved from the remaining protein. Following ATP depletion, CFTR was quantitatively recovered with all three antisera, demonstrating that all CFTR cleavage events are strictly ATP dependent (Fig. 4D).

TCA-insoluble fragments were next generated in the presence of MG132, and cytosolic and membrane-bound CFTR were recovered by immunoprecipitation and quantified by scintillation counting (Fig. 5). Under these conditions, 50% of CFTR remaining at the membrane was recovered with each of the three antisera, indicating that residual membrane-bound material consisted of very large fragments. However, cytosolic fragments are primarily 15-45 kDa in size (Fig. 3) and therefore most contain only a single epitope. Of the total cytosolic counts, 43% was recovered by immunoprecipitation, and 20%, 15% and 8% of cytosolic CFTR remained associated with N-, R- and C-domains, respectively. The CFTR N-terminus, which was most highly protected by MG132, was also the epitope recovered with the largest amount of CFTR protein in the supernatant fraction. Note that there is only one methionine residue N-terminal to the first TM segment which represents only 2.6% of total CFTR methionine content. Thus if all CFTR N-termini were simply shaved off the membrane, then the N-terminal epitope would recover <3% of total CFTR radioactivity. However, 7% of total radioactivity (20% recovery of 35% protein released into the cytosol) was recovered by N-terminus antisera when >50% of N-terminus associated radioactivity remained membrane bound. This indicates that N-terminal cytosolic fragments recovered by immunoprecipitation also contain additional TM segments that have been extracted from the membrane. This conclusion was also consistent with SDS-PAGE analysis of immunoprecipitated fragments (data not shown). Taken together, these data indicate that TCA-insoluble cytosolic fragments generated by proteasome inhibition are derived from all regions of CFTR including transmembrane domains.

Fig. 5. Cytosolic fragments are derived from multiple peptide domains. Membrane pellet and supernatant fractions were collected from CFTR degradation reactions carried out for 2 hours in the presence of 100 μM MG132. Samples were immunoprecipitated by N-terminal, C-terminal and R-domain specific antisera and quantified by scintillation counting as in Fig. 5. Data are expressed as the percentage of total radioactive counts in pellet and supernatant fractions that remained associated with indicated epitopes.

Cytosolic CFTR fragments remain sequestered by the proteasome

Given their hydrophobic nature, CFTR degradation intermediates would be highly prone to aggregation and could pose significant toxicity to cells. We therefore tested whether these fragments were released freely into the cytosol or as part of a larger complex. When supernatants were analyzed by glycerol gradient centrifugation, TCA-soluble fragments were quantitatively recovered near the top of the gradient along with a minor fraction of TCA-insoluble fragments too small to be visualized by SDS-PAGE (Fig. 6A, fractions 1-4). However, all TCA-insoluble fragments visualized by SDS-PAGE were recovered in denser fractions (10-13) that corresponded to a molecular mass of ~1000-2000 kDa (Fig. 6B). Fractions 10-13 also corresponded to the distribution of proteasomes as determined by western blotting (data not shown). We therefore tested whether degradation intermediates remained associated with the proteasome after release from the ER membrane by co-immunoprecipitation of membrane pellets and supernatants using a well-characterized antisera that recognizes intact 20S and 26S proteasomes (α_3 20S subunit) (Yang et al., 1995). The entire spectrum of cytosolic TCA-insoluble fragments specifically co-immunoprecipitated with proteasomes, whereas little residual membrane-bound CFTR was recovered (Fig. 6C). Therefore, inhibited proteasomes bind to ubiquitylated CFTR at the cytosolic face of the ER membrane where degradation is initiated (Fig. 1) (Xiong et al., 1999) and remain quantitatively associated with large fragments after they are released into the cytosol.



p97 is inhibited by hemin and contributes to in vitro CFTR degradation

Recent studies have indicated that in addition to the 19S subunit, the ternary complex p97-Ufd1-Npl4 facilitates ERAD in cells by presenting ubiquitylated substrates to the proteasome. p97 is also an abundant component of RRL, comprising ~0.1% of total protein (~100 μg/ml, data not shown). Moreover, although hemin is known to inhibit the proteasome 19S subunit, its specificity for other AAA-ATPases has not been extensively studied. We therefore used a recombinant p97 and a dominant-negative mutant that lacks ATPase activity (E305Q/E578Q) to determine whether p97 also contributes to CFTR degradation in RRL. Both purified proteins migrated as 600 kDa hexamers on glycerol gradients indicating that they were properly assembled (Fig. 7A). Hemin (40 μM) inhibited ATPase activity of purified RRL 26S proteasomes (80% inhibition) as expected (Fig. 7B). In addition, hemin also inhibited >95% of p97 ATPase activity. Mutant p97 lacked ATPase activity and any further decrease by hemin addition could not be detected. Moreover, when dominant-negative p97 (p97QQ) was added to CFTR degradation reactions, we observed a dose-dependent decrease in the production of CFTR TCA-soluble fragments such that a tenfold molar excess reduced degradation by ~50% (Fig. 7C). Thus our results are consistent with in vivo studies (Dalal et al., 2004; Gnann et al., 2004) and show that p97 also contributes to proteasome-mediated CFTR degradation in vitro.

Discussion

Polytopic proteins pose a particular challenge for the ERAD pathway because substrates must not only be unfolded, but transmembrane domains must also be extracted from the lipid bilayer, and translocated into the 20S subunit prior to degradation. Here we show that all of these steps are normally coordinated in a processive manner at the cytosolic face of the

Fig. 6. Cytosolic CFTR fragments remain stably associated with proteasomes. (A) Supernatants from degradation reactions were collected after 2 hours in the presence of 100 μM MG132 and separated by glycerol gradient centrifugation. Fractions were collected top to bottom (1-14) and TCA-soluble (○) and TCA-insoluble (●) material was quantified by scintillation counting. (B) Samples from gradients in panel A were analyzed directly by SDS-PAGE (12-17% gel) and autoradiography. Migration of molecular mass markers in the gradient are indicated at top of the gel. (C) Degradation was carried out as in A and membranes were pelleted through a 0.5 M sucrose cushion. Supernatant and pellet fractions were analyzed directly (lanes 1,4) or immunoprecipitated with preimmune sera (PIS) or antisera against the α_3 proteasome subunit.

ER membrane. In the presence of fully functional proteasomes, CFTR was released from the ER membrane solely in the form of free TCA-soluble peptide fragments. Progressive β-subunit inhibition decreased substrate cleavage but had only a modest effect on total protein released from the membrane. This resulted in excessive CFTR unfolding and the uncoordinated generation of large cytosolic TCA-insoluble fragments that were derived from multiple domains throughout the molecule. TCA-insoluble fragments were generated most efficiently when all three active β subunits were inhibited with either MG132 or a combination of clasto-lactacystin β-lactone, GPFL and leupeptin. However, partial suppression of proteolytic function (β5-subunit inhibition) with clasto-lactacystin β-lactone or ALLN also gave rise to a very small fraction of degradation intermediates. Importantly, partially degraded intermediates remained quantitatively sequestered by inactivated proteasomes after their release into the cytosol. In contrast to active site inhibitors, the non-specific AAA-ATPase inhibitor hemin primarily blocked CFTR release from the membrane, but the small percentage of fragments released were entirely TCA soluble. Taken together, these data indicate that excess proteolytic activity of the 20S subunit assures that the rate of substrate cleavage normally exceeds that of unfolding and as a result, membrane extraction is obligatorily coupled to terminal cleavage events. When protease activity is compromised, however, this balance is disrupted and substrate unfolding in the absence of cleavage gives rise to large cytosolic degradation intermediates.

In vitro CFTR degradation was ATP dependent and highly sensitive to proteasome inhibitors. This is consistent with the ATP requirement for ubiquitylation which is a prerequisite for degradation of most 26S proteasome substrates including CFTR. However, cytosolic fragments continued to be released in the presence of MG132, raising that possibility that other proteases are involved in clipping CFTR. Indeed, several non-proteasome pathways involving signal peptide peptidase (Crawshaw et al., 2004), insulin-degrading enzyme (Schmitz et al., 2004), ER-60 (Qiu et al., 2004) and other lumenal and membrane bound proteases (Loo and Clarke, 1998; Cabral et al., 2000) have been implicated in the degradation of some ERAD substrates. However, non-proteasome ERAD pathways discovered to date are independent of ATP. Thus our findings that all CFTR cleavage and fragment release were completely abolished by ATP depletion (Fig. 4D) make it less likely that alternative proteases are involved in CFTR degradation in vitro. Moreover, if ATP depletion masked secondary protease sites by stabilizing CFTR interactions with cytosolic or lumenal chaperones, e.g. Hsp40, Hsp70 or Hsp90 (Yang et al., 1993; Loo et al., 1998; Meacham et al., 1999; Meacham et al., 2001; Youker et al., 2004), then we would have expected at least some large fragments to be released when AAA-ATPases were inhibited in the presence of ATP. This we did not see, because only TCA-soluble fragments were released in the presence of hemin. Finally, given our findings that degradation intermediates can be titrated by β-subunit inhibition, and previous studies demonstrating that degradation is completely inhibited in the presence of both MG132 and Hemin (Xiong et al., 1999), the most likely explanation for our findings is that residual proteasome peptidase activity is responsible for the release of cytosolic TCA-insoluble CFTR fragments.

How then are TCA-insoluble fragments generated? The

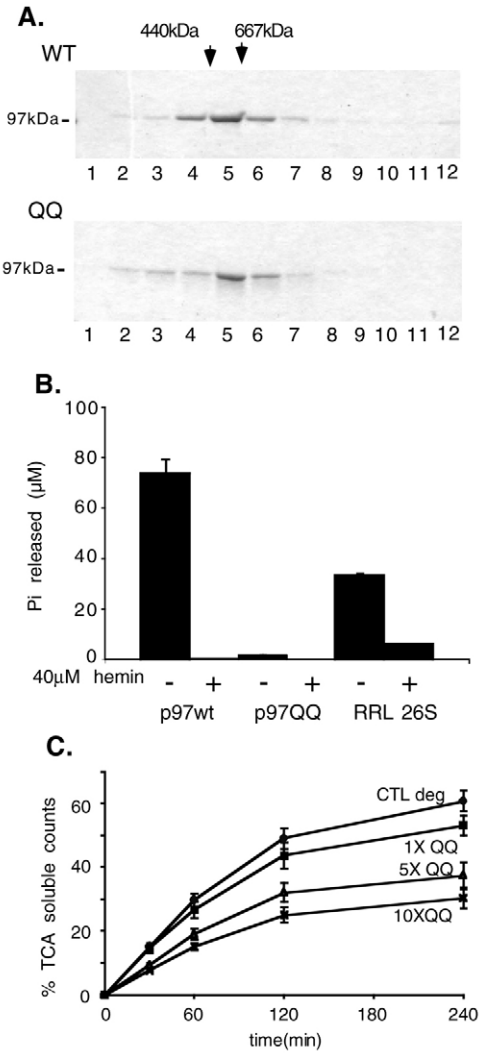


Fig. 7. p97 is inhibited by hemin and contributes to CFTR degradation. (A) Coomassie Blue-stained SDS gels of His-tagged wild type (WT) and dominant-negative mutant (QQ) p97 purified on a Ni-NTA column and run on glycerol gradients. (B) ATPase activity of purified wild-type p97, mutant p97 and 26S RRL proteasomes was determined in the presence and absence of 40 μM hemin as described in Materials and Methods. (C) CFTR degradation was carried out in the presence of indicated molar excess of dominant-negative p97 (QQ). Graph shows the fraction (mean ± s.e.m.) of newly synthesized CFTR that was converted into TCA-soluble peptide fragments.

'limited diffusion' model predicts that the size of proteasome degradation products is governed by their ability to diffuse out of the of the 20S particle (Kisselev et al., 1999; Köhler et al., 2001), which may involve repeated cycles of binding and release of the 19S caps (Babbit et al., 2005). Our results suggest a novel variation in this model in which product size can also be influenced by the relative capacities of protein unfolding and peptide cleavage. Previous studies have shown that genetic inactivation of a single β-subunit changes fragment composition but does not significantly affect product size (Dick et al., 1998; Nussbaum et al., 1998; Kisselev et al., 1999). This is consistent with results that β5-subunit inhibition (clasto-

lactacystin β-lactone and ALLN) modestly decreases the rate of CFTR degradation and has very minor effects on the fraction of TCA-insoluble fragments produced. By contrast, genetic inactivation of all β-subunit activity in the prokaryotic Lon protease not only blocks degradation but also prevents polypeptide translocation through the proteasome core (Melderen and Gottesman, 1999). We propose that MG132 inhibition of eukaryotic proteasomes represents an intermediate between these two states in which all three protease activities are severely but incompletely compromised [β1, β2 and β5 subunits exhibit <2%, 6.5% and <0.1% activity, respectively, in 100 μM MG132 (Oberdorf et al., 2001)]. This allows substrate to be unfolded and threaded into the 20S subunit where rare cleavage events can still be carried out by the weak residual β-subunit activity.

Our findings that TCA-insoluble CFTR fragments remain associated with inhibited proteasomes also suggest that β-subunit inhibitors can induce a partial block in peptide translocation similar to that observed for Lon (Groll et al., 1997; Melderen and Gottesman, 1999). As with prokaryotic ATP-dependent proteases, unfolding and membrane extraction would be expected to continue with accumulation of substrate (Groll et al., 1997; Hoskins et al., 1998; Melderen and Gottesman, 1999; Benaroudj and Goldberg, 2000; Singh et al., 2000; Navon and Goldberg, 2001; Reid et al., 2001). Thus fragment size is increased because reduced diffusion through the proteasome is compensated by a severe decrease in proteolytic capacity. Based on the size of cytosolic CFTR fragments recovered (15-45 kDa), it is likely that smaller ERAD substrates could undergo complete retro-translocation by a similar mechanism. This probably explains why intact proteins are often found in cytosolic fractions in the presence of proteasome inhibitors (McCracken and Brodsky, 1996; Wiertz et al., 1996; Yu et al., 1997; VanSlyke and Musil, 2002).

Our findings provide additional evidence that AAA-ATPases participate in unfolding and/or membrane extraction of ERAD substrates (Plemper and Wolf, 1999; Bays et al., 2001; Ye et al., 2001; Braun et al., 2002; Jarosch et al., 2002; Kobayashi et al., 2002; Meyer et al., 2002; Rabinovich et al., 2002; Ye et al., 2003; Ye et al., 2004, Mayer, 1998). Hemin has long been known to inhibit proteasome-mediated degradation in RRL by blocking proteasome 19S ATPase activity (Etlinger and Goldberg, 1980; Haas and Rose, 1981; Hough et al., 1986; Hough et al., 1987; Hoffman and Rechsteiner, 1996; Xiong et al., 1999). However, it does not affect β-subunit activity of 20S or 26S RRL proteasomes as measured by cleavage of fluorogenic substrates (Oberdorf et al., 2001). We now show that hemin also inhibits p97. Moreover, p97 facilitates CFTR degradation in vitro, which is consistent with recent in vivo studies (Dalal et al., 2004; Gnann et al., 2004). Thus our data indicate that in the presence of active site inhibitors, CFTR unfolding and extraction continues to be carried out by 19S and p97 ATPases independently of proteolytic cleavage.

In cells that contain fully functional proteasomes, degradation of polytopic proteins is processive (Mayer et al., 1998; Plemper et al., 1998; Plemper and Wolf, 1999), and cytosolic CFTR intermediates are not generally observed (Ward et al., 1995; Bence et al., 2001). However, active-site inhibitors (e.g. MG132, ALLN and lactacystin) result in CFTR accumulation in perinuclear inclusion bodies called aggresomes (Jensen et al., 1995; Ward et al., 1995; Bebök et al., 1998; Johnston et al., 1998; Wigley et al., 1999). Such inclusions characteristically lack cellular membranes, contain aggregated immunoreactive CFTR, and are highly concentrated in ubiquitin, proteasomes and cellular chaperones. The TCA-insoluble cytosolic CFTR fragments observed in this study thus probably represent aggresome precursors. They retain multiple CFTR epitopes, resemble high-molecular-weight ubiquitylated species on SDS-PAGE, and remain stably associated with the proteasome. TCA-insoluble fragments were generated much more efficiently by MG132 than by clasto-lactacystin β-lactone or ALLN. This is consistent with the greater in vivo effect of MG132 on CFTR degradation (Gelman et al., 2002) and the more pronounced stress response when compared with lactacystin or ALLN (Bush et al., 1997). The observation that all three inhibitors give rise to aggresomes in vivo, however, suggests that intact cells are more sensitive than RRL, possibly because aggregation requires only a small fraction of degradation intermediates in the face of ongoing protein synthesis. This would probably induce secondary effects by sequestering proteasomes and potentiating a general inhibition of the proteasome pathway (Bence et al., 2001; Ma et al., 2002). We propose that commonly used β-subunit inhibitors thus give rise to aggresomes by uncoupling cleavage from the ATP-dependent process of membrane extraction, thereby releasing large retro-translocated intermediates and/or intact proteins from the ER membrane.

Materials and Methods

Materials

Clasto-lactacystin β-lactone was obtained from Cal-Biochem (San Diego, CA). Nucleotides and DTT were purchased from Roche Molecular Biochemicals (Indianapolis, IN). N-terminal, C-terminal and R-domain CFTR antisera are described elsewhere (Xiong et al., 1999). Proteasome C-9 antisera against the α_3 subunit was provided by Klaus Früh (Yang et al., 1995). Trans[^{35}S]-label was purchased from ICN Pharmaceuticals (Irvine CA). Purified 26S proteasomes were kindly provided by Martin Rechsteiner (University of Utah, Salt Lake City, UT). Rabbit reticulocyte lysate, RRL, was prepared from freshly harvested reticulocytes by hypotonic lysis using a slightly modified method of Jackson and Hunt (Jackson and Hunt, 1983; Oberdorf and Skach, 2002). Lysate was digested at 24°C for 10 minutes with *Staphylococcus* S7 nuclease (Roche, Indianapolis, IN), and nuclease was inactivated by addition of 2.0 mM EGTA. Hemin was added (40 μM) for translation reactions. Canine pancreas microsomal membranes were prepared as previously described (Oberdorf and Skach, 2002). GPFL peptide was kindly provided by Christopher Cardozo (Mount Sinai School of Medicine NY, NY). Chemicals were purchased from Sigma (St Louis, MO) unless otherwise stated.

Recombinant proteins

Plasmids pQE9 p97wt and pQE9 p97QQ (E305Q/E578Q), provided by Hemmo Meyer (Yale University, New Haven, CT), were used to subclone the P97 coding sequence into *Nco*I and *Bam*HI sites of pET15b (EMD Biosciences, San Diego, CA) by PCR amplification using primers: sense, 5′-GTGGCCATGGGACACCATCAC-CATCACCATATGGCCTCTGGAGCCGAT-3′; and antisense, 5′-TGCGGATCCT-TAGCCATACAGGTCATC-3′. p97 expression was induced for 3 hours at 37°C in *E. coli* BL21(DE3) by addition of 0.4 mM Isopropyl β-D-1-thiogalactopyranoside. Cells were pelleted and lysed (2×) using a French Press (1250 psi), loaded onto a 5.0 ml Ni-NTA column (Qiagen, Valencia, CA) and washed extensively with 50 ml of 300 mM NaCl, 1 mM β-mercaptoethanol, 5% glycerol, 0.4 mM PMSF, 25 mM imidazole and 50 mM Tris-HCl (pH 7.5). His-tagged p97 was eluted with a linear 25-500 mM imidazole gradient. Fractions were pooled and dialyzed in 100 mM NaCl, 1 mM β-mercaptoethanol, and 25 mM Tris-HCl (pH 7.5), concentrated on a Centriplus YM-30 or YM-50 filter, flash frozen and stored at –80°C.

In vitro transcription/translation

CFTR mRNA was transcribed in vitro at 40°C for 1 hour in reactions containing 0.4 mg/ml plasmid DNA (plasmid pSPCFTR) (Xiong et al., 1999), 40 mM Tris-HCl (pH 7.5), 6.0 mM magnesium acetate, 2 mM spermidine, 0.5 mM each of ATP, CTP, UTP, 0.1 mM GTP, 0.5 mM GpppG (Amersham Pharmacia, Piscataway, NJ), 10 mM DTT, 0.2 mg/ml bovine calf tRNA, 0.75 U/ml RNAse inhibitor (Promega, Madison, WI), and 0.4 U/ml SP6 RNA polymerase (Epicentre, Madison, WI). Mock

transcription was identical except that no plasmid DNA was added. Translation was performed at 25°C for 2 hours in reactions containing 20% transcript mixture, 40% nuclease-treated rabbit reticulocyte lysate (Oberdorf and Skach, 2002), plus additional 1 mM ATP, 1 mM GTP, 12 mM creatine phosphate, 40 μM each of 19 essential amino acids, except methionine, 1 μCi/μl of Trans[^{35}S]-label, 40 μg/ml creatine kinase, 0.1 mg/ml tRNA, 0.2 U/μl RNAse inhibitor (Promega, Madison WI), 10 mM Tris-HCl (pH 7.5), 100 mM potassium acetate and 2 mM $MgCl_2$. Canine pancreas microsomal membranes (Walter and Blobel, 1983) were added at the start of translation (final concentration, 6.0 OD_{280}). Aurin tricarboxylic acid (50 μM) was added at t=15 minutes to synchronize protein synthesis.

Proteasome peptidase activities

Crude proteasomes were prepared by diluting RRL fivefold in 20 mM Tris-HCl (pH 7.5), 1 mM ATP, 1 mM $MgCl_2$ and 1 mM DTT and centrifuging at 290,000 ***g*** for 2 hours. Pellets were resuspended in 100 mM Tris-HCl (pH 8.0), 1 mM ATP, 1 mM DTT at dilutions yielding activity equivalent to that found in RRL (Oberdorf et al., 2001). Fluorogenic substrates cbz-LLE-AMC (CalBiochem, San Diego, CA), t-boc-LRR-AMC and suc-LLVY-AMC (Sigma) were used to monitor β1, β2 and β5 peptidase activities, respectively. Reactions were carried out at 37°C as described previously (Oberdorf et al., 2001) and terminated by adding one volume of 10% SDS followed by 18 volumes of 100 mM Tris-HCl (pH 9.0). AMC release was determined in opaque microtiter plates using a BioRad FluoroMark plate reader (Ex_{355}/Em_{460}) after correcting for background fluorescence (t=0). Purified AMC was used to generate a standard curve, and proteasome inhibition was expressed as the percentage of control activity. The length of the incubation was adjusted so that the rate of hydrolysis remained linear over the time course of the reaction.

In vitro degradation

ER membranes containing in-vitro-translated CFTR were collected by pelleting twice through 0.5 M sucrose in Buffer A (50 mM HEPES-KOH (pH 7.5) 0.1M KCl, 5 mM $MgCl_2$ and 1 mM DTT) at 180,000 ***g*** for 10 minutes as described (Oberdorf et al., 2001; Oberdorf and Skach, 2002). The membrane pellet was resuspended in Buffer A with 0.1 M sucrose and added to a degradation reaction with final concentrations of 68% reticulocyte lysate, 20% microsomal membranes, 10 mM Tris-HCl (pH 7.5), 1 mM ATP, 5 mM $MgCl_2$, 12 mM creatine phosphate, 3 mM DTT and 80 μg/ml creatine kinase. RRL used for degradation was prepared as above except that it was not treated with nuclease and no exogenous hemin was added. Because degradation activities varied between similarly prepared lysates from different animals (unpublished observation), individual lysates were tested separately, and preparations that yielded the most robust CFTR degradation (e.g. typically 50-70% in 4 hours) were used for ERAD experiments. The reasons for this variability are unknown but may reflect slight differences in degradation machinery and/or endogenous hemin levels. We favor the latter explanation because different RRL preparations also exhibited varying levels of translation in the absence of exogenous hemin (unpublished observation). Degradation was carried out at 37°C. ATP was depleted by substituting creatine kinase with hexokinase (20 U/ml) and deoxyglucose (20 mM). Where indicated, purified recombinant mutant p97 (p97QQ) was added to 0.1 mg/ml, 0.5 mg/ml or 1 mg/ml. Degradation was quantified by precipitating samples in 20% TCA and counting supernatants (Beckman LS6500 scintillation counter). Total ^{35}S incorporation was determined by direct scintillation counting. For all experiments, CFTR degradation was corrected for by subtracting counts typically released from Mock translations (~22% of total), which represent a reversible association of ^{35}S species with components of ER microsomal membranes (Oberdorf et al., 2001). TCA-soluble counts (cpm released) were expressed as a percentage of the total ^{35}S-labeled CFTR present at t=0 using the formula: % TCA counts=(cpm_{CFTR}–cpm_{Mock})/[(CFTR-incorporated ^{35}S×0.78)–($cpm_{t=0}$)]. Error bars represent the s.e.m. derived from at least three experiments.

Release of CFTR from the ER membrane

CFTR released from the ER membrane was determined by pelleting degradation samples through Buffer A as above and counting supernatant prior to and after TCA precipitation. The fraction of TCA-soluble and -insoluble CFTR released into the supernatant was calculated using the formula above. Supernatants and total products were also analyzed directly by SDS-PAGE on 7-12% or 7% gels, respectively. Gels were treated with En^{3}Hance (NEN Life Science Products, Boston, MA) fluorography, autoradiography and digitized using an UMax Powerlook III scanner and Adobe Photoshop software.

Glycerol gradient centrifugation

CFTR degradation was performed in the presence of 100 μM MG132, and membranes were removed by dilution into seven volumes of 0.1M sucrose in Buffer A and centrifugation (180,000 ***g*** for 10 minutes). Supernatant was then loaded onto a 15-40% glycerol gradient containing 100 mM Tris-HCl (pH 7.5), 100 mM KCl, 5 mM $MgCl_2$ and 1 mM DTT. Samples were centrifuged at 260,000 ***g*** for 2.5 hours and fractions were collected from top to bottom and subjected to scintillation counting before and after precipitation in 20% TCA or analysis by SDS-PAGE.

Immunoprecipitation

For CFTR immunoprecipitation, samples were diluted into 1 ml of Buffer B (0.1 M NaCl, 10 mM EDTA, 1% Triton X-100 and 0.1 M Tris-HCl pH 8.0) at specified times after initiation of degradation reaction. One μl N-terminal, C-terminal or R-domain antisera was added followed by 5 μl protein A Affi-Gel beads (BioRad, Hercules, CA). Samples were rotated overnight at 4°C, washed three times with 0.5 ml Buffer B and twice with 0.5 ml of 0.1 M NaCl, 0.1 M Tris-HCl, pH 8.0. Antigen was eluted by addition of 1.0% SDS, 0.1 M Tris-HCl (pH 8.0) and quantified by scintillation counting. Counts immunoprecipitated from mock translation were equivalent to background cpm. The percentage of CFTR counts that remained associated with each epitope was determined by: % CFTR bound to epitope=[($cpm_{t=n}$–background)×100]/($cpm_{t=0}$–background).

To determine the relative signal associated with each epitope released into the supernatant, degradation was carried out for 2 hours in the presence of 100 μM MG132. Membrane and supernatant fractions were separated by centrifugation and immunoprecipitated as above. Fragments recovered were quantified by scintillation counting and expressed as the percentage of total radioactivity in each fraction that remained associated with each epitope. Counts recovered by immunoprecipitation were corrected for IP efficiency of each antibody as determined using the following equations: % radioactivity associated with epitope=($cpm_{IP\ 2\ hours}$/IP efficiency)/(Total $cpm_{2\ hours}$) and IP efficiency (determined independently for each antibody)=$cpm_{IP\ t=0}$/$cpm_{t=0}$. Proteasomes were immunoprecipitated using C9 antisera (Yang et al., 1995) in 1% NP-40, 100 mM NaCl, 25 mM HEPES (pH 7.5), 1 mM EDTA, 2 mM ATP and 5 mM $MgCl_2$. Samples were incubated overnight, washed five times with IP buffer and beads were eluted directly with SDS loading buffer and analyzed by SDS-PAGE and autoradiography.

ATPase assay

ATPase activities of p97 and 26 proteasomes were determined as described previously (Hoffman and Rechsteiner, 1996; Song et al., 2003). Purified recombinant p97, p97QQ (0.25 μg total) or RRL 26S proteasomes (2 μg) ATP were added to 1 mM $MgCl_2$, 1 mM DTT and 20 mM HEPES (pH 7.5) with or without 40 μM hemin. Reactions were incubated at 24°C for 10 minutes. One mM ATP was then added and the reaction was incubated at 37°C for 30 minutes (p97 constructs) or 1 hour (26S), then 200 μl of 6 mM ammonium molybdate, 0.8 M HCl, 120 μM Malachite Green and 0.06% polyvinyl alcohol were added. Reactions were further incubated for 10 minutes at 24°C, and absorbance at 630 nm was measured in a 96-well plate on a Biotek plate reader. Inorganic phosphate released was calculated from a standard curve using KH_2PO_4.

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