Cell, Vol. 83, 129–135, October 6, 1995, Copyright © 1995 by Cell Press

Multiple Proteolytic Systems, Including the Proteasome, Contribute to CFTR Processing

Timothy J. Jensen,* Melinda A. Loo,* Steven Pind,† David B. Williams,† Alfred L. Goldberg,‡ and John R. Riordan*
*S. C. Johnson Medical Research Center
Mayo Clinic Scottsdale
13400 East Shea Boulevard
Scottsdale, Arizona 85259
†Department of Biochemistry
University of Toronto
Toronto, Ontario M5S 1A8
Canada
‡Department of Cell Biology
Harvard Medical School
Boston, Massachusetts 02115

Summary

The molecular components of the quality control system that rapidly degrades abnormal membrane and secretory proteins have not been identified. The cystic fibrosis transmembrane conductance regulator (CFTR) is an integral membrane protein to which this quality control is stringently applied; ~75% of the wild-type precursor and 100% of the ΔF508 CFTR variant found in most CF patients are rapidly degraded before exiting from the ER. We now show that this ER degradation is sensitive to inhibitors of the cytosolic proteasome, including lactacystin and certain peptide aldehydes. One of the latter compounds, MG-132, also completely blocks the ATP-dependent conversion of the wild-type precursor to the native folded form that enables escape from degradation. Hence, CFTR and presumably other intrinsic membrane proteins are substrates for proteasomal degradation during their maturation within the ER.

Introduction

One important function of intracellular protein breakdown is to catalyze the rapid elimination of proteins with abnormal conformations that may arise by mutations or intracellular denaturation (Etlinger and Goldberg, 1977; Goldberg, 1992). Such abnormal proteins in the cytosol are rapidly degraded by a soluble ATP-dependent pathway that involves ubiquitin and the 26S proteolytic complex (Hershko and Ciechanover, 1992). In this pathway, proteins are marked for rapid degradation by conjugation to multiple molecules of ubiquitin, which leads to their rapid hydrolysis by the 26S proteolytic complex. The proteolytic core of this structure is the 20S (700 kDa) proteasome, which contains multiple peptidase activities that function together in proteolysis (for reviews see Goldberg, 1995; Goldberg et al., 1995). Recently, several peptide aldehydes have been identified that can enter mammalian cells and inhibit the ubiquitin–proteasome pathway (Rock et al., 1994). These agents can block the rapid degradation of abnormal proteins completely as well as the degradation of short-lived, native polypeptides and the slower breakdown of the bulk of cell proteins (Rock et al., 1994). In contrast, inhibitors that affect the cysteine proteases found in lysosomes and the calpains have no such effects. Recently, it has been found that degradation of a mutant protein in the yeast endoplasmic reticulum (ER) membrane, Sec61, requires the activity of a membrane-embedded ubiquitin transferase and the proteasome for its rapid degradation (Sommer and Jentsch, 1993). Also, some surface membrane proteins in yeast undergo ubiquitination prior to endocytosis (Kölling and Hollenberg, 1994). Therefore, it is also possible that the proteasome is critical in the turnover of membrane-associated proteins, especially those with large polypeptide domains reaching into the cytosol.

The cystic fibrosis transmembrane conductance regulator (CFTR) is one such protein (Riordan et al., 1989). It is a secretory Cl^- channel that is highly conserved among vertebrates (Diamond et al., 1991), where it functions as part of a secondary active transepithelial NaCl transport system (Riordan et al., 1994). Mutations in the human CFTR gene result in cystic fibrosis (CF) (Kerem et al., 1989) due to a primary failure to hydrate exocrine secretions adequately. The most common mutation, ΔF508, present on at least one allele of 90% of patients (Collins, 1992), has the primary consequence of preventing biosynthetic maturation and transport beyond the ER (Cheng et al., 1990). However, even with wild-type CFTR, most of the immature core-glycosylated molecule is not converted to a form capable of transport beyond the ER. Conversion is only ~25% efficient; the remaining ~75% of the precursor is broken down at a very rapid rate (Cheng et al., 1990) after translation of the polypeptide is complete (Ward and Kopito, 1994). Similar effects are seen both in nonpolarized cells in which CFTR cDNAs are heterologously expressed and in epithelial cells that normally express CFTR (Lukacs et al., 1994; Pind et al., 1994). The ER degradation of ΔF508 occurs with the same kinetics as with the wild-type molecule, but ΔF508 is not converted to a form containing complex oligosaccharides (Cheng et al., 1990). The nature of the normal maturational step that enables movement of the wild-type protein out of the ER is not understood, but it does occur at the ER and is dependent on cellular ATP (Lukacs et al., 1994).

Other membrane proteins are also known to be degraded at the ER, but the process is poorly understood, and the responsible enzymes have not been identified (Klausner and Sitia, 1990). For example, some of these proteolytic events are ATP dependent (Gardner et al., 1993), but some are not (Inoue and Simoni, 1992; Wikström and Lodish, 1992); some occur within the lumen (Wikström and Lodish, 1991), while others take place on the cytoplasmic side; some exhibit inhibitor sensitivities characteristic of serine proteases (Gardner et al., 1993), whereas others do not. In fact, the most common agents used to block ER proteolysis have been frequently identi-

fied as cysteine protease inhibitors (Adeli, 1994; Inoue et al., 1991; Wileman et al., 1991), although they are also transition-state inhibitors of certain serine endoproteases and the proteasome (Rock et al., 1994). The most often used of these peptide aldehydes, N-acetyl-L-leucinyl-L-leucinyl-L-norleucinal (ALLN) and, to a much lesser extent, N-acetyl-L-leucinyl-L-leucinyl-L-methional (ALLM) have recently been shown to also inhibit the ubiquitin–proteasome pathway in intact cells, as does another peptide aldehyde, N-carbobenzoxyl-L-leucinyl-L-leucinyl-L-leucinal (MG-132; Rock et al., 1994). These inhibitors have very different potencies for inhibition of protein degradation by the proteasome. Very recently, a highly specific, irreversible inhibitor of the proteasome, lactacystin, has been discovered (Fenteany et al., 1995), and it has not been found to inhibit any other known protease. The present studies have used several of these novel inhibitors to investigate whether the proteasome may also participate in the rapid degradation or processing of CFTR during its maturation.

Results

Effect of Peptide Aldehyde Inhibitors on the Biosynthesis of Wild-Type CFTR

We had previously characterized the kinetics of degradation and maturation of wild-type CFTR heterologously expressed in Chinese hamster ovary (CHO) cells and showed that these were not significantly different from those in epithelial cells that endogenously express CFTR (Lukacs et al., 1994; Pind et al., 1994). Figure 1A shows that ALLN significantly retards the disappearance of the ~150 kDa precursor band. Although the loss of this band is markedly slowed, it is notable that all of the CFTR is eventually degraded in the continued presence of the compound, even at doses up to 100 μM. The inhibition of degradation was reversed upon removal of the inhibitor from the medium, as indicated in the last three lanes in Figure 1A. Despite the inhibition of precursor degradation, there was no apparent increase in the amount of mature CFTR protein formed. ALLM, a second peptide aldehyde inhibitor, which has been reported to be completely effective in blocking the ER degradation of 3-hydroxy-3-methylglutaryl coenzyme A reductase (Inoue et al., 1991), was also tested. As can be seen in Figure 1B, ALLM was essentially without effect on degradation of the ~150 kDa precursor. Although these compounds can potently inhibit certain cysteine and serine proteases (Wileman et al., 1991), the complete lack of inhibitory effect of ALLM suggested ALLN may be eliciting its effect by inhibition of another protease class. Recent studies by Rock et al. (1994) demonstrated that ALLN and, to a much lesser extent, ALLM also inhibit protein breakdown by purified proteasomes and proteolysis by the ubiquitin–proteasome pathway in intact cells. These observations seemed to resemble the relative influence of these compounds on the ER degradation of CFTR. Therefore, another member of this peptide aldehyde class of compounds specifically developed (Palombella et al., 1994; Goldberg et al., 1995) as proteasome inhibitors, MG-132, was tested. Figure 1C shows that the characteristic precursor–product relationship between the ~150 kDa immature band and the ~175 kDa mature band was altered in a unique and unexpected way by this compound. It completely blocked the formation of the mature CFTR band. Thus, it appears that one member of this group of agents, ALLN, causes a significant inhibition of the rapid ER degradation of immature CFTR, whereas another member, MG-132, totally blocks conversion to a maturation-competent form. Blockage of this maturation step is also seen upon ATP depletion (Lukacs et al., 1994).

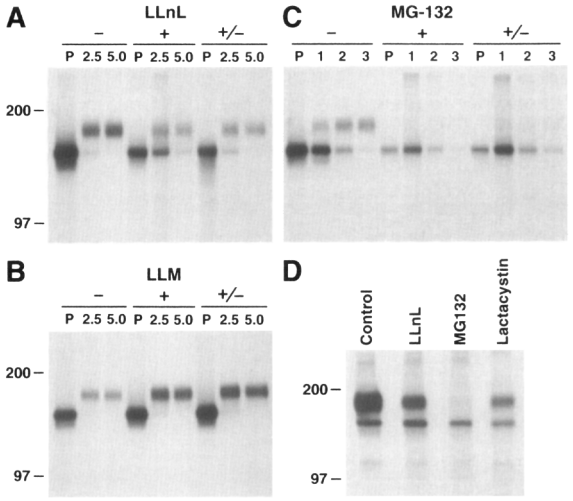

Figure 1. Effects of Protease Inhibitors on the Biosynthetic Processing of the Wild-Type CFTR Precursor

CHO cells stably expressing wild-type human CFTR were pretreated with each of the compounds for 90 min and during a further 30 min preincubation in methionine-free medium. Cells were then pulse labeled (P) for 20 min with [^{35}S]methionine and chased with methionine-containing medium. The absence and presence of the inhibitors are indicated by minus and plus signs. The designation plus/minus indicates that inhibitors were removed prior to pulse (P) and chase incubations. At the times indicated, cells were lysed and immunoprecipitated using the anti-CFTR monoclonal antibody M3A7 as described previously (Pind et al., 1994).

(A) Decreased rate of the disappearance of the pulse-labeled immature band caused by 50 μM ALLN (also known as LLnL). This effect is almost entirely lost when the drug is removed prior to the pulse (plus/minus), indicating its reversibility.

(B) The same dose of ALLM (also known as LLM) does not delay degradation or maturation of the precursor.

(C) MG-132 at 50 μM entirely prevents formation of the larger mature product.

(D) Effects of inhibitors on steady-state amounts of immature and mature CFTR. Cells were continuously labeled with [^{35}S]methionine over an 8 hr period in a medium composed of 75% methionine-free α-MEM, 20% complete α-MEM, and 5% fetal bovine serum. Cell lysates were immunoprecipitated and electrophoresed at the end of this period.

Since this series of compounds influenced both the degradation and conformational maturation of core-glycosylated CFTR in the ER, it was important to determine their steady-state effects. Figure 1D shows their effect during a continuous 8 hr labeling with [^{35}S]methionine. Consistent with its much greater impact on maturation than degradation in pulse–chase experiments, virtually no mature CFTR was present at the end of an 8 hr period in the presence of

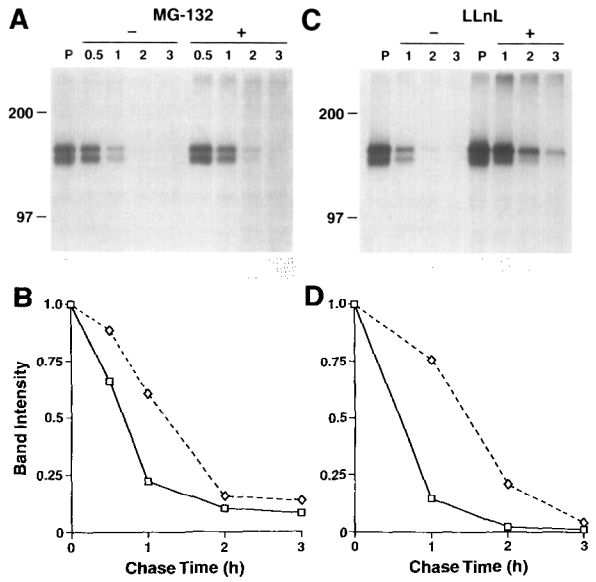

Figure 2. Effects of MG-132 and ALLN on the Degradation of the ΔF508 CFTR Precursor

The upper member of the doublet band is the counterpart of the ~150 kDa core-glycosylated band of the wild type. The lower member is the product of alternative translation initiation that is augmented in ΔF508 (S. P. et al., unpublished data). MG-132 (50 μM) reduces the rate of degradation of the precursor bands (A and B), whereas ALLN (also known as LLnL) has a stronger inhibitory effect (C and D), similar to that on the wild type. In (B) and (D), the relative band intensities normalized to those after pulse labeling (lane P) were determined by densitometry using the PDI Discovery Series. Squares and solid lines represent degradation in absence of inhibitors; diamonds and broken lines, in their presence.

MG-132; the amount of the immature species was also somewhat reduced below the control level. On the other hand, ALLN, which does not prevent maturation, left the amount of the immature species essentially unchanged and that of the mature species only slightly diminished. Since MG-132 apparently had such a strong net effect, we evaluated its influence over even longer time periods in immunoblots (see Figure 3C). The immature form progressively accumulates over these extended periods, and the amount of the mature form is diminished. To confirm that the proteasome was being affected by this treatment, we probed equivalent blots with an antibody to ubiquitin. The overall amount of ubiquitinated proteins was not much elevated until ~20 hr, when a heavy accumulation of ubiquitin conjugates was evident from the top to the bottom of the gel (data not shown). These inhibitors have been shown previously to cause a similar build up of ubiquitinated proteins in other cells by inhibition of the proteasome pathway (A. L. G. et al., unpublished data).

Inhibition of the Degradation of ΔF508 CFTR

The major defect associated with the mutation responsible for most CF is its inability to undergo the maturational conversion that occurs with the wild-type molecule (Cheng et al., 1990; Kartner et al., 1992). However, the kinetics of degradation of the immature unprocessed mutant molecule in the ER are not significantly different from those of the wild type (Lukacs et al., 1994). We evaluated the influence on this degradation of the two peptide aldehydes that had these marked effects on the wild type. Figure 2 shows that both compounds are inhibitory, with ALLN again having an apparently stronger effect than MG-132. It is notable that under conditions in which degradation is most strongly blocked (e.g., ALLN at 1 hr of chase; shown in Figure 2C), there is a significant accumulation of very high molecular weight forms of CFTR evident near the top of the gel. These species could correspond to ubiquitinated forms of CFTR. A similar accumulation of high molecular weight species was also detected with the ALLN-treated wild-type CFTR (see lane indicating 2.5 hr in Figure 1A), but not in ALLN-treated CHO cells that did not express CFTR. Despite the reduced degradation, there was no promotion of the maturation of ΔF508.

Inhibition of Escape from ER Degradation

We previously observed that when brefeldin A (BFA) was added to restrict CFTR to the ER compartment, a proportion of the immature wild-type CFTR molecules remained undegraded (Lukacs et al., 1994). This proportion was equal to that which acquired complex oligosaccharide chains in untreated cells. Hence, it was reasonable to deduce that some essential event occurred in the ER that allowed CFTR to escape endogenous proteases and to exit from this compartment. This conversion was found to be ATP dependent (Lukacs et al., 1994). Because MG-132 completely prevented the conversion of the wild-type core-glycosylated precursor to the complex-glycosylated product (see Figures 1C and 1D; Figure 3C), we determined the effect of MG-132 on this conversion that occurred in the ER of BFA-treated cells. Figure 3A clearly demonstrates that, in the presence of MG-132, the fraction of CFTR that is normally retained intact becomes completely degraded. Because this step, which we interpreted previously as conformational maturation, is blocked by MG-132, it probably involves proteolytic cleavage. One possible interpretation is that MG-132 acts on an ATP-dependent protease that mediates the conversion. A number of such enzymes have been found in bacteria and mitochondria of eukaryotic cells (Goldberg, 1992). Alternatively, the ATP-mediated step may involve Hsp70, which associates with immature CFTR (Yang et al., 1993). Dissociation of the chaperone from the cytoplasmic aspect of the ER requires ATP hydrolysis and might be necessary to allow the maturation step to occur. In a similar experiment in which ALLN instead of MG-132 was added to BFA-treated cells (Figure 3B), the result was quite different. A similar proportion of the initial pulse-labeled material was retained in the presence and absence of this peptide aldehyde, although there was significantly reduced pulse labeling of CFTR in its presence. Because the MG-132-sensitive step that is responsible for escape from complete degradation at the ER is insensitive to ALLN, this step must be different from the degradative process that is sensitive to both of these compounds (see Figure 2).

Effects of Lactacystin on CFTR Biosynthesis

Very recently, a Streptomyces metabolite with interesting cytological effects was demonstrated to be a highly specific inhibitor of multiple proteasome activities (Fenteany

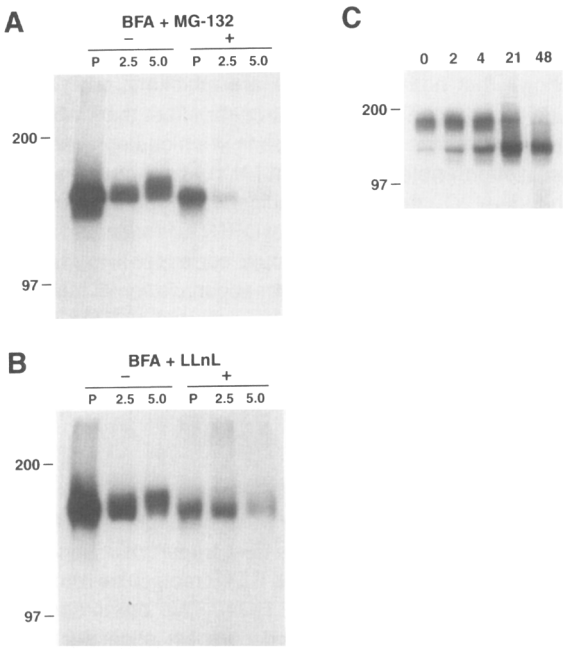

Figure 3. Effect of Peptide Aldehydes on the ER Degradation of the Wild-Type CFTR Precursor in BFA-Treated Cells and on Steady-State CFTR Levels in Untreated Cells

Experimental conditions were as in Figure 1 except that 10 μM BFA was present in all samples in (A) and (B) from the outset.

(A) MG-132 prevents the persistence of a portion of core-glycosylated CFTR in BFA-treated cells.

(B) The proportion of pulse-labeled precursor (lane P) retained after 5 hr of chase in BFA-treated cells is not diminished by ALLN (also known as LLnL), although the extent of pulse labeling is reduced.

(C) Cells were continuously exposed to MG-132 for the periods indicated and lysates immunoblotted using the M3A7 anti-CFTR antibody. The amount of mature CFTR progressively decreased and the immature form accumulated.

et al., 1995). In view of the profound but distinct effects of different peptide aldehyde inhibitors on CFTR processing, we examined the influence of lactacystin in the identical experimental paradigm. The results of one such experiment are shown in Figures 4A and 4B. Lactacystin caused a somewhat greater inhibition of precursor degradation than did ALLN. Its net effect during longer term continuous labeling (8 hr) was similar to that of ALLN (see Figure 1D). Thus, the 20S proteasome does contribute substantially to the rapid turnover of the CFTR precursor at the ER. This result provides stronger evidence that the maturational processing step that allows escape from bulk degradation must reflect another proteolytic activity that is sensitive to MG-132 but presumably distinct from the proteasome. It is very clear that lactacystin does not inhibit conversion of the immature to the mature form. Nevertheless, like the other less potent inhibitors, it also does not significantly promote the accumulation of mature forms; consequently, there must be additional quality control steps in this pathway.

Comparison with P-Glycoprotein Maturation

As shown above, characteristic features of CFTR biosynthesis are rapid breakdown of the immature form and its inefficient conversion to the mature form. A structurally related integral membrane glycoprotein, the wild-type P-glycoprotein (P-gp) multidrug transporter, does not exhibit this behavior (Loo and Clarke, 1994). As shown in Figure 4C, there is a very efficient conversion of immature P-gp into a complex glycosylated form during a pulse–chase experiment. We tested the influence of MG-132 on this maturation. While there is some slowing of the maturation of P-gp, the effect is much smaller than in the case of CFTR. It will be interesting in future experiments to determine whether the degradation of variants of P-gp created by in vitro mutagenesis that fail to mature (Loo and Clarke, 1994) is sensitive to proteasome inhibitors. However, from the present observations, the step that CFTR must undergo to avoid degradation by the proteasome and possibly additional proteases appears to be at least somewhat specific. Possibly, the ATP-requiring, MG-132-sensitive pathway involves processing of the CFTR molecule itself. However, CFTR is not believed to be cleaved N-terminally, although peptide sequencing has been performed only on the recombinant protein expressed in insect Sf9 cells (Bear et al., 1992). Antibodies against peptides as close to the N-terminus as residues 28–45 recognize mature CFTR (Kartner et al., 1991; S. P. et al., unpublished data), as do those raised against the extreme C-terminus (Cohn et al., 1991). Investigations of other possibilities, including the cleavage of chaperones or other interacting ER proteins, will be a primary goal of future work.

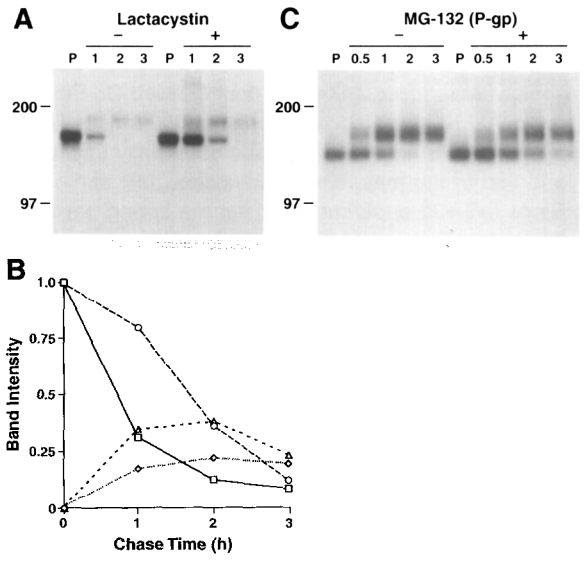

Figure 4. Effects of Proteasome Inhibitors on CFTR and P-gp Processing

(A) The proteasome-specific inhibitor lactacystin applied at 10 μM, using the same preincubation protocol described in Figure 1, strongly inhibited degradation of wild-type CFTR precursor but not its maturation. P, pulse.

(B) Graphical representation of relative band intensities in (A). Squares represent precursor degradation in the absence of drug; circles, in its presence. Diamonds represent product accumulation in the absence of drug; triangles, in its presence.

(C) MG-132 at the same concentration (50 μM) that totally blocked CFTR maturation did not abolish P-gp maturation.

Discussion

It is now clear that the proteasome is the primary site in mammalian cells for degradation of cytosolic and nuclear proteins (Rock et al., 1994). It is responsible for the degradation of many rapidly turning-over proteins in cells, especially regulatory molecules such as the cyclins (Hershko et al., 1994). It can also catalyze limited proteolytic processing that generates the active form of molecules, such as production of the 50 kDa subunit of the transcription factor, NF-κB, from the 105 kDa precursor (Palombella et al., 1994). In view of such a processing function and the fact that several membrane proteins are ubiquitinated prior to their degradation (Kölling and Hollenberg, 1994; Sommer and Jentsch, 1993) or activation (Paolini and Kimet, 1993; Hou et al., 1994), it may not be surprising that the ubiquitin–proteasome pathway is involved in the degradation and perhaps also in the proteolytic processing of CFTR. We have provided substantial pharmacological evidence that the proteasome participates in the complete hydrolysis of the ER form of CFTR. Although ER degradation has been widely assumed to involve lumenal cysteine proteases, nearly 85% of the mass of CFTR is believed to be exposed on the cytoplasmic surface of the ER, where proteasome particles also have been directly observed by immunoelectromicroscopy (Rivett, 1993). One can imagine these particles docking on the cytoplasmically exposed domains that may be marked for degradation, perhaps by ubiquitination. The abundant chaperone, Hsp70, can bind the ER form of CFTR from the cytosolic side (Yang et al., 1993), and recent studies in eukaryotic cells as well as bacteria show that Hsp70 and its cofactors in the *dnaK* family are required for rapid degradation of certain cytosolic and ER proteins (Sherman and Goldberg, 1992, 1995).

Although immature CFTR appears to be ubiquitinated, as suggested by the appearance of high molecular weight forms, at least after treatment with the more potent proteasome inhibitors, we have not yet established whether the rapid breakdown of wild-type and mutant CFTR requires ubiquitination. There are examples of ubiquitination and proteasomal degradation not being obligatorily coupled. Importantly, the rapidly turning-over STE6 surface protein, a structural relative of CFTR, is ubiquitinated, but its degradation involves endocytosis and hydrolysis in the yeast vacuole, the counterpart of the mammalian cell lysosome (Kölling and Hollenberg, 1994). Conversely, ornithine decarboxylase (Murakami et al., 1992) and perhaps other polypeptides (e.g., the model polypeptide casein; Driscoll and Goldberg, 1990) are degraded by the 26S proteasome in an ubiquitination-independent fashion.

These findings with CFTR seem to indicate that the substrate range of the proteasome includes some membrane and possibly secretory proteins at a relatively early stage of their biosynthetic processing in the ER. However, other proteolytic systems probably also contribute to ER quality control. In the cases of membrane proteins in which very little of the polypeptide is exposed on the cytoplasmic side of the ER membrane, a primary role for the proteasome would seem unlikely, although it might be possible that critical cleavages on the cytosolic side could make the lumenal parts more susceptible to proteases within the ER. It will now be of interest to determine what role the proteasome plays in the breakdown in the ER of other membrane proteins such as the asialoglycoprotein receptor (Wikström and Lodish, 1992), 3-hydroxy-3-methyl-glutaryl coenzyme A reductase, and T cell receptor α (Inoue and Simoni, 1992).

In addition to demonstrating that the proteasome contributes to the ER degradation of an intrinsic membrane protein, these experiments have provided insights into the steps involved in the handling of wild-type and ΔF508 CFTR at the ER and the sequence in which they occur. A clear inhibition of precursor degradation is caused by the proteasomal inhibitors lactacystin, ALLN, and MG-132, but in no case does this inhibition result in increased conversion to the mature form. Therefore, there must be a second degradation pathway functioning in parallel with the proteasome. It is apparently ATP independent, as it is very active in ATP-depleted cells treated with proteasome inhibitors such as lactacystin (our unpublished data). Conversion of the precursor to the native folded form capable of transport from the ER is completely ATP dependent (Lukacs et al., 1994) and inhibited entirely by MG-132 (see Figure 1C), but not by the other proteasome inhibitors tested. Hence, at least three proteolytic steps may contribute to the ER processing of CFTR. The MG-132-sensitive, ATP-requiring one seems to provide an escape route from the other two and hence must occur before they do. Normally, only a minority of the wild-type precursor molecules escape and mature; those that do not may be digested by either of the ER-associated degradative processes; if the proteasome is inhibited, the ATP-independent pathway is accelerated. These findings raise the intriguing possibility that, at the ER, the balance between rapid degradation and maturation of the core-glycosylated immature polypeptide is determined by competitive proteolytic processes whose activities may be sites of physiological regulation.

Experimental Procedures

Cells and Media

The CHO cell lines stably expressing wild-type and ΔF508 CFTR described previously (Chang et al., 1993; Pind et al., 1994; Tabcharani et al., 1991) were grown in α-minimal essential medium (MEM) containing 7% fetal bovine serum (Sigma). The B30 multidrug-resistant cells that overexpress P-gp were those developed by Kartner et al. (1985); they were grown in nucleoside-supplemented α-MEM.

Antibodies

The mouse monoclonal antibody M3A7, which recognizes an epitope between amino acid residues 1365 and 1394 on human CFTR, developed and characterized earlier (Kartner et al., 1992), was purified from ascites by chromatography on protein A–Superose (Pharmacia) and Mono Q (Pharmacia). Mouse monoclonal antibodies to BiP (grp78) and a rabbit polyclonal antibody to calnexin were obtained from StressGen, and a mouse monoclonal antibody to bovine ubiquitin was obtained from Chemicon International. The mouse monoclonal antibody C219 (Kartner et al., 1985), specific for P-gp, was from Signet Laboratories.

[^{35}S]Methionine Incorporation

Cell monolayers were starved for 30 min in methionine-free α-MEM

and then pulse labeled for 20 min with 100 μCi/ml L-[^{35}S]methionine (>800 Ci/mmol; Amersham). To chase, this medium was replaced with α-MEM containing 7% fetal bovine serum and 1 mM methionine. Pulse and chase incubations as well as preincubations were performed in a 37°C incubator with 5% CO_2.

Treatment with Protease Inhibitors

ALLN and ALLM (Sigma) and MG-132 and lactacystin (Myogenics) were dissolved in DMSO. These were added to cells usually 90 min prior to methionine starvation and, except in experiments to detect reversibility, were also present during the pulse labeling and chase periods. Final DMSO concentrations were 1% or less and, in the absence of the inhibitors, were without influence on CFTR processing.

Cell Lysis and Immunoprecipitation

Cells were solubilized in RIPA buffer consisting of 50 mM Tris–HCl (pH 7.4) containing 150 mM NaCl, 1% sodium deoxycholate, 1% Triton X-100, and 0.1% sodium lauryl sulfate. Detergents were from Sigma. This buffer contained the following protease inhibitors: leupeptin (10 μg/ml), aprotinin (5 μg/ml), and Prefabloc (50 μg/ml), all from Boehringer Mannheim, and benzamidine (100 μg/ml) and E64 (3.5 μg/ml), from Sigma. The soluble supernatant obtained after centrifugation at 15,000 × g for 15 min at 4°C was incubated at the same temperature with 1 μg/ml purified M3A7 (or C219 in the case of P-gp labeling). The complexes formed were then bound to protein G–agarose beads (GIBCO BRL) and washed four times with RIPA buffer prior to incubation at room temperature with electrophoresis sample buffer.

SDS-PAGE and Immunoblotting

Immunoprecipitates for [^{35}S]methionine-labeled cells were dissolved at room temperature in electrophoresis sample buffer as described previously (Pind et al., 1994) and separated by SDS–PAGE on 7% polyacrylamide gels that were then fixed in 30% methanol and 10% acetic acid, equilibrated in 1 M sodium salicylate, and dried for fluorography (Pind et al., 1994). For Western blotting, nonradioactively labeled cell lysates were dissolved in the same electrophoresis sample buffer, electrophoresed, and transferred to nitrocellulose membranes (Bio-Rad). The membranes were probed with the primary antibodies described above to CFTR, P-gp, BiP, calnexin, and ubiquitin, and the bound antibodies were detected by enhanced chemiluminescence (Amersham) using protocols described in detail previously (Kartner et al., 1991).

Acknowledgments

We thank Ms. Sandra Rosewell for preparation of the manuscript. MG-132 was generously provided by Myogenics, Cambridge, Massachusetts. This work was supported by grants from the National Institute of Diabetes and Digestive and Kidney Diseases of the National Institutes of Health (NIH) (P50-DK49184) to J. R. R. and by the National Institute of General Medical Sciences of the NIH (46147) to A. L. G.; J. R. R. is the Richard O. Jacobson Professor of Molecular Medicine at the Mayo Foundation.

Received August 21, 1995; revised August 31, 1995.

References

Adeli, K. (1994). Regulated intracellular degradation of apoliproprotein B in semipermeable HepG2 cells. J. Biol. Chem. *269*, 9166–9175.

Bear, C.E., Li, C., Kartner, N., Bridges, R.J., Jensen, T.J., Ramjeesingh, M., and Riordan, J.R. (1992). Purification and functional reconstitution of the cystic fibrosis transmembrane conductance regulator (CFTR). Cell *68*, 809–818.

Chang, X.B., Tabcharani, J.A., Hou, Y.X., Jensen, T.J., Kartner, N., Alon, N., Hanrahan, J.W., and Riordan, J.R. (1993). Protein kinase A (PKA) still activates CFTR chloride channel after mutagenesis of all 10 PKA consensus phosphorylation sites. J. Biol Chem. *268*, 11304–11311.

Cheng, S.H., Gregory, R.J., Marshall, J., Paul, S., Souza, D.W., White, G.A., O'Riordan, C.R., and Smith, A.E. (1990). Defective intracellular transport and processing of CFTR is the molecular basis of most cystic fibrosis. Cell *63*, 827–834.

Cohn, J.A., Melhus, O., Page, L.J., Dittrich, K.L., and Vigna, S.R. (1991). CFTR: development of high-affinity antibodies and localization in sweat gland. Biochem. Biophys. Res. Commun. *181*, 36–43.

Collins, F.S. (1992). Cystic fibrosis: molecular biology and therapeutic implications. Science *256*, 774–779.

Diamond, G., Scanlin, T.F., Zasloff, M.A., and Bevins, C.L. (1991). A cross-species analysis of the cystic fibrosis transmembrane conductance regulator: potential functional domains and regulatory sites. J. Biol. Chem. *266*, 22761–22769.

Driscoll, J., and Goldberg, A.L. (1990). The proteasome (multicatalytic protease) is a component of the 1500 kDa proteolytic complex which degrade ubiquitin conjugated proteins. J. Biol. Chem. *265*, 4789–4792.

Etlinger, J.D., and Goldberg, A.L. (1977). A soluble ATP-dependent proteolytic system responsible for the degradation of abnormal proteins in reticulocytes. Proc. Natl. Acad. Sci. USA *74*, 54–58.

Fenteany, G., Standaert, R.F., Lane, W.S., Choi, S., Corey, E.J., and Schreiber, S.L. (1995). Inhibition of proteasome activities and subunit-specific amino-terminal threonine modification by lactacystin. Science *268*, 726–731.

Gardner, A.M., Aviel, S., and Argon, Y. (1993). Rapid degradation of an unassembled immunoglobulin light chain is mediated by a serine protease and occurs in pre-Golgi compartment. J. Biol. Chem. *268*, 25940–25947.

Goldberg, A.L. (1992). The mechanism and functions of ATP-dependent proteases in bacterial and animal cells. Eur. J. Biochem. *203*, 9–23.

Goldberg, A.L. (1995). Functions of the proteasome: the lysis at the end of the tunnel. Science *268*, 522–523.

Goldberg, A.L., Stein, R., and Adams, J. (1995). New insights into proteosome function from Archaebacteria to drug development. Curr. Biol., in press.

Hershko, A., and Ciechanover, A. (1992). The ubiquitin system for protein degradation. Annu. Rev. Biochem. *61*, 761–807.

Hershko, A., Ganoth, D., Sudakin, V., Dahan, A., Cohen, L.H., Luca, F.C., Ruderman, J.V., and Eytan, E. (1994). Components of a system that ligates cyclin to ubiquitin and their regulation by the protein kinase cdc2. J. Biol. Chem. *269*, 4940–4946.

Hou, D., Cenciarell, C., Jensen, J.P., Nguyen, H.B., and Weissman, A.M. (1994). Activation-dependent ubiquitination of a T cell antigen receptor subunit on multiple intracellular lysines. J. Biol Chem. *269*, 14244–14247.

Inoue, S., and Simoni, R.D. (1992). 3-Hydroxy-3-methylglutaryl-coenzyme A reductase and T cell receptor α subunit are differentially degraded in the endoplasmic reticulum. J. Biol. Chem. *267*, 9080–9086.

Inoue, S., Bar-Nun, S., Roitelman, J., and Simoni, R.D. (1991). Inhibition of degradation of 3-hydroxy-3-methylglutaryl-coenzyme A reductase *in vivo* by cysteine protease inhibitors. J. Biol. Chem. *266*, 13311–13317.

Kartner, N., Evernden-Porelle, D., Bradley, G., and Ling V. (1985). Detection of P-glycoprotein in multidrug-resistant cell lines by monoclonal antibodies. Nature *316*, 820–823.

Kartner, N., Hanrahan, J.W., Jensen, T.J., Naismith, A.L., Sun, S., Ackerley, C.A., Reyes, E.F., Tsui, L.-C., Rommens, J.M., Bear, C.E., and Riordan, J.R. (1991). Expression of the cystic fibrosis gene in non-epithelial invertebrate cells produces a regulated anion conductance. Cell *64*, 681–691.

Kartner, N., Augustinas, O., Jensen, T.J., Naismith, A.L., and Riordan, J.R. (1992). Mislocalization of ΔF508 CFTR in cystic fibrosis sweat gland. Nature Genet. *1*, 321–326.

Kerem, B.S., Rommens, J.M., Buchanan, J.A., Markiewicz, D., Cox, T. K., Chakravarti, A., Buchwald, M., and Tsui, L.-C. (1989). Identification of the cystic fibrosis gene: genetic analysis. Science *245*, 1073–1080.

Klausner, D.R., and Sitia, R. (1990). Protein degradation in the endoplasmic reticulum. Cell *62*, 611–614.

Kölling, R., and Hollenberg, C.P. (1994). The ABC-transporter Ste6 accumulates in the plasma membrane in a ubiquitinated form in endocytosis mutants. EMBO J. *13*, 3261–3271.

Loo, T.W., and Clarke, D.M. (1994). Prolonged association of temperature-sensitive mutants of human P-glycoprotein with calnexin during biogenesis. J. Biol. Chem. *269*, 28683–28689.

Lukacs, G.L., Mohamed, A., Kartner, N., Chang, X.-B., Riordan, J.R., and Grinstein, S. (1994). Confirmational maturation of CFTR but not its mutant counterpart (ΔF508) occurs in the endoplasmic reticulum and requires ATP. EMBO J. *13*, 6076–6086.

Murakami, Y., Matsufuji, S., Kameji, T., Hayashi, S.-I., Igarashi, K., Tamura, T., Tanaka, K., and Ichihara, A. (1992). Ornithine decarboxylase is degraded by the 26S proteasome without ubiquitination. Nature *360*, 597–599.

Palombella, V.J., Rando, O.J., Goldberg, A.L., and Maniatis, T. (1994). The ubiquitin–proteasome pathway is required for processing the NF-κB1 precursor protein and the activation of NF-κB. Cell *78*, 773–785.

Paolini, R., and Kimet, J.-P. (1993). Cell surface control of the multiubiquitination and deubiquitination of high affinity immunoglobulin E receptors. EMBO J. *12*, 779–786.

Pind, S., Riordan, J.R., and Williams, D.B. (1994). Participation of the endoplasmic reticulum chaperone calnexin (p88, IP90) in the biogenesis of the cystic fibrosis transmembrane conductance regulator. J. Biol. Chem. *269*, 12784–12788.

Riordan, J.R., Rommens, J.M., Kerem, B.-S., Alon, N., Rozmahel, R., Grzelczak, Z., Zielenski, J., Lok, S., Plavsic, N., Chou, J.-L., Drumm, M.L., Iannuzzi, M.C., Collins, F.S., and Tsui, L.-C. (1989). Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. Science *245*, 1066–1073.

Riordan, J.R., Forbush, B., III, and Hanrahan, J.W. (1994). The molecular basis of chloride transport in shark rectal gland. J. Exp. Biol. *196*, 405–418.

Rivett, A.J. (1993). Proteasomes: multicatalytic proteinase complexes. Biochem. J. *291*, 1–10.

Rock, K.L., Gramm, C., Rothstein, L., Clark, K., Stein, R., Dick, L., Hwang, D., and Goldberg, A.L. (1994). Inhibitors of the proteasome block the degradation of most cell proteins and the generation of peptides presented on MHC class I molecules. Cell *78*, 761–771.

Sherman, M., and Goldberg, A.L. (1992). Involvement of the chaperonin dnaK in the rapid degradation of a mutant protein in *Escherichia coli*. EMBO J. *11*, 71–77.

Sherman, M., and Goldberg, A.L. (1995). Involvement of molecular charerones in intracellular protein breakdown. In Stress-Inducible Cellular Responses, U. Feige, R.I. Morimoto, I. Yahara, and B.S. Polia, eds. (Berlin: Birkhäuser–Springer), in press.

Sommer, T., and Jentsch, S. (1993). A protein translocation defect linked to ubiquitin conjugation at the endoplasmic reticulum. Nature *365*, 176–179.

Tabcharani, J.A., Chang, X.-B., Riordan, J.R., and Hanrahan, J.W. (1991). Phosphorylation-regulated Cl channel in CHO cells stably expressing the cystic fibrosis gene. Nature *352*, 628–631.

Ward, C.L., and Kopito, R.R. (1994). Intracellular turnover of cystic fibrosis transmembrane conductance regulator: Inefficient processing and rapid degradation of wild-type and mutant proteins. J. Biol. Chem. *269*, 25710–25718.

Wikström, L., and Lodish, H.F. (1991). Nonlysosomal, pre-Golgi degradation of unassembled asialoglycoprotein receptor subunits: a TLCK- and TPCK-sensitive cleavage within the ER. J. Cell. Biol. *113*, 997–1007.

Wikström, L., and Lodish, H.F. (1992). Endoplasmic reticulum degradation of a subunit of the asialoglycoprotein receptor *in vitro*. J. Biol. Chem. *267*, 5–8.

Wileman, T., Kane, L.P., and Terhorst, C. (1991). Degradation of T-cell receptor chains in the endoplasmic reticulum is inhibited by inhibitors of cysteine proteases. Cell Regul. *2*, 753–765.

Yang, Y., Janich, S., Cohn, J.A., and Wilson, J.M. (1993). The common variant of cystic fibrosis transmembrane conductance regulator is recognized by hsp70 and degraded in a pre-Golgi nonlysosomal compartment. Proc. Natl. Acad. Sci. USA *90*, 9480–9484.