View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Elsevier - Publisher Connector

Cell, Vol. 83, 121–127, October 6, 1995, Copyright © 1995 by Cell Press

Degradation of CFTR by the Ubiquitin–Proteasome Pathway

Cristina L. Ward,* Satoshi Omura,† and Ron R. Kopito*
*Department of Biological Sciences
Stanford University
Stanford, California 94305-5020
†The Kitasato Institute
9-1 Shirokane 5-Chome
Minato-ku, Tokyo 108
Japan

Summary

Most cases of cystic fibrosis are caused by mutations that interfere with the biosynthetic folding of the cystic fibrosis transmembrane conductance regulator (CFTR), leading to the rapid degradation of CFTR molecules that have not matured beyond the endoplasmic reticulum (ER). The mechanism by which integral membrane proteins including CFTR are recognized and targeted for ER degradation and the proteolytic machinery involved in this process are not well understood. We show here that the degradation of both wild-type and mutant CFTR is inhibited by two potent proteasome inhibitors that induce the accumulation of polyubiquitinated forms of immature CFTR. CFTR degradation was also inhibited by coexpression of a dominant negative ubiquitin mutant and in cells bearing a temperature-sensitive mutation in the ubiquitin-activating enzyme, confirming that ubiquitination is required for rapid CFTR degradation.

Introduction

Cystic fibrosis (CF) is a lethal hereditary disorder affecting approximately one in two thousand live births among populations of Caucasian or northern European descent. CF is caused by the functional absence of a plasma membrane chloride channel designated CFTR, for cystic fibrosis transmembrane conductance regulator (Welsh and Smith, 1993). The vast majority of CF cases in these populations are linked to a single genetic lesion: deletion of phenylalanine (ΔF508) from a cytoplasmically localized portion of CFTR (Kerem et al., 1990; Tsui, 1992). CFTR protein is absent from the plasma membranes of epithelial cells from patients homozygous for the ΔF508 allele (Kartner et al., 1992) and cultured cells expressing ΔF508 cDNA (Cheng et al., 1990). Unlike wild-type CFTR, which is processed by the secretory pathway and delivered to the plasma membrane in transfected cells, ΔF508 is retained and degraded in a pre-Golgi compartment (Cheng et al., 1990; Ward and Kopito, 1994; Lukacs et al., 1994). These observations have led to the hypothesis that the ΔF508 mutation leads to misfolding of CFTR and consequent retention of the misfolded protein by the quality control apparatus in the endoplasmic reticulum (ER), resulting in its rapid degradation by an unknown ER proteolysis pathway (Cheng et al., 1990; Ward and Kopito, 1994; Yang et al., 1993).

Recent studies have indicated that the folding of both wild-type and ΔF508 CFTR is inefficient and that ΔF508 molecules are rapidly degraded by a process that is kinetically and pharmacologically indistinguishable from the process involved in the degradation of misfolded wild-type immature CFTR (Ward and Kopito, 1994; Lukacs et al., 1994). Both wild-type and ΔF508 CFTR are initially synthesized as ~140 kDa core-glycosylated primary translation products that are associated with the cytosolic chaperone Hsc70 (Yang et al., 1993) and the ER chaperone calnexin (Pind et al., 1994), indicating that they span the ER membrane. A fraction (~25%–50%) of wild-type CFTR immature translation products are released from chaperone complexes, suggesting that they fold into a maturation-competent form that is rapidly exported to the Golgi apparatus (Ward and Kopito, 1994; Lukacs et al., 1994). The majority (~75%) of wild-type CFTR precursors, however, are rapidly degraded, without apparent lag following translation, by a process that is insensitive to inhibitors of lysosomal proteolysis and brefeldin A (Ward and Kopito, 1994; Lukacs et al., 1994), suggesting that it occurs in a pre-Golgi compartment. By contrast with wild-type CFTR, the vast majority (>99%) of immature, core-glycosylated ΔF508 precursors are not released from chaperone complexes (Yang et al., 1993; Pind et al., 1994), fail to acquire complex N-linked glycans, indicative of transit through the Golgi apparatus, and are degraded with kinetics identical to those of wild type (Ward and Kopito, 1994; Lukacs et al., 1994). When overexpressed, ΔF508 precursors accumulate in association with ER membranes (Yang et al., 1993). These data collectively suggest a model in which immature wild-type or ΔF508 CFTR molecules that fail to fold correctly are sorted to a degradation pathway that is ill defined but appears to have many properties in common with previously described ER degradation (Klausner and Sitia, 1990; Fra and Sitia, 1993). The effect of the ΔF508 mutation is to increase the proportion of nascent CFTR molecules that leave the folding pathway and, hence, are targeted for degradation.

In cultured cells, the ΔF508 block to maturation can be partially overcome by incubation at reduced temperatures or overexpression (Dalemans et al., 1991; Denning et al., 1992; Drumm et al., 1991), giving rise to cell surface ΔF508 molecules that form functional, cAMP-activated chloride channels. These data further confirm that the ΔF508 mutation does not necessarily interfere with the function of CFTR, but serves to target it to the ER degradation apparatus. Therefore, strategies aimed at blocking the degradation of this mutant protein have been proposed as potential therapeutics in CF management (Cheng et al., 1990). The data in this paper suggest that such strategies are unlikely to be effective. We show that lactacystin, a potent and apparently highly specific inhibitor of the 20S proteasome, blocks the degradation of immature wild-type

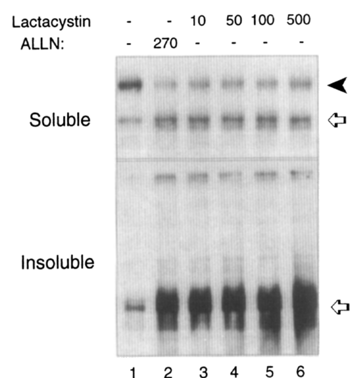

Figure 1. CFTR Degradation Is Inhibited by Inhibitors of the 20S Proteasome

Wild-type CFTR-expressing cells were incubated overnight alone (lanes 1), with ALLN (lanes 2), or with lactacystin (lanes 3–6), at the concentrations indicated (in micromolar), separated into detergent-soluble (top) and insoluble (bottom) material, and immunoblotted with CFTR antibody. Arrow indicates mobility of immature core-glycosylated form (140 kDa). Arrowhead indicates mobility of mature complex-glycosylated form (160 kDa).

and ΔF508 CFTR molecules. Inhibition of CFTR or ΔF508 proteolysis leads to the accumulation of polyubiquitinated forms of immature CFTR that though undegraded are blocked from maturation into a functional post-ER compartment. Our data demonstrate that polyubiquitination serves to direct the degradation of CFTR.

Results

Inhibitors of the 20S Proteasome Induce the Accumulation of Detergent-Insoluble Forms of Wild-Type and ΔF508 CFTR

The effect of protease inhibitors on the steady-state levels of mature (complex-glycosylated) 160 kDa and immature (core-glycosylated) 140 kDa CFTR was assessed by immunoblot analysis of HEK cells expressing wild-type CFTR. Although inhibitors of papain, trypsin, chymotrypsin, and lysosomal cathepsins did not significantly affect CFTR expression in detergent-soluble or insoluble fractions (data not shown), N-acetyl-L-leucinyl-L-leucinyl-L-norleucinal (ALLN) and lactacystin, both potent inhibitors of the 20S proteasome, led to an ~50-fold increase in steady-state levels of core-glycosylated, immature (140 kDa) CFTR (Figure 1). This accumulation is consistent with inhibition of the normally rapid ($t_{1/2} < 30$ min) degradation of CFTR precursors in a pre-Golgi compartment (Ward and Kopito, 1994; Lukacs et al., 1994). However, the proteasome inhibitors failed to increase expression of the mature (complex-glycosylated) form of CFTR, despite the increase in its apparent precursor, suggesting that immature CFTR molecules that are destined for degradation exist in a form that is not competent for export to the Golgi apparatus. Indeed, nearly all of the spared CFTR-immunoreactive material was insoluble in nonionic detergent and was frequently accompanied by the appearance of a high molecular weight smear punctuated by discrete bands (Figure 2A).

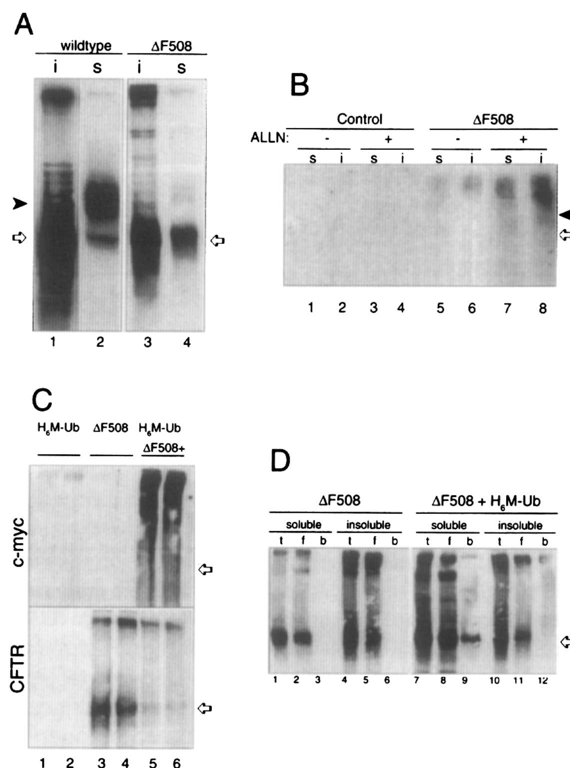

Figure 2. CFTR Is Polyubiquitinated In Vivo

(A) Ladder of wild-type (lanes 1–2) and ΔF508 (lanes 3–4) CFTR immunoreactive bands detected by immunoblotting with affinity-purified CFTR antibody. HEK cells, transfected as indicated, were treated overnight with ALLN (100 μg/ml) prior to lysis and separation into soluble (s) and insoluble (i) fractions.

(B) Immunoprecipitation of ubiquitin-immunoreactive polypeptides with CFTR antibody. Control (vector-transfected, lanes 1–4) and ΔF508 CFTR-transfected (lanes 5–8) cells, treated with (lanes 3, 4, 7, and 8) or without (lanes 1, 2, 5, and 6) ALLN, were solubilized in detergent and separated into detergent-soluble (s) and insoluble (i) fractions. CFTR immunoprecipitates were separated on 7% SDS–polyacrylamide gels and immunoblotted with a polyclonal ubiquitin antibody.

(C) High-molecular weight forms of CFTR are modified by epitope-tagged ubiquitin. HEK cells were transfected with H_6M-Ub (lanes 1–2) or ΔF508 CFTR (lanes 3–4) or cotransfected (at a 10:1 ratio) with both (lanes 5–6). Lysates were prepared, and the detergent-insoluble fractions were immunoprecipitated (after solubilization in SDS and dilution) with affinity-purified CFTR antibody. Duplicate samples (in pairs of lanes) of immunocomplexes were separated on 7.5% SDS–polyacrylamide gels and detected by immunoblotting with either c-Myc MAb (upper panel) or with CFTR antibody.

(D) Specific binding of ubiquitinated CFTR to Ni–NTA resin. Fractions from HEK cells expressing ΔF508 CFTR alone (lanes 1–6) or cotransfected with H_6M-Ub (lanes 7–12) fractionated on Ni–NTA resin as described in Experimental Procedures. Samples of total lysate (t), flow-through unbound material (f), and bound, eluted material (b), each representing equivalent fractions of the original cell lysate, were electrophoresed and immunoblotted with CFTR antibody.

Insoluble CFTR and ΔF508 Are Polyubiquitinated

Closer examination of immunoblots of insoluble CFTR and ΔF508 reveal that the immunoreactive smear can often be resolved into a ladder of bands spaced at 6.48 ± 0.45 (n = 7) kDa intervals (Figure 2A). Such a ladder of bands regularly spaced at ~7 kDa intervals is a hallmark of poly-

ubiquitination and reflects covalent attachment of multiple ubiquitin chains that serve as a degradation signal for the target protein (reviewed by Ciechanover, 1994). To test directly the hypothesis that CFTR is modified by ubiquitination, cells transfected with vector or with ΔF508 CFTR were immunoprecipitated with affinity-purified antibody to CFTR and immunoblotted with a polyclonal antibody to ubiquitin (Figure 2B). No ubiquitin immunoreactivity was detected in samples from control cells not expressing CFTR (lanes 1–4). By contrast, ubiquitin immunoreactivity was present in detergent-soluble and insoluble fractions from cells expressing ΔF508 CFTR (lanes 5–8); this signal increased significantly in ALLN-treated cells.

To characterize ubiquitinated proteins in transfected HEK cells, an NH_2-terminal His_6- and c-Myc epitope-tagged ubiquitin (H_6M-Ub) was coexpressed together with wild-type or ΔF508 CFTR. Approximately 50% of total ubiquitin in HEK cells expressing this construct was estimated by immunoblotting to contain the c-Myc epitope (data not shown). ALLN treatment of H_6M-Ub-transfected cells induced a massive increase in the steady-state level of detergent-soluble and insoluble ubiquitinated proteins (as judged from the level of c-Myc immunoreactivity [data not shown]), confirming that ALLN, a potent inhibitor of the 20S proteasome (Rock et al., 1994), inhibits the degradation of ubiquitinated proteins in HEK cells. To confirm that undegraded CFTR is also modified by polyubiquitination, cotransfected cells and control cells expressing ΔF508 CFTR or H_6M-Ub, individually, were treated with and without ALLN and separated into detergent-soluble and insoluble fractions. The insoluble fraction was immunoprecipitated with CFTR antibody (after solubilization in 1% SDS and dilution of the SDS) and the immunoprecipitates subjected to immunoblotting with CFTR antibody (Figure 2C, bottom) or c-Myc monoclonal antibody (MAb) (Figure 2C, top). Strikingly, when the immunoprecipitates were probed with a c-Myc MAb, strong immunoreactivity was observed as a smear of high molecular weight material from cells that were cotransfected with both H_6M-Ub and ΔF508 CFTR, but not from cells transfected with either CFTR or H_6M-Ub alone.

Ubiquitinated (H_6M-Ub) CFTR was also detected by Ni^{2+} chelation chromatography (Figure 2D). Binding of CFTR that was not tagged with His_6, i.e., from cells transfected only with ΔF508, to a nickel–nitrilotriacetic acid (Ni–NTA) column was negligible (lanes 3 and 6), indicating that the binding conditions used were stringent enough to ensure specificity for His_6-tagged proteins. By contrast, a significant fraction of ΔF508 from cotransfected cells bound to the column and was eluted with 1.0 M imidazole (lanes 9 and 12). Some of the detergent-soluble CFTR-immunoreactive material that bound to and was eluted from the Ni–NTA column migrated with the mobility of immature CFTR. This could suggest either that a fraction of ΔF508 was modified with short ubiquitin chains, or that some polyubiquitinated ΔF508 became deubiquitinated. However, nearly all of the material from the detergent-insoluble fraction that bound to and was eluted from the column migrated in the high molecular weight region of the gel, consistent with modification by longer ubiquitin chains. Similarly, when wild-type CFTR was coexpressed together with H_6M-Ub, immature and high molecular weight, but not mature, forms of the protein were bound to and eluted from the Ni–NTA column (data not shown). Taken together, these data strongly suggest that a fraction of CFTR and ΔF508 in HEK cells is polyubiquitinated in vivo and degraded by a ubiquitin-linked pathway.

Rapid Degradation of CFTR Requires Polyubiquitination

To demonstrate that polyubiquitination is required for CFTR and ΔF508 degradation, we coexpressed ΔF508 with a dominant negative ubiquitin mutant, K48R. The ε-amino group of the invariant Lys-48 residue of one ubiquitin molecule is the site of isopeptide linkage of other ubiquitin molecules and is essential for the formation of multiubiquitin chains (Chau et al., 1989; Finley et al., 1994). Expression of mutant ubiquitin in which this invariant lysine has been replaced by arginine (K48R) has a chain-terminating, dominant negative effect (Chau et al., 1989; Finley et al., 1994). Coexpression of ΔF508 and wild-type H_6M-Ub in HEK cells did not inhibit the degradation of ΔF508 (Figure 3A). By contrast, we observed a massive increase in both soluble and insoluble ΔF508 in cells coexpressing ΔF508 and H_6M-K48R. These data indicate that the K48R mutant ubiquitin blocks ΔF508 CFTR degradation and suggest that ubiquitination is required for CFTR degradation.

If CFTR degradation requires polyubiquitination, then K48R coexpression would be predicted to slow the kinetics of the rapid degradation of core-glycosylated CFTR. To test this prediction, HEK cells expressing ΔF508 alone or in combination with wild-type H_6M-Ub or H_6M-Ub(K48R) were pulse-labeled with [^{35}S]methionine for 15 min, then chased with excess unlabeled methionine for the times indicated (Figure 3B). In control cells transfected with ΔF508 alone (lanes 1–4) or together with wild-type H_6M-Ub (lanes 5–8), label in the 140 kDa core-glycosylated immature ΔF508 primary translation product decayed rapidly from the soluble pool. After a 3 hr chase, approximately 10% of the label originally incorporated into immature ΔF508 in control cells was recovered in the soluble fraction. Since less than 5% of the incorporated label appeared in the insoluble fraction, the data confirm that degradation accounts for the rapid ($t_{1/2}$, ~30 min) disappearance of labeled, soluble core glycosylated CFTR and ΔF508 molecules (Ward and Kopito, 1994). The kinetics of ΔF508 turnover in cells coexpressing K48R ubiquitin differed from the controls in two important respects. First, the absolute amount of [^{35}S] incorporated into immature ΔF508 during the pulse was approximately 4- to 5-fold higher than in the controls (compare lanes 9 and 1). This effect was apparently not due to increased label incorporation into immature CFTR, as the cells were transfected with equivalent amounts of plasmid, and the samples taken for immunoprecipitation were normalized to the level of total protein synthesis. Moreover, we consistently observe a similar increase in labeling of soluble immature CFTR at the end of a 15 min pulse when the pulse is performed in the presence of ALLN (data not shown). The data suggest that,

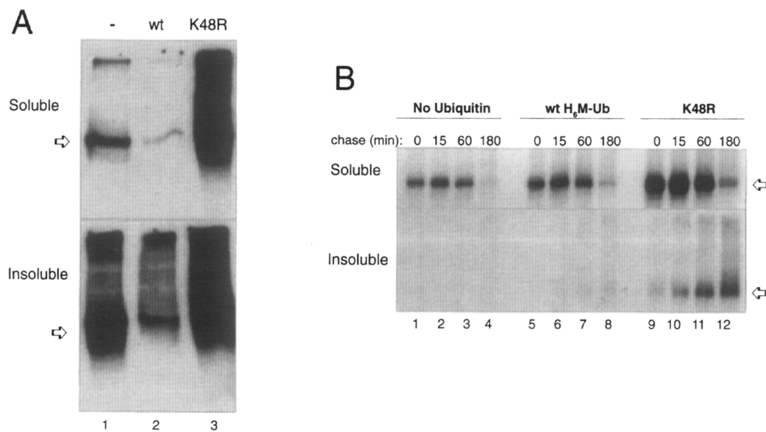

Figure 3. CFTR Degradation Is Inhibited by Dominant Negative Ubiquitin

(A) Effect of coexpression of H_6M-Ub K48R on steady-state ΔF508 CFTR levels. Cells were transfected with ΔF508 CFTR alone (lanes 1) or cotransfected (at a 10:1 ratio of Ub to ΔF508) with either wild-type H_6M-Ub (lanes 2) or H_6M-Ub in which Lys-48 was substituted with arginine (K48R; lanes 3). Samples were solubilized, separated into detergent-soluble (top) or insoluble (bottom) fractions, and immunoblotted with CFTR antibody.

(B) Effect of coexpression of H_6M-Ub K48R on the kinetics of ΔF508 CFTR turnover. HEK cells were transfected with ΔF508 CFTR alone (lanes 1–4) or cotransfected (as above) with either wild-type H_6M-Ub (lanes 5–8) or H_6M-Ub K48R (lanes 9–12). Cells were pulse-labeled with [^{35}S]methionine for 15 min and chased with excess unlabeled methionine for the times indicated. At each timepoint cells were lysed and separated into detergent-soluble (upper panel) and insoluble (lower panel) material prior to immunoprecipitation. Immunoprecipitates were then separated by SDS–polyacrylamide gel electrophoresis and visualized by fluorography.

in the absence of ALLN or K48R coexpression, a large fraction of labeled soluble immature CFTR is rapidly degraded, even during the 15 min pulse.

The second effect of K48R coexpression was on the fraction of label entering the insoluble pool. Despite the greater amount of label following the pulse in the K48R cells, the fraction of label present as soluble immature CFTR after a 3 hr chase was comparable to that of controls (~10%). Strikingly, however, in contrast with controls, a significant fraction of label in the K48R cotransfected cells was chased into the detergent-insoluble pool, both as insoluble protein with the mobility of core-glycosylated CFTR and as a high molecular weight smear. Because of this heterogeneity, it was not possible to quantitate accurately the amount of label entering the insoluble pool. We estimate by phosphorimage analysis of the gel that the majority (>70%) of the label leaving the soluble pool was recovered after a 3 hr chase in the insoluble fraction. This estimate is necessarily an underestimate, as the mutant ubiquitin accounts for only ~50% of the total ubiquitin. Together, these data indicate that coexpression of a dominant negative ubiquitin mutant effectively blocks the rapid degradation of core-glycosylated ΔF508.

Ubiquitin–protein conjugation and protein degradation are reduced in ts20 cells incubated at the nonpermissive temperature (40°C) (Kulka et al., 1988). This mutant cell line contains a thermolabile form of the ubiquitin-activating enzyme E1 that is irreversibly inactivated at elevated temperature (Kulka et al., 1988). Mutant (ts20) and wild-type (E36) cells were transfected with CFTR or ΔF508 cDNA and selected for stable expression. Expression of CFTR-immunoreactive material was assessed by immunoblotting of cell extracts grown for 24 hr at the permissive (31°C) or the nonpermissive (40°C) temperature (Figure 4). Strikingly, the accumulation of CFTR or ΔF508 was increased dramatically when cells were incubated at the nonpermissive temperature. The simplest interpretation of these results is that a functional ubiquitin pathway is required for degradation of mutant and wild-type CFTR. Similar results were observed in several independently isolated E36 and ts20 clones, suggesting that the data are not clonal artifacts. Moreover, the expression level of CFTR and ΔF508 is at least 100-fold lower in these stable lines than in the transiently transfected HEK cells, suggesting that the role of ubiquitination in CFTR degradation is not peculiar to HEK cells, nor is it likely to be a consequence simply of overexpression.

Discussion

Protein degradation is an important means by which cells control the activities of normal proteins and, in conjunction with molecular chaperones that comprise the quality control apparatus, by which they avoid the potentially toxic effects of mutant, denatured, or misfolded polypeptides. Two major proteolytic systems have been described in eukaryotic cells (reviewed by Olson and Dice, 1989). Lysosomal (or, in yeast, vacuolar) proteases are principally involved in the degradation of proteins, including plasma membrane proteins and exogenous polypeptides, en-

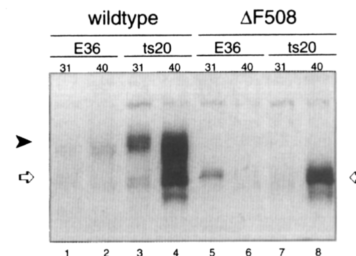

Figure 4. Accumulation of CFTR and ΔF508 in E1 Mutant Cells

E1 mutant ts20 cells (lanes 3–4 and 7–8) or parental E36 cells (lanes 1–2 and 5–6), stably expressing CFTR (lanes 1–4) or ΔF508 (lanes 5–8), were incubated for 24 hr at the permissive (31°C, odd-numbered lanes) or the nonpermissive (40°C, even-numbered lanes) temperature. Cells were harvested and immunoblotted with CFTR antibody as described in the legend to Figure 1.

gaged by the endocytic apparatus. Most short-lived cytosolic proteins are degraded by the 26S proteasome complex, which contains the multicatalytic 20S proteasome as its catalytic core and requires prior covalent attachment of multiple ubiquitin chains to the substrate (reviewed by Goldberg and Rock, 1992; Ciechanover, 1994). The degradation of proteins retained by the quality control apparatus in the ER has been proposed to be mediated by a third, distinct, ER proteolytic pathway (reviewed by Klausner and Sitia, 1990; Fra and Sitia, 1993). The data presented here constitute evidence in animal cells that the ubiquitin pathway participates in the degradation of a short-lived integral membrane glycoprotein and suggest a mechanism by which the ER quality control apparatus may be coupled to the intracellular proteolysis machinery.

Several lines of evidence support our conclusion that CFTR is degraded by the proteasome in a ubiquitin-dependent manner. First, inhibitors of the 20S proteasome, including ALLN and the recently identified compound lactacystin, interfere with CFTR degradation and lead to the accumulation of high molecular weight, polyubiquitinated forms of CFTR. Lactacystin, a Streptomyces metabolite, is a potent inhibitor of the trypsin-like, chymotrypsin-like peptidyl–glutamyl peptide–hydrolyzing activities of purified 20S proteasome in vitro (Fenteany et al., 1995). Lactacystin specifically labels the highly conserved NH_2-terminal threonine residue on the mammalian proteasome subunit X that, from the recently solved X-ray structure of the archaebacterial proteasome (Löwe et al., 1995), is proposed to be the active site nucleophile (Fenteany et al., 1995). Interestingly, the aldehyde function of ALLN, bound to the active site in the X-ray crystal structure, is situated in close proximity to and has been suggested to form a hemiacetal with hydroxyl of this threonine (Löwe et al., 1995). Although ALLN and related peptide aldehyde compounds are also potent proteasome inhibitors, they are less specific, exhibiting significant activity in inhibiting the cysteine proteases calpain and cathepsin B (Rock et al., 1994). By contrast, lactacystin is reported to have no detectable effect, even upon extended exposure, on these or other cysteine proteases or on the serine proteases trypsin and chymotrypsin (Fenteany et al., 1995). While these data cannot anticipate other uncharacterized or undiscovered proteases against which lactacystin may be active, these inhibitor studies support our conclusion that the proteasome is involved in CFTR degradation.

A second line of evidence is our demonstration that CFTR is ubiquitinated in vivo. Ubiquitin- and epitope-tagged ubiquitin were detected in CFTR immunoprecipitates, and CFTR-immunoreactive polypeptides were isolated by metal chelation chromatography only from lysates of cells expressing both CFTR and His_6-tagged ubiquitin. While these observations support the conclusion that polyubiquitination is a signal for CFTR degradation, they do not formally exclude the possibility that CFTR polyubiquitination is the consequence of inhibiting degradation. To address this issue, we coexpressed with ΔF508 CFTR a form of ubiquitin containing a mutation in the invariant Lys-48. Ubiquitin molecules bearing this mutation can covalently attach to target proteins (including wild-type ubiquitins), but since they lack the ε-amino group required for isopeptide linkage of additional ubiquitin units, they are predicted to exert a chain-terminating effect on the formation of extended multiubiquitin chains. Expression of these mutants, therefore, has a dominant negative effect on polyubiquitination, and hence, on degradation (Chau et al., 1989; Finley et al., 1994). Our data show that coexpression of K48R, but not wild-type ubiquitin, drastically slowed the kinetics of CFTR degradation, confirming that ubiquitination is required for rapid CFTR proteolysis. This conclusion is also strongly supported by the observation that CFTR and ΔF508 accumulate, at the nonpermissive temperature, in ts20 cells that lack the ubiquitin-activating enzyme E1 (Kulka et al., 1988).

Finally, the results from ts20 cells, which express at least 100-fold lower levels of CFTR than do HEK cells, also argue that our data are unlikely to be an artifact of HEK cell overexpression. We also observe a similar effect of lactacystin and ALLN on CFTR and ΔF508 degradation in stably transfected C127 mammary carcinoma cells (data not shown), which also express considerably lower CFTR levels (Marshall et al., 1994; Ward and Kopito, 1994). These data unambiguously demonstrate that polyubiquitination of both CFTR and ΔF508 is linked to rapid proteolysis; it is likely that the ubiquitinated forms of CFTR and ΔF508 are degraded in a proteasome-mediated fashion.

Degradation of membrane-spanning proteins such as CFTR by the proteasome presents a potential topological problem in that the known components of the ubiquitin–proteasome pathway are associated with the cytosolic compartment (Goldberg and Rock, 1992; Ciechanover, 1994). It is, however, important to note that CFTR, like many integral membrane proteins, has a large cytoplasmic domain. Indeed, this domain (which is predicted from topology models [Riordan et al., 1989] to constitute ~60% of CFTR mass) is the site of CF-causing mutations such as ΔF508, and the probable site of attachment of the cytosolic chaperone Hsc70 (Yang et al., 1993). It is likely, then, that misfolding within this cytosolic domain serves as a signal for ubiquitin attachment, perhaps by ubiquitin-conjugating machinery associated with the cytosolic face of the ER membrane. The ubiquitin-conjugating enzyme UBC6 has been reported to be an integral component of the Saccharomyces cerevisiae ER membrane, with its catalytic domain facing the cytosol (Sommer and Jentsch, 1993). It is reasonable to expect that other components of the ubiquitin conjugation apparatus, including mammalian homologs of this yeast enzyme, will be identified in the ER of mammalian cells. We propose that recognition by such a pathway of misfolded cytosolic domains of integral membrane proteins could serve to initiate the degradation of misfolded CFTR as well as perhaps other integral membrane proteins that are also known to be degraded in a pre-Golgi compartment. Most substrates for ER degradation identified to date are integral membrane proteins with at least one lysine residue in the putative cytoplasmic domain. It is noteworthy that deletion of the cytosolic and

membrane domains of at least one such ER-restricted short-lived protein, the α subunit of T cell receptor, abolishes its rapid degradation without influencing its retention in the ER (Shin et al., 1993).

Our data suggest that inhibition of proteasome-mediated ΔF508 proteolysis is not likely to be an effective therapy for treating ΔF508. Similarly, although dominant negative ubiquitin coexpression effectively inhibits CFTR degradation, it induces the accumulation of insoluble forms of CFTR and does not appear to increase the accumulation of soluble, mature CFTR. Although immature ΔF508 accumulates in ts20 cells incubated at the nonpermissive temperature, there is no accompanying increase in ΔF508 maturation. These data suggest that ubiquitination alone cannot be the event that commits immature ΔF508 to the degradation pathway. Additional studies will be required to elucidate the involvement of molecular chaperones and ubiquitin in determining the fate of nascent CFTR molecules.

Experimental Procedures

Plasmids, Cells, and Materials

cDNA encoding His_6-, c-Myc-tagged human ubiquitin was generated by polymerase chain reaction using pUB221, a gift from D. Finley, as the template. The ubiquitin K48R mutant construct was generated by the megaprimer method (Landt et al., 1990; Sarkar and Sommer, 1990). Both ubiquitin constructs were sequenced to verify that no errors had been introduced. Lactacystin was prepared as previously described (Omura et al., 1991). The polyclonal anti-ubiquitin antibody was a gift from A. Haas. HEK 293 cells were maintained and transfected as previously described (Lee et al., 1991). Mutant ts20 cells and the parental line E36 (Kulka et al., 1988) (provided by K. Rock) were maintained in RPMI medium supplemented with 10% fetal bovine serum. The cells were transfected by lipofection with CFTR and ΔF508 cDNA inserted into the expression vector RC/CMV (Invitrogen). Following lipofection, cells were cultured in RPMI medium supplemented with G418 (1 mg/ml) at 31°C. Clonal colonies were isolated, expanded, and assayed for CFTR expression by immunoblotting. CFTR expression levels in these cells were estimated to be 100- to 200-fold lower than in transiently transfected HEK cells.

Immunoblotting

Cell pellets from washed and transfected HEK cells were lysed in 250 μl of ice-cold buffer (10 mM Tris–HCl [pH 7.5], 5 mM EDTA, 1% Triton X-100) plus protease inhibitors (100 μM TLCK, 100 μM TPCK, 1 mM PMSF) for 30 min on ice. Insoluble material was recovered by centrifugation at 13,000 × g for 15 min at 4°C and solubilized in SDS sample buffer. DNA was sheared by repeated passage of the sample through a 25-gauge needle. Cell fractions, normalized for total protein, were separated on 7% SDS–polyacrylamide gels and electroblotted. Chemiluminescent detection was carried out with the Renaissance detection kit (New England Nuclear).

Nickel Affinity Chromatography

Cells were transfected and harvested as described above. Insoluble pellets were solubilized in 25–50 μl of 1% SDS, 10 mM Tris–HCl [pH 7.5], 10% glycerol for 10 min at 65°C, diluted to 1 ml with lysis buffer, sonicated for 20 s, and then respun. The soluble and insoluble fractions were mixed with 50 μl of a 50% slurry of Ni-NTA resin for 16–20 hr at 4°C followed by three washes in lysis buffer. Bound material was eluted in 50–100 μl of 50 mM sodium phosphate (pH 6.0), 150 mM NaCl, 1% Triton X-100 containing 1 M imidazole. Volumes loaded on SDS–polyacrylamide gels represented equivalent fractions of the original cell lysate. Gels were immunoblotted as described above.

Pulse-Chase Experiment

Cells were pulse-labeled with [^{35}S]methionine/cysteine for 15 min, harvested, and immunoprecipitated as previously described (Ward and Kopito, 1994). Loading volumes were normalized to the sample with the lowest trichloroacetic acid–precipitable cpm. Following electrophoresis, the radiolabeled bands were imaged and quantified with a phosphorimager (Molecular Dynamics).

Acknowledgments

Correspondence should be addressed to R. R. K. The authors are indebted to Daniel Finley (Harvard University) for helpful discussion and the gift of the H_6M-Ub plasmid, pUB221. We are grateful to Sumire Kobayashi for technical assistance and to Arthur L. Haas (Medical College of Wisconsin) for the anti-ubiquitin antibody. We thank Robert Simoni for stimulating discussion, and Russell Fernald for critical reading of the manuscript. This work was supported by funding from the National Institutes of Health (grant DK43994), the March of Dimes (Basic Research grant FY93-0760), and the National Cystic Fibrosis Foundation. This work was done during the tenure of an Established Investigatorship of the American Heart Association (R. R. K.). This material is based on work supported by the National Science Foundation under grant MCB-8957340.

Received June 26, 1995; revised August 29, 1995.

References

Chau, V., Tobias, J.W., Bachmair, A., Marriott, D., Ecker, D.J., Gonda, D.K., and Varshavsky, A. (1989). A multiubiquitin chain is confined to specific lysine in a targeted short-lived protein. Science *243*, 1576–1583.

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.

Ciechanover, A. (1994). The ubiquitin–proteasome proteolytic pathway. Cell *79*, 13–21.

Dalemans, W., Barbry, P., Champigny, G., Jallat, S., Dott, K., Dreyer, D., Crystal, R.G., Pavirani, A., Lecocq, J.P., and Lazdunski, M. (1991). Altered chloride ion channel kinetics associated with the ΔF508 cystic fibrosis mutation. Nature *354*, 526–528.

Denning, G.M., Anderson, M.P., Amara, J.F., Marshall, J., Smith, A.E., and Welsh, M.J. (1992). Processing of mutant cystic fibrosis transmembrane conductance regulator is temperature-sensitive. Nature *358*, 761–764.

Drumm, M.L., Wilkinson, D.J., Smit, L.S., Worrell, R.T., Strong, T.V., Frizzell, R.A., Dawson, D.C., and Collins, F.S. (1991). Chloride conductance expressed by ΔF508 and other mutant CFTRs in *Xenopus* oocytes. Science *254*, 1797–1799.

Fenteany, G., Standaert, R.F., Lane, W.S., Chois, 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.

Finley, D., Sadis, S., Monia, B.P., Boucher, P., Ecker, D.J., Crooke, S.T., and Chau, V. (1994). Inhibition of proteolysis and cell cycle progression in a multiubiquitination-deficient yeast mutant. Mol. Cell. Biol. *14*, 5501–5509.

Fra, A., and Sitia, R. (1993). The endoplasmic reticulum as a site of protein degradation. Sub-Cell. Biochem. *21*, 143–168.

Goldberg, A.L., and Rock, K.L. (1992). Proteolysis, proteasomes and antigen presentation. Nature *357*, 375–379.

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*, 321–327.

Kerem, B., Zielenski, J., Markiewicz, D., Bozon, D., Gazit, E., Yahav, J., Kennedy, D., Riordan, J.R., Collins, F.S., Rommens, J.M., and Tsui, L. (1990). Identifications of mutations in regions corresponding to the two putative nucleotide (ATP)-binding folds of the cystic fibrosis gene. Proc. Natl. Acad. Sci. USA *87*, 8447–8451.

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

Kulka, R.G., Raboy, B., Schuster, R., Parag, H.A., Diamond, G., Ciechanover, A., and Marcus, M. (1988). A Chinese hamster cell cycle mutant arrested at G2 phase has a temperature-sensitive ubiquitin-activating enzyme, E1. J. Biol. Chem. *263*, 15726–15731.

Landt, O., Grunert, H.P., and Hahn, U. (1990). A general method for rapid site-directed mutagenesis using the polymerase chain reaction. Gene *96*, 125–128.

Lee, B.S., Gunn, R.B., and Kopito, R.R. (1991). Functional differences among nonerythroid anion exchangers expressed in a transfected human cell line. J. Biol. Chem. *266*, 11448–11454.

Löwe, J., Stock, D., Jap, B., Zwickl, P., Baumeister, W., and Huber, R. (1995). Crystal structure of the 20S proteasome from the archaeon *T. acidophilum* at 3.4 Å resolution. Science *268*, 533–539.

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

Marshall, J., Fang, S., Ostedgaard, L.S., O'Riordan, C.R., Ferrara, D., Amara, J.F., Hoppe, H., IV, Scheule, R.K., Welsh, M.J., Smith, A.E., and Cheng, S.H. (1994). Stoichiometry of recombinant cystic fibrosis transmembrane conductance regulator in epithelial cells and its functional reconstitution into cells in vitro. J. Biol. Chem. *269*, 2978–2995.

Olson, T.S., and Dice, J.F. (1989). Regulation of protein degradation rates in eukaryotes. Curr. Opin. Cell Biol. *1*, 1194–1200.

Omura, S., Matsuzaki, K., Fujimoto, T., Kosuge, K., Furuya, T., Fujita, S., and Nakagawa, A. (1991). Structure of lactacystin, a new microbial metabolite which induces differentiation of neuroblastoma cells. J. Antibiotics *44*, 117–118.

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.

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.

Sarkar, G., and Sommer, S.S. (1990). The "megaprimer" method of site-directed mutagenesis. Biotechniques *8*, 404–407.

Shin, J., Lee, S., and Strominger, J.L. (1993). Translocation of TCR α chains into the lumen of the endoplasmic reticulum and their degradation. Science *259*, 1901–1904.

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

Tsui, L.-C. (1992). The spectrum of cystic fibrosis mutations. Trends Genet. *8*, 392–398.

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.

Welsh, M.J., and Smith, A.E. (1993). Molecular mechanisms of CFTR chloride channel dysfunction in cystic fibrosis. Cell *73*, 1251–1254.

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.