The Mechanism Underlying Cystic Fibrosis Transmembrane Conductance Regulator Transport from the Endoplasmic Reticulum to the Proteasome Includes Sec61β and a Cytosolic, Deglycosylated Intermediary*

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Endoplasmic reticulum (ER) degradation pathways can selectively route proteins away from folding and maturation. Both soluble and integral membrane proteins can be targeted from the ER to proteasomal degradation in this fashion. The cystic fibrosis transmembrane conductance regulator (CFTR) is an integral, multidomain membrane protein localized to the apical surface of epithelial cells that functions to facilitate Cl⁻ transport. CFTR was among the first membrane proteins for which a role of the proteasome in ER-related degradation was described. However, the signals that route CFTR to ubiquitination and subsequent degradation are not known. Moreover, limited information is available concerning the subcellular localization of polyubiquitinated CFTR or mechanisms underlying retrograde dislocation of CFTR from the ER membrane to the proteasome either before or after ubiquitination. In the present study, we show that proteasome inhibition with clasto-lactacystin β-lactone (4 μM, 1 h) stabilizes the presence of a deglycosylated CFTR intermediate for up to 5 h without increasing the core glycosylated (band B) form of CFTR. Deglycosylated CFTR is present under the same conditions that result in accumulation of polyubiquitinated CFTR. Moreover, the deglycosylated form of both wild type and ΔF508 CFTR can be found in the cytosolic fraction. Both the level and stability of cytosolic, deglycosylated CFTR are increased by proteasome blockade. During retrograde translocation from the ER to the cytosol, CFTR associates with the Sec61 trimeric complex. Sec61 is the key component of the mammalian co-translational protein translocation system and has been proposed to function as a two way channel that transports proteins both into the ER and back to the cytosol for degradation. We show that the level of the Sec61-CFTR complexes are highest when CFTR degradation proceeds at the greatest rate (approximately 90 min after pulse labeling). Quantities of Sec61-CFTR complexes are also increased by inhibition of the proteasome. Based on these results, we propose a model in which complex membrane proteins such as CFTR are transported through the Sec61 trimeric complex back to the cytosol, escorted by the β subunit of Sec61, and degraded by the proteasome or by other proteolytic systems.

The cystic fibrosis transmembrane conductance regulator (CFTR) was among the first membrane proteins for which the role of the proteasome in ER-associated proteolysis was described (1, 2). CFTR is an integral multidomain membrane protein localized to the apical surface of epithelial cells that functions to facilitate Cl⁻ transport (3). Certain mutations in the CFTR gene, including the frequent ΔF508 mutation, lead to rapid degradation of the protein prior to maturation beyond the endoplasmic reticulum (4). Wild-type CFTR folding and processing is inefficient (5). In vitro, up to 75% of newly synthesized wild-type molecules are degraded without maturing beyond the ER in a fashion kinetically similar to the degradation of ΔF508 CFTR. Proteasome inhibition impedes CFTR degradation without increasing the post-ER processed form, but the inhibition is not complete, indicating the involvement of other proteolytic pathways (1, 2). More recently it has been demonstrated in cell-free systems that CFTR ubiquitination is a co-translational process, and that the fate of the protein may be established while the polypeptide chain is still elongating on the ribosomes. (6). However, the signals that route CFTR either to maturation or to ubiquitination and subsequent degradation, and the differences between the wild type and mutant protein degradation are not known. Moreover, no information is available concerning mechanisms underlying CFTR retrograde dislocation, before or after ubiquitination, from the ER membrane to the proteasome.

Recent studies indicate that both soluble and integral membrane proteins can be targeted from the ER membrane to the cytosol, where degradation is catalyzed by the proteasome (7–12). This retrograde transport involves Sec61 (11), an ER integral membrane protein complex that has been shown to be the key component of the co-translational protein translocation machinery (13, 14). The existence of a protein-conducting channel that leads proteins across the ER membrane was proposed more than 20 years ago (15), but the structure and function of the heterotrimeric protein complex was revealed only recently (13, 16). Polypeptide chains associate with Sec61 during their co-translational insertion into ER membranes (17), but the dimensions of the translocation channel formed by the Sec61 heterotrimeric complex are not sufficient to accommodate multiple membrane-spanning domains simultaneously (16). Therefore, the mechanisms of insertion and subsequent integration into the ER membrane or the excision and retrograde transport of these proteins through the channel remains unclear. It has been proposed that the heterotrimeric complex may form oli-

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gromers suitable to contain polytopic membrane proteins (16), but an association between Sec61 and a polytopic membrane protein has not been shown previously in mammalian cells. Very recently, models have been suggested in which membrane proteins are selected for degradation from the ER because they never leave or reversibly enter protein-conducting channels such as Sec61 (18). It has also been shown that certain glycoproteins are deglycosylated by a peptide:N-glycanase prior to degradation (10, 11, 19). The deglycosylation process may take place in the cytosol, or in the microsomes, since the existence of enzymes with similar activity has been shown in both compartments (20, 21).

Based on the above information, we designed experiments using CFTR, which contains 12 membrane-spanning domains, to investigate the role of the Sec61 complex in ER-associated degradation of the wild type and ΔF508 mutant proteins. We show that CFTR interacts with the Sec61 complex and that this interaction participates in proteasomal degradation of both the wild type and ΔF508 mutant proteins. Proteasome inhibition not only increases the ubiquitinated forms of CFTR but also results in the appearance of a deglycosylated intermediate that is present in the cytosol. Our results confirm the hypothesis that deglycosylation and removal from the ER membrane precedes proteasomal destruction of CFTR and have important implications for cytosolic dislocation and degradation of other complex membrane-localized proteins.

EXPERIMENTAL PROCEDURES

Cell Lines, Antibodies, and Inhibitors—HeLa cells were obtained from the American Type Culture Collection (ATCC) and were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum. Anti-CFTR C-terminal polyclonal antibody was produced as described (22) or provided by David Bedwell (University of Alabama at Birmingham). Anti-Sec61β polyclonal rabbit antibody was a gift from Tom Rapoport and Walter Mothes (Harvard Medical School) and used as described (11). Anti-ubiquitin antibodies were purchased from Calbiochem. N-Acetyl-leu-leu-norleucinal (ALLN) (Sigma), calpain inhibitor I was used to inhibit the proteasome at a concentration of 50–100 μM (5, 6). Lactacystin β-lactone was purchased from Calbiochem and used at a 2–3 μM concentration. Protease inhibitor mixture tablets (Complete Mini, Boehringer Mannheim) were used in all cell lysis and homogenization buffers according to the manufacturer’s protocol.

CFTR Expression System—A vaccinia/T7 expression protocol was used to express wild-type or ΔF508 CFTR in HeLa cells previously for studies of wild type and mutant CFTR processing (23, 24). CFTR processing using this method closely resembles what has been observed in other systems (4, 5, 25). Viruses were propagated in HeLa cells (ATCC CCL2) and titrated in HuTK-143B cells (ATCC CFL8303). Virus stocks were stored at −70 °C. T7 RNA polymerase-expressing vaccinia virus (vTF-7) was a gift from Dr. B. Moss (National Institute of Health). A plasmid expressing wild-type CFTR or ΔF508 CFTR under the regulatory control of the T7 promoter was obtained from Dr. D. Bedwell (26). Briefly, CFTR or ΔF508 CFTR genes were cloned into the NcoI site of the pTM1 vector (27). The resulting clones were used to transiently express CFTR by co-infecting with vTF-7 vector. The same clones were used to build recombinant vaccinia viruses expressing CFTR in the presence of T7 RNA polymerase, according to published protocols (28, 29). Expression of CFTR was confirmed by Western blotting, immunocytochemistry, and functional assay. Prior to immunoprecipitation, cells were plated on six-well trays. 10–12 h later, vTF-7 and virus containing the CFTR were added at a multiplicity of infection of 1–2.

Pulse-Chase Analysis, Immunoprecipitation, and Electrophoresis—Cells were labeled with 100–200 μCi of [35]S-methionine for 15–60 min. Incorporation was terminated by washing the cells with phosphate-buffered saline and replacing the medium with nonradioactive Dulbecco’s modified Eagle’s medium (with methionine). Before lysis, cells were placed on ice and rinsed at 4 °C (2.5 mM HEPES, pH 7.6, 10 mM CaCl2). Ice-cold lysis buffer (500 μl, containing 1% digitonin in 2.5 mM HEPES, pH 7.6, 10 mM CaCl2) was then added in all experiments for co-immunoprecipitation of CFTR with Sec61.

Direct immunoprecipitation of CFTR was carried out after lysing the cells in radioimmune precipitation buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-Cl, pH 8.0). The cells were scraped, lysed for 30 min on ice, and then centrifuged at 14,000 rpm for 10 min. Immunoprecipitation was performed for 2 h at 4 °C. Immunoprecipitated proteins were washed and separated by SDS-polyacrylamide gel electrophoresis. In some experiments, proteins from an initial immunoprecipitation with anti-Sec61β or anti-CFTR antibody were redissolved in SDS and diluted 1:10 in radioimmune precipitation buffer before a second immunoprecipitation was carried out on the re-solubilized protein as described (11) using anti-CFTR C-terminal monoclonal antibody and protein G-Sepharose (Amersham Pharmacia Biotech).

Western Blotting—Metabolically labeled and immunoprecipitated proteins were transferred onto Trans-Blot® transfer medium (polyvinylidene difluoride membrane; 0.2 μm) (Bio-Rad) membranes (transferred at 300 mA for 120 min). Membranes were blocked and probed with an anti-ubiquitin monoclonal antibody (Calbiochem) and developed with alkaline phosphatase-labeled anti-mouse IgG antibody using nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate substrate (Boehringer Mannheim).

Cell Fractionation—Cells were lysed in ice-cold homogenization buffer (150 mM sucrose, 10 mM triethanolamine, 10 mM acetic acid, 1 mM EDTA, pH 7.4), protease inhibitor mixture tablet (Complete Mini and ALLN) and homogenized with a Dounce homogenizer. Fractionation was as described by Wiertz et al. (30). Briefly, homogenates were centrifuged at 1000 × g for 10 min. Supernatants were then transferred and centrifuged at 10,000 × g for 30 min. Supernatant from the 10,000 × g centrifugation was spun at 100,000 × g for 60 min in an ultracentrifuge to clarify a cytosolic fraction. Membrane pellets were washed with HEPES buffer (2.5 mM HEPES, pH 7.6, 10 mM CaCl2), and then 500 μl of lysis buffer (2.5 mM HEPES, pH 7.6, 10 mM CaCl2 plus 1% digitonin) was added to each sample. After 30 min of lysis on ice, the samples were centrifuged at 14,000 × g for 10 min. Immunoprecipitation was carried out as described above.

Endoglycosidase H Treatment—Immunoprecipitated samples were treated with endoglycosidase H (Boehringer Mannheim) for 1 h at 37 °C according to manufacturer’s protocol in order to remove high mannose type glycan chains from core glycosylated CFTR.

Analysis—Gels were placed on Phosphor Screen (Molecular Dynamics) and analyzed with PhosphorImager (Molecular Dynamics). Images were further characterized and densitometry was carried out using IPLab Spectrum (Signal Analytics Corp.).

RESULTS

Proteasome Inhibition Results in the Appearance of Unglycosylated CFTR—Wild-type and ΔF508 CFTR were expressed in HeLa cells. 10 h later, cells were pulse-labeled with [35]S-methionine, followed by chase (0–5 h). Lysates were subjected to immunoprecipitation using anti-CFTR NBD1 antibody. CFTR appeared as a doublet of approximately 140 kDa in either wild-type (Fig. 1A) or ΔF508 (Fig. 1B) cell lysates immediately after the pulse (T = 0 h). In the absence of proteasome inhibition, the lower molecular weight form in the doublet markedly decreased without any increase in the upper band by 1–3 h of chase. Fully glycosylated CFTR (band C) could be detected by 3 h specifically in cells expressing wild-type CFTR (Fig. 1A). No mature CFTR was found in ΔF508 samples (Fig. 1B). According to earlier data (4), CFTR appears in three different forms in living cells, including band A (nonglycosylated), band B (core glycosylated), and band C (fully glycosylated) forms. Because protein translation into the ER membrane and core glycosylation are believed to occur co-translationally in mammalian cells (14, 31), we propose that full-length, nonglycosylated CFTR is likely to represent a previously glycosylated form that has been deglycosylated. As seen in Fig. 1, A and B, proteasome inhibition increases the amount of the lower molecular weight form of CFTR without increasing the amount of core glycosylated CFTR. We interpret this result to indicate that unglycosylated CFTR is an intermediary that exists after CFTR core
glycosylation but before proteasomal degradation. Proteasome inhibition also increased the amount of polyubiquitinated forms of CFTR as judged by Western blotting (Fig. 2A). [35S]methionine-labeled cell lysates were immunoprecipitated with anti-CFTR antibody, separated on 6% polyacrylamide gels, transferred to polyvinylidene difluoride membranes, and developed with an anti-ubiquitin (mouse monoclonal) antibody (A) and by autoradiography (B). Proteasome inhibition resulted in increased amounts of polyubiquitinated forms of CFTR, as shown in panel A by Western blotting, in which anti-CFTR polyclonal antibody immunoprecipitated samples were probed with anti-ubiquitin monoclonal antibody. In the same samples, autoradiography (B) indicated the appearance of the lower molecular weight form of CFTR (band A, CHO−) specifically after proteasome inhibition. M, molecular weight marker.

FIG. 2. Increase of polyubiquitinated CFTR corresponds with an increase in the lower molecular weight (band A) form of CFTR. Proteasome inhibitor treated cells (4 μM clasto-lactacystin β-lactone given for 1 h before the pulse) and control (nontreated) HeLa cells expressing wild type (wt) or ΔF508 CFTR (Δ) were labeled with [35S]methionine, immunoprecipitated with anti-CFTR NBD1 antibody, and then separated on 8% SDS-polyacrylamide gel electrophoresis. Gels were transferred onto a polyvinylidene difluoride membrane and developed with anti-ubiquitin (mouse monoclonal) antibody (A) and by autoradiography (B). Proteasome inhibition resulted in increased amounts of polyubiquitinated forms of CFTR, as shown in panel A by Western blotting, in which anti-CFTR polyclonal antibody immunoprecipitated samples were probed with anti-ubiquitin monoclonal antibody. In the same samples, autoradiography (B) indicated the appearance of the lower molecular weight form of CFTR (band A, CHO−) specifically after proteasome inhibition. M, molecular weight marker.

Increased amounts of polyubiquitinated CFTR and the appearance of a lower molecular weight (deglycosylated) form of CFTR were specific to proteasome inhibitor-treated cells expressing either wild type or ΔF508 CFTR. To confirm that the lower molecular weight form was a deglycosylated intermediate of CFTR, immunoprecipitated samples were treated with endoglycosidase H to remove high mannose-type glycans. Disappearance of the higher molecular weight form (band B) of CFTR was observed after Endo H treatment (Fig. 3).

Deglycosylated CFTR Intermediates Can Be Detected in the Cytosolic Fraction—To further verify the nature and subcellular localization of the deglycosylated form of CFTR, we combined metabolic labeling, pulse-chase, and subcellular fractionation. First, [35S]methionine-labeled cells were homogenized and fractionated as described (30). As presented in Fig. 4A, three fractions were collected: 1000 g pellet or crude fraction (Cr) containing nonhomogenized cells; 100,000 g x g pellet or membrane fraction (M); and 100,000 g x g supernatant (C) or cytosolic fraction. Each fraction was immunoprecipitated using anti-CFTR antibody. Immediately after a 15-min labeling with 100 μCi of [35S]methionine, CFTR localized to the membrane fractions (T1 time point, Cr and M). After a 30-min chase, very low levels of deglycosylated CFTR appeared in the cytosolic fraction specifically in proteasome inhibitor treated cells (Fig. 4A, T2, fraction C). No CFTR was detected in cytosolic fractions at the T1 time point immediately after the pulse (Fig. 4A, T1, wild type and ΔF508 CFTR). In Fig. 4B, cell homogenates were separated into four fractions: 1000 g x g pellet (Cr), 10,000
CFTR Degradation and Role of the Sec61 Complex

FIG. 4. Deglycosylated CFTR in the cytosol persists after proteasome inhibition. A, cells expressing wild type or ΔF508 CFTR (with and without clasto-lactacystin β-lactone) were labeled with [35S]methionine for 15 min and homogenized, and three fractions (1000 × g pellet, crude fraction (Cr); 100,000 × g pellet, membrane fraction (M); 100,000 × g supernatant, cytosolic fraction (C)) were collected at two time points (T1, immediately after pulse; T2, 30 min after pulse). Samples were immunoprecipitated with anti-CFTR NBD1 polyclonal antibody and separated by SDS-polyacrylamide gel electrophoresis. Both wild type and ΔF508 CFTR were present in the membrane fraction at the T1 time point. Proteasome inhibitor pretreatment resulted in the appearance of the deglycosylated intermediate (CHO−). This lower molecular weight form was most readily detected at T2, and the cytosolic fraction. B, cells expressing wild type or ΔF508 CFTR (in the presence (+) or absence (−) of clasto-lactacystin β-lactone) were labeled with [35S]methionine for 60 min and homogenized, and four fractions (1000 × g crude (Cr); 10,000 × g membrane (M1); 100,000 × g membrane (M2); 100,000 × g supernatant (C)) were collected at three time points (T1, immediately; T2, 1 h after pulse; T3, 3 h after pulse). Deglycosylated forms of wild type and ΔF508 CFTR (CHO−) were present in both proteasome inhibitor-treated and -untreated cytosolic fractions by the T1 time point. The wild type and ΔF508 cytosolic, deglycosylated intermediates persisted longer and were present at higher levels after proteasome inhibition. The core glycosylated (band B, CHO+) and fully processed forms (band C) restricted to the membrane fractions and not detected in the cytosol as expected (Fig. 4A and B). As a control, the transferrin receptor (an endogenous integral membrane protein) and β-galactosidase (a cytosolic protein control) were expressed in HeLa cells as described in methods, and immunoprecipitated from each fraction. As expected, the transferrin receptor was found exclusively in the membrane fractions, while β-galactosidase was recovered only from the 100,000 × g supernatant or cytosolic fraction (data not shown).

CFTR Associates with Sec61, and the Amount of CFTR Bound to Sec61 Is Increased after Inhibition of the Proteasome—Based on previous studies indicating that a major histocompatibility complex I/Sec61 association could be detected only in the presence of proteasome inhibition (11), we pretreated cells with 100 μM ALLN for 1 h before and during the pulse labeling period. The effect of this inhibitor is reversible (1, 2). Therefore, after its removal, labeled proteins proceed down the proteasome degradation pathway. After a 15-min pulse, cells were chased for 30, 90, and 150 min, based on the hypothesis that a Sec61/CFTR interaction occurs during CFTR synthesis and ER processing. To detect Sec61-CFTR complexes, cells were lysed in a low salt digitonin lysis buffer (a buffer shown previously to leave Sec61 complexes intact), and immunoprecipitated with anti-Sec61b polyclonal antibody (Fig. 5A). Similar samples immunoprecipitated with anti-CFTR antibody were used as positive controls to detect CFTR under the same conditions (Fig. 5C). Proteasome inhibitor pretreatment resulted in the appearance of both the core glycosylated and deglycosylated forms of CFTR when samples were immunoprecipitated with anti-CFTR antibody. Anti-Sec61b antibody immunoprecipitated the same doublet form of CFTR, indicating that CFTR physically interacts with Sec61 and that the complexes could be co-immunoprecipitated. Cells expressing control (vaccinia T7) proteins without CFTR were studied as negative controls.

The differences in the interaction of wild type and ΔF508 CFTR with the Sec61 complex were analyzed by densitometry. Sec61 complexed to ΔF508 CFTR was detected earlier and persisted slightly longer than Sec61 complexed to wild type CFTR (Fig. 5A). The interaction with the Sec61 complex was strongest over a time period when CFTR degradation proceeded at the highest rate (Fig. 5, compare B and D). This result further supports the specificity of the Sec61/CFTR interaction, since the level of CFTR in association with Sec61 was increased at a time point when total CFTR amount had substantially decreased (T = 90 min; Fig. 5). If the interactions with Sec61 were not specific, we would have expected that the levels of complexes would be the greatest at the T = 30 min time point when CFTR levels were maximal.

To verify that the protein co-immunoprecipitated with Sec61 is CFTR, we performed an experiment in which anti-Sec61 immunoprecipitated proteins were redissolved in 2% SDS, diluted in radiouimmunoprecipitation buffer, and subjected to a second immunoprecipitation using anti-CFTR C-terminal antibody. Lysates from comparably treated cells without the CFTR construct were tested as negative controls. ΔF508 CFTR-transfected samples, immunoprecipitated with anti-CFTR antibody, were used as positive controls to verify that CFTR proteins could be reimmunoprecipitated from radiouimmunoprecipitation buffer-diluted samples. Both wild-type and ΔF508 CFTR could be reimmunoprecipitated from protein samples resolubilized from Sec61 complexes (data not shown).

Characterization of Proteasome Inhibitor Effects on CFTR and Sec61-CFTR Complexes—To test whether the Sec61/CFTR interaction related specifically to protein synthesis and ER
Cells expressing wild-type or ΔF508 CFTR.

**Fig. 5.** Interactions of wild-type and ΔF508 CFTR with Sec61. Cells expressing wild-type or ΔF508 CFTR were pulse-labeled with 200 μCi of [35S]methionine and studied at the time points shown. Lysates were immunoprecipitated with anti-Sec61β antibody (A) or with anti-CFTR antibody (C). Proteasome inhibitor (ALLN) with a reversible effect was present before and during the labeling period to increase steady-state levels of band A (CHO−) CFTR. During the chase phase, the inhibitor was removed so that CFTR degradation could be examined. CFTR band B (CHO+) and CFTR band A (CHO−) were co-immunoprecipitated with Sec61 until 90 min of the chase period, after which the complex was barely detectable (Fig. 5A). ΔF508 and wild-type CFTR were most rapidly degraded over 150 min, a result in agreement with previous studies of CFTR band B proteolysis in the absence of proteasome blockade (Fig. 5C). Densitometry was used to study the relative amount of CFTR co-immunoprecipitated with Sec61 at each time point (Fig. 5D) and also to detect wild-type and ΔF508 CFTR degradation rates (Fig. 5D). −, cells expressing pVTF-7 protein without CFTR.

**Fig. 6.** Effect of proteasome inhibitor (ALLN, 100 μM) on the appearance of CFTR co-immunoprecipitated with anti-Sec61 antibody. Omitting ALLN resulted in a significant decrease of CFTR co-immunoprecipitated with Sec61. Only the higher molecular weight form (band A CHO+ + CFTR) was found in complex with Sec61 in the absence of proteasome inhibitors. Immunoprecipitation was carried out at 90 min into the chase, a time point when Sec61-CFTR complexes were most prevalent (Fig. 5A).

**Fig. 7.** CFTR-Sec61 complexes are present in both membrane and cytosolic fractions. To localize CFTR-Sec61 complexes, wild type and ΔF508 CFTR-expressing cells were labeled with [35S]methionine for 30 min. Cell fractionation, followed by direct CFTR immunoprecipitation with anti-CFTR antibody or co-immunoprecipitation using anti-Sec61β antibody, were carried out after a 60-min chase. CFTR was found in both membrane and cytosolic fractions after immunoprecipitation with anti-CFTR NBD1 antibody. CFTR was also co-immunoprecipitated with Sec61β from both the membrane and in the cytosolic fractions. −, cells expressing pVTF-7 protein, without CFTR expression.

**DISCUSSION**

Involvement of the proteasome in ER-related selective protein degradation has been shown for CFTR (1, 2), unassembled T-cell receptor (10, 18), major histocompatibility complex class I protein (11, 30), and the secretory protein ApoB (32). However, the ways in which complex membrane proteins like CFTR are removed from the ER membrane and the signals that route CFTR for processing or degradation are not known. Neither proteasome blockade nor inhibition of ubiquitination lead to increased maturation of wild-type or ΔF508 CFTR but instead cause the accumulation of CFTR otherwise targeted for degradation (1, 2). CFTR has also been shown to be ubiquitinated during the elongation of the polypeptide chain on the ribosome (6). These past experiments therefore suggest that interactions determining the fate of wild-type or ΔF508 CFTR occur early in biosynthesis and do not depend upon the subsequent addition of ubiquitin side chains. Sec61 participates in co-translational protein synthesis in mammalian cells. Taken together with the present studies, it is very likely that CFTR is co-translationally bound to Sec61 at a very early step in CFTR protein synthesis.

The failure of proteins to integrate into the ER membrane may act as a signal for ubiquitination and subsequent proteasomal destruction. In our experiments, as in earlier studies, an
increase in polyubiquitinated CFTR was observed in the presence of clasto-lactacystin β-lactone (Fig. 2). In concert with the accumulation of polyubiquitinated CFTR, we also detected the accumulation of a deglycosylated CFTR intermediate, indicating that this intermediate exists along the pathway leading to the proteasome (Fig. 2). Because these experiments do not exclude the possibility that some CFTR was produced off of the native pathway and also targeted the proteasome, perhaps as a result of the transient expression system, we performed experiments using a cell line stably expressing CFTR under the regulatory control of an inducible promoter (4). Proteasome inhibition resulted in the accumulation of a deglycosylated product similar to the data presented here. We conclude therefore that the lower molecular weight form of CFTR is a deglycosylated intermediate that is produced from the core glycosylated form. The deglycosylation process is believed to take place in the cytosol by a peptidase:N-glycanase (33). Therefore, portions of CFTR suitable for glycosylation that are exported to the luminal side of ER may have to enter the cytosol for deglycosylation to occur. Microsomal peptide:N-glycanase could also contribute to deglycosylation, since enzymes with similar activity to the cytosolic peptide:N-glycanase have also been reported in microsomes (20, 21). In either case, the deglycosylated form of CFTR is targeted for degradation (Figs. 1 and 4), either by residual proteasome activity or by other degradative pathways (34).

It has not been clear from earlier experiments (11) whether proteins selected for degradation detach from the Sec61 channel or are escorted to the proteasome by Sec61. From co-immunoprecipitation studies combined with cell fractionation (Fig. 7), our experiments suggest that CFTR can be co-immunoprecipitated in complex with Sec61β from the cytosol. This result establishes that at least some CFTR has been cut out of the membrane in a complex with Sec61β. The role of each of the Sec61 complex subunits in the translocation defect of yeast mutants has been studied earlier (35, 36). Only Sec61p (Sec61α in mammals) and Ssa1p (Sec61γ in mammals) have been found essential to complement a protein transport defect across the ER membrane (13, 35, 36), while the role of the third component of the heterotrimer (Sec61β) was not found to be necessary for protein insertion and integration into the lipid bilayer. Our results demonstrate that Sec61β accompanies CFTR into the cytosol if proteasome function is inhibited. Although the role of this particular Sec61 subunit in ER membrane protein insertion is not known, the present studies point to a function related to ER-associated proteolysis.

In summary, our findings demonstrate the existence of a pathway in which polytopic ER membrane-localized proteins such as CFTR can be targeted to the proteasome through cytosolic deglycosylated intermediates bound to the ER membrane protein, Sec61β. Our findings support the notion that a general pathway, which has been proposed for proteins with a single membrane-spanning motif, also applies to complex polytopic membrane proteins such as CFTR. Characterization of the intermediary complex and its components offers a means by which biosynthesis of membrane proteins and their degradation in the proteasome can be more fully understood.

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