Cystic Fibrosis Mutations Lead to Carboxyl-terminal Fragments That Highlight an Early Biogenesis Step of the Cystic Fibrosis Transmembrane Conductance Regulator*

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Inefficient delivery of the cystic fibrosis transmembrane conductance regulator (CFTR) to the surface of cells contributes to disease in the majority of cystic fibrosis patients. Analysis of cystic fibrosis-associated missense mutations in the first nucleotide binding domain (NBD1), including A455E, S549R, Y563N, and P574H, revealed reduced levels of mature CFTR with elevated levels of carboxyl-terminal polypeptide fragments of 105 and 90 kDa. These fragments appear early in biogenesis and degrade rapidly in four distinct cell types tested including the bronchial epithelial IB3–1 cell line. They were detected at highest levels with CFTRA455E where the 105-kDa fragment accounted for 40% of newly synthesized polypeptide but for only 20 and 7% of nascent wild type and mutant ΔF508 proteins, respectively. The bands represent core- and unglycosylated forms of the same CFTR fragment supporting that precursor forms are correctly inserted into the membrane of the endoplasmic reticulum. Proteolytic cleavage would be predicted to occur on the cytosolic face of the endoplasmic reticulum within the NBD1-R domain segment, but pharmacological testing did not support involvement of the 26 S proteasome. The examined missense mutations in NBD1 manifest differently than the major mutant, ΔF508, and highlight a critical conformational aspect of biogenesis of CFTR.

Cystic fibrosis (CF)1 is a recessive disorder with impaired vectorial transport in the epithelia of sweat gland ducts and of the respiratory, gastrointestinal, and genitourinary tracts. Over 800 mutations (1) identified in patients lead to a spectrum of phenotypes that include the classical picture of CF with chronic pulmonary disease, pancreatic insufficiency, elevated sweat electrolytes, and male sterility. Some of the mutations lead to the pancreatic sufficient form or to the notably milder form that involves congenital bilateral absence of the vas deferens as its major clinical manifestation (2). The affected gene product, the cystic fibrosis transmembrane conductance regulator (CFTR), functions as a protein kinase A and ATP-regulated, multidomain chloride channel (3–6). Associated activities include regulatory roles in ATP transport, in amiloride sensitive Na+ transport, and in the action of outwardly rectifying chloride channels (7–9).

Both family studies (10) and CF mouse models (11) have emphasized the importance of genetic background in modulation of disease, but the nature of mutations or their combinations are major contributors to the tissue phenotype. The precise defects have not been elucidated for many mutations, but deficiencies can be broadly classified into at least six groups (2, 13, 14) resulting in the reduction of mature CFTR at the cell surface or impaired regulation and/or conductance of chloride.

A multidomain membrane protein, such as CFTR, attains its overall native conformation by co- and post-translational folding in the endoplasmic reticulum (ER) (15). These processes have been shown to be inefficient for wild type CFTR (CFTRwt), as only 20–50% of nascent polypeptides mature beyond the ER (16, 17). Studies of the major mutation, ΔF508, have revealed near complete degradation of the core-glycosylated mutant protein such that no mature protein is detectable under normal growth conditions (16–19).

A number of the components of the ER and associated proteins have been implicated in monitoring the production and elimination of misfolded wild type (wt) and mutant CFTR. Prolonged interactions between CFTRA508 and two chaperones, Hsp70 and calnexin, have been shown to occur in comparison to wt (20, 21). More recently, it has also been demonstrated that Hsp40 and Hsp90 contribute to the folding of CFTR (22–24). Whether the interactions of these molecular chaperones prevent the removal of specific forms of incompletely folded polypeptides or help to promote degradation is not known, although it was noted that interruption of Hsp90 binding seems to lead to accelerated degradation of both the wt and ΔF508 forms (22).

Misfolded proteins are commonly targeted for degradation via the lysosomal proteolytic system or the cytoplasmic degradative system involving the proteasome (25, 26). Lysosomal degradation does not appear to contribute substantially to the degradation of either core-glycosylated wt or ΔF508 CFTR (16). Proteasomal degradation of most proteins is dependent on the covalent attachment of multiple ubiquitin molecules and elim-
ination by the 26 S proteolytic complex (for review see Ref. 27). Proteasome inhibitor sensitivity and the detection of ubiquitin CFTR conjugates has demonstrated that immature wt and ΔF508 CFTR are degraded, at least in part, by the ubiquitin-proteasome pathway (28, 29).

Although the observations with the major mutation outline the significance of CFTR maturation and provide an explanation for the absence of mutant protein at the cell surface, many aspects of CFTR biogenesis remain unknown. Further, although many CF mutations show trafficking problems (30–33), few have been examined in detail. To gain additional insight into the determinants of CFTR gain we have analyzed the production and processing of a series of CF-associated NBD1 missense mutations and have identified a group that show defects that appear distinct from CFTRΔF508. The identified pathway also appears with CFTRwt and thus highlights a mechanism that contributes to the notable inefficiency of CFTR maturation.

**EXPERIMENTAL PROCEDURES**

**Construction of CFTR Expression Plasmids**—The CFTR mutants A455E, S549R, P574H, and Y563N were generated as described previously (35). To generate carboxy-terminal HA (CHA) epitope (YPKDVPDYA) infrane, prior to the natural stop codon (amine acid 1480) of the CFTR cDNA. All polymerase chain reaction-generated fragments were confirmed by sequencing after insertion into recipient vectors. Expression vector (pCMV) constructions and CHA-CFTR mutants were generated by shuttling appropriate restriction fragments between untagged and tagged cDNA versions and confirmed by sequencing or restriction digestion analysis.

**Cell Culture and Transfections—**HEK293, COS-7, and CHO-duk cells were grown at 37 °C with 5% CO2. Transfected with CHA-CFTR were split 24 h post-transfection and cultured on 12-mm circular coverslips in Dulbecco’s modified Eagle’s medium supplemented with 10% PBS and antibiotics. Subconfluent cells grown in 100-mm plates were transfected according to the SuperFect reagent provided by the manufacturer (Qiagen), using 10 μg of the appropriate CF expression plasmid, 250 ng of pCMV-β-galactosidase reporter plasmid, and 30 μl of the SuperFect reagent in 3 ml of medium containing PBS. After an 8-h incubation at 37 °C with 5% CO2, the cells were rinsed with PBS and incubated for an additional 16 h in normal culture medium prior to harvest or as indicated in individual experiments.

**Cell Lysis, Deglycosylation, Electrophoresis, and Immunoblotting—**Transfected cells were washed with ice-cold PBS and solubilized in 2 ml of RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% deoxycholate, 1% phenylmethylsulfonyl fluoride, and 1 mg/ml trypsin inhibitor) for 20 min on ice. Following centrifugation at 15,000 × g for 15 min at 4 °C, the supernatant was removed and used for immunoblot analysis or immunoprecipitation. Protein concentrations were determined (Bradford assay; Bio-Rad) using BSA as a standard, and the total protein concentrations were adjusted to 1 mg/ml. 25 μg of protein were loaded on each gel lane for the Western blots. To assess the amount of RIPA insoluble CFTR, the insoluble remains were resuspended in 90 μl of RIPA (minus SDS) and treated with 100 units of DNase (Roche Molecular Biochemicals) for 15 min at room temperature followed by incubation in the presence of 1% SDS for 15 min at 55 °C. The SDS concentration was then diluted to 0.1% with RIPA (minus SDS), and any remaining insoluble material was removed by centrifugation. Proteins from equal proportions of cells were used in the comparison of the pellets to the supernatants.

Endoglycosidase H and N-glycosidase F (New England BioLabs) digests were performed as specified by the manufacturer. In brief, 25 μg of protein was digested with 100 units of enzyme for 2 h at 37 °C. The deglycosylated protein was then prepared for SDS-PAGE (with 50% reaction load per lane) by precipitating with trichloroacetic acid and sodium deoxycholate as described (38).

Protein samples were separated by SDS-PAGE on 7.5% gels and transferred to Hybond-C super membranes (Amersham Pharmacia Biotech). Immunoblotting was performed as described previously (39) using monoclonal anti-CFTR antibodies (M3A7 and L12B4) (40, 41) or a 1:10,000 dilution of monoclonal anti-HA antibody, HA.11 (BabCO). Immunoreactive protein was detected using enhanced chemiluminescence of horseradish peroxidase-conjugated anti-mouse secondary antibody (Amersham Pharmacia Biotech). Immunoblot Labeling, Proteasome Inhibition, and Immunoprecipitation—Metabolic labeling and immunoprecipitation were carried out essentially as described (21). Cells transfected on 100-mm plates were split 24 h post-transfection onto four 60-mm plates and grown for an additional 24 h. After incubation in methionine- and cysteine-free α-MEM for 30 min at 37 °C, the transfected cells were pulsed in the presence of 140 μCi/ml [35S]methionine and [35S]cysteine (>1000 Ci/mmol; Amersham Pharmacia Biotech) for 15 min at 37 °C. After the pulse period, cells were washed twice with growth medium and chased at 37 °C in complete α-MEM; minimal essential media supplemented with 10% FBS for the times indicated.

Proteasome inhibitor studies were performed using 50 μM each of MG-132 and ALLN (Calbiochem) and 10 μM lactacystin (Calbiochem). HEK293 cells transfected with the indicated CFTR expression constructs were preincubated with or without inhibitors for 60 min and metabolically labeled in the presence or absence of the inhibitors as described above.

Metabolically labeled CFTR was isolated by immunoprecipitation with M3A7 and L12B4 antibodies as a mixture as described previously (39). Imunoprecipitated proteins were separated by SDS-PAGE as above; the gels were then fixed in 10% acetic acid and 40% ethanol, soaked for 30 min in Amplify (Amersham Pharmacia Biotech), and dried. Labeled proteins were visualized by exposing the gels to Kodak BIOMAX MR film at −70 °C for 1–3 days. The radioactivity associated with the immunoprecipitated CFTR bands was quantified using a PhosphoImager with ImageQuant software (Molecular Dynamics).

**Indirect Immunofluorescence of Epitope-tagged CFTR—**HEK293 cells transfected with CHA-CFTR were split 24 h post-transfection and cultured on 12-mm circular coverslips in Dulbecco’s modified Eagle’s medium supplemented with 10% PBS and antibiotics for an additional 24 h at 37 °C. The cells were fixed and permeabilized in 4% paraformaldehyde (0.1% Triton X-100, respectively, for 30-min durations at room temperature. Nonspecific binding sites were blocked with 1% BSA in phosphate-buffered saline (PBS-BSA) for 30 min, and the cells were subsequently incubated with HA.11 and diluted 1:1000 in PBS-BSA for 1 h at room temperature. Cells were washed with PBS-BSA and incubated with 1:1000 donkey anti-mouse fluorescein isothiocyanate-conjugated secondary antibody (Jackson ImmunoResearch Laboratory Inc.) for 1 h at room temperature. Cells were washed twice with PBS-BSA, once with PBS, and mounted with Vectashield mounting medium (Vector Laboratories). Indirect immunofluorescence was examined with a Nikon E1000 fluorescence microscope. Images were captured using a CCD camera and IPLab Spectrum software (Scanalytics).

**RESULTS**

**Bioisotopic Processing of Epitope-tagged CFTR—**N-Linked oligosaccharide modification of CFTR can be followed by the rapid conversion of the nascent polypeptide (band A) to a core-glycosylated form (band B) with subsequent conversion to a complex-glycosylated mature form (band C) (18). Complex glycosylation reflects the trafficking of core-glycosylated CFTR through the cis-medial-Golgi to the plasma membrane. Band B and the predominant band C forms of CFTRwt are recognized in both heterologous and endogenous expression systems. Many CFTR polypeptides with CF-associated mutations fail to undergo correct processing such that only the core-glycosylated forms are identified (30–33, 42). To rapidly assay the fate of mutants and analyze their patterns of degradation, a HA epitope for high affinity monoclonal antibodies was introduced into the coding region of CFTR at the carboxy terminus (Fig. 1A). A similarly positioned epitope has been introduced by others to achieve large scale purification of CFTR leading to functional chloride channels in recombinant systems (43).

Immunoblot analysis was used to directly compare the glycosylation states of wt and mutant CFTR versions (Fig. 1B) to those with the CHA epitope (Fig. 1C). At steady state, the level of expression of bands B and C in HEK293 cells was comparable for tagged and untagged CFTRwt. Analysis of the S549R
The occurrence of the 90-kDa band, called band B or carboxyl-terminal polypeptides were present in the membrane. CFTR immunoreactivity was detected by using the M3A7 anti-CFTR antibody in B and the HA.11 anti-HA antibody in C as the primary antibodies. The location of the immature (band B) and mature (band C) forms of CFTR are indicated at the right. Migration of the molecular mass standards are indicated at the left. The filled and open arrowheads correspond to the carboxyl-terminal 105- and 90-kDa bands, respectively. The asterisk indicates an 80-kDa carboxyl-terminal fragment that was also detected in proportions consistent with the steady state amounts of the 90- and 105-kDa bands; however, no direct relationship could be established with metabolic labeling experiments.

We also examined the subcellular distribution of the CHA-CFTRs in COS-7 cells using indirect immunofluorescence microscopy (Fig. 2). Predominant levels of CHA-CFTRwt appear at the cell surface, in contrast to CHA-ΔF508 and CHA-A455E, as described in alternate reports with untagged versions (18, 44). The mutants reside intracellularly, with a reticular, ER-like distribution. Consistent with the observed steady state glycosylation pattern, CHA-S549R shows both cell surface and ER-like staining. These results affirm that wt and mutant CHA-tagged CFTRs reflect the behavior of their untagged counterparts.

Identification of Carboxyl CFTR Derivatives—In addition to full-length CFTR forms, both the M3A7 and the anti-HA antibodies revealed products with relative molecular masses of 105 and 90 kDa that were most prominent in detergent extracts of HEK293 cells expressing the A455E and S549R mutants (Fig. 1, B and C, lanes 4 and 5). These polypeptides were detected at lower steady state levels in cells expressing CFTRwt or CHA-CFTRwt (lanes 2) and were negligible for CFTRΔF508 or CHA-ΔF508 (lanes 3). The placement of the epitope tag establishes that the fragments contain the carboxyl terminus with an apparent molecular mass that would include at least the complete R domain, the second series of transmembrane spanning segments, and the second nucleotide binding domain (NBD2) (Fig. 1A). The results with the monoclonal antibody M3A7 (Fig. 1B) are consistent with this interpretation as its epitope resides in NBD2 (40).

Three sets of experiments were designed to confirm that the identified and presumed degradation intermediates do not reflect a peculiar facet of overexpression in HEK293 cells. The first involved an analysis of the extract preparation to examine the compartmentalization of CFTR, the second involved analysis of the intermediates at reduced expression levels with the A455E mutant, and the third involved expression in alternate cell types. CFTRwt is solubilized and can be extracted with RIPA buffer containing a mixture of ionic and non-ionic detergents (16, 21). Because the formation of protein aggregates may differ between mutants (45), we used a high concentration of SDS to extract the RIPA insoluble polypeptides. Immunoblot analysis indicated that less than 10% of total immunoreactive band B or carboxyl-terminal polypeptides were present in the original CHA-A455E pellet (Fig. 3A). In addition, overexposure of an immunoblot revealed that CHA-CFTRwt and ΔF508 pellets did not contain appreciable levels of either the 105- and 90-kDa bands (Fig. 3B). The occurrence of the 90-kDa band, most prominent with the A455E mutant, does suggest that it is relatively less soluble.

A series of parallel transfections were carried out using the standard conditions described for Fig. 1 and using 1/10 and...
Detergent extracts of CHO-duk terminal CFTR fragments. Most prominent accumulation of the 105- and 90-kDa bands between the cell types, CHA-A455E consistently revealed the detergent extracts indicated that although the levels varied bronchial epithelium IB3–1 cell lines. Immunoblot analysis of CHA-A455E, or with mixtures of pCMV and CHA-A455E to maintain expression. HEK293 cells were transfected with pCMV (vector alone), CHA-A455E, or with mixtures of pCMV and CHA-A455E to maintain constant plasmid-lipid ratios for transfection. Total protein extracts were prepared and analyzed as in Fig. 1, films were exposed for chemiluminescent detection for the times indicated.

Transient transfections were carried out with CHA-tagged CFTRwt, A5F08, and A455E in COS-7, CHO-duk–, and CF bronchial epithelium IB3–1 cell lines. Immunoblot analysis of detergent extracts indicated that although the levels varied between the cell types, CHA-A455E consistently revealed the most prominent accumulation of the 105- and 90-kDa bands (Fig. 4, A–C). The 90-kDa band was difficult to detect in the detergent extracts of CHO-duk– and IB3–1 cells reflecting both a lower steady state level and the reduced solubility of this fragment (Fig. 4, A, B and C, and data not shown). Consistent with the observations made in HEK293 cells, reduced levels of the intermediates were identified for CFTRwt in all cell types. Together, these results confirm that solubility differences between the CFTRs and overexpression do not account for the varied accumulation of the carboxyl-terminal fragments.

Transitively transfected IB3–1 cells were metabolically labeled with [35S]methionine and [35S]cysteine for 3 h. CFTR polypeptides were immunoprecipitated with anti-CFTR antibodies (M3A7 and L12B4), separated by SD8-PAGE, and visualized by fluography.

Fig. 4. Carboxyl fragments occur in different cell types. Whole cell protein extracts were prepared from transfected (A) COS-7, (B) CHO-DUK–, and (C) IB3–1 cells and analyzed as in Fig. 1, C and D. Transiently transfected IB3–1 cells were metabolically labeled with [35S]methionine and [35S]cysteine for 3 h. CFTR polypeptides were immunoprecipitated with anti-CFTR antibodies (M3A7 and L12B4), separated by SD8-PAGE, and visualized by fluography.

With the steady state immunoblot analyses and suggest that the 105- and 90-kDa fragments reflect a feature of CFTR biogenesis that is bypassed by the major mutant because of its specific folding defect and degradation fate (16–18, 20). Together, these data suggest that the observed carboxyl fragments reflect common processes in multiple cell types including those of epithelial origin and that differences in accumulation of the carboxyl fragments between CHA-CFTRs are intrinsic to the polypeptide being expressed. Analysis of Biogenesis and Turnover of Untagged Wild Type and Mutant CFTR—Given their carboxyl-terminal constitution, the 105- and 90-kDa immunoreactive polypeptides were suspected to be degradation products, although it was not immediately apparent when or how they were generated. CFTR degradation events may occur during the folding of the nascent polypeptide at the ER, upon modification in the Golgi, during vesicular transport to the cell surface and/or subsequent to its incorporation into the plasma membrane. Rapid turnover of band B and band C forms have been shown (14, 16, 17). We therefore analyzed the intermediates using metabolic pulse-chase analyses to determine appearance and turnover kinetics. CFTRwt is initially synthesized as a 140-kDa core-glycosylated, immature protein and is inefficiently converted to the 160-kDa complex-glycosylated, mature protein (16–18). In HEK293 cells (Fig. 5), using a 15-min pulse, mature protein became evident only after a minimum chase period of 1 h (left panel). In contrast, no mature forms were detected for the A455E mutation (right panel) or with prolonged pulse and/or chase periods (data not shown). The 105- and 90-kDa carboxyl-terminal fragments were evident for both CFTRwt and A455E as early as the completion of the pulse (15 min) and rapidly disappeared during the chase. These untagged proteins were detectable with a CFTR antibody mixture (M3A7 and L12B4) and were consistently most prominent for the A455E mutant. Detection within the pulse period of 15 min clearly reveals that the carboxyl-terminal fragments result from an immature CFTR intermediate as opposed to a mature band C form.
CFTR Biogenesis Defects

**Fig. 5.** Carboxyl-terminal fragments appear early in biogenesis. Transfected cells were metabolically labeled with radioactive amino acids using a 15-min pulse and chase times as indicated. Cells were lysed, and CFTR was immunoprecipitated with a mixture of the M3A7 and L12B4 antibodies. Proteins were separated by SDS-PAGE and visualized by fluorography. Identical results were obtained by immunoprecipitation with the anti-HA antibody (not shown). The relative levels of the 105-kDa band with CFTRwt, A455E, and ΔF508 were determined by phosphorimage analysis. The percentage of radioactivity incorporated was calculated as the counts of the 105-kDa band over the sum of the counts of band B plus the 105-kDa band from five experiments.

Quantitative phosphorimage analysis of five experiments indicated that the radioactivity incorporated into the 105-kDa band corresponds to 38.1 ± 4.0% of total nascent CFTR-A455E (Fig. 5 and data not shown). In contrast, the 105-kDa band in CFTRwt corresponded to 18.4 ± 2.7% of nascent protein. For CFTRAF508, a weak 105-kDa band corresponded to only 7.2 ± 1.8% (data not shown) of nascent polypeptide. The half-life of the 105-kDa intermediate of both CFTRwt and A455E was determined to be very similar to that of band B (τ₁/₂ ~ 30–40 min) (16, 17) emphasizing that this fragment is susceptible to proteolysis.

**Fig. 6.** Deglycosylation analysis of the degradation bands. RIPA soluble protein extracts of CHA-S549R were treated with N-glycosidase F (N-Glyc F) and endoglycosidase H (Endo H). Immunoblot analysis using the anti-HA antibody as described revealed that the 105-kDa band is the core glycosylated form of the 90-kDa band.

**DISCUSSION**

**Carboxyl Fragments Reflect Early Recognition at the ER Membrane**—The carboxyl-terminal fragments that have been identified reveal remnants of a feature of early CFTR biogenesis that limits the delivery of CFTR to the cell surface. The timing of appearance, localization, and glycosylation features indicate that the process is active when CFTR is associated with the ER. It is imposed on the wt protein but is more prominent in the presence of missense mutations such as A455E or S549R. These altered amino acids are both within NBD1 but are separated by nearly 100 residues so it is unlikely
that specific cis proteolytic sites have been directly affected. Further, it is evident that for enhanced proteolytic activity, the mutated amino acid need not be present in the resulting carboxyl fragments as size estimation would preclude this for at least the A455E mutant. Our findings are consistent with the subjection of CFTR to a “quality control” surveillance mechanism that eliminates improperly or unfavorably folded protein (48). A number of conformational changes are imposed on nascent ER membrane-bound polypeptides during the folding of the individual domains, the assembly of interdomain interactions, or during post-translational modification. We predict that the mutations lead to an increased fraction of folding intermediates or of blocked conformations that are recognized and subsequently removed by a proteolytic mechanism that leads to the carboxyl remnants.

Newly synthesized proteins including apolipoprotein B100 have been shown in vitro to be exposed to the cytosol and targeted to the proteasome co-translationally (49). A number of conformational changes are imposed on nascent ER membrane-bound polypeptides during the folding of the individual domains, the assembly of interdomain interactions, or during post-translational modification. We predict that the mutations lead to an increased fraction of folding intermediates or of blocked conformations that are recognized and subsequently removed by a proteolytic mechanism that leads to the carboxyl remnants.

Distinction of the DF508 Mutation and Formation of the CFTR Carboxyl Fragments—The degradation process and fate of the carboxyl fragments appear interesting from several perspectives. Foremost, the formation of the carboxyl fragments from nascent wt and mutant CFTR indicates a fate distinct to the formation of the carboxyl fragments. We pharmacological studies, however, do not support a role for the major cytoplasmic degradation system in either their formation or direct elimination and thus imply that an alternate mechanism is responsible. We cannot determine whether the mechanism employs the proteasome at later stages of degradation, subsequent to the loss of identifying epitopes. The protease involved is unknown but the absence of an effect with ALLN would also indicate that it is not a neutral cysteine protease.

Degradation of misfolded ER membrane proteins via a cytoplasmic pathway would require retrograde transport from the membrane of the ER back into the cytosol (51). To enable such processes, the Sec61 membrane protein complex, which is the major constituent of the co-translational protein translocation machinery, may be utilized (51–53). Recent evidence suggests that Sec61β, a subunit of the Sec61 complex, does interact with CFTR and may participate in the elimination of both wt and ΔF508 CFTR (46). It would therefore be interesting to closely examine its interaction with the S549R and A455E CFTR mutants.

Distinction of the ΔF508 Mutation and Formation of the CFTR Carboxyl Fragments—The degradation process and fate of the carboxyl fragments appear interesting from several perspectives. Foremost, the formation of the carboxyl fragments from nascent wt and mutant CFTR indicates a fate distinct
from nascent ΔF508 molecules. The carboxyl fragments were difficult to detect for CFTRΔF508 in all cell lines tested. The proximity of the amino acid deletion to a proteolytic site may account for this; however, analysis of cells expressing CFTR versions with both the ΔF508 mutation and either S549R or A455E mutations retain the formation of prominent levels of the carboxyl-terminal fragments (data not shown). It is possible that for the major mutation, discrete conformational intermediates or aggregates form with consequent burial of the proteolytic site or sites such that the generation of the carboxyl polypeptides is diminished unless provoked by an alternate mutation.

The nature or number of steps of the proteolytic process involved in the formation of the carboxyl fragments remains unknown. The placement of the carboxyl-terminal epitope tag together with the sites of N-glycosylation provide two definitive inclusion features for the observed fragments, but the large size and the distorted migration of the immature CFTR polypeptides in SDS-PAGE limits a precise determination of the amino end. The observed sizes do indicate that at least one cleavage occurs within the latter portion of NBD1 or at the NBD1-R domain boundary and would thus be predicted to occur on the cytoplasmic face of ER-associated CFTR. Predicted proteolytic cleavage sites for general proteases do occur within this region, but preference of one over another is not readily rationalized.

**Mutations and Complexity of CFTR Maturation**—Our results indicate the specificity of the processes involved in the folding pathway of the cytosolic domains but also outline the complexity of the effects of individual mutations on CFTR biogenesis. In attempting to correlate the abundance of the carboxyl degradation bands with the formation of band C, it was evident from the S549R mutation that moderate levels of complex-glycosylated CFTR can be generated even though the production of 90- and 105-kDa products is prominent. We interpret this to indicate that conformational or folding defects that manifest very early are not necessarily problematic at later maturation stages. This is consistent with the findings of the wt protein but less discernible for some mutations such as A455E.

The A455E, Y563N, and P574H mutations do appear to contribute to the overall inefficient maturation of CFTR. Their prominence is enhanced with missense mutations in NBD1 but are notably reduced with the major CF mutation, the F508 molecule. The carboxyl fragments were detected for the major mutation, discrete conformational inter-

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