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F508del CFTR with two altered RXR motifs escapes from ER quality control but its channel activity is thermally sensitive

Tamás Hegedűs [a], Andrei Aleksandrov [a], Liying Cui [a], Martina Gentzsch [a], Xiu-Bao Chang [b], John R. Riordan [a,*]

[a] *Department of Biochemistry and Biophysics and Cystic Fibrosis T&R Center, UNC, 5011 Thurston-Bowles Bldg., Chapel Hill, NC 27510-7248, USA*
[b] *Mayo Clinic College of Medicine, Mayo Clinic, Scottsdale, AZ 85259, USA*

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Abstract

Most cystic fibrosis (CF) patients carry the F508del mutation in the CFTR chloride channel protein resulting in its misassembly, retention in the endoplasmic reticulum (ER), and proteasomal degradation. Therefore, characterization of the retention and attempts to rescue the mutant CFTR are a major focus of CF research. Earlier, we had shown that four arginine-framed tripeptide (AFT) signals in CFTR participate in the quality control. Now we have mutated these four AFTs in all possible combinations and found that simultaneous inactivation of two of them (R29K and R555K) is necessary and sufficient to overcome F508del CFTR retention. Immunofluorescence staining of BHK cells expressing this variant indicates that it matures and is routed to the plasma membrane. Acquisition of at least some wild-type structure was detected in the pattern of proteolytic digestion fragments. Functional activity at the cell surface was evident in chloride efflux assays. However, single channel activity of the rescued mutant measured in planar lipid bilayers diminished as temperature was increased from 30 to 37 °C. These findings support the idea that absence of Phe 508 causes not only a kinetic folding defect but also steady-state structural instability. Therefore effective molecular therapies developed to alleviate disease caused by F508del and probably other misprocessing mutants will require overcoming both their kinetic and steady-state impacts.

Keywords: CFTR; Arg-based retention signal; ER exit; Membrane protein assembly; Thermal instability

1. Introduction

The CFTR protein is a member of the ABC family consisting of two large transmembrane domains and two ATP binding cassettes (ABC domains). In contrast to most of the other ABC transporters, CFTR is a chloride channel that mediates the passive movement of the anion and is tightly regulated by phosphorylation through its unique R domain [1–3]. This chloride channel is the product of the gene mutated in patients with cystic fibrosis [4,5]. Although over 1,300 different mutations are known in the *CFTR* gene, approximately 90% of the patients carry the F508del mutation [6]. This mutant is not correctly assembled resulting in ER retention and degradation of the protein [4,7,8]. Efforts to redirect F508del CFTR to the plasma membrane with agents and conditions (osmolites, low temperature) promoting protein folding are partially effective but not readily applicable in vivo [9,10]. In order to develop effective treatments, elucidation of steps in CFTR maturation and quality control (QC) is necessary and hence these processes are being extensively studied [11–13].

ER export of membrane proteins may be prevented by mutations in either cytoplasmic or luminal domains and in one model, quality control mechanisms on either side of the ER membrane operate sequentially [14]. The best known of any cellular mechanism of protein conformation sensing is the so-called calnexin cycle, in which that ER luminal chaperone retains core-glycosylated glycoproteins according to their glucosylation state, which is in turn dependent on conformation-sensitive glucosyl transferases and glucosidases [15].

* Corresponding author. Tel.: +1 919 967 1320; fax: +1 919 966 5178.
E-mail address: john_riordan@med.unc.edu (J.R. Riordan).

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However while calnexin binds to core-glycosylated forms of both wild-type and F508del CFTR, this interaction is not responsible for either ER exit of the former or retention of the latter [13]. In contrast to intensively studied membrane glycoproteins such as VSV-G [16], very little of the CFTR polypeptide is exposed to the luminal compartment and disease-associated mutations in the short extracytoplasmic loops do not prevent ER exit [17]. Many missense mutations detected in patients like F508del are in sequences coding for luminal domains with which Hsp70 and Hsp90 and their co-chaperones interact and influence the processing of the protein [11].

Although the molecular details of conformational state recognition are not known, short sequences identified in secretory and membrane proteins serve as signals for forward transport from the ER [18] or retention and retrieval [19,20]. A transmembrane motif in the nicotinic acetyl choline receptor was reported to be responsible for monitoring its assembly [21]. Several diverse ER export signals [22–25] have been identified including a diacidic motif active in CFTR as well as several other membrane proteins[26]. Most known ER retention signals are constituted by basic amino acids, e.g., RRXX or KKXX at the carboxyl termini of many monotopic membrane proteins [19]. Apparently distinct arginine-based motifs were found to be retention signals in the cytoplasmic regions of polytopic membrane proteins including K_{ATP} [20,27], NMDA [22] and kainate receptors [28], glutamate receptor 1B [29,30] and cardiac sodium channel [31].

We found that mutagenesis of four arginine based RXR motifs simultaneously in F508del CFTR promoted its export from the ER [32]. To determine which of these are responsible for the ER retention of the mutant protein, we have mutated these sequences in all possible combinations. Only combinations in which the codes in the N-terminal tail (residues 29–31) and just C-terminal of the NBD1 signature sequence (residues 553–555) were found to enable F508del maturation. Properties of the rescued mutant protein were evaluated in pulse-chase and protease digestion experiments and function was demonstrated by chloride efflux and single channel measurements. Single channel activity was found to be highly temperature sensitive, indicating that escape from the ER quality control by this means did not coincide with overcoming the thermolability of the mutant protein.

2. Materials and methods

2.1. Expression of CFTR constructs

pNut vector containing the full length human CFTR cDNA [33] served as a template for site-directed mutagenesis by polymerase chain reaction (PCR). The PCR was performed according to standard Stratagene protocols using the same oligonucleotides employed to make the R29K and R555K substitutions previously [32]. BHK-21 cells were transfected and grown as described previously [11].

2.2. PAGE and Western blotting

Cells were lysed and subjected to SDS gel electrophoresis on 7.5% polyacrylamide gels. Proteins were blotted to nitrocellulose membranes which were probed with monoclonal antibody 596 [34]. HRP secondary antibody was used before chemiluminescent film detection (Pierce).

2.3. Protein N-glycosidase F treatment

To remove N-linked oligosaccharide chains, membranes prepared from BHK cells expressing our constructs were diluted in buffer containing 100 mM sodium phosphate (pH 7.5), 0.1% SDS, 50 mM β-mercaptoethanol, 0.75% NP-40, and protease inhibitors (1 mg/ml leupeptin, 2 mg/ml aprotinin, 50 mg/ml Pefabloc, 121 mg/ml Benzamidine, 3.5 mg/ml E64). After addition of 10 mU Protein N-Glycosidase F (PNGaseF), the reaction mixture was incubated 6 h at 37 °C. PNGaseF was obtained from ProZyme, CA, USA.

2.4. Confocal microscopy

BHK cells expressing the Extope constructs (see Results and [35]) were grown on collagen coated chamber slides, fixed, and labeled with anti-HA monoclonal antibody (16B12, Babco) without permeabilization. FITC labeled secondary Ab (Molecular Probes) was used for detection by confocal microscopy.

2.5. Pulse chase experiments

Pulse chase experiments were performed exactly as we described earlier [11]. Briefly, plates with equal cell density were incubated with methionine-free α-MEM for 30 min, then loaded with ^{35}S-Met for 20 min. After varying chase periods (0, 1, 2, 3, 4, and 6 h) cells were solubilized in NP40 buffer (0.09% NP40, 50 mM Tris, 150 mM NaCl, pH 7.4, protease inhibitors), and CFTR was immunoprecipitated with mAb 596 immobilized on Dynabeads. After SDS-PAGE and transfer the autoradiogram was quantified using ImageJ software.

2.6. Functional measurements

Chloride efflux and single channel measurements were performed as described in [32,36]. Briefly, cells grown on 6 well plates were loaded with $^{36}Cl^-$ for 60 min at room temperature in 0.5 ml buffer (20 mM HEPES, pH 7.4, 11 mM glucose, 2 mM $CaNO_3$, 2 mM KNO_3, 135 mM $NaNO_3$) containing 1 μCi of the isotope. Before stimulation, efflux buffer were collected and replaced three times in 90 s intervals to determine the basal chloride flux. That was followed by sampling with the same rate using efflux buffer supplemented with 10 μM forskolin, 100 μM DiBu-cAMP, and 1 mM IBMX to activate CFTR. The radioactivity was measured in a TopCount-NXT Microplate Scintillation Counter (Packard). Efflux rates were calculated from the $^{36}Cl^-$ specific activity and normalized to the cell number.

Single channel experiments were done as described in [36,37]. Microsomal membrane vesicles were prepared from BHK cells expressing wild-type CFTR or the other variants. Vesicles were pretreated with 100 U/ml PKA (Promega) for 15 min (2 mM Na_2ATP, 5 mM $MgCl_2$, 250 mM sucrose, 10 mM HEPES, pH 7.2), then fused to the preformed lipid bilayer spontaneously. The two chambers contained symmetrical solution (300 mM Tris–HCl, 3 mM $MgCl_2$, 1 mM EGTA, pH 7.2), which were thermostated and stirred. CFTR single channel function was recorded under voltage clamp conditions at V_m=−75 mV. Therefore in the all single channel records shown, the lower current level corresponds to the open state, whereas the upper one corresponds to the closed state. In the course of single channel recording the temperature in the chamber was gradually increased from +30 °C to +35 °C at a rate of 1 °C/min.

2.7. Tryptic digestion

Membranes isolated from BHK cells expressing WT, F508del, or 2RK F508del CFTR were diluted to 1 mg/ml in digestion buffer (0.5 mM EGTA, 40 mM TRIS, 50 mM KCl, 0.5 mM DTT, pH 7.4). The digestion was performed with 0.05, 0.1, 0.5, 1.0 μg trypsin or chymotrypsin in 50 μl reaction volume for 10 min on ice. The proteolysis was terminated with protease inhibitors (1 mg/ml leupeptin, 2 mg/ml aprotinin, 50 mg/ml Pefabloc, 121 mg/ml Benzamidine, 3.5 mg/ml E64), and the samples were immediately denatured in 2× sample buffer.

3. Results

3.1. Alteration of two arginine-framed tripeptide motifs of CFTR F508del is sufficient for maturation

It was shown earlier that arginine-framed tripeptide (AFT) motifs of CFTR take part in the ER quality control [32]. R→K substitution at one of the two flanking arginines in individual motifs was without effect on ER-retained F508del CFTR. In contrast, with simultaneous mutation of all four arginine-based motifs the mutant protein escaped ER quality control, became glycosylated, and showed functional chloride channel activity in the plasma membrane [32].

In order to determine which particular RXR motifs are involved in the ER retention of F508del CFTR, we generated all possible combinations of substitutions at the four sites. Since CFTR maturation is very sensitive to mutations in its cytoplasmic domains, the less conserved arginines were replaced in each RXR motif at position 1 or 3 (R29, R516, R555, R766; Fig. 1). These constructs were expressed in BHK-21 cells, the cells were lysed and electrophoresed. The maturation level of all single, double, triple, and quadruple mutants was followed by Western blot with a monoclonal anti-CFTR antibody as shown in Fig. 2A. Since the CFTR is a glycoprotein, the mature form with complex glycosylation migrates at higher molecular weight. We reported earlier that among the single mutants only the R29K substitution produced a very low level maturation and this is not visible on the exposure in Fig. 2A. The other three single mutations also did not result in any detectable maturation. Of the double combinations only the 29/555 substitutions permitted maturation revealing that those arginine-framed motifs are primarily responsible for the ER retention of F508del CFTR. Of the triple combinations only 29/555/766 and 29/516/555, both containing the essential two, produced mature CFTR, while the others (29/516/766 and 516/555/766) did not (Fig. 2A).

To confirm the complex glycosylation of the 2RK F508del CFTR (29,555) mature band, membranes prepared from wild-type, F508del, or 2RK F508del expressing BHK cells were treated with PNGase F. Western blots show that the migration of 2RK F508del CFTR is increased by this treatment (Fig. 2B) verifying that it had acquired complex oligosaccharide chains. Interestingly, this mobility shift caused by the glycosidase digestion is more complete with rescued mutant than with the wild-type. This may reflect better accessibility to the glycosidase of the oligosaccharide chains of the still incompletely folded rescued mutant protein (see below).

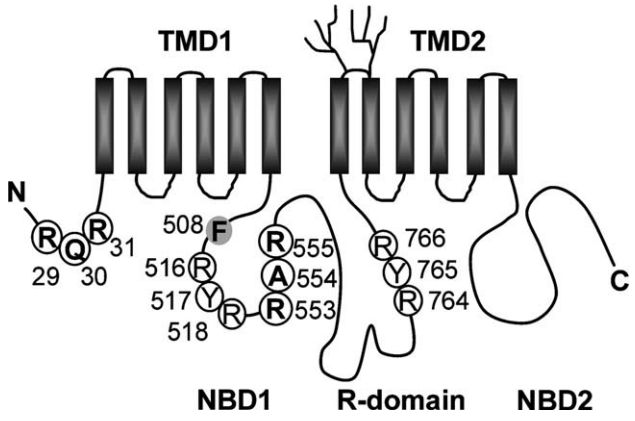

Fig. 1. Schematic representation of CFTR with the relative positions of the arginine-framed tripeptides and F508del.

3.2. Plasma membrane appearance of 2RK F508del CFTR

Since the acquisition of complex oligosaccharide chains indicates only that the protein had at least reached the Golgi, we employed immunofluorescence microscopy to determine if it also had progressed to the plasma membrane. To facilitate these experiments, we employed constructs with an epitope inserted in an extracytoplasmic loop enabling detection at the external surface of unpermeabilized cells. These constructs contain an HA tag inserted in the second extracytoplasmic loop (34). Plasma membrane expression of these "Extope" CFTR channels can be followed with an anti-HA antibody without cell permeabilization. The maturation of the Extope proteins was similar to that of the untagged proteins according to the glycosylation patterns (Fig. 3A). In the case of both WT and 2RK F508del CFTR, cell surface associated signals were detectable, demonstrating that they had been routed similarly (Fig. 3B, C). In contrast, cells expressing F508del CFTR did not show any staining (Fig. 3D).

3.3. Biosynthetic stability of the mature 2RK F508del CFTR

In order to kinetically monitor the biosynthetic maturation and stability of the 2RK F508del CFTR protein, pulse chase experiments were performed as described in Materials and methods. Compared to F508del CFTR, where no conversion from immature to mature form could be detected (not shown, see [17]), a substantial amount of the 2RK F508del CFTR was converted from a core-glycosylated to a complex-glycosylated form (Fig. 4; approximately 9% of the total compared to the 25% of the WT [17,38]). A significant proportion of the mature protein is still present after 6 h, but the half life seems to be considerable shorter than that of the wild type protein (16 h, see [17,38]).

3.4. Chloride channel function of 2RK F508del CFTR

We assessed the functional capability of F508del protein that evaded ER quality control due to inactivation of the two significant motifs, first by measuring the cAMP activated Cl^- efflux from BHK cells preloaded with the radioactive isotope (Fig. 5A). In WT and 2RK F508del CFTR expressing cells, a rapid rate of chloride efflux could be observed upon stimulation, while F508del expressing cells showed no detectable change in efflux rate after addition of the stimulatory cocktail. Thus, the mutant protein that reaches the cell surface apparently exhibits some chloride channel activity as detected in this flux assay performed at room temperature.

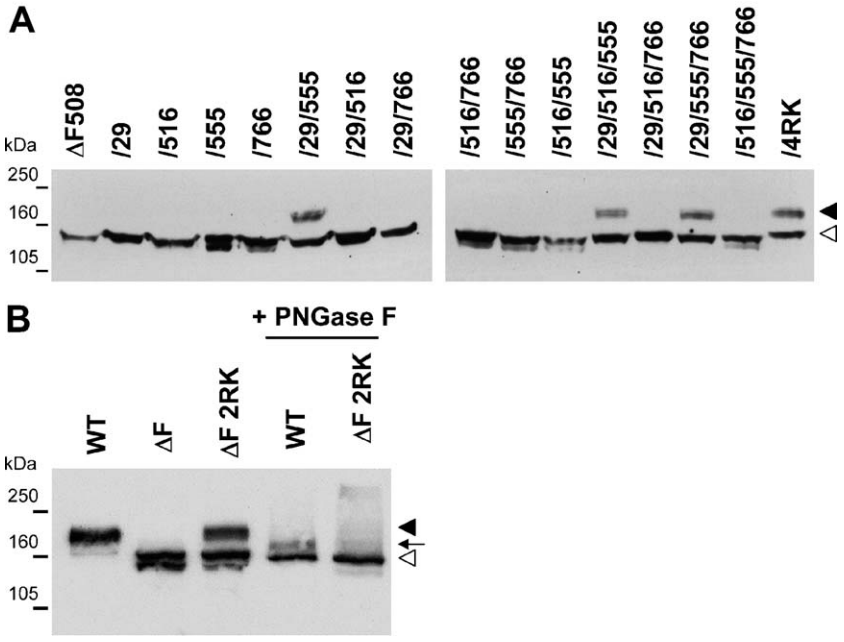

Fig. 2. Maturation of different combinations of Arg-based signal substitutions. (A) Effects of R→K mutations in all possible combinations on F508del CFTR maturation detected in Western blots. BHK cells expressing each of the variants were lysed and subjected to SDS-PAGE, blotted to nitrocellulose, and detected with anti-CFTR monoclonal Ab 596. (B) Change in electrophoretic mobility of WT and 2RK F508del CFTR by PNGase F. Treatment was performed as described in Materials and methods. Closed arrowhead: complex glycosylated mature band C; open arrowhead: core glycosylated band B; arrow: WT CFTR with partially digested oligosaccharide chains.

This channel activity was also monitored by single channel current measurements in planar lipid bilayers after fusion of membrane vesicles from 2RK F508del CFTR expressing cells. Current records obtained at 25 °C were essentially indistinguishable form those obtained previously with 4RK F508del CFTR, in which all four arginine-based motifs in the protein had been mutated [32]. Unitary conductance in both cases was not different from wild-type. Gating kinetics were altered with both mean open and mean closed times prolonged. This behavior is evident at the beginning of the lower tracing in Fig. 5 at temperatures from 30 °C to 31 °C. Strikingly, however, as temperature is continuously increased, channel activity of 2RK F508del CFTR is progressively lost. This lability is clearly due to the F508del mutation and not the 2RK substitutions because it is not observed in wild-type CFTR in which all four motifs are mutated (middle tracing). In the wild-type with or without (upper tracing) these Arg substitutions, gating is accelerated

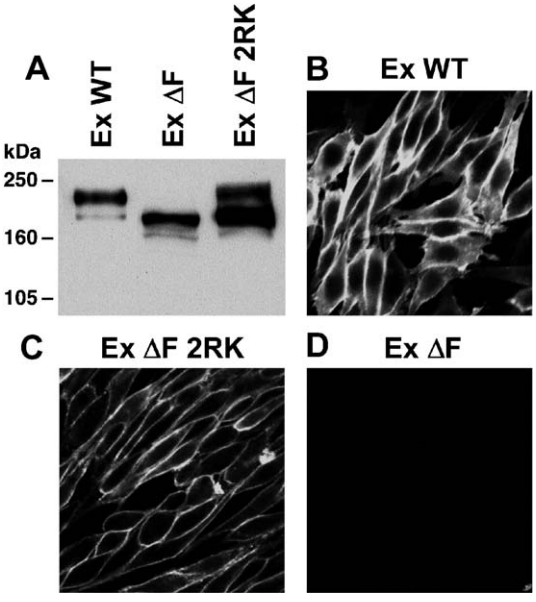

Fig. 3. 2RK F508del CFTR reaches the cell surface. In order to detect the protein at the cell surface, constructs with an HA epitope in an extracellular loop (Extope) were utilized. (A) Western blot shows that the proteins in membranes isolated from BHK cells are similar to those without the insert. (B–D) BHK cells expressing the CFTR Extope constructs were grown on collagen coated slides, fixed, labeled with anti-HA mAb, and then with an anti-mouse secondary antibody conjugated with Alexa 488.

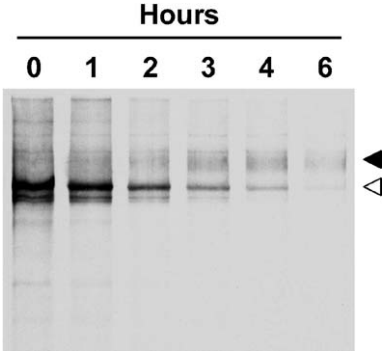

Fig. 4. Biosynthetic stability of the 2RK F508del. BHK cells were pulsed with ^{35}S-Met for 20 min, and chased for the times indicated in hours. After solubilization the metabolically labeled CFTR was immunoprecipitated, run on SDS gel, and transferred to nitrocellulose membrane. The radioactivity was detected by autoradiograpy. Closed arrowhead: complex glycosylated mature band C; open arrowhead: core glycosylated band B.

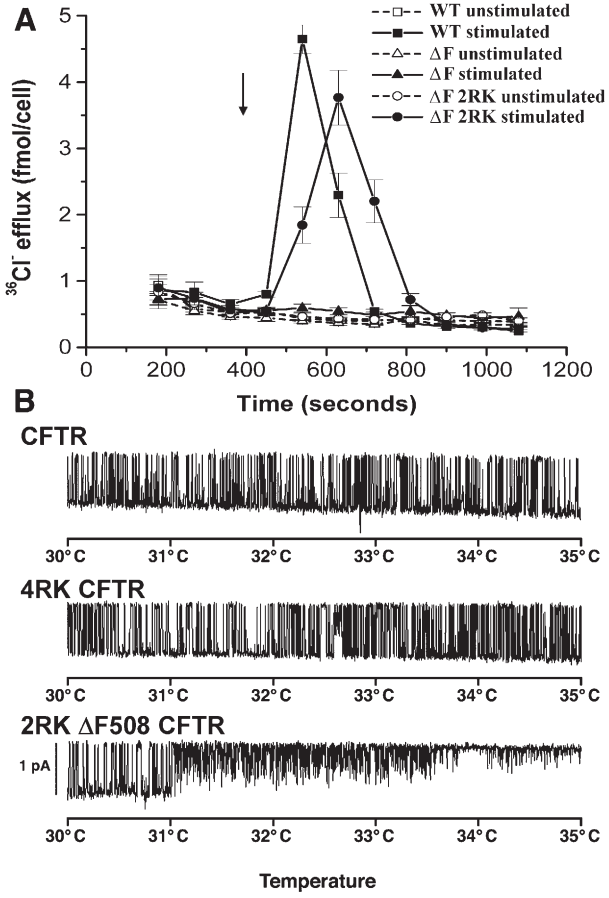

Fig. 5. Chloride channel function of the 2RK F508del CFTR protein. (A) $^{36}Cl^-$ efflux from BHK cells expressing WT, F508del, or 2RK F508del. Cells were grown in 6-well culture plates, loaded with $^{36}Cl^-$ after reaching confluency, then 0.5 ml/well chloride free efflux buffer was added. The buffer was collected and replaced three times in 90 s intervals, then the sampling was continued similarly using efflux buffer supplemented with a stimulation cocktail containing forskolin, DiBu cAMP, and IBMX. Initiation of the stimulation is indicated by an arrow. Radioactivity of the collected samples was determined in a scintillation counter. (B) Rescued 2RK F508del CFTR single channel is temperature sensitive. Membrane vesicles from cells expressing wild type CFTR without (upper tracing) or with all for arg-based signals substituted (middle tracing), or from 2RK F508del CFTR (lower tracing) were fused to lipid bilayers. Currents were recorded as temperature was increased at a rate of 1 °C/min.

with increasing temperature as expected and the single channel conductance is increased. These findings reveal that the rescued F508del protein cannot support the open channel structure at temperatures of ~32 °C and higher, at least in a planar lipid bilayer.

3.5. Conformational stability of the rescued 2RK F508del CFTR

The apparently increased oligosaccharide chain accessibility to PNGase F (Fig. 2B), the decreased biosynthetic stability of 2RK F508del CFTR (Fig. 4), and the functional instability at higher temperatures (Fig. 5B) suggest that this rescued mutant retains some degree of conformational instability. Proteolytic enzymes are commonly used tools for testing correct folding of proteins, since they usually have increased access to the disturbed structure [39]. We utilized limited proteolysis as an additional means to detect differences in the stability of the mature WT and 2RK F508del forms and the immature F508del form. Membranes from BHK cells with the same total protein concentration, expressing the channel variants at comparable expression levels, were digested with different concentrations of trypsin or chymotrypsin on ice for 10 min (see Materials and methods). The samples were electrophoresed and blotted to nitrocellulose membranes which were probed with mAb 596 (Fig. 6). Under these conditions the mature wild-type CFTR was highly resistant to tryptic digestion as the mature band started to disappear only at high trypsin concentrations resulting in the appearance of a prominent band at ~90 kDa. In contrast F508del CFTR showed high sensitivity to trypsin, as its immature band was completely digested at the lowest concentration of trypsin used in our experiments, generating a ~65 kDa band and smaller fragments which then also disappeared. These dramatically different sensitivities were also apparent with chymotrypsin digestion with the appearance of a distinct set of smaller bands. 2RK F508del CFTR yielded a digestion pattern which appeared to be a combination of those from the wild-type and F508del. It is reasonable to assume that this may be due to the presence of both immature and mature forms of 2RK F508del CFTR. However, beyond this quite obvious relationship, it also appeared that the mature, more slowly migrating band in 2RK F508del CFTR succumbed to digestion by both proteases somewhat more readily than the comparable mature band in the wild-type. Furthermore, the primary detectable digestion products of the mature forms disappeared at lower protease concentrations in the case of the 2RK F508del CFTR than the wild-type. Thus although much less sensitive than the immature wild-type or mutant, the F508del CFTR that has escaped the ER and gained some functional capacity may remain less compact structurally and hence slightly less resistant to proteolysis.

4. Discussion

Arginine-based sorting motifs have been identified in a number of membrane proteins that are normally exported from the ER to begin trafficking to the cellular location where they function. They are accessible to components of the COPI coat complex when they are not masked by intermolecular associations in the case of multi-subunit complexes or possibly domain assembly in monomeric membrane proteins [20]. Hence, when correct assembly is not achieved because of inappropriate subunit stoichiometry or defective folding, the polypeptide bearing the exposed motif is retained or retrieved to the ER. In the variety of polytopic membrane proteins in which these signals operate, neither their position in the sequence nor proximity to the membrane seem to be crucial. However, the number of arginine-based motifs in a retained unit may be significant as suggested by the apparent role of valency in their effectiveness in retention of the Kir 6.2 subunits of K_{ATP} [40]. This may be due to the fact that two motifs can be bound simultaneously by dimer 14-3-3 proteins which effectively

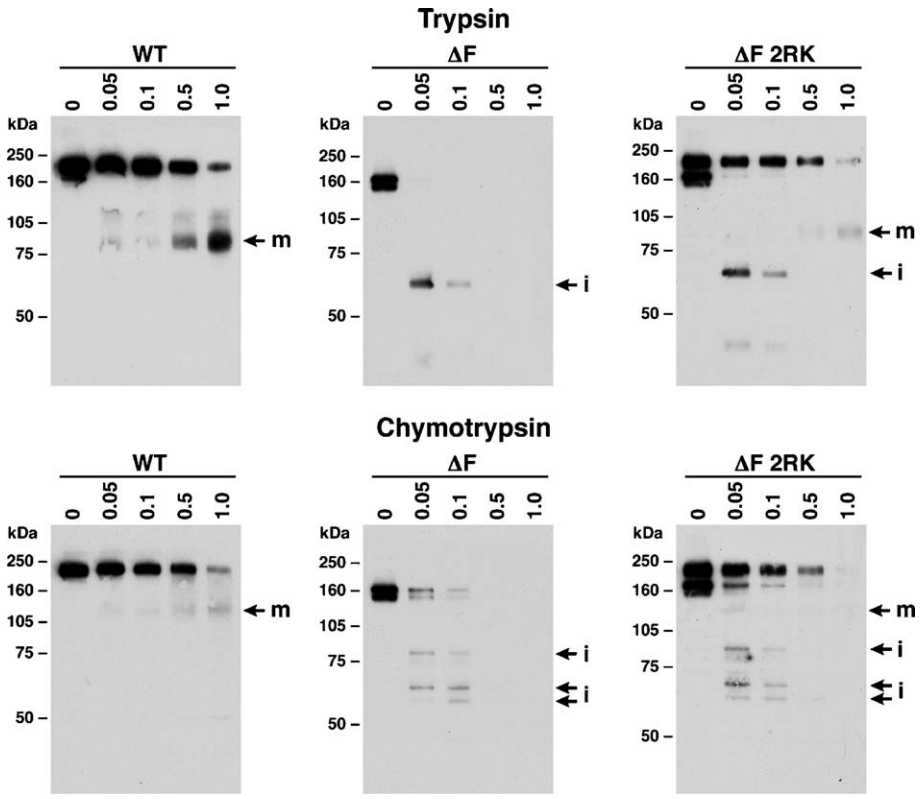

Fig. 6. Conformational stability of the 2RK F508del. Limited proteolysis of wild type, F508del, and 2RK F508del were performed on ice with membranes isolated from stably expressing BHK cell lines. Reaction mixtures contained 50 μg total membrane protein with similar levels of the different CFTR proteins and 0, 0.05, 0.1, 0.5, 1 μg trypsin (top) or chymotrypsin (bottom). The digestion was started with protease addition and stopped after 10 min with excess protease inhibitor mixture. Labels of fragments derived from mature or immature bands are *m* or *i*, respectively.

competes for COPI binding, thereby overcoming retention when the two sites become appropriately aligned. These findings suggested to us a possible basis for the apparent requirement for more than one of the four motifs for retention of F508del CFTR [32] and motivated us to determine exactly how many and which ones were responsible. The finding that only a specific pair, one in the N-terminal cytoplasmic tail and one in NBD1 were required is at least loosely compatible with the concept that the two motifs may need to become aligned to enable interaction with the same bivalent binding partner. Indeed there is some evidence that the N-terminal tail and NBD1 may need to interact for CFTR channel function [41]. However, in the case of CFTR, this putative bivalent arginine-based motif binding partner is apparently not a 14-3-3 protein as we were unable to detect any phosphorylation-independent association of these proteins with F508del CFTR (with or without substitution of the two motifs) or with wild-type CFTR (not shown). It remains possible that other bivalent competitors of COPI binding by the individual sites may be involved.

Of the two arginine-based motifs identified, the first satisfies one of the features of the consensus determined by combinatorial peptide screening [24] as there is an aromatic residue (tyrosine) at the −1 position and the second at least does not have negatively charged residues at either the −1 or −2 positions, a feature that appears to inactivate these signals [20].

Our data do not prove that the two arginine-based tripeptide sequences which when mutated result in partial rescue of F508del CFTR, do so by preventing specific binding to a retrieval mechanism component. Alternative possibilities include just compensatory conformational influences of replacing the arginines at the two locations with the smaller lysine residues. In fact Teem et al. [42] had found that the R555K substitution alone restored a small F508del CFTR chloride current. However as shown we could not detect any maturation with this mutation alone despite a substantial amount when it was combined with R29K. We had originally detected a slight effect of R29K alone as well [32]. Whether or not there are small effects of the individual substitutions below or near the threshold of our detection, there clearly is a large increment when the two are combined. There is precedent for substitutions at two separate positions in NBD1 itself having a synergistic effect in restoring F508del CFTR chloride conductance, presumably by a direct conformational impact on the domain [43]. However, none of the nominal suppressor mutations of F508del CFTR misfolding including those at R555 significantly change the 3D structure of NBD1 [44].

It has been shown recently that exposure of an acidic exit code within NBD1 is required for ER export of wild-type CFTR [45]. Although the hiding of this exit code in the F508del NBD1 could be responsible for the failure of ER export, this does not exclude a role for retention/retrieval mechanisms as well.

Regardless of the mechanism whereby replacing arginines in these two motifs enables escape of F508del CFTR from ER

quality control, the mutant protein rescued in this way remains thermolabile at a functional level. This is consistent with the findings of Sharma et al. [46] that the F508del mutation causes a temperature-sensitive stability defect in the polypeptide and not just a kinetic defect in folding. They showed that after the rescue of F508del CFTR by growth of cells at low temperature, the lifetime of the mature mutant protein itself was much shorter than the wild-type. However they did not follow the rate of its loss of function at higher temperatures as we did. Both of these issues and their relationship will need to be examined with F508del CFTR rescued by small molecule correctors such as those recently reported [47]. Moreover, the 2RK F508del CFTR construct is a useful tool to test small molecules as stabilizers of F508del CFTR that reached the plasma membrane.

Acknowledgements

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