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The ΔF508 Mutation Decreases the Stability of Cystic Fibrosis Transmembrane Conductance Regulator in the Plasma Membrane

DETERMINATION OF FUNCTIONAL HALF-LIVES ON TRANSFECTED CELLS*

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Deletion of the phenylalanine at position 508 of the cystic fibrosis transmembrane conductance regulator (CFTR) is the most prevalent mutation in cystic fibrosis (CF). This mutation (ΔF508CFTR) leads to a reduced cAMP-sensitive Cl^- conductance in epithelial cells. While the mutant protein can function as a Cl^- channel, it seems to be misprocessed and unable to accumulate at normal levels in the plasma membrane. Under conditions where the biosynthetic block of ΔF508CFTR is not complete, the residence time of ΔF508CFTR in the plasma membrane is a critical determinant of the cAMP-sensitive Cl^- conductance. To assess the stability of the mutant and wild-type CFTR, we compared their functional half-lives at the plasma membrane of transfected Chinese hamster ovary cells. The plasma membrane Cl^- conductance was assessed by patch-clamp recordings and/or by fluorimetric determinations of the membrane potential. Accumulation of ΔF508CFTR in the plasma membrane was promoted by growing the transfected cells at reduced temperature (24–28 °C), and was verified by immunoblotting and by detecting the appearance of a plasmalemmal cAMP-activated Cl^- conductance. Subsequently increasing the temperature to 37 °C inhibited further delivery of newly synthesized ΔF508CFTR to the surface membrane. By studying the time dependence of the disappearance of the Cl^- conductance, the functional half-life of the mutant protein at the plasma membrane was determined to be <4 h, which is considerably shorter than the half-life of wild-type CFTR (>24 h). The latter was estimated by terminating protein synthesis or secretion with cycloheximide or brefeldin A, respectively. Inhibition of protein synthesis did not alter the rate of disappearance of ΔF508CFTR at 37 °C, validating the difference in turnover between mutant and wild-type CFTR. These results indicate that the structural abnormality of ΔF508CFTR affects not only the delivery of the protein to the plasma membrane, but also its stability therein. Moreover, they suggest that overcoming the processing block at the endoplasmic reticulum may not suffice to restore normal Cl^- conductance in CF.

Cystic fibrosis (CF),[1] a lethal hereditary disease, is caused by mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR; Refs. 1–3). Recent studies provided convincing evidence that CFTR can function as a cAMP-activated Cl^--selective channel in the plasma membrane and also in endosomes (4–12).

The most prevalent mutation in CF is the deletion of phenylalanine at position 508 of CFTR (ΔF508CFTR). The abnormal electrolyte transport in epithelia afflicted with this mutation likely results from impaired Cl^- permeability. Accordingly, in apical membranes of CF epithelia and in the plasmalemma of most cells transfected with ΔF508CFTR, stimulation of protein kinase A fails to elicit the increase in Cl^- conductance that is observed when wild-type CFTR is present (see Refs. 13–15 for review). The precise mechanism underlying the defective permeability of ΔF508CFTR-expressing cells is incompletely understood. A decreased channel density at the plasma membrane and/or inability of the channels to respond to cAMP could account for the abnormal Cl^- permeability. In fact, an abnormal activation pattern of ΔF508CFTR was demonstrated in high expression systems or at reduced temperature, where the protein is apparent in the plasma membrane (16–18). On the other hand, in insect cells the mutant protein can be activated normally and following purification and reconstitution into phospholipid bilayers, wild-type and ΔF508CFTR displayed identical behavior in response to protein kinase A (19). Despite this apparent discrepancy, there is general agreement that ΔF508CFTR is at least partly functional and responsive to cAMP. This lends credence to the hypothesis that cellular mislocalization, and not impaired ion transport, is the primary cause of the abnormal behavior of ΔF508CFTR. Defective intracellular traffick-

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[1] The abbreviations used are: CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; CHO, Chinese hamster ovary; E_m, membrane potential; E_{Cl^-}, Cl^- equilibrium potential; NMG, *N*-methylglucammonium; CTP-cAMP, 8-(4-chlorophenylthio)-adenosine 3′:5′-cyclic monophosphate; IBMX, 3-isobutylmethyl-1-xanthine; CHX, cycloheximide; BFA, brefeldin A.

ing of ΔF508CFTR was first observed by Cheng *et al.* (7) and subsequently confirmed by other studies of immunolocalization and biosynthesis in primary and transfected cells (7, 18, 20–26). In contrast to wild-type CFTR, which becomes fully glycosylated and is predominantly present in the plasma membrane, ΔF508CFTR is only core-glycosylated, accumulating instead in intracellular compartments (7, 18, 20–26). Mislocalization of ΔF508CFTR has been attributed to inappropriate folding of the protein, a concept supported by the temperature sensitivity of the defect (Ref. 18; see below).

The steady-state density of a protein at the plasma membrane is determined by the relative rates of delivery from internal compartments and of internalization and/or degradation. While delivery of ΔF508CFTR from the biosynthetic pathway to the plasma membrane is thought to be reduced (7, 18, 20–24), little is known about the rate of removal of ΔF508CFTR from the plasma membrane. To assess the stability of mutant and wild-type CFTR, we estimated the relative rates of their disappearance from the plasma membrane, using a functional assay of Cl^- permeability. The results suggest that ΔF508CFTR has a considerably shorter half-life than wild-type CFTR, which may contribute to the defective Cl^- permeability in CF.

EXPERIMENTAL PROCEDURES

Cell Lines—Chinese hamster ovary cells (K1 cells), which do not express endogenous CFTR, were stably transfected with cDNA encoding for wild-type CFTR (Q1 and Q2 cells), ΔF508CFTR (Δ508 cells), or with the vector (pNUT) alone, as described before (10). Cells were maintained in monolayer or suspension culture in α-minimal essential medium supplemented with 7% fetal calf serum. CFTR- and ΔF508CFTR-transfected cells were grown at 37 or 28 °C in the presence of 10 or 200 μM methotrexate, respectively.

Fluorimetric Membrane Potential Measurements—The anionic fluorescent probe *bis*-oxonol $DiSBAC_2(3)$ was used to estimate the membrane potential (E_m) of cells in suspension at 37 °C, as described earlier (27). For calibration, E_m was manipulated by adding gramicidin to cells in isotonic media containing varying ratios of K^+ and choline$^+$. E_m was calculated as in Ref. 27. Fluorescence intensity varied approximately linearly with E_m in the −60 to 0 mV range (see Fig. 1, *inset*).

An increase in Cl^- conductance tends to shift E_m toward E_{Cl-}, the Cl^- equilibrium potential. To facilitate detection of CFTR activity, the difference between E_m and E_{Cl-} was maximized by replacement of the extracellular Cl^- (145 mM) with the impermeant anions glutamate$^-$ or cyclamate$^-$. Under these conditions activation of CFTR induces a pronounced depolarization. Influx of Na^+ was ruled out as the source of the depolarization by replacing it with an impermeant cation, NMG^+.

Patch-clamp Studies—Single channel currents were recorded essentially as described (5). Bath and pipette solutions contained (in mM) 140 NaCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 glucose, 10 HEPES, pH 7.2. Single-channel current amplitudes were obtained by examination of amplitude histograms generated using the pCLAMP FECHAN analysis program (5). Whole cell currents were recorded by the method of Hamill *et al.* (28), using pipettes of tip resistance between 10 and 20 MΩ. The pipette filling solution contained (in mM) 110 sodium gluconate, 20 NaCl, 8 $MgCl_2$, 5 EGTA, 10 glucose, 2 ATP, 10 HEPES, pH 7.2. The bath solution was: 137 NaCl, 3 $MgCl_2$, 1 $CaCl_2$, 10 glucose, 10 HEPES, pH 7.2. Where indicated, gluconate substitution was used to lower the Cl^- concentration to 36 mM. The holding potential was −60 mV. For analysis of current-voltage relationships the potential was stepped between −90 and +90 mV in 15-mV increments. Voltage steps were applied for 200 ms at 800-ms intervals. In each experiment currents were recorded at the same holding potential three times, averaged, and analyzed using the CLAMPFIT program. The currents were normalized per unit capacitance, to account for variations in cell size. All electrophysiological measurements were conducted at room temperature (22–24 °C).

Detection of CFTR and ΔF508CFTR by Immunoblotting—A crude microsomal fraction from ≈10^9 transfected cells, obtained as described (12), was depleted of lysosomes and mitochondria by fractionation on a Percoll gradient (25% Percoll, 28,000 × *g* for 100 min). The fractions containing endoplasmic reticulum, Golgi, and plasmalemmal markers were combined and polypeptides were separated by SDS-polyacrylamide gel electrophoresis on 7.5% gels. Immunoblotting was performed as described using monoclonal antibody M3A7 (21). M3A7 recognizes the core-glycosylated and mature forms of both CFTR and ΔF508CFTR (21). Data are presented as the mean ± 1 S.E. of the number of experiments indicated in parentheses.

Immunofluorescence—CFTR expressing CHO cells were stained with a rabbit IgG directed against mannosidase II (kindly provided by Drs. K. Moremen and M. Farquhar) as described (29). A fluoresceinated secondary antibody was used for visualization. [^{35}S]Methionine incorporation into cellular proteins was measured as in Ref. 30.

RESULTS AND DISCUSSION

Functional Localization of CFTR and ΔF508CFTR—To monitor the presence of CFTR and ΔF508CFTR in the plasma membrane, a fluorimetric method was implemented for the estimation of Cl^- conductance in populations of transfected cells. Membrane potential changes in response to protein kinase A activation were recorded in cell suspensions using the *bis*-oxonol $DiSBAC_2$ (3). As shown in Fig. 1*A*, addition of forskolin to CFTR expressing cells (Q1) elicited a sizable depolarization. Several lines of evidence indicate that the depolarization is attributable to a cAMP-regulated Cl^--conductive pathway: 1) the depolarization was considerably greater when the cells were suspended in low [Cl^-] media (54.8 ± 5.2 mV, n = 7) than at normal [Cl^-] (18–24 mV), as expected from the change in E_{Cl-} (Fig. 1*A*); 2) in K^+-rich solutions, which shift the resting E_m near 0 mV, forskolin induced a depolarization at low external [Cl^-], but the cells hyperpolarized towards E_{Cl-} at physiological [Cl^-] (Fig. 1*A*); and 3) similar results were obtained in cells treated with CTP-

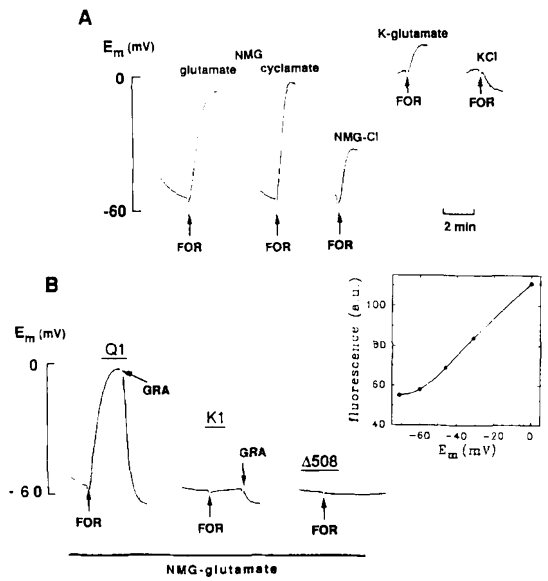

FIG. 1. **Detection of plasma membrane cAMP-sensitive Cl^- permeability by membrane potential (E_m) measurement.** E_m was measured fluorimetrically using *bis*-oxonol ($DiSBAC_2(3)$; 200 nM). *A*, CFTR-expressing CHO cells (Q1; 10^6/ml) were suspended in a medium containing 145 mM of the indicated salt, 10 mM glucose, and 10 mM HEPES, pH 7.3. Where indicated, adenylate cyclase was activated with 20 μM forskolin (*FOR*). *B*, wild-type CHO cells (K1) or cells expressing CFTR (Q1) or ΔF508CFTR (Δ508) were suspended in NMG-glutamate solution. Where indicated, forskolin (20 μM) or gramicidin (2 μM) were added. Traces in *A* and *B* are representative of three experiments. *Inset*, calibration of the fluorescence intensity of *bis*-oxonol *versus* E_m. The latter was varied by suspending cells in media with varying ratios of K^+ and choline$^+$, followed by addition of gramicidin.

cAMP and IBMX (not illustrated). The potential change appears to be mediated by CFTR since it was not observed in nontransfected (K1; Fig. 1*B*) or mock transfected cells (not shown). In accordance with these findings, patch-clamp determinations in the whole cell configuration revealed the appearance of a Cl^--selective conductance after treatment of Q1 cells with forskolin or CTP-cAMP[2] (see below). These findings, which are consistent with earlier single channel measurements documenting the existence of cAMP-activated Cl^- channels in CFTR expressing CHO cells (10), indicate that the fluorescence assay provides a sensitive measure of the presence of CFTR at the plasma membrane.

The assay was used next to determine whether a protein kinase A-activated Cl^- conductance is detectable in the membrane of ΔF508CFTR-transfected cells grown at 37 °C. While the resting potential of these cells was normal, neither forskolin (Fig. 1*B*) nor CTP-cAMP (not shown) were able to elicit a depolarization. These data imply that the level of expression of functional ΔF508CFTR at the plasma membrane is below the detection limit of the fluorimetric method. In agreement with these findings, forskolin failed to increase the whole cell current in patch-clamped ΔF508CFTR cells (not illustrated). As suggested earlier, functional ΔF508CFTR may not be detectable due to inadequate routing to the plasma membrane (7, 18, 20). Consistent with this notion, in cells grown under standard conditions only the core glycosylated form of ΔF508CFTR (approximate $M_r \approx 150{,}000$) was detectable by immunoblotting, while the fully glycosylated form was not observed (see *inset* in Fig. 3*A*).

Expression of ΔF508CFTR in the Plasma Membrane—Our inability to detect ΔF508CFTR in the plasma membrane of cells grown at 37 °C precluded studies of its turnover. To circumvent this obstacle, we exploited the temperature sensitivity of the expression of ΔF508CFTR. As described recently (18), the intracellular transport block that prevents delivery of ΔF508CFTR to the surface membrane is overcome by reducing the temperature. In agreement with the results of Denning *et al.* (18) in 3T3 and C127 cells, we found that after incubation of ΔF508CFTR-transfected CHO cells at <30 °C for 24–48 h, forskolin induced a sizable depolarization (26.2 ± 2.0 mV, $n = 19$), suggestive of elevated Cl^- permeability (Figs. 2*A* and 3*A*).[3] Similar results were obtained with IBMX alone or in combination with CTP-cAMP (Fig. 2*B*). The effect was apparent after 12 h at 28 °C and increased modestly between 24 and 48 h (Fig. 3*A*). The depolarization was independent of the nature of the extracellular cation, but was instead proportional to the transmembrane $[Cl^-]$ gradient (Fig. 2*B*). Moreover, in these temperature-shifted cells, a low conductance channel was detected after forskolin treatment by patch clamping in the cell-attached configuration (Fig. 3*B*). In 9 of 17 trials, we detected channels with conductance (8.4 pS), gating, and voltage dependence resembling those reported for CFTR and ΔF508CFTR (10, 19). In contrast, in cells kept at 37 °C the small conductance anion channel was not detected in 9 trials. These findings suggest that the biosynthetic processing block was overcome by reducing the temperature, allowing ΔF508CFTR to exit the endoplasmic reticulum, proceeding to the Golgi and subsequently to the plasma membrane. In agreement with this notion, the mature glycosylated form of ΔF508CFTR (approximate $M_r \approx 170{,}000$) was detected in immunoblots of light microsomes of temperature-shifted cells (Fig. 3*A*, *inset*).

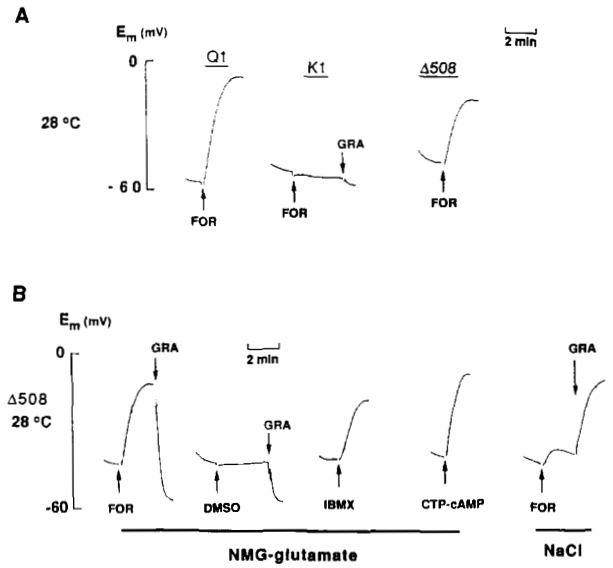

FIG. 2. **Effect of reduced temperature on the cAMP-sensitive Cl^- permeability.** *A*, Q1, K1, and Δ508 cells were grown at 28 °C for 48 h. Cl^- permeability was then estimated fluorimetrically by suspending the cells in NMG-glutamate medium at 37 °C and challenging with forskolin (20 μM) as in Fig. 1. *B*, Δ508 cells were grown at 28 °C for 48 h and resuspended at 37 °C in either NMG-glutamate or NaCl media, as indicated. Forskolin (20 μM), IBMX (0.2 mM), CTP-cAMP (0.5 mM), and gramicidin (2 μM) were added where specified. Traces are representative of at least three experiments.

As illustrated in Figs. 2*A* and 3*A*, the membrane potential and responsiveness to cAMP of cells expressing wild-type CFTR (Q1) or of untransfected (K1) or mock-transfected cells (not shown) were not significantly affected by incubation at lower temperature for up to 48 h, underlining the specificity of the effects observed in ΔF508CFTR-transfectants.

Comparison of the Functional Half-life of CFTR and ΔF508CFTR in the Plasma Membrane—The turnover of wild-type and mutant CFTR were assessed by measuring the cAMP-induced increase in Cl^- permeability after blocking protein synthesis with cycloheximide (CHX). This approach has been used successfully earlier for the estimation of half-lives of various plasma membrane and intracellular proteins (*e.g.* Ref. 30), suggesting that CHX does not interfere acutely with protein degradation and/or internalization. CHX (20 μg/ml) inhibited [^{35}S]methionine incorporation into proteins in CHO cells expressing CFTR and ΔF508CFTR by 95 and 94%, respectively (average of two experiments each with five determinations).

Despite the nearly complete inhibition of protein synthesis, the magnitude of the depolarization induced by forskolin in CFTR transfectants remained nearly constant for up to 24 h (Fig. 4*A*), suggesting that the rate of disappearance of wild-type CFTR from the membrane is comparatively slow. Similar results were obtained when the cells were kept at 24 °C for 48 h before CHX treatment (data not shown). Because the extent of depolarization is a complex function of the permeabilities and concentrations of several ions, the Cl^- conductance of these cells was estimated more directly by patch clamping in the whole cell mode. This electrophysiological approach enabled us to control the intracellular ionic composition and to

[2] Forskolin (20 μM) apparently evoked maximal activation of CFTR, since no significant difference could be detected in the extent of depolarization or whole cell current upon stimulation with forskolin alone or in combination with 0.5 mM CTP-cAMP and 0.2 mM IBMX (data not shown).

[3] The resting potential of ΔF508CFTR-transfected cells incubated at 24 °C was slightly depolarized (≈10 mV) compared to that of cells maintained at 37 °C. The mechanism underlying this depolarization is presently not understood.

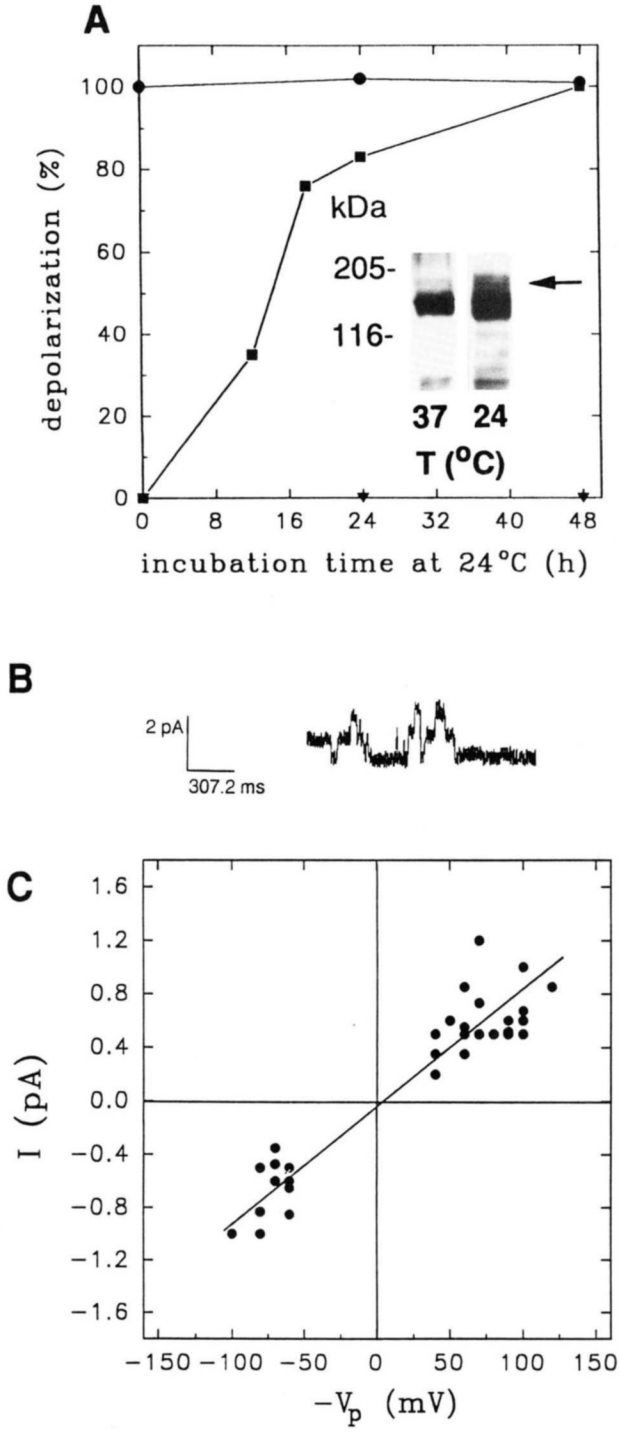

FIG. 3. *A*, expression of cAMP-dependent Cl^- conductance during incubation at 24 °C. K1 cells (*triangles*), Q1 cells (*circles*), and Δ508 cells (*squares*) were preincubated for the indicated periods at 24 °C and tested at 37 °C for protein kinase A-activated depolarization in NMG-glutamate medium as described in the legend to Fig. 2. The extent of depolarization is expressed as percentage of the depolarization detected after incubation of the cells at 24 °C for 48 h. The average values (two separate experiments) for the maximum depolarization were: K1, 0 mV; Q1, 55 mV; Δ508, 34 mV. *Inset*, immunoblot analysis using antibody M3A7 of the light microsomal fraction obtained from ΔF508CFTR expressing cells grown for 48 h at either at 37 or 24 °C. Note that the fully mature form of ΔF508CFTR (≈170 kDa; *arrow*) is detectable only at 24 °C. *B*, single channel currents recorded in the cell-attached configuration in ΔF508CFTR-expressing cells after incubation at 24 °C for 48 h, followed by treatment with forskolin (10 μM). The holding potential was −80 mV. Upward deflections indicate outward Cl^- current. *C*, current-voltage relationship of single-channel events recorded as in Fig. 3*B* in 4 cells stimulated with forskolin. The *straight line* was fitted by least squares.

estimate the magnitude and selectivity of the conductance in both the resting and stimulated states. When normalized per unit capacitance, the whole cell currents were not significantly altered by a 24-h treatment with CHX whether before or after stimulation with forskolin (Fig. 4*B*). The Cl^- selectivity of the forskolin-induced conductance was validated by assessing the effects of ionic substitution on the reversal potential (Fig. 4*C*). Reduction of extracellular [Cl^-] from 145 to 36 mM, the concentration present intracellularly, shifted the reversal potential from −36 mV to near 0 mV, as predicted. These results are in full agreement with data obtained by membrane potential measurements and support the notion the CFTR has a long residence time in the plasma membrane of CFTR-expressing CHO cells. Similar observations were made with CaCo-2 cells, an intestinal cell line that expresses CFTR at a level comparable to that of CHO-Q1 cells, as determined by immunoblotting (Fig. 5, *top panel*). After treatment with CHX for 24 h, the forskolin-induced depolarization in low Cl^- medium was not significantly impaired (91.3 ± 2.5%, $n = 7$; Fig. 5, *middle panel*). These data imply that the relatively long half-life of CFTR is not a consequence of heterologous expression in CHO cells, but is rather a property of CFTR itself, at least in some cell types.

Insertion of newly synthesized proteins into the plasma membrane can alternatively be inhibited by preventing their exit from the endoplasmic reticulum and the Golgi using the fungal metabolite brefeldin A (BFA, Ref. 31). Treatment with BFA for 24 h, at concentrations (5 μg/ml) reported to disrupt the integrity of the Golgi complex in several cell lines and to inhibit protein secretion by >85% (32, 33), did not decrease the magnitude of the protein kinase A-activated depolarization in CFTR expressing CHO (not shown) cells or in CaCo-2 cells (Fig. 5, *bottom panel*). The effectiveness of BFA was verified by detecting the distribution of mannosidase II, a marker of the Golgi apparatus, by immunofluorescence. In CFTR expressing CHO cells and CaCo-2 cells, the perinuclear accumulation of mannosidase II, characteristic of Golgi localization, was eliminated by treatment with BFA, being replaced by a diffuse distribution characteristic of endoplasmic reticulum (not illustrated). The similarity of the results obtained with CHX and BFA supports the notion that, in CHO and CaCo-2 cells, the functional half-life of CFTR at the plasma membrane is greater than 24 h.

Next, the half-life of ΔF508CFTR was determined. The protein was first allowed to accumulate at the plasma membrane by growing the cells at reduced temperature, as above. The temperature was then raised to 37 °C and CHX was added to inhibit further synthesis. At increasing time intervals, the protein kinase A-sensitive Cl^- permeability was determined fluorimetrically (Fig. 4*D*). As illustrated in Fig. 4*A*, the forskolin induced depolarization disappeared rapidly when further synthesis and insertion were precluded. After 8 h, the response was 10% of that recorded in control cells, kept at reduced temperature throughout this period to promote continued insertion (Fig. 4, *A* and *D*). From four similar experiments we conclude that the functional half-life of ΔF508CFTR in the membrane of CHO cells is ≤4 h. This is significantly shorter than the value determined above for wild-type CFTR under comparable experimental conditions.

The inability of ΔF508CFTR to reach the membrane at 37 °C enabled us to assess its turnover without reliance on

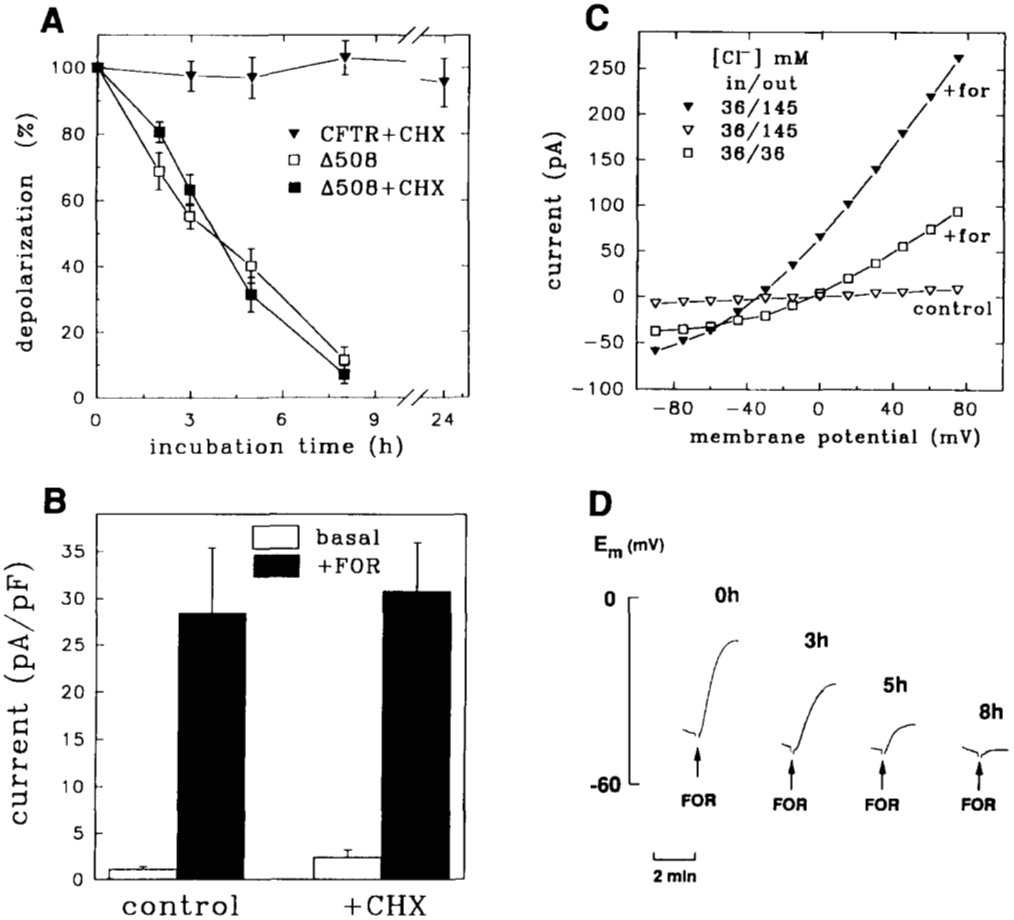

FIG. 4. *A*, comparison of the functional stability of CFTR and ΔF508CFTR in the plasma membrane. Determination of functional half-lives by fluorimetric measurement of the forskolin-induced depolarization in NMG-glutamate (as in Fig. 2). *Triangles*, CFTR-expressing (Q1) cells were incubated with 20 μg/ml CHX for the periods indicated and then challenged with forskolin. *Squares*, ΔF508CFTR-expressing cells were grown at 24–28 °C for 24–36 h to accumulate plasmalemmal ΔF508CFTR. The cells were then transferred to 37 °C in the presence (*solid squares*) or absence (*open squares*) of CHX (20 μg/ml) for the periods specified in the *abscissa*. Cl^- permeability was finally determined from the forskolin-induced depolarization. *B*, protein kinase A-mediated current activation in CFTR-expressing (Q1) cells before and after CHX treatment. Cells were patch-clamped in the whole cell mode as described under "Materials and Methods." Where indicated CHX (20 μg/ml) was present for 24 h. Current values were averaged from 8–9 cells before (basal) and after forskolin (20 μM, *+FOR*) stimulation at +75 mV holding potential. *C*, whole cell currents recorded from a Q1 cell incubated for 24 h with CHX (20 μg/ml). *Open triangles*, unstimulated cell, extracellular $[Cl^-]$ = 145 mM. *Solid triangles*, forskolin-stimulated cell, extracellular $[Cl^-]$ = 145 mM. *Open squares*, forskolin-stimulated cell, extracellular $[Cl^-]$ = 36 mM. Pipette (intracellular) $[Cl^-]$ = 36 mM. Data are representative of 3–8 determinations. *D*, temperature shift induces the rapid disappearance of the cAMP-activated Cl^- permeability in ΔF508CFTR cells. Plasmalemmal accumulation of ΔF508CFTR was accomplished by incubation at 28 °C for 24–36 h. The cAMP-sensitive Cl^- permeability was then measured as in Fig. 2 at the indicated times after shifting the temperature to 37 °C. Representative of three to four experiments.

pharmacological agents (*i.e.* CHX). First ΔF508CFTR was allowed to accumulate in the membrane at reduced temperature. Next, the temperature was raised to 37 °C and membrane potential was monitored. Internalization and/or degradation of the protein without concomitant insertion resulted in a progressive rapid loss of activity (Fig. 4*A*). Importantly, the rate of disappearance was indistinguishable from that noted when CHX was present (Fig. 4*A*). This implies that CHX did not appreciably alter endocytosis or proteolysis acutely, validating the measurements obtained in wild-type CFTR-transfectants and CaCo-2 cells. The results also indicate that at 37 °C the rate of delivery of ΔF508CFTR to the membrane is much slower than the rate of retrieval.

To our knowledge, the stability of the mature glycosylated ΔF508CFTR has only been estimated by Denning *et al.* (18). Their results suggested that the total pool of mature ΔF508CFTR has a half-life of 7 h, a value greater than estimated for ΔF508CFTR at the plasma membrane (4 h). In contrast, the turnover of wild-type CFTR reported here is somewhat slower than the values described by others using immunoprecipitation of the protein or monitoring its function in cells treated with antisense oligonucleotides (7, 34, 35). Wagner *et al.* (34) reported that 92% of CFTR is still present 12 h after addition of antisense oligonucleotides to T84 cells and Sorscher *et al.* (35) observed no significant decrease in CFTR activity after 4 h in sweat duct cells. While precise quantitation was not provided, Cheng *et al.* (7) indicated that CFTR was clearly detectable 8 h after radiolabeling and chasing. The basis of this apparent discrepancy is not immediately obvious, but differences in the cell types or transfection methods used could be responsible. We feel that differences in the detection methods used are less likely to be involved, since pulse-chase experiments followed by immunoprecipitation, a method similar to that used by Cheng *et al.* (7), yielded half-lives in excess of 12 h for CHO cells (CHO-

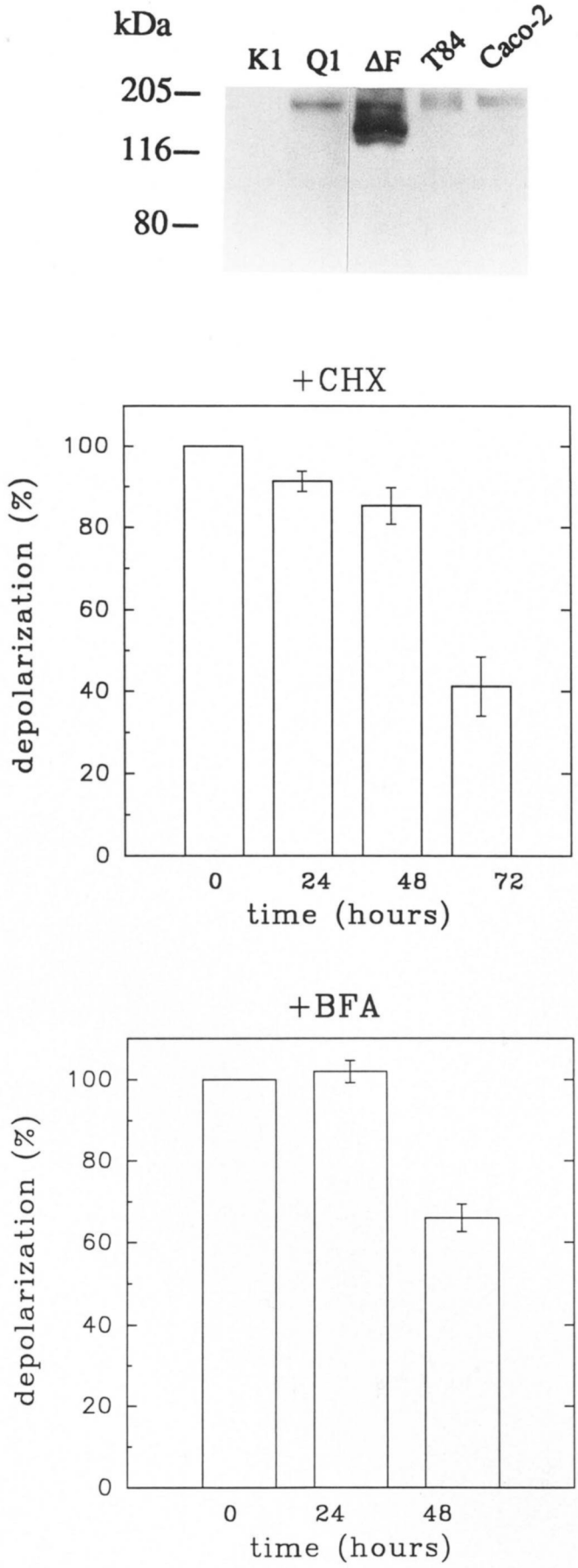

FIG. 5. *Top panel*, expression levels of CFTR in different cell lines. Whole cells were lysed with RIPA buffer and the extracts were subjected to SDS-polyacrylamide gel electrophoresis (7.5%). After transferring to nitrocellulose, proteins were blotted with the anti-CFTR monoclonal antibody M3A7. Blots were developed with ECL (Amersham) using a secondary goat anti-mouse antibody labeled with horseradish peroxidase. Lanes labeled *K1*, *Q1*, *CaCo-2*, and *ΔF* were loaded with 50 μg of protein from CHO-K1, CHO-Q1, CaCo-2 cells, and ΔF508CFTR-transfected cells kept at 24 °C for 48 h, respectively. The remaining lane was loaded with 25 μg of protein from T84 cells. *Middle* and *bottom panels*, effect of CHX (*middle*) and BFA (*bottom*) on the forskolin-induced depolarization of CaCo-2 cells. CaCo-2 cells were treated for the indicated time with either CHX (20 μg/ml) or BFA (5 μg/ml). After trypsinization and washing, cells were suspended in NMG-glutamate medium. The cAMP-sensitive Cl conductance was then determined by monitoring the membrane potential changes induced by addition of forskolin (20 μM) or CTP-cAMP (0.5 mM) plus IBMX (0.2 mM), as described in the legend to Fig. 2. The extent of depolarization is expressed as percentage of the depolarization detected in the absence of the drugs.

Q2 cells)[4] transfected with wild-type CFTR.[5]

In principle, it is conceivable that the long functional half-life of CFTR following inhibition of protein synthesis is the result of continued replenishment of the plasmalemmal pool by delivery of CFTR from the endoplasmic reticulum and/or Golgi compartments. However, this possibility is unlikely since the CFTR pool in the endoplasmic reticulum and Golgi is relatively small. We could not detect significant amounts of nonglycosylated or core-glycosylated CFTR in immunoblots of whole cell extracts of CHO-Q1 and CaCo-2 cells (Fig. 5, *top panel*).

Moreover the transit time of CFTR through the biosynthetic pathway, determined by pulse-chase experiments using [^{35}S]methionine and immunoprecipitation, is fast (≤2.5 h; see also Ref.7) and therefore unable to account for continued delivery after inhibition of protein synthesis. Finally, impairment of CFTR delivery from the reticulum and Golgi to the membrane using brefeldin A yielded results comparable to those obtained with cycloheximide. Nevertheless, our data cannot distinguish between long residence time at the plasma membrane and effective recycling from an endocytic compartment back to the membrane, nor can they rule out the existence of a sizable post-Golgi storage compartment. In any case, the behavior of these pathways would be different in wild-type and ΔF508CFTR, accounting for the differential functional stability of the two proteins.

In summary, our data suggest that after reaching the plasma membrane, ΔF508CFTR turns over more rapidly than its wild-type counterpart at 37 °C, possibly due to changes in its structure and stability, similar to those reported for synthetic peptides corresponding to a region of the N-terminal nucleotide binding domain including residue 508 (36). Three mechanisms could account for the decreased residence time of ΔF508CFTR. First, the mutant protein may be internalized more rapidly. Second, internalized CFTR may recycle back to the membrane more effectively than ΔF508CFTR either through the endosomal compartment, the trans-Golgi network, or both. In this regard, CFTR has been suggested to play a role in accelerating exocytosis of endomembranes (37). Finally, ΔF508CFTR may be degraded (or inactivated) more readily, either by accelerated routing to lysosomes or by increased exposure to cytosolic proteases. Further experiments are required to define the underlying mechanism.

The present findings imply that therapeutic interventions intended solely to increase delivery of ΔF508CFTR to the plasmalemma may be ineffective, if internalization/inactiva-

[4] The CHO-Q2 cell line was used because it has an expression level of CFTR that is higher than that of CHO-Q1 cells.

[5] S. Pind, J. Riordan, and D. Williams, unpublished observations.

tion of the mutant protein occurs relatively rapidly. Additional measures may be necessary to prolong the residence time of the protein at the plasma membrane.

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