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Cystic Fibrosis: Channel, Catalytic, and Folding Properties of the CFTR Protein

Fabian S. Seibert,[2] Tip W. Loo,[2] David M. Clarke,[2] and John R. Riordan[1,3]

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The identification and characterization of the CFTR gene and protein have provided not only a major impetus to the dissection of the molecular pathophysiology of cystic fibrosis (CF) but also a new perspective on the structure and function of the large superfamily of membrane transport proteins to which it belongs. While the mechanism of the active vectorial translocation of many hydrophobic substrates by several of these transporters remains nearly as perplexing as it has for several decades, considerable insight has been gained into the control of the bidirectional permeation of chloride ions through a single CFTR channel by the phosphorylation of the R-domain and ATP interactions at the two nucleotide binding domains. However, details of these catalytic and allosteric mechanisms remain to be elucidated and await the replacement of two-dimensional conceptualizations with three dimensional structure information. Secondary and tertiary structure determination is required both for the understanding of the mechanism of action of the molecule and to enable a more complete appreciation of the misfolding and misprocessing of mutant CFTR molecules. This is the primary cause of the disease in the majority of the patients and hence understanding the details of the cotranslational interactions with multiple molecular chaperones, the ubiquitin-proteasome pathway and other components of the quality control machinery at the endoplasmic reticulum could provide a basis for the development of new therapeutic interventions.

KEY WORDS: Cystic fibrosis; CFTR; chloride channel; phosphorylation ATPase; protein folding; proteolysis; proteasome; ER quality control.

INTRODUCTION

Cystic fibrosis (CF) is the most common fatal genetic disease among Caucasians with an incidence of one in every 2000 live births (Quinton, 1990). Every twentieth person is a carrier of the disorder, but heterozygotes are asymptomatic. CF has been categorized as a polyexocrinopathy (Davis and di Sant' Agnese, 1980) because it affects a multitude of exocrine organs, including the airways, pancreas, sweat ducts, reproductive tract, salivary glands, duodenum, and liver. With the exception of sweat ducts, the recurring theme in all organs is obstruction of passages by mucus, which inflicts such problems as malnutrition due to the inability to release pancreatic enzymes and infertility because Wolffian ducts and the cervical os are plugged (Boat *et al.*, 1989). Most of these complications are well controlled by modern medicine, but one fatal problem remains—the occurrence of persistent bacterial infections in CF lungs concurrent with the mucus build-up. The associated immune response leads to parenchymal destruction and progressive loss of lung function (Birrer, 1995). As a result, respiratory tract disease accounts for three quarters of all hospital admissions and more than 90% of the morbidity and mortality in CF (Penketh *et al.*, 1987).

[1] Mayo Graduate School of Medicine and Department of Biochemistry and Molecular Biology, S. C. Johnson Medical Research Center, Mayo Clinic, Scottsdale, Arizona 85259.
[2] Medical Research Council Group in Membrane Biology, Departments of Medicine and Biochemistry, University of Toronto, Toronto, Ontario, Canada, M5S 1A8.
[3] To whom correspondence should be addressed.

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CF is inherited in an autosomally recessive fashion, so that the disease should result from alteration(s) in a single gene product. However, the wide range of associated symptoms made it difficult to pinpoint an underlying biochemical defect which would guide in the search for the gene mutated in CF patients. In fact, after many years of results which seemed difficult to reconcile, the affected gene was identified by positional cloning, rather than biochemical techniques (Rommens *et al.*, 1989; Riordan *et al.*, 1989; Kerem *et al.*, 1989). From its primary amino acid sequence the gene product, named the cystic fibrosis transmembrane conductance regulator (CFTR), was suggested (Riordan *et al.*, 1989) and largely confirmed (Cheng *et al.*, 1990; Denning *et al.*, 1992a,b; Chang *et al.*, 1994, 1995; Chen and Zhang, 1996) to consist of two structurally similar halves. Each half contains six hydrophobic transmembrane helices (TMs) and ends in a C-terminal cytoplasmic nucleotide binding fold (NBF), with Walker A and Walker B motifs (Walker *et al.*, 1982) for possible interaction with ATP. The two parts are interconnected by a highly charged regulatory domain or R-domain, rich in consensus sequences for phosphorylation by the cAMP-dependent protein kinase (PKA) and protein kinase C (PKC). On the cytoplasmically exposed side of the protein the TMs are linked to each other by cytoplasmic loops (CLs), which vary between 55 and 65 amino acids in length. Very little of the protein appears to be exposed at the exterior surface, except the regions between TMs 1 and 2 and between TMs 7 and 8, with the later containing two N-linked glycosylation sites.

Thus, structurally CFTR resembles members of the ABC Superfamily of transporters (Doige and Ames, 1993), but also functionally, because the protein was found to be regulated by hormonally induced phosphorylation (Tabcharani *et al.*, 1991) as well as cellular energy levels (Anderson *et al.*, 1991a). However, further investigations proved that CFTR thus far is the only member of this Superfamily that adapted the transporter motif to act as an ion channel (Drumm *et al.*, 1990; Rich *et al.*, 1990; Anderson *et al.*, 1991b; Bear *et al.*, 1992). In addition to its role in anion translocation, the molecule has also been implicated in the regulation of an epithelial amiloride-sensitive sodium channels (Stutts *et al.*, 1995; Ismailov *et al.*, 1996), an outwardly rectifying chloride channel (Egan *et al.*, 1992; Schwiebert *et al.*, 1995), and several other channels (Loussouarn *et al.*, 1996; Mall *et al.*, 1996; McNicholas *et al.*, 1996; Zhang *et al.*, 1996).

To date, more than 500 gene alterations have been observed in the CFTR of CF patients (Tsui, 1997). Some mutations may affect any of the additional proposed functions of the molecule, but most impact the chloride channel in one way or another. Amino acid substitutions in the TMs were observed to modify channel conductance (Sheppard *et al.*, 1993), whereas mutations in the NBFs and CL3 affect its regulation (Drumm *et al.*, 1991; Anderson and Welsh, 1992; Seibert *et al.*, 1996a). In addition, many gene alterations, including the most common ΔF508 deletion, induce mis-processing of CFTR (Cheng *et al.*, 1990; Gregory *et al.*, 1991; Kartner *et al.*, 1992; Champigny *et al.*, 1995; Sheppard *et al.*, 1995; Cotten *et al.*, 1996; Seibert *et al.*, 1996b) or destabilize the mRNA (Hamosh *et al.*, 1991) so that the channel never reaches its site of action. Therefore, in an attempt to tackle CF, detailed understanding of CFTR's channel properties, its gating, and its maturation will be invaluable. The present paper attempts to provide a small glimpse of what is known about these parameters of the CFTR protein.

THE CFTR ANION CHANNEL

Chloride channel properties of CFTR have been studied in cells which endogenously express the protein, as well as in various heterologous expression systems and in its reconstituted form (Cliff and Frizzel, 1990; Tabcharani *et al.*, 1990; Anderson *et al.*, 1991a; Bear *et al.*, 1991; Kartner *et al.*, 1991; Bear *et al.*, 1992; Haws *et al.*, 1992; Gray *et al.*, 1993; Becq *et al.*, 1993; Gunderson and Kopito, 1994; Hanrahan *et al.*, 1994; Xie *et al.*, 1995). CFTR was found to be activated by cAMP in the whole-cell configuration (Rich *et al.*, 1990) and by the catalytic subunit of PKA in the presence of ATP in the excised patch configuration (Tabcharani *et al.*, 1991). Typical for CFTR are its loosely coordinated gating and flickery kinetics at hyperpolarizing potentials on-cell (but not in the excised state). CFTR is insensitive to chloride channel blockers including DIDS, IAA-94 (Tabcharani *et al.*, 1990; Kartner *et al.*, 1991), and DNDS (Haws *et al.*, 1992) when applied to the extracellular side of the protein, although cytoplasmic DIDS and DNDS can block the channel in a voltage-dependent manner (Linsdell and Hanrahan, 1996a). The protein is responsive to extracellular and intracellular DPC (Anderson *et al.*, 1991a; McDonough *et al.*, 1994) and is significantly inhibited by the sulfonylureas, glibenclamide and tolbutamide (Sheppard and Welsh, 1992; Schultz

et al., 1996a; Venglarik *et al.*, 1996). CFTR channels show slight outward rectification of the current–voltage relationship during on-cell recordings, but are nonrectifying upon patch excision into symmetrical chloride concentrations (Cliff and Frizzell, 1990; Kartner *et al.*, 1991). The outward rectification on-cell has recently been attributed to a fast, voltage-dependent block by intracellular large anions and osmolytes (Linsdell and Hanrahan, 1996b). Collectively, it was found that the channel has a conductance between 6 and 11 pS, depending on the applied cell systems, recording temperatures, and ion concentrations. Thus far unexplained are several conductance substates, which appear to occur more frequently in reconstitution systems than in excised patches. The stability of the various conductance substates is influenced by the ionic composition of surrounding solutions (Tao *et al.*, 1996). The calculated K_m for CFTR is 35–40 mM chloride. Estimates about the selectivity of CFTR for different anions initially were conflicting and dependent completely on the laboratory in which they were measured. The issue was eventually resolved when Tabcharani *et al.* (1992) demonstrated that iodide blocks the pore, so that the iodide : chloride permeability ratio is >1 before the block and <1 after the block takes place. Consistently these two anions are more permeable than bromide or fluoride. The use of various sized anions and measurement of their permeability suggested that CFTR has a pore diameter of approximately 0.6 nm (Linsdell and Hanrahan, 1996a).

Tabcharani *et al.* (1993) provided elegant evidence that CFTR functions as a multi-ion pore. If channels translocate several ions simultaneously, the destabilization resulting from ion–ion repulsions allows rapid ion movement despite high affinity binding. Thus, in solutions containing two permeant ions, conductance of a single-ion pore should increase linearly as the mole fraction of the more permeant species is increased, whereas conductance of a multi-ion pore may decrease relative to results for pure solutions of either ion (Tabcharani *et al.*, 1993). This anomalous mole fraction effect can be observed for CFTR, but is abolished if residue Arg 347 is mutated to Asp, suggesting that this site is involved in the interaction with permeant anions. If Arg 347 is mutated to His, the anomalous mole fraction behavior can be turned on and off, simply by changing the pH of the bath solutions (the imidazole group of histidine is positively charged at low pH and uncharged at high pH) (Tabcharani *et al.*, 1993). The results could not be confirmed by Hipper *et al.* (1995).

Thus, the "functional pore" of the CFTR channel has been well characterized, but little is known about the "structural pore." CF-associated as well as novel mutations in the TMs have been shown to modify CFTR's ion selectivity (Anderson *et al.*, 1991b), its conductance (Sheppard *et al.*, 1993), and its interaction with permeant anions (Tabcharani *et al.*, 1993) and inhibitors (McDonough *et al.*, 1994), so that it is fairly well established that the TMs contribute to the ion conductive pathway. Interestingly, the CLs which connect the TMs on the intracellular side of the protein and therefore might be part of the pore-forming unit, appear not to be directly involved in the anion movement as CF-associated amino acid substitutions and deletions within the CLs have little effect on the conductance of CFTR, but rather modify its response to regulatory stimuli and the stability of conductance substates (Xie *et al.*, 1995, 1996; Cotten *et al.*, 1996; Seibert *et al.*, 1996a,b). In contrast, residues in the extracellular loop 1 (EL1) influence the selectivity of the channel. When the three residues that are different in EL1 between human CFTR and *Xenopus* CFTR are changed to the *Xenopus* versions in the human background molecule, the altered human CFTR shows selectivity characteristics of *Xenopus* CFTR (Price *et al.*, 1996).

The question of which residues within the TMs actually face the pore was addressed by accessibility studies of introduced cysteines to sulfhydryl-specific methanethiosulfonate reagents (Akabas *et al.*, 1994; Cheung and Akabas, 1996) and DPC blockade (McDonough *et al.*, 1994) for TMs 1, 6, and 12. Similar studies can and most likely will be performed for the remaining TMs, but they will only indicate which residues of the TMs are exposed on the water-accessible surface of the channel, without giving any information about the pore arrangement. Within the boundaries of these findings, various configurations of the channel can be envisioned, such as a dodeca-helical pore, two separate hexa-helical arrangements of each half, or even proximal interactions between TMs of the two homologous halves of the molecule. Cross-linking studies similar to those performed on P-glycoprotein could give further insights (Loo and Clarke, 1996b); however, elucidation of exact arrangements will have to await the eventual application of 3-D analysis to membrane proteins.

Very little evidence has been presented regarding multimeric requirements for CFTR activity, although Marshall *et al.* (1994) believe that the minimal functional unit is a monomer. This would be in agreement

with findings for the related P-glycoprotein (Loo and Clarke, 1996a). It is conceivable that the two homologous halves may indicate that CFTR is a dimer within itself. In fact, when only the amino-terminal half of CFTR is expressed, it occasionally forms a chloride channel with conductance properties comparable to full-length CFTR (Sheppard *et al.*, 1994; Ostedgaard *et al.*, 1997), as does a renal CFTR isoform that terminates C-terminally to the R-domain (Morales *et al.*, 1996). Migration on sucrose density gradients suggests that such half-proteins act as multimers (Sheppard *et al.*, 1994). The N- and C-terminal transmembrane domains were seen to associate in co-precipitation experiments (Ostedgaard *et al.*, 1997).

CATALYTIC PROPERTIES OF CFTR

From the primary amino acid sequence it was apparent that CFTR contains Walker A (G-X-X-G-X-G-K-T/S) and B (R/K-X_{7-8}-h_4-D) motifs (Walker *et al.*, 1982) in each NBF in addition to ten perfect dibasic consensus sites for potential phosphorylation by PKA (R/K-R/K-X-S*/T*; asterisk indicates potential site of phosphorylation; Kennelly and Krebs, 1991), nine of which are clustered within the central R-domain. This molecule therefore was highly likely to be regulated by ATP binding/hydrolysis and/or phosphorylation/dephosphorylation. Indeed, subsequent studies demonstrated that both modes of regulation occur and that one cannot activate the channel without the other.

In the whole-cell system, phosphorylation and dephosphorylation appear to serve as the molecular switch to gate the channel. If recombinant CFTR is expressed in epithelial or nonepithelial cells, chloride channel activity that is absent in control cells can be evoked in all cases by treatment with cAMP agonists (Drumm *et al.*, 1990; Gregory *et al.*, 1990; Anderson *et al.*, 1991a; Kartner *et al.*, 1991). That the cAMP-mediated effect occurs via PKA was seen by direct activation through PKA injection into CFTR expressing oocytes (Bear *et al.*, 1991), as well as by single-channel patch-clamping: cAMP-induced channel activity, observed in the on-cell recording mode, runs down upon patch excision, presumably due to the presence of membrane-associated phosphatases. If PKA is added to the cytosolic bath of the inside-out membrane patch, chloride channel activity is regained in the presence of ATP, which can be further elevated with nonspecific phosphatase inhibitors and almost completely eliminated by alkaline phosphatase (Tabcharani *et al.*, 1991).

However, cAMP cannot stimulate transepithelial anion conductance in ATP-depleted cells. Only when ATP levels are raised close to intracellular physiological levels (5 mM; Williamson and Corkey, 1969) is the cAMP response restored (Bell and Quinton, 1993). Similarly, kinetic analysis by Venglarik *et al.* (1994) and Winter *et al.* (1994) showed that the P_0 of CFTR in excised inside-out patches increases with increasing levels of ATP concentrations (0.1–3 mM). Both effects are most likely due to direct interactions of ATP with CFTR rather than due to invoked phosphorylation because they occur at ATP concentrations which are too high to be mediated by PKA which has a K_m for ATP of ~7 μM (Sugden, *et al.*, 1976). In fact, initial proof that ATP is required in the gating of CFTR for tasks other than as a phosphorylation substrate was provided by Anderson *et al.* (1991c) who were able to maintain CFTR in a phosphorylated state in excised patches so that the effects of ATP on regulation could be observed in isolation. This showed that phosphorylation by PKA is necessary but not sufficient to open the channel. Once phosphorylated, channels require cytosolic ATP for opening. Phosphorylated CFTR channels close if the ATP is removed but can be reactivated by re-adding ATP. However, addition of ATP alone does not open the channel.

Initial indications that the ATP-interacting domain of CFTR is indeed an NBF came from the observation that a synthetic peptide, corresponding to the central 67 amino acids of NBF1, is bound by numerous adenine nucleotides (Thomas *et al.*, 1991). This observation was extended to recombinant NBF1 (Hartman *et al.*, 1992; Ko *et al.*, 1993) and eventually to full-length CFTR (Travis *et al.*, 1993). On full-length CFTR the photoactivatable ATP-analog 8-azidoadenosine 5′-triphosphate (8-N_3ATP), which substitutes for ATP in the activation of CFTR and therefore interacts at the active site, shows half-maximal labeling at 10 μM in the presence of magnesium and at 100 μM in the absence of magnesium. ATP is able to prevent photolabeling with half-maximal inhibition at 1 mM, a concentration which is comparable with the concentration of ATP required for chloride channel activity in sweat duct or T84 cells (Quinton and Reddy, 1992; Bell and Quinton, 1993). ADP inhibits at half-maximal concentrations of 10 mM, while AMP cannot inhibit. The study of Travis *et al.* (1993) was not able to differentiate whether binding occurs at one or both NBFs. However, similarly to NBF1 peptides, NBF2

peptides (synthetic and recombinant) can also be bound by adenine nucleotides (Ko *et al.*, 1994; Randak *et al.*, 1995).

Is ATP binding at the NBFs sufficient for the activation of CFTR, or is the high energy phosphobond of the molecule utilized as an energy source? ATP hydrolysis appears to be necessary for CFTR activation since channel opening cannot be initiated by nonhydrolyzable ATP analogues such as ATPγS or magnesium-free ATP (Anderson *et al.*, 1991c). ATPγS is often found to serve as an adequate substrate for PKA-mediated phosphorylation, but not for ATPase reactions. Reddy and Quinton (1996b) refined these findings, showing that both hydrolytic and nonhydrolytic interactions regulate CFTR *in vivo*. Subsequent to initial hydrolytic activation, nonhydrolyzable analogues can activate CFTR in the absence of ATP. It was suggested that ATP hydrolysis may be required to induce a conformational change in CFTR that involves the conversion of a deactivated state to a thermodynamically unfavorable activated state in which allosteric nonhydrolytic ATP binding then stabilizes activated CFTR. The two steps could involve the two NBFs (Reddy and Quinton, 1996b).

Although ATP hydrolysis is required, it has proven very difficult to demonstrate ATPase activity of the NBFs biochemically. Many attempts have failed (Ko *et al.*, 1994), until Ko and Pedersen (1995) were able to observe ATPase activity of a fusion NBF1 protein, stabilized by the presence of the maltose binding protein. The observed V_{max} of ~30 nmol/mg/min is very low compared to other ATPases such as the Ca^{++}-ATPase (600 nmol/mg/min; Racker, 1985) or P-glycoprotein (300–1650 nmol/mg/min; Sharom *et al.*, 1995), although an indication of the significance of the data is given by a negative effect due to Walker A mutations K464H and K464L. Recently, Li *et al.* (1996) confirmed low-level ATPase activity of reconstituted CFTR with an ATP turnover number of 1–2/sec/CFTR in the presence of 1 mM ATP. Interestingly, since none of the major ATPase inhibitors thus far have been successful in the inhibition of full-length CFTR (Schultz *et al.*, 1996b), the mechanisms of ATP hydrolysis by CFTR may not comply with known pathways utilized by other ATPases.

Initial functional indications that both NBFs may be involved in the regulation of the channel came from single-channel studies of CF-associated and novel mutations which are predicted to lie within these domains. Mutations in the Walker A lysine of either NBF1 or NBF2 decrease the potency with which Mg ATP stimulates the channel (Anderson and Welsh, 1992). Furthermore, substitutions for the conserved glycine and aspartate in the Walker motifs of NBF1 produce a discernible reduction in the sensitivity of CFTR to activating conditions. In contrast, analogous mutations in NBF2 have the opposite effect, actually increasing sensitivity over that seen with wild-type CFTR (Smit *et al.*, 1993).

Building on these concepts, Hwang *et al.* (1994) utilized whole-cell and single-channel measurements to show that a nonhydrolyzable analog of ATP, 5′-adenosine(β,γ-imino)triphosphate (AMP-PNP), cannot activate CFTR by itself, but when added to CFTR that is already activated by ATP does prolong this open state. The "locking open" only is possible for fully PKA-phosphorylated channels, but not for partially phosphorylated channels for which the low level of phosphorylation is achieved with the phosphatase inhibitor okadaic acid or continued run-down (Hwang *et al.*, 1993). Delineating the various functional states, a model was proposed in which ATP hydrolysis at one NBF (NBFA) opens the channel. CFTR rapidly closes, unless full phosphorylation and ATP binding at the second NBF (NBFB) induce stability of the open state. Upon ATP hydrolysis at NBFB (which is not possible if AMP-PNP is bound), CFTR closes. One might expect that AMP-PNP also should interact with NBFA to increase the frequency of long closures between bursts. The fact that this is not observed is interpreted as a reflection of different affinities of the two NBFs for AMP-PNP. In agreement with AMP-PNP findings, nonhydrolyzable pyrophosphate also prolongs channel openings in the presence of ATP (Carson *et al.*, 1995a).

Evidence that the proposed NBFA of Hwang *et al.* (1994) corresponds to NBF1 and the proposed NBFB corresponds to NBF2 came from mutagenesis studies which showed that alteration of the conserved Walker A lysine in NBF1 decreases the frequency of bursts in single-channel tracings whereas parallel mutations in NBF2 and mutations in both NBFs simultaneously prolong bursts of activity, as well as decrease the frequency of bursts (Carson *et al.*, 1995b). None of the mutations alter binding profiles of 8-N_3ATP. Careful rate analysis of transition steps between the various states substantiated the conclusions, but also demonstrated that the two NBFs have some overlap in function, with NBF1 mutations somewhat destabilizing the open state and NBF2 mutations somewhat decreasing the access rate of the open state (Wilkinson *et al.*, 1996). It thus appears that there must be a significant amount of interaction between the two domains.

When studying catalytic properties of CFTR, one has to remember that phosphorylation and dephosphorylation of CFTR determine whether the channel can open due to ATP catalysis or not. Widespread disagreement remains regarding the identity of the dephosphorylating phosphatase(s), which in fact may be cell type specific (Becq *et al.* 1993; Berger *et al.*, 1993; Hwang *et al.*, 1993; Fischer *et al.*, 1995; Reddy and Quinton, 1996a). On the other end of the spectrum PKA has uniformly been reported as the most potent activator of CFTR identified thus far, although the underlying molecular events are poorly understood. *In vitro* phosphorylation by PKA occurs to a stoichiometry of ~5–6 mol of phosphate per mole of CFTR, with all labeling residing within the R-domain, mostly on six of the nine dibasic consensus sites (Picciotto *et al.*, 1992; Dulhanty and Riordan, 1994a). In agreement, Cheng *et al.* (1990) found that *in vivo* serines 660, 737, 795, and 813 are highly PKA-labeled whereas additional unidentified residues account for only a small amount phosphate labeling. Interestingly, mutation of one to three of the four major sites to alanines has little effect on CFTR function, whereas removal of all four sites (4SA-CFTR) strongly reduces activation by PKA (Cheng *et al.*, 1990). Thus, regulation of CFTR is degenerate, i.e., more than one site is normally involved, but no one site is essential. Surprisingly, even simultaneous removal of all ten dibasic PKA consensus sites (10SA-CFTR) still allows activation by the kinase to at least 30% of wild-type levels (Chang *et al.*, 1993; Rich *et al.*, 1993). The residual responsiveness of 10SA-CFTR in part is mediated by minute levels of phosphorylation on the monobasic PKA consensus site Ser 753 (Seibert *et al.*, 1995). Note that mutagenesis of phosphorylation sites decreases the channels' open probability (P_0) without affecting their conductance or ion selectivity. Larger decreases in the P_0 as more phosphorylation sites are removed, lower activation of partially phosphorylated channels (Hwang *et al.*, 1994), and the finding that increased levels of cAMP (Drumm *et al.*, 1991) elicit higher whole-cell anion conductances indicate that a multiple site mechanism is employed to allow a graded response to hormonal stimulation, rather than a single site switch. The degenerate nature of this mechanism may also be reflected by the absence of CF-causing mutations in phosphorylation sites (Tsui *et al.*, 1997).

Importantly, CFTR contains numerous consensus sites for potential interaction with PKC. *In vitro*, PKC incorporates approximately 2 mol of phosphate per mole of CFTR (Picciotto *et al.*, 1992). In fact, recent studies demonstrated that basal PKC labeling is essential to allow the regulation via phosphorylation by PKA (Dechecchi *et al.*, 1993; Jia *et al.*, 1997). However, no information is available regarding the underlying mechanism or the functional sites involved. Additional kinases that were reported to modulate the function of CFTR are the tyrosine kinase $p60^{c\text{-}src}$ (Fischer and Machen, 1996) and the cGMP-dependent protein kinase (French *et al.*, 1995; Vaandrager *et al.*, 1997).

How does the R-domain regulate CFTR activity? The level of phosphorylation appears to determine the likelihood that a channel can open and in the absence of phosphorylation CFTR cannot be stimulated. That the channel can be closed by a recombinant unphosphorylated R-domain protein, but not by the phosphorylated form (Ma *et al.*, 1996), reinforces that phosphorylation is the important step in relieving the closed state. Furthermore, constitutive activity of an 8SD mutant (Rich *et al.*, 1993) indicates that the negative charge of the introduced phosphates plays some role. However, charge introduction alone may not be sufficient since the 8SD mutant is still less active than phosphorylated wild-type CFTR (Gadsby and Nairn, 1994). Both Pichiotto *et al.* (1992) and Dulhanty and Riordan (1994a) observed a mobility shift in the electrophoresis of phosphorylated R-domain which appears to be the result of a conformational change. In fact, this conformational change can be readily detected by C.D. spectroscopy (Dulhanty and Riordan, 1994a) and may be necessary to move the whole molecule into an activation-competent conformation. The need for charge introduction and conformational change are not mutually exclusive possibilities and may both prove to be important. In search for a functional model, deletion of a large part of the R-domain (Δ708-835) renders CFTR constitutively open in the presence of ATP (Rich *et al.*, 1991; Anderson *et al.*, 1991c). Therefore, the R-domain could be thought to inhibit channel activity like a cork that sits in the bottle neck and blocks flux; however, in such an analogy the observation is overlooked that CFTR with the R-domain deleted stays closed until ATP is added (Anderson *et al.*, 1991c), indicating that this domain is not just "blocking" the channel. In fact, two recent observations suggest that the unphosphorylated R-domain does not block access to the channel, but more likely to the NBFs, or that phosphorylated R-domain promotes ATP binding at the NBFs: (i) ATPase activity of reconstituted CFTR is elevated 2- to 3-fold in the presence of PKA by decreasing the K_m for this activation from 1 to 0.3 mM without increasing the V_{max} (Li *et al.*,

1996), and (ii) mutation of dibasic PKA consensus sites increases the ATP concentration required for half-maximal activity, without significantly altering the final achieved open probability (Mathews *et al.*, 1997). Physical interactions between the regulatory domains may be indicated by the observation that NBF1 and R-domain can be co-precipitated if co-expressed in the baculovirus expression system (Gruis *et al.*, 1996). What is even less understood is how the regulating domains then pass information to the pore-forming domains of CFTR, although functional evidence indicates that the CLs may be involved in such processes (Xie *et al.*, 1995, 1996; Cotten *et al.*, 1996; Seibert *et al.*, 1996a,b).

FOLDING AND BIOSYNTHETIC MATURATION OF CFTR

Intracellular targeting and processing of the CFTR protein have received much attention, but mostly from a single perspective. The reason for this focus is that 70% of all CF chromosomes contain the ΔF508 mutant allele. In fact, the ΔF508 mutation is found on at least one chromosome of 90% of the affected individuals (Sferra and Collins, 1993). Cheng *et al.* (1990) demonstrated that ΔF508-CFTR cannot function normally because the protein never reaches its site of action. The absence of ΔF508-CFTR from the apical surface of sweat ducts from patients was confirmed by Kartner *et al.* (1992) by immunocytochemistry of cryosections from rapidly frozen skin biopsies. Misprocessing was seen as lack of glycosylation and absence of a mature band C, as defined by Gregory *et al.* (1990): when CFTR is separated by SDS-PAGE, by *in vitro* translation a primary translation product is observed with an apparent molecular mass of 130 kDa (band A), observed in cells as a core-glycosylated 135-kDa species (band B), and a diffusely migrating 150–170 kDa version that represents mature, fully glycosylated CFTR (band C). This banding pattern is very typical; however, recent lower molecular weight bands from cells have been confused with unglycosylated band A but actually represent also core-glycosylated forms which result from utilization of alternate sites of initiation of translation (Carroll *et al.*, 1995; Pind *et al.*, 1994, 1995).

Importantly, ΔF508-CFTR has been seen to exhibit wild-type like channel activity when reconstituted into planar lipid bilayers (Li *et al.*, 1993), by whole-cell measurements in the maturation-capable baculovirus-insect cell expression system (Li *et al.*, 1993), or by patch clamping at the ER membrane (Pasyk and Foskett, 1995). Additional reports indicated that the ΔF508 mutation somewhat decreases the P_0 of CFTR chloride channels (Dalemans *et al.*, 1991; Denning *et al.*, 1992a). Nonetheless, such residual function indicates that promotion of processing may provide one therapeutic approach to CF treatment. In cell culture systems several treatments showed some success for this approach, such as exposure to reduced temperatures (Denning *et al.*, 1992b) or application of so-called chemical chaperones, including glycerol, deuterated water, dimethylsulfoxide, and trimethylamine N-oxide (Brown *et al.*, 1996; Sato *et al.*, 1996). It is theorized that both temperature and chemical chaperone treatments allow a misfolded protein to adopt a wild-type conformation, thereby resulting in its release from the ER (Brown *et al.*, 1996). A third method that enables some proteins to escape from the ER is the flooding of the control machinery by stimulating over-expression with sodium butyrate (Cheng *et al.*, 1995). These methods promote ΔF508-CFTR movement to the cell surface at levels which are sub-wild-type. Such concentrations, however, may be sufficient since expression as low as 10% of normal has been reported to restore normal chloride currents (Johnson *et al.*, 1992).

Why is ΔF508-CFTR incapable of escaping the ER? Improper trafficking of gene products occurs in many different diseases, including Tay-Sachs disease and α_1-antitrypsin deficiency, and is often attributed to misfolding of the protein (Thomas *et al.*, 1995). In the case of CF, misfolding is also commonly stated as the reason for retention in the ER of mutants such as ΔF508-CFTR. Frequently cited proof are reduced stability of ΔF508-CFTR at the plasma membrane (Lukacs *et al.*, 1993), slightly altered circular dichroism spectra, and reduced stability of a synthetic peptide representing a portion of NBF1 which contains the ΔF508 mutation (Thomas *et al.*, 1992; Thomas and Pedersen, 1993), and alleviation of the processing defect due to reduced temperatures (Denning *et al.*, 1992b) and chemical chaperones (Sato *et al.*, 1996; Brown *et al.*, 1996). A recent study by Qu and Thomas (1996) with recombinant NBF1 indicated that the ΔF508 mutation significantly reduces the folding yield at a variety of temperatures as well as the rate of folding.

Whatever the molecular defect is that results from the ΔF508 mutation, it can be partially corrected with revertant mutations. Using STE6-CFTR chimeras as

a model system, Teem *et al.* (1993) were able to identify two mutations, NBF1-located R553M and R553Q, which when introduced into ΔF508-CFTR partially restored the function of these chimeras. This may indicate that the Phe 508 region of the sequence normally interacts with the Arg 553 region, or that upon the deletion of the Phe 508 new interactions take place with the Arg 553 region (Teem *et al.*, 1993). Interestingly, in one patient a combination of the ΔF508 and the R553Q mutations was found on the same chromosome and this patient showed a mixed phenotype of severe lung and pancreatic disease, but normal sweat chloride (Dork *et al.*, 1991).

It is notable that even wild-type CFTR protein shows very inefficient processing in pulse-chase experiments when expressed in COS-7, CHO, mouse epitheloid C127, and pig kidney epithelial LLCPK1 cells. Only a relatively small fraction of the core-glycosylated band B matures to form the fully glycosylated band C (Cheng *et al.*, 1990; Marshall *et al.*, 1994). Initially, this was attributed to overpowering of the cellular machinery in the applied heterologous expression systems. However, careful studies by Lukacs *et al.* (1994), Pind *et al.* (1994), and Ward and Kopito (1994) demonstrated that inefficient processing is inherent in CFTR synthesis even in cells that endogenously express CFTR, such as the epithelial cell lines T84, HT-29, Caco-2 (human colon carcinomas), and Calu-3 (human lung adenocarcinoma). Depending on the cell line, 50–80% of the newly synthesized wild-type CFTR molecules are degraded by endogenous proteases resulting in a half-life of approximately 35 min. In the case of ΔF508-CFTR all the newly synthesized protein is degraded, but the half-life is not significantly different from the wild-type situation; furthermore, mutant and wild-type CFTR both show the same rate of synthesis (Ward and Kopito, 1994). The degradation appears to be ER-localized because proteolysis of wild-type and ΔF508-CFTR proceeds after disruption of vesicular transport between ER and Golgi by exposure to brefeldin A, and both ΔF508-CFTR and its breakdown products are detected in the ER after cellular fractionation, but are absent from the Golgi and plasmalemmal fractions (Lukacs *et al.*, 1994).

Lukacs *et al.* (1994) demonstrated that the fraction of wild-type CFTR which is properly processed must be present in a modified state and that the transition from the protease-susceptible to the protease-unsusceptible form is energy dependent. If transport from the ER to the Golgi is inhibited with brefeldin A, the same fraction of wild-type CFTR does not become degraded as in the control situation. In both cases the protease-resistant fraction of CFTR molecules has a half-life of ~24 h. When brefeldin A is removed, normal processing can proceed. Thus, the processing of the high-mannose, N-linked oligosaccharides into a complex structure is not necessary for stabilization of core-glycosylated CFTR. If cellular ATP is depleted ≥96%, the transition from the newly synthesized, protease susceptible intermediate to the protease-resistant, transport-competent form occurs with reduced efficiency.

It was proposed that the two species of core-glycosylated CFTR (maturation-competent and maturation-incompetent) may differ in their tertiary conformation and/or in the state of association with other components of the ER (Lukacs *et al.*, 1994). This hypothesis was further developed by Ward and Kopito (1994) who suggested that the inefficiency of maturation of wild-type CFTR reflects the kinetics of CFTR folding, which generally occurs in the context of molecular chaperones (Welch and Brown, 1996). Thus far newly synthesized CFTR has been observed to complex with the chaperones Hsp70 and calnexin, but not with the ER-lumen chaperones BiP and Grp94. For Hsp70 limited evidence was provided that wild-type CFTR dissociates from the chaperone before its transport to the Golgi, whereas the complex formed between ΔF508-CFTR and Hsp70 is retained in the ER, and ΔF508-CFTR is rapidly degraded in a pre-Golgi nonlysosomal compartment (Yang *et al.*, 1993). Based on co-immunoprecipitation and co-sedimentation through glycerol density gradients, the association with a second chaperone, calnexin, is transient (less than 2.5 h) but of roughly equal duration for wild-type and ΔF508-CFTR (Pind *et al.*, 1994). When a pulse-chase experiment is performed and the cell lysate from selected chase times is separated on glycerol gradients, the distributions of immature wild-type CFTR and ΔF508-CFTR are initially very similar and both patterns involve association with calnexin as observed with anti-calnexin antibodies. After 45- and 90-min chases, the distribution of ΔF508-CFTR across the gradient is unchanged, indicating that it remains included in complexes with calnexin throughout its lifetime. In contrast, the distribution of immature wild-type CFTR is shifted toward the lighter end of the gradient to a position intermediate between the initial immature-CFTR/calnexin complex and calnexin-free mature CFTR. However, since at these later time points immature wild-type CFTR recovered in anti-calnexin immunoprecipitates exhibits a distribution similar to that of immature wild-type CFTR in anti-CFTR

immuniprecipitates, it is suggested that complete dissociation from calnexin is not responsible for the shift to the intermediate position. The shift must represent a step in the maturation of CFTR that is unattainable by ΔF508-CFTR, but it is not known what it is (Pind *et al.*, 1994).

Proteins retained in the ER are eventually degraded (Klausner and Sitia, 1990). In the case of CFTR this degradation is very rapid and appears at least in part to be mediated by the ubiquitin-proteasome pathway because inhibitors of this pathway, N-acetyl-L-leucinyl-L-leucinyl-L-norleucinal (ALLN) and lactacystin, lead to a severalfold increase in steady-state levels of the immature band B for both wild-type and ΔF508-CFTR. However, band B increase is not accompanied by an increase in the mature band C. Some of the spared material is polyubiquitinated and insoluble in nonionic detergents. Pulse-chase experiments suggest that the build-up of band B is the result of slowed degradation (Jensen *et al.*, 1995; Ward *et al.*, 1995). Interestingly, a different member of the same inhibitor class, MG-132, completely blocks the formation of the mature band C (Jensen *et al.*, 1995). One can speculate that the proteolytic event which is blocked by MG-132 corresponds to the ATP-dependent step described by Lukacs *et al.*, (1994) that is needed to move core-glycosylated CFTR into the protease-resistant state that eventually can mature from the ER. It could also be that the core-glycosylated wild-type CFTR that shows a shift on the glycerol gradient of Pind *et al.* (1994), but is still associated with calnexin, is the result of this same proteolytic step. Possibly, this is the point in CFTR maturation where chaperone association and CFTR maturation/degradation converge and do so differently for wild-type CFTR and mutants such as ΔF508-CFTR. However, this is pure speculation and thus far no CFTR cleavage is known to occur during its synthesis, suggesting that if the cleavage is necessary, it must be of chaperones or other interacting ER proteins. In addition to the two proteolytic pathways identified by ALLN/lactacystin and MG-132, there must be one more proteolytic pathway because after initial slowing of CFTR degradation with ALLN/lactacystin the protein is still degraded. This third pathway is ATP-independent since it is very active in ATP-depleted cells (Lukacs *et al.*, 1994; Jensen *et al.*, 1995).

OUTLOOK

In addition to the ΔF508 mutation, many more gene alterations have been reported to affect CFTR's processing characteristics. Because a majority of the maturation-incompetent mutants retain at least some residual function (Li *et al.*, 1993; Champigny *et al.*, 1995; Sheppard *et al.*, 1995; Cotten *et al.*, 1996; Seibert *et al.*, 1996a) and because most CF patients express a mis-processed CFTR variant from at least one chromosome, a therapy which allows the protein to escape the ER seems highly desirable. Such a proposal is not unrealistic, as it has recently been reported for the structurally related P-glycoprotein that all mis-processed variants examined could be promoted to the cell surface by exposure to specific substrates or modulators of the transporter (Loo and Clarke, 1997). Furthermore, regardless of whether this type of manipulation, gene therapy, or protein replacement will eventually be utilized to deliver CFTR to the plasma membrane, it may in any case be beneficial to induce maximal activity of the molecules that reach their site of action. Therefore, the mechanisms of CFTR function have to be elucidated further. Significant knowledge has been accumulated about the individual modes of channel regulation, but thus far very little is known of how the regulatory domains communicate with each other and with the pore-forming unit. Such investigations should also be illuminating from a basic science point of view, since CFTR is the only chloride channel known that uses the structural motif of transporters to regulate anion movement across a transmembrane pore.

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