Dimeric cystic fibrosis transmembrane conductance regulator exists in the plasma membrane

Mohabir RAMJEESINGH, Jackie F. KIDD, Ling Jun HUAN, Yanchun WANG and Christine E. BEAR[1]

Programme in Structural Biology and Biochemistry, Physiology and Biochemistry Departments, Faculty of Medicine, Research Institute, Hospital for Sick Children, University of Toronto, Toronto, ON, Canada M5G 1X8

CFTR (cystic fibrosis transmembrane conductance regulator) mediates chloride conduction across the apical membrane of epithelia, and mutations in CFTR lead to defective epithelial fluid transport. Recently, there has been considerable interest in determining the quaternary structure of CFTR at the cell surface, as such information is a key to understand the molecular basis for pathogenesis in patients harbouring disease-causing mutations. In our previous work [Ramjeesingh, Li, Kogan, Wang, Huan and Bear (2001) Biochemistry **40**, 10700–10706], we showed that monomeric CFTR is the minimal functional form of the protein, yet when expressed in Sf9 cells using the baculovirus system, it also exists as dimers. The purpose of the present study was to determine if dimeric CFTR exists at the surface of mammalian cells, and particularly in epithelial cells. CFTR solubilized from membranes prepared from Chinese-hamster ovary cells stably expressing CFTR and from T_{84} epithelial cells migrates as predicted for monomeric, dimeric and larger complexes when subjected to sizing by gel filtration and analysis by non-dissociative electrophoresis. Purification of plasma membranes led to the enrichment of CFTR dimers and this structure exists as the complex glycosylated form of the protein, supporting the concept that dimeric CFTR is physiologically relevant. Consistent with its localization in plasma membranes, dimeric CFTR was labelled by surface biotinylation. Furthermore, dimeric CFTR was captured at the apical surface of intact epithelial cells by application of a membrane-impermeable chemical cross-linker. Therefore it follows from the present study that CFTR dimers exist at the surface of epithelial cells. Further studies are necessary to understand the impact of dimerization on the cell biology of wild-type and mutant CFTR proteins.

Key words: biotinylation, chemical cross-linker, cystic fibrosis transmembrane conductance regulator, non-dissociative electrophoresis.

INTRODUCTION

CFTR (cystic fibrosis transmembrane conductance regulator) belongs to the superfamily of ATP-binding-cassette transporter proteins [1]. Of this family of membrane proteins, CFTR is the only member that exhibits intrinsic function as a chloride channel [2]. Mutations in the CFTR gene cause cystic fibrosis (CF), a disease affecting the epithelia of multiple organs, including the airway, gastrointestinal tract, sweat ducts, pancreatic ducts and the reproductive tract. Although the pathogenesis of CF lung disease remains a subject of intense debate [3,4], the primary defect leading to intestinal disease has been clearly linked to defective chloride conductance [5].

Recent crystal structures support the concept that ion channels are typically multimeric. For example, the selectivity filter of the KcsA (potassium channel from *Streptomyces lividans*) channel is formed by the symmetrical interaction of four polypeptides [6,7]. ClC chloride channels are dimeric, yet in contrast with the potassium channel structure, the selectivity filter and fast gating mechanism reside within each polypeptide [8,9]. Based on these findings, it is tempting to hypothesize that the chloride channel function of CFTR may also be mediated by multimers of this protein. However, although controversy remains [10,11], there is compelling evidence suggesting that monomeric CFTR is fully functional as a chloride channel [12,13]. For example, co-expression of wild-type CFTR with a pore mutant, i.e. CFTR S341A ($Ser^{341} \rightarrow Ala$), leads to the appearance of two distinct conductances, rather than a hybrid conductance path [12]. Further, we developed methods for the purification and reconstitution of monomeric CFTR and determined that it exhibited normal chloride channel activity [13].

The quaternary structure of CFTR that mediates its function at the apical surface of epithelial cells is yet to be determined. Although a monomer may be the minimal functional unit of CFTR, there are several studies that suggest that CFTR molecules may exist as dimers at the cell surface. Patch-clamp studies have characterized the apparent co-operativity amongst multiple CFTR channels [14–17]. Images of CFTR expressed in the plasma membrane of *Xenopus* oocytes obtained by electron microscopy were consistent with a dimeric structure [18]. To date, few biochemical studies have directly assessed the quaternary structure of CFTR at the cell surface. Mostly, biochemical studies have assessed CFTR structure in the total cellular pool, which includes CFTR protein at the cell surface as well as in the biosynthetic and degradative pathways. Our studies showed that both monomeric and dimeric CFTR can be detected in Sf9 insect cells [13] using various methods including gel filtration, non-dissociative PAGE and chemical cross-linking. Other studies, using alternative biochemical approaches such as immunoprecipitation, failed to find any evidence for dimeric CFTR [12,19]. This lack of consistency may reflect variability in the cell types studied, the extent of CFTR solubilized by different detergents as well as the particular cellular pool of CFTR studied.

The goal of the present study was to assess directly whether dimeric CFTR can be detected at the plasma membrane in mammalian cells stably expressing the protein and in epithelial cell lines that express CFTR endogenously.

Abbreviations used: BS^3, bis(sulphosuccinimidyl) suberate; CF, cystic fibrosis; CFTR, CF transmembrane conductance regulator; CHO, Chinese-hamster ovary; DTT, dithiothreitol; PFO, pentadecafluoro-octanoic acid.

[1] To whom correspondence should be addressed (e-mail bear@sickkids.on.ca).

EXPERIMENTAL

Materials

PFO (pentadecafluoro-octanoic acid), manufactured by Fluorochem (Old Glossop, Derbyshire, U.K.), was obtained from Oakwood Products (West Columbia, SC, U.S.A.). EZ-link™ sulpho-NHS-SS-Biotin, BS^3 [bis(sulphosuccinimidyl) suberate] and avidin beads were obtained from Pierce (Rockford, IL, U.S.A.). T_{84} and Caco-2 cells were purchased from A.T.C.C. (Manassas, VA, U.S.A.). Stably transfected CHO (Chinese-hamster ovary) cells were a gift from G.L. Lukacs (Hospital for Sick Children, Toronto, ON, Canada).

PFO/PAGE, SDS/PAGE and immunoblotting

SDS/PAGE, PFO/PAGE and Western blotting were performed as described previously [13]. For immunoblotting, the protein was transferred to a nitrocellulose membrane and probed with an anti-CFTR polyclonal antibody generated against a fusion protein corresponding to the predicted NBD2 (nucleotide-binding domain 2) and C-terminus of CFTR (amino acids Asn^{1197}-Leu^{1480}). Immunopositive bands were visualized by enhanced chemiluminescence (Amersham Biosciences, Oakville, ON, Canada).

Preparation of membrane fractions

CHO cells stably expressing CFTR were grown to confluence in 10 cm dishes. Cells were then harvested and pelleted at 1000 ***g*** for 5 min. The supernatant was removed and the cells were gently resuspended in PBS (150 mM NaCl/10 mM sodium phosphate, pH 7.4) and protease inhibitors (Roche Diagnostics, Mannheim, Germany). Resuspended cells were disrupted using a French press (French® Pressure Cell Press; SLM-AMINCO Spectronic Instruments, New York, U.S.A.) at 6.89 MPa. EDTA was added to yield a final concentration of 1 mM, and cells were centrifuged at 500 ***g*** for 10 min at 4 °C. To prepare a crude plasma membrane, the supernatant was spun at 100 000 ***g*** for 2 h. To prepare highly purified plasma membranes, the supernatant was layered over 35 % (w/v) sucrose in 50 mM Tris/HCl, and centrifuged at 100 000 ***g*** for 1 h at 4 °C. The interface was resuspended in 25 mM sucrose, 50 mM Tris/HCl (pH 7.5) and centrifuged at 100 000 ***g*** for 30 min at 4 °C. The membrane pellet was washed with PBS (115 mM NaCl/50 mM sodium phosphate, pH 7.5), and centrifuged at 125 000 ***g*** for 20 min at 4 °C.

Separation of CFTR monomers and multimers by gel-filtration chromatography

Total cell lysates, total membranes or purified plasma membranes were solubilized using 4 % (w/v) PFO as the detergent in a buffer solution also containing 25 mM sodium phosphate, 0.5 mM EDTA and 1 mM DTT (dithiothreitol) at pH 7.5. A 500 μl aliquot containing 10 μg of CFTR protein was applied to a Superose 6 column (120 cm × 1 cm), and fractions were eluted with 25 mM sodium phosphate, 100 mM NaCl, 4 % PFO, 0.5 mM EDTA and 1 mM DTT at pH 7.5 at a flow rate of 0.2 ml/min. The multimeric status of the CFTR protein in each reconstituted fraction was assessed immediately by PFO/PAGE, as described previously [13].

Cell-surface biotinylation

Cells were grown to confluence in 10 cm dishes. After confluence was reached, cells were washed twice with PBS (pH 7.2) at 4 °C and then washed once in solution A [PBS (pH 7.8) with 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$]. After this, cells were incubated with 1 mg/ml EZ-link™ sulpho-NHS-SS-Biotin in solution A for 30 min at 4 °C. Biotinylation was quenched by washing the cells twice with PBS (pH 7.8) containing 1 % BSA. Then, cells were washed twice with solution B (50 mM Tris, pH 7.4) and finally lysed with 4 % PFO in solution B.

Isolation of biotinylated proteins on streptavidin beads

Solubilized biotinylated cells were sheared through a pipette tip and then incubated with washed avidin beads at room temperature (24 °C) for 30 min. Beads were then washed five times with buffer A (100 mM NaCl/25 mM sodium phosphate/4 % PFO; pH 8.0). Biotinylated protein was eluted from the beads after incubating for 30 min at room temperature in buffer B (100 mM NaCl/25 mM sodium phosphate/5 mM DTT/5 % 2-mercaptoethanol/4 % PFO; pH 8.0). Beads were centrifuged at 9300 ***g*** for 1 min and the supernatant containing the protein was harvested and subjected to PFO/PAGE or SDS/PAGE and Western blotting as described above.

Chemical cross-linking

Stably transfected CHO cells or Caco-2 cells were grown to confluence in 10 cm dishes. Cells were harvested by trypsinization, washed and then resuspended in PBS (pH 8.0). The cell-impermeant cross-linker BS^3 was prepared fresh in PBS (pH 8.0) and added to the cells to reach a final concentration of 1 or 5 mg/ml. Cells were then incubated with shaking for 1 h at 4 °C. After the reaction, Tris base was added to a final concentration of 20 mM and incubated for 15 min at room temperature to quench the reaction. After this, cells were washed with PBS, solubilized in 2 % SDS and then subjected to SDS/PAGE as described above.

RESULTS

CFTR exists as monomers and dimers in mammalian cells

We have defined conditions for the optimal solubilization, separation and analysis of dimeric and monomeric CFTR expressed in Sf9 cell membranes in our previous studies [13]. Briefly, we showed that the anionic detergent 4 % PFO permits the solubilization of CFTR monomers and CFTR-containing complexes. Further, as reported previously, PFO-solubilized CFTR monomers and dimers can be separated by gel filtration. Finally, the substitution of SDS with PFO permits analysis of the oligomeric structure of CFTR and membrane proteins by gel electrophoresis [20]. In the present study, we applied these methods to the study of the oligomeric structure of CFTR in mammalian cells.

Whole cell lysates of stably transfected CHO cells, solubilized in 4 % PFO, were subjected to gel filtration on a Superose 6 column. This column had been previously calibrated using the intrinsic membrane protein AE1 (monomers and dimers) and the ryanodine receptor 2 monomer in the same detergent, as shown in Figure 1. The fractions containing CFTR protein were assessed by dot-blot analysis, and pixel intensity was determined using NIH Image software to provide an estimate of the relative quantities of CFTR in each fraction. The elution profile is shown in Figure 1(A). Fraction 54 mainly contained monomeric CFTR (approx. 165 kDa), whereas fractions 47–49 contained multimeric CFTR, migrating as predicted for the dimeric protein (approx. 330 kDa) as assessed by PFO/PAGE (Figure 1B, upper panel). This method for molecular sizing suggests that CFTR molecules

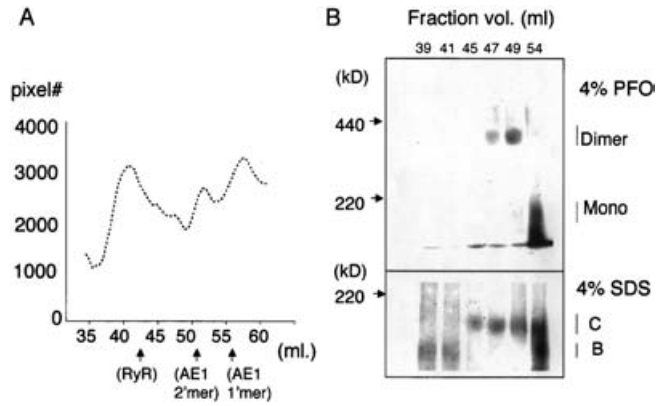

Figure 1 Monomeric and multimeric complexes containing CFTR can be separated by gel filtration

PFO-solubilized lysate of CHO cells stably expressing CFTR was passed through a Superose 6 column, previously calibrated using the membrane proteins: RyR (ryanodine receptor; 565 kDa), AE1 (dimer, 180–200 kDa) and AE1 (monomer, 90–100 kDa). The elution profile for CFTR was determined by dot-blot analysis, using a polyclonal antibody directed against CFTR, and the pixel intensity was quantified using NIH Image software (**A**). Specific eluted volumes were analysed by non-dissociative PFO/PAGE (**B**, upper panel) and by SDS/PAGE (**B**, lower panel). Both the Western blots were probed using an anti-CFTR polyclonal antibody generated against a fusion protein, corresponding to the predicted NBD2 and C-terminus of CFTR (amino acids 1197–1480). PFO/PAGE reveals separation of monomeric and dimeric CFTR. SDS/PAGE resolves whether CFTR is immature (band B) or mature (band C). The results are representative of three studies.

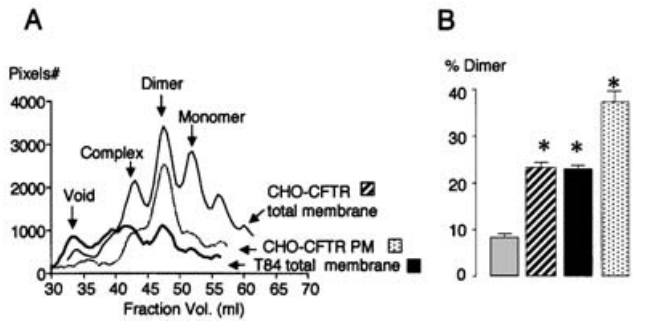

Figure 2 Dimeric CFTR is enriched in the plasma membrane of transfected CHO and T_{84} cells

Total cellular membranes, prepared from stably transfected CHO cells, T_{84} cells or purified plasma membranes of CHO cells, were solubilized in PFO and applied to a calibrated Superose 6 column. (**A**) The elution profiles were determined as described in the legend to Figure 1. The percentage of eluted protein migrating as expected for dimeric CFTR (see Figure 1), relative to the total eluted CFTR, was estimated using NIH Image software for 2–3 separate runs. (**B**) Percentage of dimeric CFTR relative to the total CFTR immunoreactivity. Error bars represent the means $\pm$ S.E.M. for different protein preparations. Light grey bar, total cell lysate; hatched bar, total cellular membranes prepared from transfected CHO cells; solid bar, total cellular membranes prepared from T_{84} cells; dotted bar, purified plasma membranes. The percentage of dimeric CFTR is significantly enriched in total cellular membrane and purified plasma membrane preparations (indicated by asterisks) from total cell lysates ($P < 0.001$), as assessed using ANOVA (Prism software).

associate directly to form dimeric structures; however, it remains formally possible that we may be capturing heteromeric complexes that contain CFTR and migrate as expected for CFTR dimers.

The fractions analysed by PFO/PAGE were subjected to SDS/PAGE. This is mostly dissociative of multimeric complexes, yet permits the resolution of the immature (band B) and mature (band C) proteins (Figure 1B, lower panel). SDS/PAGE analysis revealed that the fractions that contain dimeric CFTR contained primarily the mature band C form of the protein, whereas the monomeric fractions contained both immature (band B) and mature (band C) proteins. These findings are consistent with the suggestion that dimeric CFTR is derived from the cell surface.

There was no detectable CFTR in fractions 39–41 as assessed by PFO/PAGE, consistent with the idea that CFTR protein can also exist in multimeric complexes larger than those predicted for dimeric protein which fail to enter the gel. Analysis of the same fractions by SDS/PAGE revealed that CFTR exists primarily in the immature form, with a lesser amount of mature CFTR in larger complexes. These findings suggest that immature CFTR may be interacting in macromolecular complexes.

CFTR dimers exist at the cell surface of mammalian cells

To assess further the subcellular distribution of CFTR monomers and complexes, we compared the size distribution of CFTR-positive fractions eluted from the above Superose 6 column after the application of total cellular membrane or purified plasma membrane preparations (Figure 2B). Three major peaks were detected in fractions separated from the total membrane preparation obtained from CHO cells stably expressing CFTR, corresponding to a monomeric, a dimeric and a larger CFTR-containing complex. Two predominant peaks and one minor peak were evident in the elution profile resulting from the application of total membranes harvested from T_{84} cells. The two major peaks co-migrate with a larger macromolecular complex and the dimeric form of the protein. The minor peak corresponds to the migration predicted for monomeric CFTR. Unfortunately, the protein was insufficient in each fraction of these experiments to conduct analysis of the glycosylation status of CFTR as performed for our studies using total cell lysates. Comparison with the elution profile generated using total CHO cell lysates reveals that macromolecular, dimeric and monomeric CFTRs are relatively enriched in the total membrane preparations obtained from stably transfected CHO cells and from epithelial cells that endogenously express the protein.

A predominant peak corresponding to the predicted mass for dimeric CFTR was detected in the elution profile, obtained from the plasma membranes purified from stably transfected CHO cells. This elution profile also exhibits a shoulder co-migrating with the larger CFTR-containing peak, described previously in the profile from total membranes, as well as a shoulder co-migrating with monomeric CFTR. These findings support the claim that CFTR dimers may be enriched in the plasma membrane of mammalian cells. Quantitative analysis of the relative enrichment of the predicted dimeric CFTR is shown in Figure 2(B). Gel filtration was performed 2 or 3 times for each preparation and the enrichment of dimeric CFTR was assessed as the area under the peak eluting as fractions 46–49 relative to the area under the entire elution profile. There is a significant enrichment of dimeric CFTR in total cellular membrane preparations ($23 \pm 1\%$) and plasma membranes ($37 \pm 3\%$) relative to cell lysates ($8 \pm 1\%$).

Dimeric CFTR can be detected at the cell surface using biotinylation and non-dissociative electrophoresis

We employed surface biotinylation as an alternative approach towards characterizing the quaternary structure of CFTR at the cell surface. EZ-link™ sulpho-NHS-SS-Biotin was used to derivatize extracellular lysine residues on the plasma membrane of CHO

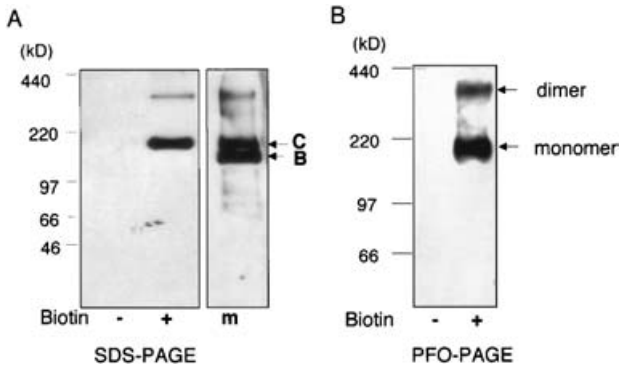

Figure 3 Cell-surface biotinylation labels both CFTR dimers and monomers

(**A**) Intact CHO cells stably expressing CFTR were subjected to surface biotinylation, and the biotinylated protein was captured using streptavidin beads, released under reducing conditions and analysed by SDS/PAGE and Western blotting using the CFTR-specific polyclonal antibody. Band C CFTR was biotinylated and precipitated by the beads (lane +). No CFTR was pulled down from cells not previously subjected to cell-surface biotinylation (lane −). A crude membrane preparation obtained from untreated cells was analysed by SDS/PAGE and Western blotting, and the migration of band B and band C CFTR proteins is indicated in lane m. (**B**) PFO/PAGE shows that both monomeric and dimeric CFTR proteins are precipitated by streptavidin beads after cell-surface biotinylation (lane +).

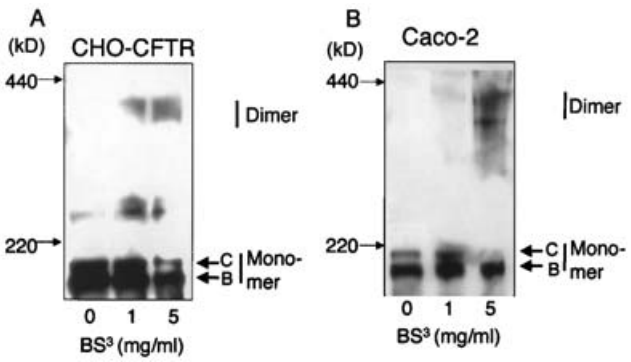

Figure 4 Mature CFTR can be cross-linked within a complex on the surface of intact cells

The membrane-impermeant cross-linking reagent (BS^3) was applied at different concentrations to the extracellular surface of either a monolayer of CHO cells expressing CFTR or a monolayer of confluent Caco-2 cells. Cells were then lysed and analysed by SDS/PAGE and Western blotting with the CFTR-specific polyclonal antibody. (**A**) In CHO cells treated with 1 and 5 mg/ml BS^3, complexes migrating as predicted for dimeric CFTR could be detected, whereas only the monomeric protein (bands B and C) was detected in untreated cells. (**B**) Complexes migrating as predicted for dimeric CFTR could also be detected in the Caco-2 cells treated with 1 and 5 mg/ml BS^3. Mature monomeric band C protein was depleted in both cell types at the highest BS^3 concentration.

cells stably expressing CFTR. Cells were then lysed in PFO and biotinylated proteins, captured using streptavidin beads. The resulting proteins were then subjected to either SDS/PAGE or PFO/PAGE and probed using a CFTR-specific polyclonal antibody. As shown in Figure 3(A), only the mature band C protein, migrating as a 170–180 kDa protein, was biotinylated, supporting the reliability of this assay for reporting the modification of CFTR residing exclusively at the cell surface. Analysis of biotinylated CFTR by PFO/PAGE revealed two bands, corresponding to monomeric and dimeric CFTR (Figure 3B). The stoichiometry of these two forms of the protein cannot be determined using this method of analysis, as PFO/PAGE is partially dissociative of multimeric structures; yet these results support the concept that CFTR dimers exist at the cell surface.

The short-chain cell-impermeant cross-linker BS^3 cross-links CFTR dimers on the cell surface

Previous experiments involved the study of the multimeric structure of CFTR after detergent solubilization. To capture the structure of CFTR in intact cells, we applied the cell-impermeant cross-linker BS^3 to the cell surface (Figure 4A). BS^3 is a short-chain cross-linker with a spacer arm of 11.4 Å (1 Å = 0.1 nm) and has been used in previous studies of the oligomeric structure of membrane proteins [13,21,22]. Intact, transfected CHO cells were incubated with the bifunctional cross-linker and then harvested, solubilized and subjected to SDS/PAGE. As shown in Figure 4(A), in the absence of cross-linker, CFTR runs as two bands: immature band B at approx. 150 kDa and complex glycosylated band C at approx. 170–180 kDa. In the presence of 1 mg/ml BS^3, there is evidence of cross-linked CFTR running as predicted for dimeric CFTR (330 kDa). The amount of cross-linked protein, migrating as a 330 kDa complex, is further increased at 5 mg/ml BS^3. Conversely, the amount of monomeric mature CFTR (band C) decreased with this higher cross-linker concentration. These results are consistent with previous surface-biotinylation studies and suggest that CFTR exists as a dimeric complex in the plasma membrane.

BS^3 was also applied to the apical surface of fully confluent Caco-2 cells, an intestinal epithelial cell line that expressed CFTR endogenously to determine whether multimeric CFTR could be captured in epithelial cells. As in the case of the studies employing CHO cells, we found that CFTR could be cross-linked as a complex migrating as expected for dimeric protein (Figure 4B). Further, the disappearance of the fully mature band C form of CFTR (but not the immature form) coincides with the appearance of the multimeric complex, suggesting that in epithelial cells CFTR dimers may exist at the cell surface.

DISCUSSION

Results of the present study suggest that dimeric CFTR can be detected both in a mammalian heterologous expression system and in epithelial cells that endogenously express the protein. As our previous studies showed that monomeric CFTR is fully functional [13], the biological significance of CFTR dimerization remains to be determined.

The present study has shown that dimeric CFTR is relatively enriched in the plasma membrane. On the other hand, in total cell lysates of mammalian cells, homodimers of CFTR represent a relatively small proportion of the total cellular pool of this protein. We estimate from an integration of the elution profile in Figure 1 that homodimers of CFTR can be detected in only 7–8 % of the total cellular pool of this protein. The remainder of the immunoreactive protein migrated as monomeric protein or in larger heteromeric complexes. The percentage of dimeric protein enriched in purified plasma membrane preparations is 37 %, suggesting that this structure is derived primarily from the cell surface. Therefore these estimates suggest that dimeric CFTR is a physiologically relevant structure and, further, provide a plausible explanation for the relative lack of detectable dimeric CFTR in previous biochemical studies of total cell lysates [12].

CFTR dimerization may facilitate its interaction with other proteins localized at the cell surface. CFTR has been shown to interact with other proteins to form macromolecular complexes in the apical membrane of epithelial cells. For example, Wang et al.

[23] showed that CFTR interacts with the PDZ-binding protein CAP70 (CFTR-associated protein 70) in the apical surface of airway epithelia. Recently, a functional complex comprised of the β-adrenergic receptor EBP-50, and CFTR was characterized on the surface of epithelial cells [24]. In the present study, we have described a novel biochemical approach that permits isolation and analysis of such macromolecular complexes and allows us to determine the impact of the quaternary structure of CFTR on such interactions.

We have described a method for solubilizing and isolating CFTR-containing complexes from epithelial cells using the detergent PFO. PFO is highly effective in extracting CFTR from cellular membranes, yet it is less disruptive of higher order complexes than SDS [20]. These detergent properties permit the assessment of structures, namely the larger macromolecular CFTR complexes that may not be as effectively solubilized in milder detergents such as Nonidet P40, CHAPS or Triton X-100 [20], or alternatively may be disrupted by treatment with stronger detergents such as SDS. Defining the molecular composition of these larger complexes will be the focus of our future work.

Furthermore, the present study highlights the importance of future assessment of the impact of heterodimerization between wild-type and mutant versions of CFTR. Biochemical, physiological and cellular studies of various CF-related mutations reveal that certain disease-causing mutations can have an effect on the molecular phenotype by various mechanisms, e.g. by affecting processing and maturation, cell-surface stability and/or channel function [25]. Our future studies will focus on examining the effect of co-expressing wild-type CFTR and various mutants on the relative biosynthesis, stability and channel function of both proteins. Our finding that dimeric CFTR is primarily comprised of mature protein predicts that dimerization may be rate-limiting for maturation, or alternatively may occur in post-endoplasmic reticulum compartments. In the former case, CFTRΔ508, a mutant known to be misprocessed and retained in the endoplasmic reticulum [26–29], would be expected to affect the expression of wild-type protein after co-expression. However, Chen et al. [12] showed that the processing and maturation of wild-type CFTR to form band C was unaffected by co-expression with the major mutant CFTRΔ508. These findings may favour our latter proposal and prompt a detailed investigation of the subcellular site at which dimerization occurs.

We are grateful for the technical assistance from Andrew Bulos. This work was supported by an operating grant to C. E. B. by the Canadian Cystic Fibrosis Foundation. J. F. K. was supported by a Kinsmen Fellowship Award from the Canadian Cystic Fibrosis Foundation.

REFERENCES

1 Riordan, J. R., Rommens, J. M., Kerem, B., Alon, N., Rozmahel, R., Grzelczak, Z., Zielenski, J., Lok, S., Plavsic, N., Chou, J. L. et al. (1989) Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. Science **245**, 1066–1073

2 Sheppard, D. N. and Welsh, M. J. (1999) Structure and function of the CFTR chloride channel. Physiol. Rev. **79**, S23–S45

3 Zabner, J., Smith, J. J., Karp, P. H., Widdicombe, J. H. and Welsh, M. J. (1998) Loss of CFTR chloride channels alters salt absorption by cystic fibrosis airway epithelia *in vitro*. Mol. Cells **2**, 397–403

4 Boucher, R. C. (2002) An overview of the pathogenesis of cystic fibrosis lung disease. Adv. Drug Deliv. Rev. **54**, 1359–1371

5 Clarke, L. L., Grubb, B. R., Gabriel, S. E., Smithies, O., Koller, B. H. and Boucher, R. C. (1992) Defective epithelial chloride transport in a gene-targeted mouse model of cystic fibrosis. Science **257**, 1125–1128

6 Jiang, Y. and MacKinnon, R. (2000) The barium site in a potassium channel by X-ray crystallography. J. Gen. Physiol. **115**, 269–272

7 Doyle, D. A., Morais Cabral, J., Pfuetzner, R. A., Kuo, A., Gulbis, J. M., Cohen, S. L., Chait, B. T. and MacKinnon, R. (1998) The structure of the potassium channel: molecular basis of K^+ conduction and selectivity. Science **280**, 69–77

8 Dutzler, R., Campbell, E. B., Cadene, M., Chait, B. T. and MacKinnon, R. (2002) X-ray structure of a CIC chloride channel at 3.0 Å reveals the molecular basis of anion selectivity. Nature (London) **415**, 287–294

9 Dutzler, R., Campbell, E. B. and MacKinnon, R. (2003) Gating the selectivity filter in CIC chloride channels. Science **300**, 108–112

10 Zerhusen, B., Zhao, J., Xie, J., Davis, P. B. and Ma, J. (1999) A single conductance pore for chloride ions formed by two cystic fibrosis transmembrane conductance regulator molecules. J. Biol. Chem. **274**, 7627–7630

11 Raghuram, V., Mak, D. D. and Foskett, J. K. (2001) Regulation of cystic fibrosis transmembrane conductance regulator single-channel gating by bivalent PDZ-domain-mediated interaction. Proc. Natl. Acad. Sci. U.S.A. **98**, 1300–1305

12 Chen, J. H., Chang, X. B., Aleksandrov, A. A. and Riordan, J. R. (2002) CFTR is a monomer: biochemical and functional evidence. J. Membr. Biol. **188**, 55–71

13 Ramjeesingh, M., Li, C., Kogan, I., Wang, Y., Huan, L. J. and Bear, C. E. (2001) A monomer is the minimum functional unit required for channel and ATPase activity of the cystic fibrosis transmembrane conductance regulator. Biochemistry **40**, 10700–10706

14 Kartner, N., Hanrahan, J. W., Jensen, T. J., Naismith, A. L., Sun, S. Z., Ackerley, C. A., Reyes, E. F., Tsui, L. C., Rommens, J. M., Bear, C. E. et al. (1991) Expression of the cystic fibrosis gene in non-epithelial invertebrate cells produces a regulated anion conductance. Cell (Cambridge, Mass.) **64**, 681–691

15 Krouse, M. E. and Wine, J. J. (2001) Evidence that CFTR channels can regulate the open duration of other CFTR channels: cooperativity. J. Membr. Biol. **182**, 223–232

16 Fischer, H. and Machen, T. E. (1994) CFTR displays voltage dependence and two gating modes during stimulation. J. Gen. Physiol. **104**, 541–566

17 Pasyk, E. A. and Foskett, J. K. (1995) Mutant (δ F508) cystic fibrosis transmembrane conductance regulator Cl^- channel is functional when retained in endoplasmic reticulum of mammalian cells. J. Biol. Chem. **270**, 12347–12350

18 Eskandari, S., Wright, E. M., Kreman, M., Starace, D. M. and Zampighi, G. A. (1998) Structural analysis of cloned plasma membrane proteins by freeze-fracture electron microscopy. Proc. Natl. Acad. Sci. U.S.A. **95**, 11235–11240

19 Marshall, J., Fang, S., Ostedgaard, L. S., O'Riordan, C. R., Ferrara, D., Amara, J. F., Hoppe, Ht., Scheule, R. K., Welsh, M. J., Smith, A. E. et al. (1994) Stoichiometry of recombinant cystic fibrosis transmembrane conductance regulator in epithelial cells and its functional reconstitution into cells *in vitro*. J. Biol. Chem. **269**, 2987–2995

20 Ramjeesingh, M., Huan, L. J., Garami, E. and Bear, C. E. (1999) Novel method for evaluation of the oligomeric structure of membrane proteins. Biochem. J. **342**, 119–123

21 Angers, S., Salahpour, A., Joly, E., Hilairet, S., Chelsky, D., Dennis, M. and Bouvier, M. (2000) Detection of β2-adrenergic receptor dimerization in living cells using bioluminescence resonance energy transfer (BRET). Proc. Natl. Acad. Sci. U.S.A. **97**, 3684–3689

22 Hall, R. A., Hansen, A., Andersen, P. H. and Soderling, T. R. (1997) Surface expression of the AMPA receptor subunits GluR1, GluR2, and GluR4 in stably transfected baby hamster kidney cells. J. Neurochem. **68**, 625–630

23 Wang, S., Yue, H., Derin, R. B., Guggino, W. B. and Li, M. (2000) Accessory protein facilitated CFTR–CFTR interaction, a molecular mechanism to potentiate the chloride channel activity. Cell (Cambridge, Mass.) **103**, 169–179

24 Naren, A. P., Cobb, B., Li, C., Roy, K., Nelson, D., Heda, G. D., Liao, J., Kirk, K. L., Sorscher, E. J., Hanrahan, J. et al. (2003) A macromolecular complex of β2-adrenergic receptor, CFTR, and ezrin/radixin/moesin-binding phosphoprotein 50 is regulated by PKA. Proc. Natl. Acad. Sci. U.S.A. **100**, 342–346

25 Tsui, L. C. and Durie, P. (1997) Genotype and phenotype in cystic fibrosis. Hosp. Pract. (Off. Ed.) **32**, 115–118, 123–129, **134**

26 Zhang, F., Kartner, N. and Lukacs, G. L. (1998) Limited proteolysis as a probe for arrested conformational maturation of δ F508 CFTR. Nat. Struct. Biol. **5**, 180–183

27 Kartner, N., Augustinas, O., Jensen, T. J., Naismith, A. L. and Riordan, J. R. (1992) Mislocalization of δ F508 CFTR in cystic fibrosis sweat gland. Nat. Genet. **1**, 321–327

28 Denning, G. M., Anderson, M. P., Amara, J. F., Marshall, J., Smith, A. E. and Welsh, M. J. (1992) Processing of mutant cystic fibrosis transmembrane conductance regulator is temperature-sensitive. Nature (London) **358**, 761–764

29 Cheng, S. H., Gregory, R. J., Marshal, J., Paul, S., Souza, D. W., White, G. A., O'Riordan, C. R. and Smith, A. E. (1990) Defective intracellular transport and processing of CFTR is the molecular basis of most cystic fibrosis. Cell (Cambridge, Mass.) **63**, 827–834

Received 8 May 2003/23 June 2003; accepted 24 June 2003
Published as BJ Immediate Publication 24 June 2003, DOI 10.1042/BJ20030683