Stable dimeric assembly of the second membrane-spanning domain of CFTR (cystic fibrosis transmembrane conductance regulator) reconstitutes a chloride-selective pore

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Structural information is required to define the molecular basis for chloride conduction through CFTR (cystic fibrosis transmembrane conductance regulator). Towards this goal, we expressed MSD2, the second of the two MSDs (membrane-spanning domains) of CFTR, encompassing residues 857–1158 in Sf9 cells using the baculovirus system. In Sf9 plasma membranes, MSD2 migrates as expected for a dimer in non-dissociative PAGE, and confers the appearance of an anion permeation pathway suggesting that dimeric MSD2 mediates anion flux. To assess directly the function and quaternary structure of MSD2, we purified it from Sf9 cells by virtue of its polyhistidine tag and nickel affinity. Reconstitution of MSD2 into liposomes conferred a 4,4'-di-isothiocyanostilbene-2,2'-disulphonate-inhibitable, chloride-selective electrodiffusion pathway. Further, this activity is probably mediated directly by MSD2 as reaction of its single cysteine residue (Cys⁸⁶⁶) with the thiol modifying reagent, *N*^α(3-maleimidylpropionyl)biocytin, inhibited chloride flux. Only

MSD2 dimers were labelled by *N*^α(3-maleimidylpropionyl)biocytin, supporting the idea that only dimeric MSD2 can mediate anion flux. As a further test of this hypothesis, we conducted a second purification procedure, wherein purified dimeric and monomeric MSD2 proteins were reconstituted separately. Only proteoliposomes containing stable MSD2 dimers mediated chloride electrodiffusion, providing direct evidence that dimeric MSD2 mediates chloride channel function. In summary, we have shown that the second membrane domain of CFTR can be purified and functionally reconstituted as a chloride channel, providing a tool for probing the structural basis of chloride conduction through CFTR.

Key words: cystic fibrosis transmembrane conductance regulator (CFTR), chloride-selective channel, cysteine modification, dimer, glycosylation, second membrane-spanning domain.

INTRODUCTION

CFTR (cystic fibrosis transmembrane conductance regulator) is a phosphorylation and nucleotide-regulated chloride channel and this function is essential for maintaining proper hydration of the fluid lining epithelia in multiple hollow organs including the gastrointestinal tract, airways and other ducted organs [1,2]. Mutations resulting in altered expression and/or loss of the chloride channel function of CFTR cause cystic fibrosis, a disease with the distinct feature of thick, sticky mucus obstructing the airways, pancreatic ducts and, in severe cases, the intestine. An understanding of the molecular basis for the chloride channel function of CFTR would identify structural features amenable for therapeutic modification and define the molecular changes underlying disease caused by specific mutations.

The CFTR gene codes for a polypeptide possessing two halves, each half possessing a putative MSD (membrane-spanning domain) and a putative NBD (nucleotide-binding domain). This modular organization plus the presence of conserved motifs in the NBDs places CFTR in the ABC (ATP-binding cassette) superfamily of membrane proteins [3]. At present, there is little information available regarding the structure of CFTR. On the other hand, recently, there has been remarkable progress in our understanding of the structure of bacterial members of the ABC superfamily of transporters. The structures of MsbA (lipid A transporter) [4] and BtuCD (a vitamin B₁₂ transporter) [5] have been solved at 4.5 and 3.2 Å (1 Å = 0.1 nm) resolution respectively. As predicted

from previous biochemical studies, these structures revealed that the intact transporter comprises two MSDs and two NBDs, with each domain being conferred by a distinct polypeptide. For both structures, the substrate translocation pathway is formed at the interface between the two MSDs. Conformational changes in the membrane domains required for transporter function are presumably regulated by long-range signals transmitted via conformational changes initiated at the NBDs.

CFTR is a chloride channel, unlike most of the members of the ABC superfamily, and the conduction properties of the native CFTR pore have been investigated intensively by patch-clamp electrophysiology [6–10]. Whole-cell and single-channel patch-clamp studies reveal that the phosphorylation and nucleotide-regulated CFTR conduction path is non-rectifying in the presence of symmetrical chloride concentrations and exhibits the following relative anion conductances: Cl[−] > NO₃[−] > Br[−] > F[−] ≥ I[−] > SCN[−] [6]. Unitary conductance is approx. 8–10 ps. Chloride conduction through CFTR is blocked by three classes of compounds including: disulphonic stilbenes, the arylamino-benzoates and sulphonylurea compounds [7]. Blocking by DIDS (4,4'-di-isothiocyanostilbene-2,2'-disulphonate) occurs only when the drug is applied to the cytoplasmic side of the membrane [8].

The structural basis for chloride conduction through CFTR remains incompletely understood. One would predict, on the basis of the structures of prokaryotic ABC proteins [4,5], that the translocation path for chloride ion may be formed at the interface between

Abbreviations used: ABC, ATP-binding cassette; CFTR, cystic fibrosis transmembrane conductance regulator; DIDS, 4,4'-di-isothiocyanostilbene-2,2'-disulphonate; MPB, *N*^α(3-maleimidylpropionyl)biocytin; MSD, membrane-spanning domain; NBD, nucleotide-binding domain; NEM, *N*-ethylmaleimide; PFO, pentadecafluorooctanoic acid; ProP, proline transporter.

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Figure 1 Boundaries of MSD2 domain

MSD2 domain used in these studies extends from residue 857 to 1158 and includes putative helical segments 7–12, intracellular loops 3 and 4 and extracellular loops 4–6.

the two MSDs of CFTR (each comprising six putative helical segments). The contribution of the residues in MSD1 and particularly the sixth putative transmembrane helix has been studied most extensively either through mutagenesis and/or through monitoring the accessibility of substituted cysteine residues along its length using thiol reagents [9–15]. Multiple residues appear to contribute to anion binding in TM6, including Lys³³⁵ to Ile³⁴⁴ [9,16]. Studies by Gong and Lindsell [17] and Smith et al. [13] highlighted the potential importance of the positively charged residues, Arg³³⁴ and Lys³³⁵, in forming an external vestibule, which attracts anions towards the selectivity filter. The region, which determines selectivity, appears to be confined to a narrower segment (comprising residues Phe³³⁷ to Ser³⁴¹ [18–20]). Therefore residues have been identified in MSD1, which play a critical role in determining the pore properties of CFTR. In fact, a dimer of MSD1 was shown to be sufficient to reconstitute the native conduction properties of CFTR in studies of a ‘half molecule’ (including MSD1–NBD1 and R domains) by Sheppard et al. [21].

The role for specific helices or residues in the second MSD for anion conduction is somewhat less clear. A construct coding for the second half of the CFTR (i.e. MSD2–NBD2) was expressed by Devidas et al. [22] in *Xenopus* oocytes and was found to confer an anion selective path, albeit lacking the selectivity exhibited by the full-length protein. Gupta et al. [23] reported that mutations in TM12 (Thr¹¹³⁴, Met¹¹³⁷ and Ser¹¹⁴¹) altered anion binding within the pore, but had relatively minor effects on the anion selectivity and unitary conductance of the channel. Similarly, mutations at Ser¹¹¹⁸ in TM11 [24] and Thr¹¹³⁴ in TM12 altered the affinity and/or voltage dependence of blockade by *N*-phenylanthranilic acid or 5-nitro-2-(3-phenylpropylamino) benzoic acid [18,24]. McCarty and co-workers [18,24,25] suggested that Thr¹¹³⁴ (in TM12) plays an indirect role in determining selectivity. Therefore these studies suggest that MSD2 contributes to the functional properties of the CFTR pore although the molecular basis and the nature of its contribution is less clear than that for MSD1.

In the present study, we describe our progress in the purification and reconstitution of the second MSD of CFTR encompassing residues 857–1158 (MSD2; Figure 1), including putative transmembrane helices 7–12 plus intracellular loops 3 and 4 and extracellular loops 4–6. This domain was expressed in Sf9 cells using the baculovirus system and purified by virtue of its polyhistidine tag. Our studies suggest that MSD2 domains dimerize and that this stable structure can mediate anion permeation following reconstitution into phospholipid liposomes. Therefore this domain may be amenable to future studies of the structural requirements for chloride permeation through CFTR.

METHODS

Construct

MSD2 is a PCR product of CFTR spanning from 2569 to 3474 nt, inserted into the pFastBac vector (Invitrogen, ON, Canada), for generation of recombinant baculovirus and expression in Sf9 cells. This construct is engineered to possess three glycine residues as a linker and a His₆ tag at its C-terminus for the purpose of purification. The inserted amino acid sequence was checked and was found to be 100% identical with the native sequence.

Antibodies

MSD2 proteins were detected on nitrocellulose using a monoclonal anti-His antibody (Qiagen, Mississauga, ON, Canada) and full-length CFTR was detected using a specific polyclonal antibody directed against the second NBD and the C-terminus (residues from Asn¹¹⁹⁷ to Leu¹⁴⁸⁰) [26].

Membrane preparation

Sf9-baculovirus expression system was used for the production of MSD2-His protein. Frozen Sf9 cell pellet from 200 ml cell suspension, expressing recombinant MSD2-His proteins, was thawed and resuspended in PBS and protease inhibitors. Cells were then disrupted three times using a French Press (Spectronic Instruments, NY, U.S.A.) at 1000 lb/in² (8.4 MPa) and centrifuged at 500 *g* for 10 min at 4 °C. A crude membrane preparation was obtained by centrifugation of the supernatant at 100 000 *g* for 2 h at 4 °C. Pure plasma membranes of Sf9 cells were prepared as described previously [27].

Purification of MSD2-His and CFTR-His

The methods for purification, reconstitution and phosphorylation of CFTR-His have been described in detail elsewhere [26]. For the purification of MSD2, crude membrane preparations containing MSD2-His were solubilized overnight in 8% (w/v) PFO (pentadecafluoro-octanoic acid) and 25 mM phosphate buffer (pH 8.0). Purification of MSD2 was performed using nickel-affinity chromatography. The solubilized and filtered sample was applied to a freshly generated Ni²⁺-nitrilotriacetate column (10 ml) at a rate of 2 ml/min. A pH gradient, pH 6.0–8.0 (generated with 25 mM phosphate, 100 mM NaCl and 4% PFO), was then applied to the column using an FPLC, and 5 ml fractions were collected.

Analysis and reconstitution of MSD2

Fractions were analysed by dot-blot, and 400 µl of the immunopositive fraction was concentrated (Microcon YM-50; Millipore, Bedford, MA, U.S.A.), run on SDS/PAGE [4–12% (w/v) gel] (Novex, Carlsbad, CA, U.S.A.) and silver-stained to analyse the purity and quantity of MSD2 protein in the different fractions, as described previously [27a]. Fractions containing only MSD2 were concentrated in a Centricon YM-50 concentrator (molecular mass cut-off, 50 kDa; Millipore) to a final volume of approx. 100 µl. Concentrated samples in 4% PFO, 25 mM phosphate and 100 mM NaCl were diluted 1:10 in a buffer containing 8 mM Hepes, 0.5 mM EGTA (pH 7.2) and concentrated further to a final volume of 300–500 µl to reduce the PFO concentration to 0.4%. After rough quantification of MSD2 in the concentrated sample, using a modified Lowry method [27b], the protein was mixed with liposomes to yield a protein/lipid ratio of 1:20.

Methods describing liposome preparation are reported elsewhere [26,27]. Briefly, a concentrated MSD2-His sample in 0.4 % PFO was mixed with a sonicated liposome preparation containing phosphatidylethanolamine/phosphatidylserine/phosphatidylcholine/ergosterol (5:2:1:1 by wt.; phospholipids from Avanti Polar Lipids, Birmingham, AL, U.S.A.). The concentrated MSD2-lipid mixture was then dialysed in a Spectra/Por dialysis membrane (Spectrum Laboratories, Rancho Dominguez, CA, U.S.A.; molecular mass cut-off, 50 kDa) overnight at 4 °C against 4 litres of a buffer containing 8 mM Hepes and 0.5 mM EGTA (pH 7.2).

Separation of dimeric MSD2 from monomeric MSD2

Concentrated samples of purified MSD2 from the nickel column were applied to a Superose 6 column (1.5 cm × 50 cm) and fractions containing monomeric or dimeric MSD2 eluted using 25 mM phosphate buffer, 100 mM NaCl and 1 % PFO (pH 7.0). For subsequent functional analyses, monomeric and dimeric MSD2 were reconstituted as above.

Iodide efflux

Untransfected and transfected Sf9 cells were iodide-loaded according to established methods using a 1 ml Ringers nitrate loading buffer at pH 7.4 containing 136 mM NaCl, 4 mM KNO₃, 2 mM CaNO₃ · 4H₂O, 2 mM MgNO₃ · 6H₂O, 11 mM glucose and 20 mM Hepes. The cells were incubated for 1 h at room temperature (24 °C), and then loosely spun down at 500 g for 3 min. A 50 µl aliquot of cells was then washed with 3 × 1 ml of Ringers nitrate efflux buffer [110 mM NaNO₃/4 mM KNO₃/2 mM CaNO₃ · 4H₂O/2 mM MgNO₃ · 6H₂O/11 mM glucose/20 mM Hepes (pH 7.4)] with the osmolarity adjusted to 300 mosmol with added sucrose. After each wash, cells were pelleted at 1500 g for 15 s. Efflux of cellular iodide was assessed continuously after resuspending the cells with 5 ml of the Ringers nitrate efflux buffer or hypotonic solutions (osmolarity adjusted to 228 mosmol). Iodide efflux (measured as a change in mV) was assessed over a 5 min period, using an iodide-sensing electrode (Fisher, Nepean, ON, Canada). Changes in voltage were acquired using the FETCHEX data acquisition program (pCLAMP 6.04; Axon Instruments, Foster City, CA, U.S.A.) and results were analysed using FETCHAN software.

Chloride electrodiffusion

We used a concentrative tracer uptake assay, developed by Goldberg and Miller [28], to characterize the chloride conductance properties of reconstituted CFTR. Proteoliposomes were preloaded with 150 mM KCl and centrifuged through Sephadex G-50 columns equilibrated with glutamate-containing salts: 125 mM potassium glutamate, 25 mM sodium glutamate, 10 mM glutamic acid and 20 mM Tris glutamate (pH 7.6), to replace external chloride. Uptake was initiated and quantified by the addition of 1.0 µCi/ml ³⁶Cl⁻. Intravesicular ³⁶Cl⁻ was assayed at various time points following separation of liposomes from the external media using a mini anion-exchange column (Dowex 1).

CD

Purified MSD2 in 4 % PFO was diluted to 0.4 % PFO with 8 mM Hepes (pH 7.0). CD measurements were recorded on a Jasco J-720 spectropolarimeter, using a cuvette with a 0.1 cm path length. Scans were executed at room temperature. The resultant spectrum

for MSD2 was an average of four scans recorded between 260 and 190 nm, with noise reduction and baseline subtraction to minimize background absorbance by buffer components. Mean residue ellipticity $[\theta] = \theta M_{\text{mrw}} / (10cl)$ deg · cm² · dmol⁻¹, where θ stands for ellipticity in mdeg, M_{mrw} the mean residue molecular mass of the protein, c the concentration of the protein (in mg/ml) and l the path length of the CD cell.

Labelling of MSD2 and CFTR with MPB

Reconstituted MSD2 or CFTR protein (100 µl) was centrifuged at 100 000 g for 30 min in an airfuge, washed twice with PBS (pH 6.0) and labelled with MPB [*N*^α-(3-maleimidylpropionyl)biocytin] (Molecular Probes, Eugene, OR, U.S.A.) as described before with some modifications [29]. Briefly, the proteoliposomes, re-suspended in 100 µl of PBS (pH 6.0), were labelled in 120 µM MPB for 15 min at 25 °C. The reaction was stopped by adding 2-mercaptoethanol to a final concentration of 20 mM. The labelled proteoliposomes were pelleted at 100 000 g and washed twice in an airfuge. The proteoliposomes were analysed by Western blot and the labelled protein detected using avidin-peroxidase conjugate by enhanced chemiluminescence following blotting of SDS/PAGE (12 or 6 % gels) of the labelled MSD2 or CFTR respectively.

Labelling of carbohydrates on MSD2

Periodate-induced biotinylation was performed directly on nitrocellulose strips following blotting by SDS/PAGE (12 % gel) of reconstituted MSD2 fractions [30]. All the steps were performed at 25 °C. The nitrocellulose strips were blocked with 4 % (w/v) BSA solution for 1 h and rinsed three times with PBS, and incubated with freshly prepared solution of potassium periodate (2.5 mM PBS) for 30 min followed by three washes with PBS. Periodate-treated blots were then incubated with biocytin hydrazide (3 µg/ml in PBS; Pierce, Rockford, IL, U.S.A.) for 1 h followed by three washes with PBS. The biotinylated bands were detected with avidin-peroxidase conjugate by enhanced chemiluminescence.

RESULTS

MSD2 is expressed as a dimer in Sf9 plasma membranes and confers a permeation pore for anions

The nominal second MSD of CFTR encompassing residues 857–1158 (MSD2; Figure 1) including a polyhistidine tag at its C-terminus was expressed in Sf9 cells using the baculovirus expression system. This putative domain includes the putative transmembrane helices 7–12 plus intracellular loops 3 and 4 and extracellular loops 4–6. Two polypeptides, migrating on SDS/PAGE as predicted for monomeric and dimeric MSD2 with the apparent masses of 25–28 and 60–66 kDa respectively, were conferred by MSD2 expression (Figure 2A, i). A single prominent band migrating as a dimer was detected in purified Sf9 cell plasma membranes, analysed by non-dissociative PFO/PAGE (Figure 2A, ii). These results suggest that MSD2 dimers are stably expressed at the cell surface. Furthermore, this structure functions as an anion channel since expression of MSD2 conferred the appearance of an anion permeation path, determined by using the iodide-efflux assay (Figures 2B and 2C). The half-maximal iodide flux (measured as a change in voltage) was significantly greater in Sf9 cell cultures expressing MSD2 than in untransfected cells (27.6 ± 1.8 versus 12.3 ± 1.9 mV respectively; Figure 2C).

Figure 2 MSD2 expression in Sf9 cells confers the appearance of an anion efflux pathway

(A) (i) Analysis of MSD2 in total cell lysate obtained from Sf9 cells infected using recombinant virus. Two bands were detected, corresponding to monomeric (28 kDa) and dimeric (60 kDa) MSD2 in infected, but not in uninfected, cells using an antibody directed against the polyhistidine tag. (ii) Plasma membranes prepared from infected Sf9 cells were analysed by non-dissociative PFO/PAGE and MSD2 migrated as a dimer. (B) Iodide efflux (in mV measured using an iodine-sensing electrode) was determined in suspensions of Sf9 cells expressing MSD2 (+ MSD2) or untransfected cells (- MSD2). (C) The half-maximal iodide efflux (in mV measured using an iodine-sensing electrode) was significantly greater in cells expressing MSD2 (solid bar) than in untransfected Sf9 cells (open bar). Scale bars indicate means $\pm$ S.E.M. from three separate transfections.

Figure 3 Purification of MSD2 from Sf9 cells

(A) Crude membranes prepared from Sf9 cells expressing MSD2 were solubilized in 8% PFO, filtered and applied to a Ni^{2+} -nitrilotriacetate column and MSD2 protein eluted using a pH gradient. MSD2 protein elution was monitored by dot-blot analysis using an anti-His antibody. Pixel number was assessed using NIH imaging software. (B) Silver stained gel showing purity of MSD2 eluted in fraction numbers 38–40. MSD2 migration was as predicted for monomeric and dimeric proteins.

Purified, reconstituted MSD2 exists as a dimer in phospholipid liposomes and mediates chloride-selective electrodiffusion

Polyhistidine-tagged MSD2 was purified from crude membranes solubilized in PFO (8%) by metal affinity, and eluted using a pH gradient as shown in Figure 3(A). A silver-stained gel after

SDS/PAGE of eluted fractions revealed that MSD2, migrating primarily as a dimer with a minor proportion of monomeric protein, was eluted at pH 6.8 (Figure 3B). The identity of the eluted protein as MSD2 was confirmed by sequencing of the N-terminus. We confirmed that the isolated, purified domain adopted an α -helical conformation using CD. The CD spectrum of purified

Figure 4 CD spectrum of purified MSD2

MSD2 purified from crude membranes was soluble in 0.4% PFO in Hepes buffer. CD measurements were recorded using a cuvette with a 0.1 cm path length. Each spectrum is an average of four scans executed at room temperature (25 °C).

MSD2 in 0.4% PFO exhibited the characteristic, double minima at 208 and 222 nm of the α -helical structure (Figure 4).

Purified MSD2 was reconstituted into phospholipid liposomes and the quaternary structure of the reconstituted protein was determined by size-exclusion chromatography on a Superose 12 column (Figure 5). MSD2 proteoliposomes solubilized in PFO were applied to a gel filtration column, previously calibrated using PFO-solubilized membrane proteins, ProP (proline transporter; 50 kDa protein) and rhodopsin (27 kDa protein). MSD2 protein was eluted as two peaks, with the predominant peak eluting slightly ahead of ProP and the second slightly behind rhodopsin. This elution pattern is consistent with dimeric MSD2 being the predominant species with a lesser amount of monomeric MSD2 also being present.

CFTR expressed in Sf9 cells has been shown to possess oligomannose glycosylation [31]. Oligosaccharides are attached

to residues Asn⁸⁹⁴ and Asn⁹⁰⁰ in the fourth extracellular loop of the full-length protein [32] and we reasoned that if MSD2 is properly folded it also would possess attached oligosaccharides. Figure 5(B) shows that only the dimeric MSD2 structure is labelled by biocytin hydrazide, a probe which labels galactose and other sugars in glycoconjugates on periodate treatment, supporting the idea that the dimeric rather than the monomeric structure is properly folded.

The chloride channel function of reconstituted MSD2 was assessed using an electrodiffusive flux assay. In this assay, the liposomes were loaded with KCl and suspended in a potassium glutamate-containing solution. The outward chloride gradient leads to the generation of a positive, intraliposomal potential. $^{36}\text{Cl}^-$ added to the medium should be accumulated into the proteoliposome interior in response to this driving force only if the reconstituted protein mediates chloride electrodiffusion and there are no contaminating proteins forming non-selective or cation-selective shunt pathways to dissipate the electrical potential. As shown in Figure 6(A), empty proteoliposomes do not accumulate $^{36}\text{Cl}^-$, whereas there is a time-dependent uptake of $^{36}\text{Cl}^-$ by proteoliposomes bearing MSD2, suggesting that MSD2 can mediate anion-selective flux. In support of this suggestion, we found that the anion channel inhibitor, 100 μM DIDS, inhibited $^{36}\text{Cl}^-$ electrodiffusion significantly (Figure 6B). The relative anion conductance through MSD2 was assessed using an approach described previously by Miller and co-workers [33]. Various potassium salts, including chloride, nitrate, bromide and fluoride in 10 mM concentrations were added individually to the bath solution of proteoliposomes exposed to the gradient described above. Bath addition of anions that can be conducted through the pore will result in the dissipation of the above gradient and inhibition of $^{36}\text{Cl}^-$ accumulation. Alternatively, $^{36}\text{Cl}^-$ accumulation could also be inhibited by anions that permeate but bind tightly to block the pore, thereby inhibiting the generation of an electrical driving force. As shown in Figure 6(C), the addition of potassium

Figure 5 Molecular mass and carbohydrate analysis of MSD2

(A) Elution profile from a Superose 12 (HR10/30) purified MSD2 (—) was compared with that of other membrane proteins of known mass, i.e. rhodopsin (27 kDa; ----) and the ProP (50 kDa; - - - -). All purified proteins were solubilized in PFO and fractionated on a Superose 12 column. Pixel number was determined by dot-blot and analysed by NIH Image software. Rhodopsin protein was assessed by spectrophotometry at 280 nm. (B) Purified MSD2, analysed by Western blotting using the α -His antibody, revealed a dominant band migrating as predicted for dimeric MSD2 (**) and another band migrating as predicted for monomeric MSD2 (*). Glycoconjugate analysis of the same sample of MSD2 using biocytin hydrazide after SDS/PAGE showed that only the dimeric structure is glycosylated.

Figure 6 MSD2 functions as a chloride-selective channel that is inhibited by DIDS

(A) Time course of electrodiffusive $^{36}\text{Cl}^-$ accumulation (nmol) by empty phospholipid liposomes (▲) or by proteoliposomes containing purified MSD2 (3 $\mu\text{g}/\text{assay}$, ■). Means $\pm$ S.E.M. from three different purifications assayed in triplicate. (B) Bar graph shows $^{36}\text{Cl}^-$ accumulation by MSD2 containing proteoliposomes after 60 min with or without DIDS (100 μM). DIDS inhibition, expressed relative to untreated controls assayed in triplicate, is statistically significant (* $P < 0.05$). (C) Bar graph shows relative inhibition of $^{36}\text{Cl}^-$ accumulation after the addition (10 mM) of different salts. Inhibition, expressed relative to control (10 mM KCl, $n = 8$), is inversely related to conductance. Separate studies ($n = 3-8$) were conducted for each salt. Accumulation was determined at 60 min after initiation of uptake.

salts inhibited $^{36}\text{Cl}^-$ accumulation in the following order: $\text{Cl}^- > \text{NO}_3^- = \text{Br}^- > \text{F}^-$, consistent with published reports of their relative conductance through the native CFTR pore [6]. Iodide and thiocyanate also prevented $^{36}\text{Cl}^-$ accumulation, possibly reflecting inhibition of the channel pore as reported previously for the channel function of the intact CFTR protein [23,34]. Therefore reconstituted MSD2 functions as an anion-selective pore with properties similar to those reported for the full-length protein.

Cysteine modification inhibits electrodiffusion through MSD2 as well as the purified full-length CFTR protein

To verify that the chloride-selective conductance detected in our flux assay of MSD2 containing proteoliposomes was due to MSD2 and not a contaminant, we took advantage of the fact that MSD2 has one cysteine residue in putative TM7. This cysteine residue is accessible and can be labelled using MPB in the reconstituted dimeric MSD2 protein as shown in Figure 7(A). Labelling with MPB was performed (pH 6.0) to ensure the specificity of the maleimido functional group for thiols [35]. MPB (120 μM) labelling significantly inhibited $^{36}\text{Cl}^-$ electrodiffusion as shown in Figure 7(B). These findings suggest that MSD2 directly mediates chloride flux and, further, that a cysteine residue (Cys⁸⁶⁶) may reside close to the pore formed by MSD2 dimers.

The cysteine modification studies described above prompted us to determine whether the intact CFTR protein can be similarly labelled with MPB. As shown in Figure 7(C), purified, intact CFTR protein is also labelled by 120 μM MPB and the chloride electrodiffusion mediated by the whole protein is inhibited significantly. These findings suggest that there are cysteine residues accessible to the modification in the whole protein and, further, that one of these may be Cys⁸⁶⁶.

MSD2 dimers are functional as anion channels

Several of our findings indirectly suggested that dimeric rather than monomeric MSD2 is functional as an anion channel. For

Figure 7 MPB labelling of purified MSD2 and CFTR inhibits their chloride flux activity

(A) Only the dimeric, purified and reconstituted MSD2 is labelled by MPB as detected by avidin-horseradish peroxidase and chemiluminescence. (B) Bar graph shows $^{36}\text{Cl}^-$ electrodiffusion by empty liposomes (empty bar), proteoliposomes containing MSD2 (solid bar) and MPB-treated proteoliposomes (hatched bar). Means $\pm$ S.E.M. are shown from four different preparations. MSD2 proteoliposomes (but not MPB-treated proteoliposomes) mediate significantly more flux than empty liposomes (*). (C) Purified full-length CFTR is also labelled by MPB. (D) Bar graph shows $^{36}\text{Cl}^-$ electrodiffusion by empty liposomes (empty bar), proteoliposomes containing CFTR (solid bar) and MPB-treated CFTR-proteoliposomes (hatched bar). Means $\pm$ S.E.M. from triplicate studies are shown. CFTR proteoliposomes (but not MPB-treated proteoliposomes) mediate significantly more flux than empty liposomes (*).

example, the dimeric rather than the monomeric protein is glycosylated, suggesting that dimeric protein is properly folded. Secondly, only the dimeric form of the protein is labelled by the

Figure 8 Separation of dimeric from monomeric MSD2 on a Superose 6 column

(A) Elution profile of purified MSD2 in 1% PFO. Fraction volume was 0.5 ml. (B) Western-blot analysis of eluted fractions was analysed by 12% PFO/PAGE, which shows clear separation between monomeric, dimeric and high-order structures.

Figure 9 Purified dimeric MSD2 mediates cumulative chloride ion uptake

(A) PFO/PAGE of reconstituted fractions (64, 65) and (57, 58) showing preservation of monomeric and dimeric structures respectively. (B) SDS/PAGE of reconstituted fractions (57, 58) showing stability of MSD2 dimer in SDS. (C) Bar graph shows that dimeric MSD2 (D) mediates $^{36}\text{Cl}^-$ electrodiffusion, whereas monomeric MSD2 (M) fails to mediate flux. Ctl, control. Means $\pm$ S.E.M. values are shown from triplicate samples each containing 7.4 μg of protein.

thiol reagent MPB and treatment with this reagent is inhibitory to channel function. However, to directly compare the channel competence of the two structures, we separated monomeric and dimeric proteins by gel filtration, separately reconstituted each structure and compared their capacity to mediate chloride electrodiffusion using the flux assay described previously.

In Figures 8(A) and 8(B), we show that detergent-solubilized monomers, dimers and higher-order structures of MSD2 can be separated by gel filtration on a Superose 6 column. Fractions containing purified monomers (64, 65) or dimers (57, 58) were separately reconstituted into proteoliposomes. Each structure was stably retained after reconstitution as determined by PFO/PAGE (Figure 9A). The dimeric structure was also stable in SDS as shown in Figure 9(B). Finally, we determined that proteoliposomes-bearing dimeric MSD2 but not monomeric MSD2-

mediated $^{36}\text{Cl}^-$ electrodiffusion, providing direct evidence that the dimer of this domain is the functional structure (Figure 9C).

DISCUSSION

The second MSD of CFTR encompassing the putative helical segments 7–12 plus intervening intracellular and extracellular loops was expressed, purified and reconstituted as monomers and dimers in phospholipid liposomes. Only the dimeric structure mediated anion-selective electrodiffusion by proteoliposomes and this function exhibited certain properties previously associated with the native CFTR pore. For example, the relative ranking of anion conductances determined for MSD2, i.e. $\text{Cl}^- > \text{NO}_3^- = \text{Br}^- > \text{F}^-$ is consistent with the ranking reported for the native

CFTR pore [6]. Further, $^{36}\text{Cl}^-$ electrodiffusion through MSD2 was inhibited by iodide and thiocyanate, consistent with the reported ability of these anions to block chloride conductance through CFTR [23,34]. Overall, our findings suggest that the purified, reconstituted MSD2 domain may provide a useful tool for our future studies of the pore structure of CFTR.

We used the thiol-modifying reagent, MPB, as a probe of MSD2 structure and function. MPB has been used extensively in studies of membrane protein topology because it is membrane impermeant (at concentrations of approx. 100–125 μM) and only labels water-exposed residues [35]. MSD2 possesses one cysteine residue at position 866 and, surprisingly, this residue is only accessible to labelling by MPB in the dimeric rather than monomeric structure. These findings are consistent with our assessments of glycosylation status and channel function and suggest that only dimeric MSD2 is properly assembled to form a pore. Further, these results show that residue 866 (in predicted TM7) may be lining the pore vestibule. Similarly, the channel function of intact CFTR protein, purified and reconstituted in phospholipid liposomes, is inhibited by MPB, showing that cysteine residues (possibly including Cys⁸⁶⁶) are also accessible in the full-length protein and reside within regions which are important for channel function. These findings are consistent with patch-clamp studies of CFTR as described by Cotten and Welsh [36], which showed that native cysteine residues are accessible to modification by the thiol-modifying reagent, NEM (*N*-ethylmaleimide). In previous studies, addition of 1 mM NEM led to channel inhibition [36]. The marked inhibition caused by 120 μM MPB in the present study may reflect a similar mechanism of action and its enhanced solubility is relative to NEM. Together, these findings support our claim that dimeric MSD2 assembles to form a chloride pore that reconstitutes certain features of the pore in the native protein.

The studies of the voltage-sensing region of voltage-gated potassium channels by Jiang et al. [37] provides compelling rationale for expressing membrane protein fragments with the goal of faithfully reconstituting their native structure. Jiang et al. [37] showed that the overall fold of the voltage-sensing region in the intact channel was preserved in a truncated version of the protein, containing the isolated voltage sensor. However, this approach has generated apparently conflicting results in various studies of CFTR, possibly reflecting differences in the expression systems used and the particular domain boundaries chosen. For example, Chan et al. [38] found that expression of the second half of CFTR (i.e. residues 634–1480) in *Xenopus* oocytes failed to confer chloride channel activity. The authors showed that this fragment was not complex glycosylated, suggesting that it was not properly processed and inserted into the plasma membrane. On the other hand, in the Sf9 expression system, we detected MSD2 protein (residues 857–1158) in purified plasma membrane preparations and, further, could measure its function in intact Sf9 cells. Therefore apparent discrepancy between these studies probably reflects the more stringent quality control for protein processing in *Xenopus* oocytes relative to the Sf9-baculovirus expression system.

Finally, our studies suggest that MSD2 can form a chloride-selective pore following purification and reconstitution as a dimer, providing a potentially valuable tool for future studies of the structural basis for the chloride-channel function of CFTR. However, it is unlikely that the native pore of the intact CFTR protein is formed exclusively by this domain. As mentioned previously in the Introduction section, residues within putative helical segments derived from MSD1 have been studied extensively in the context of the intact protein and have been shown to have a critical role in determining the pore properties of CFTR [9,13,16–21]. Self-assembled segments derived from MSD1 also appeared to be

capable of conferring chloride-channel function in certain studies [21]. In our future studies, we plan to employ the techniques described in the present studies of MSD2 to assess the structural and functional properties of MSD1 homodimers and heterodimers of MSD1 and MSD2. Ultimately, MSD1–MSD2 heterodimers are expected to reconstitute the native pore with the highest fidelity.

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