Association of Domains within the Cystic Fibrosis Transmembrane Conductance Regulator†

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ABSTRACT: The cystic fibrosis transmembrane conductance regulator (CFTR) is a Cl^- channel composed of two membrane-spanning domains (MSD), two nucleotide-binding domains (NBD), and an R domain. To understand how these domains interact, we expressed various constructs of CFTR containing membrane-spanning and/or cytosolic domains either separately or together. We then tested for functional association of these domains using the SPQ halide-efflux assay or physical association using coimmunoprecipitation experiments. Coexpression of the amino-terminal half (MSD1, NBD1, and the R domain) and the carboxy-terminal half (MSD2 and NBD2) of CFTR generated functional Cl^- channel activity whereas expression of either alone did not give a signal with the SPQ assay. This result suggests that the two halves associate in the membrane. Using domain-specific antibodies, we found that either half of CFTR could coimmunoprecipitate the other, suggesting a physical association. Coimmunoprecipitation persisted between halves missing the NBDs, the R domain, or the amino-terminal tail. Moreover, constructs from MSD1 containing only the first and second transmembrane sequences and intervening extracellular loop were sufficient for interaction with MSD2. These data suggest that interactions between the two membrane-spanning domains of CFTR may mediate association between the two halves of the protein.

The cystic fibrosis transmembrane conductance regulator (CFTR)[1] is a Cl^- channel that is regulated by phosphorylation and by intracellular ATP (Welsh et al., 1992; Collins, 1992; Riordan, 1993). The amino acid sequence of CFTR predicts a protein that is composed of two halves, each consisting of a membrane-spanning domain (MSD) and a nucleotide-binding domain (NBD), linked by a unique regulatory (R) domain (Riordan et al., 1989). Numerous studies support this domain structure and have begun to provide insight into the structure and function of individual domains. Studies of CFTR topology suggest that each of the two MSDs consist of six membrane-spanning helices (Chang et al., 1994; Denning et al., 1992), and patch-clamp analysis of wild-type and mutant CFTR indicates that residues within the MSDs contribute to the formation of the Cl^- channel pore (Sheppard et al., 1993; Tabcharani et al., 1993; Akabas et al., 1994). The cytoplasmic R domain regulates the activity of the channel: when the R domain is phosphorylated by cAMP-dependent protein kinase, the channel is able to open. The cytoplasmic NBDs also regulate channel activity: binding and probably hydrolysis of MgATP by the NBDs control the opening and closing of phosphorylated channels. For reviews of these data, see: Welsh et al., 1992; Collins, 1992; Riordan, 1993; Gadsby & Nairn, 1994.

Previous studies suggest that the function of the individual domains influences the overall function of the protein. For example, the function of the NBDs and the R domain may influence the MSDs to control the opening and closing of the channel pore (Welsh et al., 1992; Collins, 1992; Riordan, 1993; Gadsby & Nairn, 1994). In addition, the R domain may influence the activity of the NBDs (Rich et al., 1991; Hwang et al., 1994; Carson et al., 1995). The physical interactions that underlie these functional effects are not understood. In other members of the traffic ATPase or ATP binding cassette (ABC) transporter family, physical interactions among domains are required for activity (Mimura et al., 1991; Hyde et al., 1990). This is most apparent in family members in which individual domains are composed of separate proteins that interact to form an active complex; examples include the histidine (Bishop et al., 1989) and maltose (Panagiotidis et al., 1993) transporters.

The goal of this study was to identify domains or sequences within domains that physically interact. Such interactions are likely important in stabilizing the protein. To accomplish this goal, we first obtained functional evidence that the two halves of CFTR associate. We then tested for physical interactions between specific domains of CFTR by coimmunoprecipitation with domain-specific antibodies. Using progressively truncated variants of both the amino (N)- and carboxy (C)-terminal halves of CFTR, we found that association occurs through the MSDs.

EXPERIMENTAL PROCEDURES

Materials. 6-Methoxy-*N*-(3-sulfopropyl)quinolinium (SPQ) was purchased from Molecular Probes, Inc. (Eugene, OR). Recombinant vaccinia virus vTF7-3 was obtained from

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[1] Abbreviations: CFTR, cystic fibrosis transmembrane conductance regulator; SPQ, 6-methoxy-*N*-(3-sulfopropyl)quinolinium; MSD, membrane-spanning domain; NBD, nucleotide-binding domain; R domain, regulatory domain; M1, membrane-spanning sequence 1; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; IBMX, 3-isobutyl-1-methylxanthine; MEM, minimum essential medium; MDR, multidrug resistant protein; TCR, T-cell receptor.

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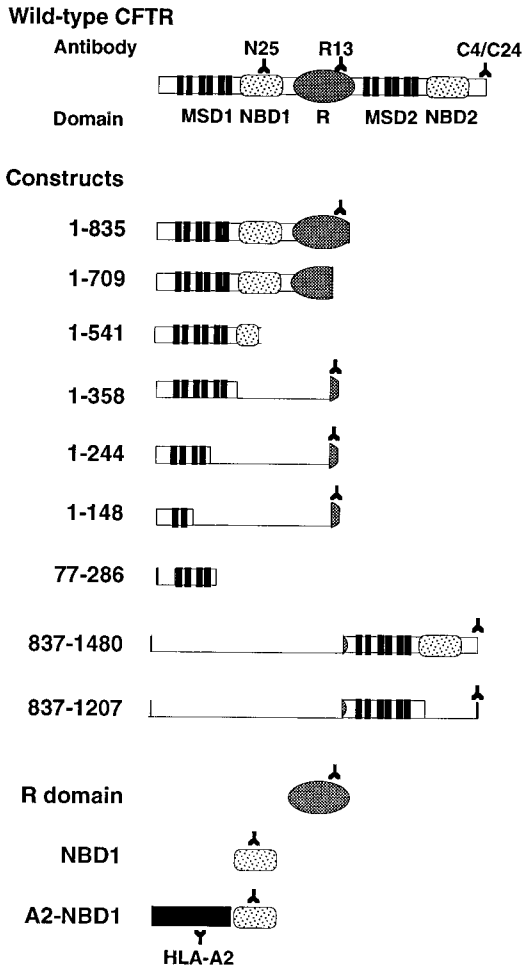

FIGURE 1: Domain structure of wild-type CFTR and the various constructs. A representation of the domains present in wild-type CFTR is shown at the top, and constructs are indicated below. The amino acids contained in some constructs are shown to the left of the model. The NBD1 construct contained amino acids 404−589, and the R domain construct contained amino acids 590−835. The construction of the A2-NBD1 is described in the Experimental Procedures. The locations of epitopes for the antibodies used in the study are shown above the appropriate domains of each CFTR construct and below the A2 domain.

ATCC (Rockville, MD) and lipofectin from Gibco Life Technologies (Gaithersburg, MD). Protein A−agarose beads were purchased from Pierce (Rockford, IL). [^{35}S]Methionine (cell labeling grade) was purchased from Amersham (Arlington, IL). Rabbit anti-mouse IgG was purchased from Organon Teknika (Durham, NC). Sheep anti-mouse IgG-HRP was from Amersham (Arlington Heights, IL) and SuperSignal from Pierce (Rockford, IL). TNT kit was purchased from Promega Corp. (Madison, WI). Other chemicals were analytical grade.

Site-Directed Mutagenesis. CFTR mutants were constructed in the vaccinia virus expression plasmid pTM1-CFTR4 as previously described (Kunkel, 1985; Gregory et al., 1990). Successful incorporation of the desired mutation into pTM1-CFTR4 was verified by restriction analysis and DNA sequencing around the site(s) of mutation.

Models corresponding to wild-type CFTR and to each of the variants used in this study are shown in Figure 1. We name each variant by the first and last amino acid number in the construct. Thus the N-terminal half is called 1−835 and includes MSD1, NBD1, and the R domain (defined as the boundaries of exon 13; amino acids 590−836). Two N-terminal constructs which correspond to CF-associated mutations were made by truncation to 1−709 (which includes MSD1, NBD1, and part of the R domain) and 1−541 (which includes MSD1 and the first half of NBD1) (Fanen et al., 1992; Kerem et al., 1990). The N-terminal constructs 1−358 (which includes M1 through M6), 1−244 (which includes M1 through M4), and 1−148 (which includes M1 through M2) were made by inserting the codons for the R domain sequence 722−745 (which includes the epitope for antibody R13 and the adjacent protein kinase A consensus site) after the last indicated amino acid. Thus these 3 constructs can be recognized by R13. We also made the N-terminal construct 77−286 which includes M1−M4 with the interconnecting extra- and intracellular loops, but lacks the N-terminal sequence from amino acids 2−76. Each of the N-terminal truncations was constructed by introducing a stop codon after the last numbered amino acid.

The C-terminal construct 837−1480 was made by deleting amino acids 6−836. This variant includes the six membrane-spanning segments (M7−M12) of MSD2, NBD2, and the full C-terminus. The smaller C-terminal construct 837−1207 was made by further deleting amino acids 1208−1465. This variant includes only M7−M12 plus the most C-terminal 15 amino acids of CFTR which constitute the epitope for the antibody C4. Thus, this variant lacks NBD2 and most of the cytoplasmic C-terminal amino acids.

The R domain construct was made by inserting amino acids coded by exon 13 of CFTR (Riordan et al., 1989) (amino acids 590−835) into the pTM1 vector. Likewise, the NBD1 construct was made by inserting the sequence of NBD1 of CFTR (Riordan et al., 1989) (amino acids 404−589) into the pTM1 vector.

For the A2-NBD1 chimera, we used a chimeric pcDNA3-A2-CD45 which contains the extracytoplasmic and transmembrane domains of A2 fused to the cytoplasmic domain of CD45 (Hovis et al., 1993). We substituted the sequence coding for NBD1 of CFTR (amino acids 398−596) for those of the cytoplasmic domain of CD45. Successful incorporation of the NBD1 into pcDNA3-A2 was verified by restriction analysis and DNA sequencing around the site(s) of mutation. This construct can be immunoprecipitated with polyclonal antibody made against synthetic peptide corresponding to amino acids 522−537 in the NBD1 of CFTR and can be immunoblotted using monoclonal antibody recognizing the extracellular domain of HLA-A2 (clone CR11) (a kind gift of C. Lutz).

CFTR Expression Systems. In Vitro Translation. Before expressing variants in cells, each construct was *in vitro* translated using the TNT system (Promega) to verify the expected molecular weight. We then confirmed that the protein was integrated into the membrane by isolating the protein from the high-speed microsomal membrane pellet (200000*g* pellet). In addition, the C-terminal constructs isolated from the membrane pellet had electrophoretic mobilities that reflected glycosylation at N894/900 in the extracellular loop between M7 and M8 (Cheng et al., 1990). We confirmed that each *in vitro* translated protein was specifically immunoprecipitated using the appropriate domain-specific antibody.

Cells. We transiently expressed wild-type and mutant CFTR in HeLa cells using the vaccinia virus/bacteriophage T7 hybrid expression system (Rich et al., 1990; Elroy-Stein et al., 1989). For some experiments, we used a double-infection protocol with one recombinant virus expressing the bacteriophage T7 RNA polymerase (vTF7-3) and a second

Table 1: Antibodies Used in This Study

antibody	epitope (aa)	domain	ref
R13	729−736	R domain of CFTR (13-1)	Gregory et al., 1990
N25	522−537	NBD1 of CFTR	this paper
C4	1476−1480	C-terminus of CFTR	Denning et al., 1992; Ostedgaard & Welsh, 1992
C24	1476−1480	C-terminus of CFTR (24-1)	Marshall et al., 1994
HLA-A2		extracellular of HLA-A2	Hovis et al., 1993

recombinant virus containing either wild-type or mutant CFTR cDNA under control of the T7 promoter. In other experiments, we used the cationic lipid lipofectin to transfect vTF7-3-infected cells with either wild-type or mutant CFTR plasmid. Similar results were obtained with both methods. Each dish was transfected with the same total amount of DNA. Cells were maintained in culture, and recombinant vaccinia viruses expressing wild-type and mutant CFTR were prepared as previously described (Rich et al., 1990).

Metabolic Labeling, Solubilization, and Immunoprecipitation. Six to eighteen hours after infection/transfection, cells were incubated in methionine-free HeLa media (Sigma) for 20−30 min, and then labeled with [^{35}S]methionine (Amersham) (200 μCi/$\sim$(3−5) $\times$ 10^5 cells) for 1−2 h. Cells were then washed three times with phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 0.1 mM phenylmethanesulfonyl fluoride (PMSF), pH 7.3) and solubilized in 1% recrystallized digitonin in lysis buffer (50 mM Tris, 150 mM NaCl, 0.3 μM aprotinin, 0.1 mM PMSF, pH 7.4) (Ostedgaard & Welsh, 1992). Proteins were solubilized with 1% digitonin; we previously showed that digitonin was more effective than 1% TX-100, 1% CHAPS, or 2.5% β-octyl glucoside for solubilization and subsequent immunoprecipitation of full-length CFTR (Ostedgaard & Welsh, 1992). In addition, digitonin was the most effective detergent when solubilization and immunoprecipitation of halves of CFTR were evaluated. Total cell lysates were centrifuged at 200000*g* for 30 min, and the supernatant of soluble proteins was retained. Proteins were immunoprecipitated from the supernatants immediately after centrifugation to eliminate any potential artifacts caused by freezing and thawing of samples. Within an experiment, the expressed constructs were immunoprecipitated from equivalent volumes of the soluble fraction with the indicated domain-specific CFTR antibodies. Soluble proteins were incubated with the indicated antibody for 2−10 h. Rabbit anti-mouse IgG and protein A−agarose were then added to precipitate antibody-specific proteins. Protein A precipitates were washed 3 times with 1% digitonin in lysis buffer, and once with lysis buffer alone, and incubated 10 min at room temperature with 2$\times$ sample buffer before electrophoresis. Greater than 90% of the specific constructs were immunoprecipitated with the initial immunoprecipitation. Immunoprecipitated proteins were separated on SDS−PAGE, En^{3}Hanced (NEN, Boston, MA), dried, and autoradiographed.

For simplicity, we have shortened the names of the antibodies used in this study to reflect the epitope domains of CFTR, as shown in Table 1. R13 (13-1) and C24 (24-1) were generous gifts of Genzyme Corp. (Framingham, MA). R13, C4, C24, and HLA-A2 are mouse monoclonal antibodies; N25 is a rabbit polyclonal antibody.

Western Blotting. For visualizing immunoprecipitated proteins by immunoblotting, constructs were expressed in HeLa cells, solubilized, and immunoprecipitated as above. Immunoprecipitated proteins were electrophoresed, transferred to nitrocellulose (Schleicher and Schuell), blocked with 5% bovine serum albumin (BSA) in PBS, and immunoblotted with the following antibodies diluted as indicated in Tris-buffered saline (137 mM NaCl, 2.7 mM KCl, 25 mM Tris, pH 8.0)/0.05% Tween 20 (Pierce) (TTBS): R13 (1:1000), C24 (1:3000), and HLA-A2 (1:100) for 2 h. Membranes were washed 2$\times$ with TTBS and then probed with sheep anti-mouse IgG (1:5000) for 1 h. After 2 washes in TTBS, reactive bands were visualized using chemiluminescence (Pierce).

Membrane Preparation. For some studies, membranes were isolated by differential centrifugation before solubilization. Cells were harvested in lysis buffer in the absence of detergent, briefly homogenized with a pestle, and then disrupted with 15 strokes of a 25 gauge needle, followed by 15 strokes of a 30 gauge needle. The extent of disruption was monitored under low power microscopy. Unbroken cells, nuclei, and large mitochondria were pelleted by low speed centrifugation (1000*g*) for 5 min. The low speed supernatant was centrifuged at 200000*g* for 30 min to form a supernatant of cytosol and a high-speed membrane pellet. This membrane fraction was then solubilized with 1% digitonin in lysis buffer and centrifuged at 200000*g* for 30 min to separate soluble from insoluble proteins. The high-speed soluble supernatant was used for immunoprecipitation. Results using the soluble high-speed supernatant were comparable to those using the soluble proteins from the total cell lysate.

SPQ Assay of Channel Function. Cells were assayed for Cl^- channel function using the SPQ halide efflux assay as previously described (Rich et al., 1993). Twelve to sixteen hours after transfection, cells were loaded with the halide-sensitive fluorophore SPQ by including 10 mM SPQ (diluted in Eagle's MEM) in the media for 8−10 h prior to study. SPQ fluorescence was initially quenched by incubating cells for 20−45 min in a buffer containing in mM: 135 NaI, 2.4 K_2HPO_4, 0.6 KH_2PO_4, 1 $MgSO_4$, 1 $CaSO_2$, 10 Hepes (pH 7.4), and 10 dextrose. After measuring fluorescence for 2 min, the 135 mM NaI buffer was replaced with 135 $NaNO_3$ (0 min in Figure 2). Five minutes after anion substitution, forskolin (20 μM) and IBMX (100 μM) were added to increase intracellular cAMP levels and stimulate the channels. Fluorescence of SPQ was quantitated as previously described (Rich et al., 1993). Three different transfected cultures were studied for each condition, and experiments were performed in duplicate for each transfection. Data shown are representative of the 40% of cells in each field with the largest response.

RESULTS

To begin to test the hypothesis that domains within CFTR may interact, we expressed 1−835 (the N-terminal half of CFTR which includes the N-terminus, MSD1, NBD1, and the R domain) and 837−1480 (the C-terminal half of CFTR which includes MSD2, NBD2, and the C-terminus) as separate proteins and asked whether we could detect

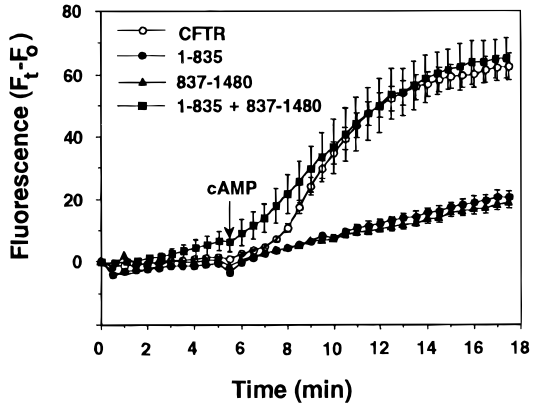

FIGURE 2: Functional analysis of the N-terminal and the C-terminal halves by SPQ halide efflux. The change in SPQ fluorescence is shown for HeLa cells expressing wild-type CFTR ($n = 39$, where n = number of cells), 1−835 ($n = 41$), 837−1480 ($n = 40$), and coexpressing 1−835 and 837−1480 ($n = 39$). NO_3^- was substituted for I^- in the bathing media at 0 min. Five minutes later (at the arrow) cells were stimulated by addition of 20 μM forskolin and 100 μM IBMX to increase intracellular cAMP levels (cAMP). Data are mean ±SEM of fluorescence at time t (F_t) minus the baseline fluorescence (F_0, the average fluorescence measured in the presence of I^- for 2 min prior to ion substitution). In some cases, the standard error bars are smaller than the symbols. Note that because the SPQ signal is not quantitative (Rich et al., 1990), the similar curves in Figure 1 for the combination of the two halves and wild-type protein do not indicate equal levels of activity.

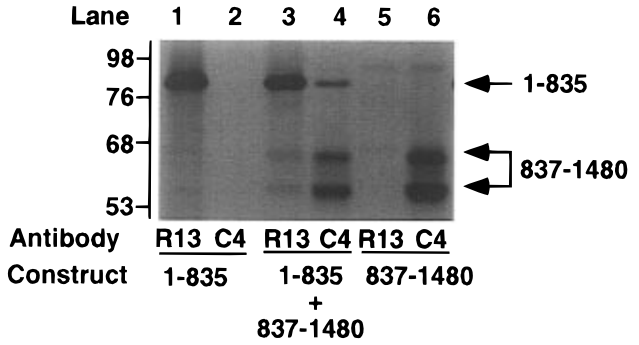

FIGURE 3: Coimmunoprecipitation of the N- and C-terminal halves of CFTR. The constructs 1−835 and 837−1480 were expressed in HeLa cells, metabolically labeled with [^{35}S]methionine, solubilized in 1% digitonin in lysis buffer, and immunoprecipitated from the high-speed supernatants with the indicated antibodies. Immunoprecipitated proteins were separated by SDS−PAGE and detected by autoradiography. The immunoprecipitated proteins are indicated by the arrows on the right. The upper band of 837−1480 represents glycosylated protein, and the lower band represents nonglycosylated protein. Lane numbers are shown at the top. The antibody used in each immunoprecipitation and the construct(s) expressed are indicated at the bottom.

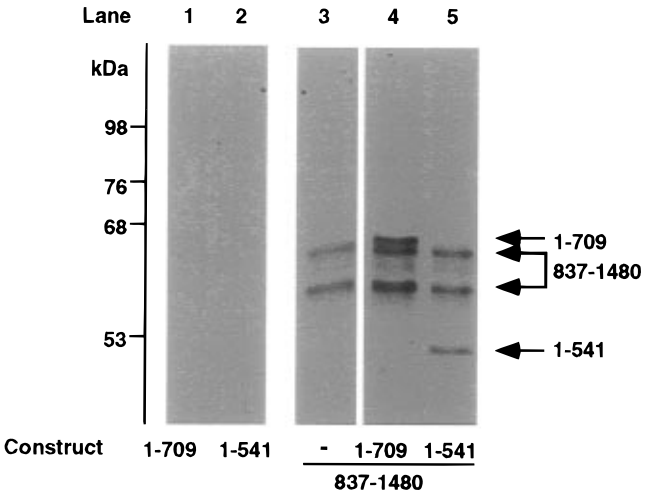

FIGURE 4: Coimmunoprecipitation of 1−541 and 1−709 with 837−1480. Constructs were expressed separately or coexpressed, and treated as indicated in the legend of Figure 3. Proteins were immunoprecipitated with antibody C4. Lane numbers are shown at the top, and the constructs are indicated at the bottom.

regulated Cl^- channel activity. Figure 2 shows that when we expressed either construct alone, we could not detect channel activity with the SPQ halide efflux assay. However, when we coexpressed the two halves, we observed cAMP-stimulated anion channel activity like that observed with wild-type CFTR. These results suggested that the two halves of CFTR associate to form a channel.

In a previous study, we were able to measure regulated Cl^- channel activity when we expressed the N-terminal half (1−835) of the channel alone (Sheppard et al., 1994). However, the frequency with which we observed such channels was very low, and they could be observed only with the more sensitive patch-clamp technique, never with the less sensitive SPQ halide-efflux assay. These results have been reproduced with a similar construct by Morales et al. (1996). Moreover, we found that expression of the C-terminal half did not generate channels that could be detected by the SPQ assay or by single-channel patch-clamp (Sheppard et al., 1994). Thus the difference between our previous results and our present data is a quantitative one; the N-terminal half by itself is very inefficient at generating Cl^- channels while coexpression of the two halves generated much greater channel activity than expression of either half alone.

To demonstrate that the two halves of CFTR physically associate, we expressed 1−835 (the N-terminal half) or 837−1480 (the C-terminal half) either separately or together and tested for coimmunoprecipitation. Figure 3 shows that both constructs were produced by HeLa cells and were specifically immunoprecipitated by domain-specific antibodies. When expressed alone, 1−835 was immunoprecipitated with antibody to R domain (R13) (lane 1), but not with antibody to C-terminus (C4) (lane 2). When expressed alone, 837−1480 was immunoprecipitated by C4 (lane 6), but not R13 (lane 5). 837−1480, which contains the consensus glycosylation sites at N894 and N900, appeared as two bands. The higher molecular weight band disappeared and the lower band was unaltered after treatment with *N*-glycanase or with endoglycosidase H (not shown). This indicates that the upper band is a core glycosylated form of the protein and that the lower molecular weight band is a nonglycosylated form of the protein. When 1−835 and 837−1480 were coexpressed, both proteins were coimmunoprecipitated by both antibodies (lanes 3 and 4) although coimmunoprecipitation was less efficient with R13 than with C4. These results suggest that the two halves of CFTR generate functional channels because they physically associate within the cell.

To begin to identify those regions of the protein that are responsible for the association, we coexpressed the C-terminal half with progressively shorter portions of the N-terminal half. Figure 4 shows results with 1−709 which retains approximately half of the R domain, and the shorter construct, 1−541, which retains a portion of NBD1. As expected, when these two constructs were expressed alone, they were not recognized by antibody C4 (1−709, lane 1; 1−541, lane 2). However, when 1−709 or 1−541 was coexpressed with 837−1480, they were coimmunoprecipitated by C4 (lanes 4 and 5). These data indicate that neither

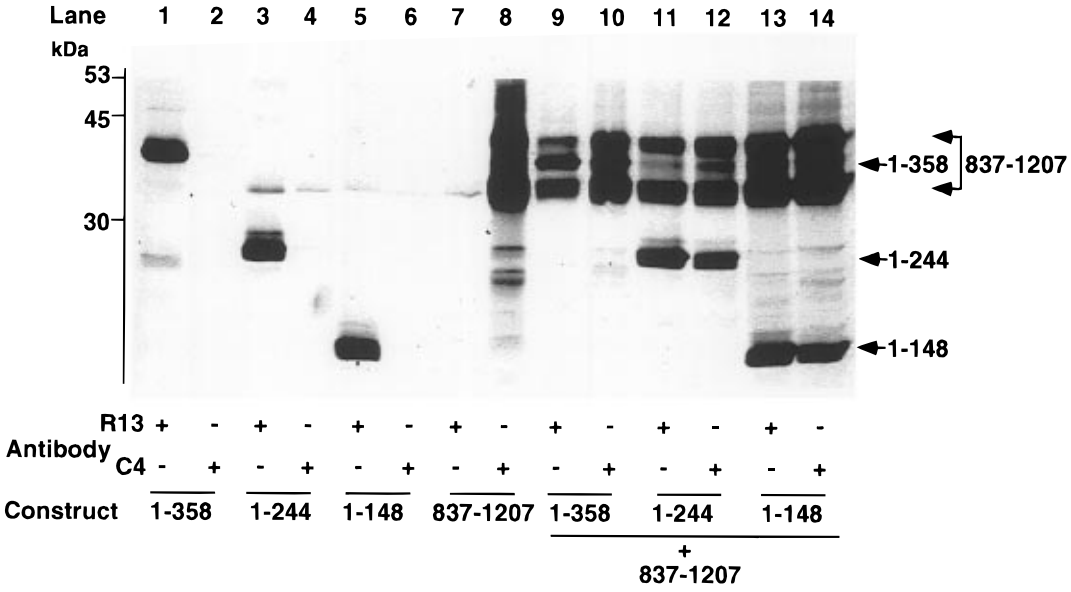

FIGURE 5: Coimmunoprecipitation of 1−358, 1−244, and 1−148 with 837−1207 from a membrane fraction. Constructs were expressed either alone or together in HeLa cells and metabolically labeled with [^{35}S]methionine. A high-speed (200000*g*) membrane pellet was isolated from the total cell lysate, solubilized with 1% digitonin in lysis buffer, and proteins immunoprecipitated from the high-speed supernant with the indicated antibodies. Similar results were obtained using total cell lysates. Lane numbers are shown at the top, and the antibodies and constructs used in each lane are indicated at the bottom.

the R domain nor the portion of NBD1 beyond amino acid 541 is required for physical association of the two halves of the protein.

To define further the regions required for association, we evaluated three N-terminal constructs with progressive truncations of transmembrane segments, 1−358 (M1−M6), 1−244 (M1−M4), and 1−148 (M1−M2), and a shorter C-terminal construct, 837-1207, which contains only M7−M12 plus the C-terminal residues 1466−1480 recognized by antibody C4. We expressed these N- and C-terminal halves which lacked cytosolic domains either separately or together. To ensure that coimmunoprecipitations occurred between proteins integrated into the membrane, we tested for association between proteins solubilized from high-speed (200000*g*) membrane fractions.

Figure 5 shows that when we expressed 1−358, 1−244, or 1−148 alone, each was immunoprecipitated by R13 (lanes 1, 3, and 5), but not antibody C4 (lanes 2, 4, and 6). When expressed alone, 837−1207 was immunoprecipitated by antibody C4 (lane 8), but not by antibody R13 (lane 7). However, when each of the N-terminal truncations was coexpressed with the C-terminal truncation, immunoprecipitation of either the N- or the C-terminal protein coimmunoprecipitated the other (lanes 9−14). The only result that was not clear in this experiment was coimmunoprecipitation of 1−358 with 837−1207 with antibody C4 (lane 10), because 1−358 migrates at the same position as one of the bands in 837−1207 (lanes 10, 12, and 14). However, evidence that 1−358 and 837−1207 associate is provided in lane 9 where antibody R13 coimmunoprecipitated 837−1207. Coimmunoprecipitation of the different MSD constructs suggests that association occurred through the transmembrane segments or the loops that join these transmembrane sequences, or through the N-terminus.

To determine if the N-terminal cytosolic sequence is necessary for association, we used the 77−286 construct which contains M1−M4 and the connecting loops, but not the N-terminus. We coexpressed 77−286 with the C-terminal construct 837−1207. We again tested for association between proteins integrated into the membrane by using proteins solubilized from high-speed (200000*g*) membrane fractions. Figure 6 shows that, as expected, 77−286 was not recognized by antibody C4 (lane 1). However, when 77−286 was coexpressed with 837−1207, immunoprecipitation of 837−1207 coimmunoprecipitated 77−286 (lane 2). These results indicate that association between the two halves occurs in proteins which have been incorporated into membranes and that the N- and C-termini, the NBDs, and the R domain are not necessary for association between the two halves. Instead, it appears that transmembrane segments M1−M2 and M7−M12 themselves, and/or the connecting intra- and extracellular loops, are sufficient for the physical association between the first and second halves of CFTR.

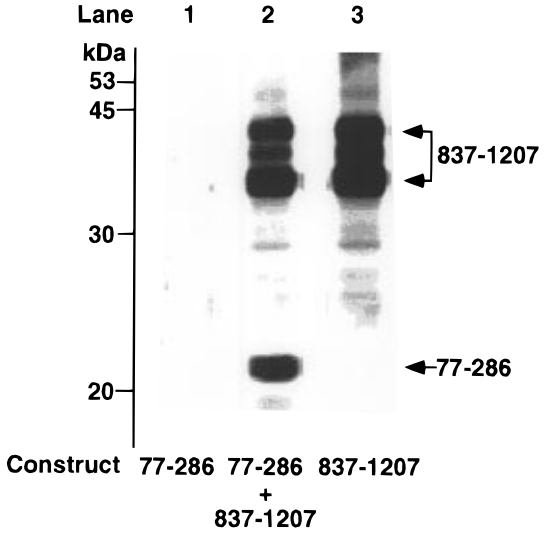

FIGURE 6: Coimmunoprecipitation of 77−286 with 837−1207 from a membrane fraction as described in Figure 5. Proteins were immunoprecipitated with antibody C4. Lane numbers are shown at the top, and the constructs expressed are indicated at the bottom.

To show that coimmunoprecipitation of N-terminal constructs with C-terminal constructs reflects associations initiated in the cell and not an artificial association resulting from

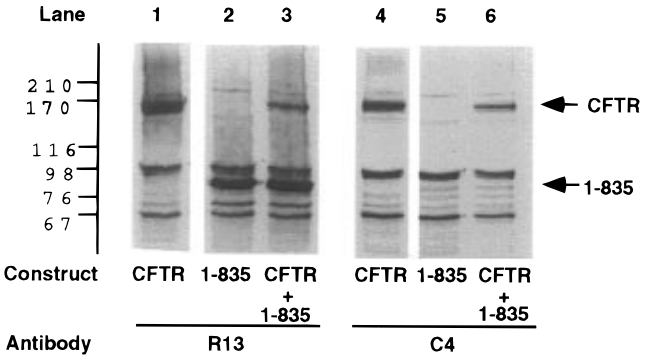

FIGURE 7: Full-length CFTR does not coimmunoprecipitate with 1−835. Constructs were expressed in FRT (Fischer rat thyroid) cells and subsequently treated as indicated in legend of Figure 3. Proteins were immunoprecipitated with antibody R13 (lanes 1−3) or antibody C4 (lanes 4−6). Lane numbers are shown at the top, and the expressed constructs are indicated at the bottom.

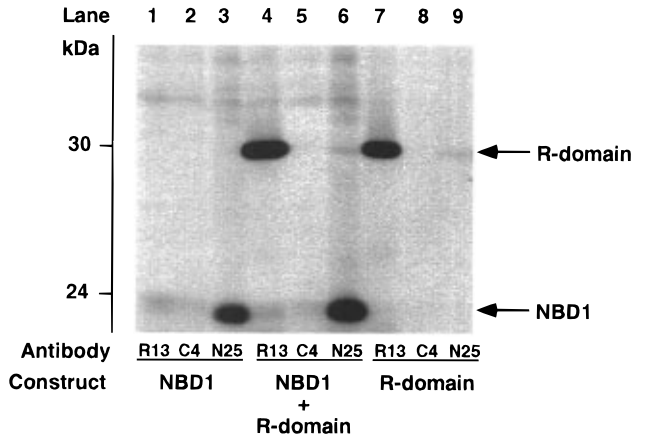

FIGURE 8: Cytosolic domains NBD1 and R domain do not coimmunoprecipitate. Constructs were expressed separately or coexpressed, and treated as indicated in legend of Figure 3. Proteins were immunoprecipitated with antibodies R13, C4, or N25. Lane numbers are shown at the top, and the expressed constructs and antibodies are indicated at the bottom.

solubilization and coimmunoprecipitation, we expressed the C-terminal and N-terminal constructs separately. They were then solubilized and combined before immunoprecipitation. Under these conditions, we observed no coimmunoprecipitation (not shown), suggesting that associations must take place within the cell and that, once the cells have been disrupted by solubilization, the association can no longer form.

We considered the possibility that association of the halves of CFTR could be the result of nonspecific interactions following expression of these constructs. We earlier published studies demonstrating that truncated versions of CFTR (CFTRΔR or CFTR1419X) did not coimmunoprecipitate with full-length wild-type CFTR (Marshall et al., 1994), suggesting this was not the case. We did several additional studies addressing this issue. First, the N-terminal half of CFTR, 1−835, did not coimmunoprecipitate with full-length wild-type CFTR (Figure 7). This result suggests that 1−835 is not just a "sticky" protein that associates with any polytopic membrane protein. Second, when the R domain and NBD1 were expressed under conditions identical to those used for constructs containing membrane-spanning domains, no coimmunoprecipitation was observed despite high levels of expression (Figure 8).

Third, we expressed portions of MSD1 and MSD2 (1−148 and 837−1207, respectively) with a chimeric protein which contains the extracellular and membrane-spanning domains of the HLA-A2 protein fused to NBD1 of CFTR (A2-NBD1). We immunoprecipitated these proteins and then detected them by immunoblotting. When 1−148 was expressed alone, it was recognized on a blot by R13 only after immunoprecipitation with R13 (Figure 9, lanes 1−3). Likewise, 837−1207 was recognized on a blot by antibody C24 only after immunoprecipitation with C4 (lanes 6−8). Further, A2-NBD1 was recognized by the A2 blotting antibody (HLA-A2) only after immunoprecipitation with N25 (lanes 11−13). When coexpressed, 1−148 coimmunoprecipitated 837−1207 (lane 9) and 837−1207 coimmunoprecipitated 1−148 (lane 4). However, neither construct was immunoprecipitated when coexpressed with A2-NBD1 and immunoprecipitated with N25 (lanes 5 and 10). Finally, A2-NBD1 was not coimmunoprecipitated with 1−148 using R13 (lane 14) or with 837−1207 using C4 (lane 15). Similarly, 1−244 and 1−358 were coimmunoprecipitated with 837−1207, but not with A2-NBD1 (not shown). These data suggest that coimmunoprecipitation of membrane-spanning domains of CFTR results from specific associations rather than nonspecific interaction between membrane proteins.

The most common CF-associated mutant, ΔF508, is misprocessed; it is retained in the endoplasmic reticulum and does not travel to the plasma membrane (Cheng et al., 1990). We asked if the defect that causes retention would also prohibit association of an N-terminal half that contained the ΔF508 mutation, 1−835ΔF508, with the C-terminal half, 837−1480. As shown in Figure 10A, 1−835ΔF508 was coimmunoprecipitated with 837−1480 (lane 5). This result suggests that the ΔF508 mutation does not disrupt interactions between domains.

Coimmunoprecipitation between transmembrane segments suggested the possibility that mutations in transmembrane regions themselves might alter the association between halves. We made an N-terminal construct that contained the CF-associated mutation G91R (Guillermit et al., 1993) in M1 (1−835G91R) and expressed it alone or with the C-terminal half, 837−1480. Figure 10B shows that 1−835G91R was coimmunoprecipitated with 837−1480 (lane 3). Two other naturally occurring mutations in M1, E92K and G85E (Nunes et al., 1993; Zielenski et al., 1991), also failed to disrupt associations between halves. These results suggest either that M1 or this limited sequence of M1 is not the site of transmembrane association or that mutation of these three M1 residues is not sufficient to disrupt an association in this region.

DISCUSSION

To form a channel, all of the domains of CFTR must pack together in a specific and stable arrangement. The question of how this occurs and how the interactions are stabilized is a general one for all members of the traffic ATPase/ABC transporter family, as well as for other ion channels and transporters. Our data indicate that, in CFTR, the N- and C-terminal halves can associate in the cell to generate functional channels. A similar result has been observed with the multidrug resistance transporter, MDR1 (Loo & Clarke, 1994), and with STE6, the yeast pheromone transporter (Berkower & Michaelis, 1991). Coexpression of the N- and C-terminal halves of MDR and STE6 generates drug-stimulatable ATPase activity and a-factor-dependent mating, respectively.

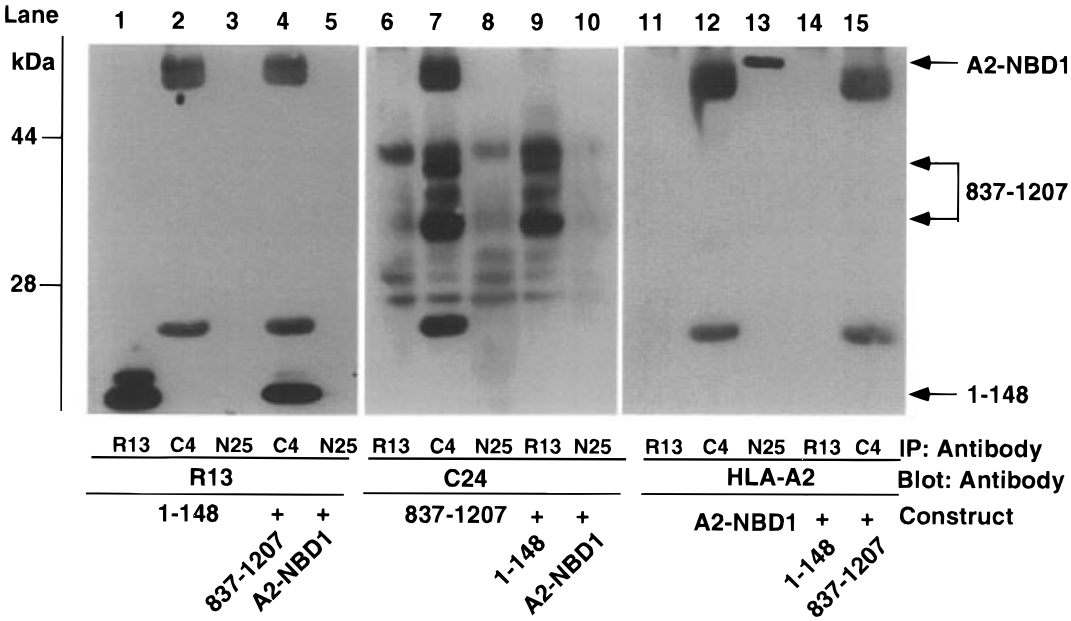

FIGURE 9: Constructs containing CFTR membrane domains coimmunoprecipitate with each other but not with non-CFTR membrane domains. The chimeric construct A2-NBD1, and the CFTR constructs 1−148 and 837−1207, were expressed separately or coexpressed, immunoprecipitated with the indicated antibodies (R13, C4, or N25), electrophoresed, transferred to nitrocellulose, and immunoblotted with the indicated antibodies (R13, C24, or HLA-A2). Lane numbers are shown at the top, and the expressed constructs are indicated at the bottom. Bands at 55 and 22 kDa in lanes 2, 4, 7, 12, and 15 represent heavy and light chains of monoclonal antibody C4. Heavy and light chains of monoclonal antibody R13 are not detected because lower concentrations of R13 were used for immunoprecipitation.

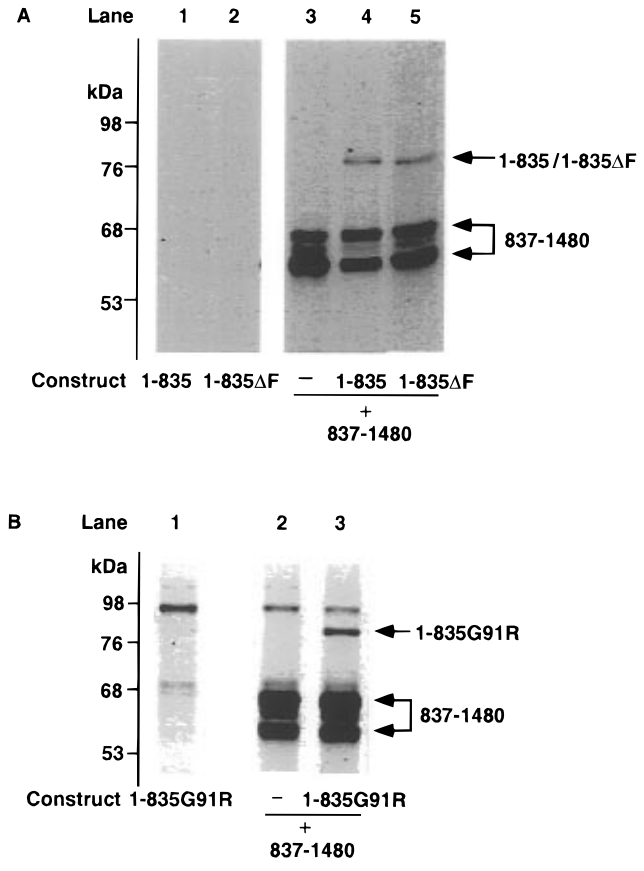

FIGURE 10: (A) Coimmunoprecipitation of 1−835ΔF508 with 837−1480. (B) Coimmunoprecipitation of 1−835G91R with 837−1480. Constructs were expressed separately or coexpressed, and treated as indicated in legend of Figure 3. Proteins were immunoprecipitated with antibody C4. Lane numbers are shown at the top, and the expressed constructs are indicated at the bottom.

Our results provide insight into the domains of CFTR that are responsible for the physical associations that form the channel. The data indicate that associations between the N- and C-terminal halves of CFTR can be attributed primarily to interactions between MSD1 and MSD2. In fact, M1 and M2 with the intervening extracellular loop were sufficient for association with MSD2. This does not preclude, however, interactions between other membrane sequences which we have not studied. Persistence of these associations after solubilization and coimmunoprecipitation suggests a fairly strong interaction. Furthermore, previous studies showing that full-length CFTR does not coimmunoprecipitate with a CFTR variant truncated at the C-terminus or with the CFTRΔR construct indicate that the coprecipitations of halves is not due to nonspecific membrane protein interactions (Marshall et al., 1994). Likewise, we have been unable to show coimmunoprecipitation of full-length CFTR with the N-terminal half, association of NBD1 with the R domain, or association of MSD1 or MSD2 with an unrelated membrane protein. The lack of association of full-length CFTR with the N-terminal half contrasts with results obtained with STE6 where the full-length protein can be immunoprecipitated by either an N- or a C-terminal half (Berkower et al., 1996). In STE6, association of mutant full-length STE6 with a normal N- or C-terminal half restored mating function. Our results suggest that expression of normal half molecules or individual domains would not be sufficient to restore function in mutated CFTR. Because coimmunoprecipitation persisted in variants missing the NBDs, the R domain, and the N- and C-termini, it appears that these cytosolic domains are not required for association. However, we cannot exclude the possibility that additional interactions occur between cytosolic domains that are transient or are not maintained during solubilization and coimmunoprecipitation. For instance, it has recently been shown that NBD1 and NBD2 as well as MSD1 and MSD2 may be sites of association between halves of P-glycoprotein (Loo & Clarke, 1995).

Our observation that association between domains of CFTR does not involve the N-terminus contrasts with studies in both voltage-gated K^+ channels and the acetylcholine

receptor where interactions through the N-terminus are responsible for the initial association of subunits (Li et al., 1992; Verrall & Hall, 1992). However, in those channels, such associations alone are unlikely to be entirely responsible or sufficient for construction of the final three-dimensional organization of these channels (Becq et al., 1993; Babila et al., 1994; Green & Claudio, 1993). Because CFTR is not an oligomer of several associated subunits, there is no reason to expect similar associations via the N-terminus (Marshall et al., 1994).

Although our data point to the MSDs as the site of association, they do not allow us to determine whether interactions between transmembrane sequences or between intracellular or extracellular loops are responsible. Studies of glycophorin A and the T-cell receptor (TCR) provide a precedent for associations that occur between transmembrane sequences. The transmembrane segment of glycophorin A has been shown to be responsible for dimerization of the functional protein (Lemmon et al., 1992). Klausner and colleagues (Manolios et al., 1990) demonstrated that residues within the transmembrane domain of the α subunit of TCR are responsible for association with other members of the TCR complex, and they identified specific charged residues within the transmembrane domain that are required for the interaction. Interestingly, CFTR contains 11 charged residues within the predicted bounds of the 12 transmembrane sequences (Riordan et al., 1989).

Associations between residues within specific transmembrane or adjacent loop sequences of CFTR would provide a mechanism for establishing and stabilizing the highly ordered structure required to form a Cl^- channel. Our data suggest that further identification of interacting sites within the MSDs is feasible and could provide a better understanding of the organization and function of the CFTR Cl^- channel.

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