A Single Conductance Pore for Chloride Ions Formed by Two Cystic Fibrosis Transmembrane Conductance Regulator Molecules

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The cystic fibrosis transmembrane conductance regulator (CFTR) is a cAMP-dependent protein kinase (PKA)- and ATP-regulated chloride channel, whose gating process involves intra- or intermolecular interactions among the cytosolic domains of the CFTR protein. Tandem linkage of two CFTR molecules produces a functional chloride channel with properties that are similar to those of the native CFTR channel, including trafficking to the plasma membrane, ATP- and PKA-dependent gating, and a unitary conductance of 8 pico-siemens (pS). A heterodimer, consisting of a wild type and a mutant CFTR, also forms an 8-pS chloride channel with mixed gating properties of the wild type and mutant CFTR channels. The data suggest that two CFTR molecules interact together to form a single conductance pore for chloride ions.

CFTR is a multi-functional protein, which provides the pore of a linear conductance chloride channel (1–5) and also functions to regulate other membrane proteins (6, 7). Mutations in CFTR leading to defective regulation or transport of chloride ions across the apical surface of epithelial cells are the primary cause of the genetic disease of cystic fibrosis (8–10). Comprehensive genotype-phenotype studies have indicated possible contributions of protein-protein interactions to the severity of the disease (11), but little is known on the stoichiometry of CFTR and the ΔR channels. Our rationale is as follows. If a monomer of CFTR is sufficient to form an 8-pS chloride channel, the dimeric CFTR molecules would form either a 16-pS chloride channel or two 8-pS chloride channels that may gate independently or together. On the other hand, if two CFTR molecules are required to function as a chloride channel, we expected the tandem construct to form a single 8-pS chloride channel, provided that the linker sequence does not affect the CFTR channel function. Furthermore, we predicted that the wt-ΔR (or ΔR-wt) channel should exhibit mixed properties of the wt and ΔR channels.

EXPRESSMENT PROCEDURES

Subcloning of CFTR cDNAs—The wild type and ΔR (708–835) CFTR cDNAs were cloned into the NheI/XhoI sites of the pCEP4 expression vector (14, 17). The tandem constructs, wt-wt, wt-ΔR, ΔR-wt, and ΔR-ΔR, were generated in three steps. First, site-directed mutageneses were used to remove the stop codon and to introduce a BassHI restriction site at the 3’ end of the CFTR cDNA, to create C-BassH. Second, similar approach was taken to remove the Kozak sequence and to introduce a BassHII site at the 5’ end of the CFTR cDNA, to yield N-BassH. Third, the entire CFTR cDNA from N-BassH was released from the pCEP4 vector through digestion with BassHII and XhoI and ligated into the BassHII/XhoI sites of the C-BassH, to create the wt-wt dimer. This represents a direct head-to-tail linkage of two CFTR cDNAs. A double stranded oligonucleotide containing the recognition sequence for thrombin (underlined), Arg-Ala-Ala-Ser-Leu-Val-Pro-Arg-Gly-Ser-Gly, Gly-Gly-Gly, was ligated to the BassHII site of wt-wt, to yield the wt-wt construct.

Expression of CFTR in HEK 293 Cells—The human embryonic kidney (HEK 293) cells were used for expression of CFTR proteins. The different CFTR cDNAs were introduced into the HEK 293 cells using the LipofectAMINE reagent. Two days after transfection, the cells were used for Western blot assay, SPQ measurement, or isolation of membrane vesicles followed by reconstitution studies in the lipid bilayer membranes, as described previously (14, 15).

SPQ Assay of Chloride Transport—The CFTR-mediated chloride transport was measured by SPQ assay with HEK 293 cells expressing the wt, ΔR, wt-wt, ΔR-wt, and ΔR ΔR CFTR proteins, following the procedure described previously (14). Basically, the cells were loaded with SPQ dye (Molecular Probes) using hypotonic shock, and chloride flux across the plasma membrane was measured upon stimulation with 10 μM forskolin.

Lipid Bilayer Reconstitution of CFTR Channel—The procedure for single channel measurements of CFTR using the lipid bilayer reconstitution technique has been described elsewhere (17). Briefly, microsomal membrane vesicles were isolated from HEK 293 cells transiently expressing either the wt-, ΔR-, wt-wt, wt-ΔR, ΔR-wt, or wtΔR ΔR proteins and added to the cis (intracellular) solution containing 200 mM KCl, 2 mM Mg-ATP, 10 mM HEPES-Tris (pH 7.4). The trans solution contained 50 mM KCl, 10 mM HEPES-Tris (pH 7.4). To study the PKA-dependent regulation of the CFTR channel, 100 units/ml of the catalytic subunit of PKA was added to the cis solution. Single channel currents were recorded using an Axopatch 200A patch clamp unit (Axon Instruments). Data acquisition and pulse generation were performed with a 486 Computer and a 1200 Digidata A/D-D/A converter. The currents were sampled at 1–2.5 ms/point and filtered at 100 Hz. Single channel analysis were performed with the pClamp7 software.

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 RESULTS AND DISCUSSION

Fig. 1A shows a Western blot of CFTR expressed in HEK 293 cells. Both fully glycosylated (~170 kDa) and core glycosylated (~140 kDa) proteins can be detected in cells transfected with the wt-CFTR cDNA (lane 1). The corresponding bands for the ΔR-CFTR run at apparent molecular masses of ~150 and ~120 kDa (lane 2), reflecting the deletion of 128 amino acids from the R domain (amino acids 708–835). The wt protein has molecular masses of ~340 and ~280 kDa (lane 4), as expected for a dimer of the wt-CFTR. Similarly, the wt-ΔR (lane 5), ΔR-wt (lane 6), and ΔR-ΔR (lane 7) proteins can all be expressed in HEK 293 cells, with the expected size as dimers. To be able to manipulate the oligomerization state of the CFTR proteins, we engineered an enzymatic digestion site for thrombin in the linker sequence of the wt-dimer. This construct is named wt-e-wt (Fig. 1A, lane 10). Digestion of wt-e-wt with thrombin resulted in reduction of the apparent size from dimer to monomers (lane 9), whereas thrombin had no effect on the wt monomer (lane 8) or the wt-wt dimer (lane 11).

SPQ assays indicate that the wt-wt dimer, similar to the wt monomer, supports chloride transport in HEK 293 cells upon stimulation with forskolin (Fig. 1B). Those cells expressing the ΔR and ΔR-wt proteins exhibit basal chloride transport activities without stimulation with forskolin, which is consistent with the studies of Rich et al. (16). Interestingly, the cells expressing wt-ΔR have basal chloride transport activity in the absence of forskolin, which became significantly higher upon stimulation by forskolin (relative changes in fluorescence per minute, 0.095 ± 0.010, −forskolin; 0.203 ± 0.007, +forskolin, n = 65). These results indicate that CFTR dimers can traffic properly to the plasma membrane of HEK 293 cells.

To study the single channel functions of the CFTR dimers, microsomal membrane vesicles containing the wt-wt or ΔR-ΔR proteins are incorporated into the lipid bilayer membrane. Fig. 2A shows representative current traces from the wt, wt-wt, ΔR, and ΔR-ΔR channels, and their corresponding current-voltage relationships are plotted in Fig. 2B. As can be seen, all four constructs give rise to chloride channels with unitary conductances of ~8 pS. In 9 out of 13 experiments with wt-wt, and 8 out of 12 experiments with ΔR-ΔR, we only observed openings of a single channel (not two channels) in the bilayer membrane. Similar to the wt channel, opening of the wt-wt channel absolutely requires the presence of both ATP and PKA in the cytosolic solution; and similar to ΔR, opening of the ΔR-ΔR channel is independent of PKA phosphorylation. Interestingly, the activity of the wt-wt channel appears to be significantly lower than that of the wt channel (Fig. 2C). Studies from other laboratories have shown that the amino- and carboxyl-terminal tails of CFTR contribute to the overall function of the CFTR channel (19, 20). We speculate that the head-to-tail connection in the dimeric construct probably constrains the movement of the amino- and carboxyl-terminal portions of CFTR, reducing activity of the wt-wt channel (see also Fig. 4).

Thus, it appears that the dimeric constructs of CFTR form functional chloride channels with conduction properties that are indistinguishable from the monomers of CFTR, i.e. all of them have single channel conductance of ~8 pS. The dimeric constructs could in principle have two separate pores with
late added to the extracellular solution completely inhibited activities of wt- channels (see Fig. 2). Diary plot of channel open probability (P_o) as a function of time with the wt-ΔR channel (C) and the ΔR-ΔR channel (D). P_o was calculated as the fractional time occupied by the open state during the 5-s test pulse to -100 mV. The plot showed that PKA phosphorylation increased activity of the wt-ΔR channel, but it did not affect open probability of the ΔR-ΔR channel. 3 mM diphenyl carboxylate added to the extracellular solution completely inhibited activities of both wt-ΔR and ΔR-wt channels. The plot shown in C is representative of four other experiments with wt-ΔR and three other experiments with ΔR-wt and that in D is representative of four other experiments with the ΔR-ΔR channel.

Fig. 3. PKA dependence of the wt-ΔR and ΔR-wt channels. Selected single channel currents through the wt-ΔR (A) and ΔR-wt (B) channels at -100 mV test potential, in the absence of PKA (PKA) and presence of PKA (+PKA) in the cis solution. Notice the fast gating kinetics of the wt-ΔR and ΔR-wt channels compared with the wt and ΔR channels (see Fig. 2A). Diary plot of channel open probability (P_o) as a function of time with the wt-ΔR channel (C) and the ΔR-ΔR channel (D). P_o was calculated as the fractional time occupied by the open state during the 5-s test pulse to -100 mV. The plot showed that PKA phosphorylation increased activity of the wt-ΔR channel, but it did not affect open probability of the ΔR-ΔR channel. 3 mM diphenyl carboxylate added to the extracellular solution completely inhibited activities of both wt-ΔR and ΔR-wt channels. The plot shown in C is representative of four other experiments with wt-ΔR and three other experiments with ΔR-wt and that in D is representative of four other experiments with the ΔR-ΔR channel.

conductance of 8 pS for chloride ions, and because of some physical constraint due to the linker sequence, opening of one pore could prevent opening of the other pore, which would result in the overall appearance of a single CFTR channel. The other possibility is that the 8-pS channel normally recorded in single channel measurements (2, 4, 5, 14, 17) actually represents dimeric complexes of CFTR that naturally assemble in the cell surface membrane. Data from the following sets of experiments support the latter hypothesis.

The heterodimers of CFTR, wt-ΔR and ΔR-wt, also form functional chloride channels with unitary conductance of 8 pS, which display mixed gating properties of the wt and ΔR channels (Fig. 3). First, opening of the wt-ΔR and ΔR-wt channels exhibit bursting kinetics, but unlike either the wt or ΔR channels, these bursting patterns are interrupted by fast closing transitions (compare traces of Fig. 3, A and B, with Fig. 2A). The wt channel has an average open lifetime of τ_o = 96.0 ± 9.3 ms, and the ΔR channel has a τ_o = 54.2 ± 6.5 ms (17), whereas the wt-ΔR channel has a τ_o = 24.4 ± 5.8 ms (n = 9), and the ΔR-wt channel has a τ_o = 32.4 ± 3.4 ms (n = 7). Second, open probability of the wt-ΔR and ΔR-wt channels display a clear PKA dependence (Fig. 3C). The channels exhibit constitutive activity in the absence of PKA, which becomes significantly higher upon PKA phosphorylation (Fig. 2C). In contrast, open probabilities of the ΔR and ΔR-ΔR channels are completely independent of PKA phosphorylation (Fig. 3D).

Fig. 4. Effect of thrombin on the wt-e-wt CFTR channel. A, selected current traces at -100 mV from a single wt-e-wt channel in the presence of 2 mM ATP and 100 units/ml of PKA (control), and 3 min after the addition of 10 units of thrombin to the intracellular solution (+thrombin). Treatment of the wt-e-wt channel with thrombin resulted in significant increase of P_o from 0.186 ± 0.045 (n = 11, control) to 0.456 ± 0.064 (n = 7, +thrombin), without affecting the distribution of single channel conductance states, i.e. the number of channels in the bilayer remained unchanged. B, time-dependent effect of thrombin on the wt-e-wt channel. Following stable incorporation of a single wt-e-wt channel in the bilayer membrane, 10 units of thrombin was first added to the cis solution. The second addition of thrombin was 40 units.

Taken together, we have shown that the dimeric constructs of CFTR can be expressed in the plasma membrane of HEK 293 cells, and these CFTR dimers form functional chloride channels with unitary conductance of 8 pS, similar to the native CFTR channel (2, 4). The gating properties of the wt-wt and ΔR-ΔR channels are similar to the wt and ΔR channels, whereas the wt-ΔR and ΔR-wt channels exhibit mixed properties of the wt and ΔR channels. The fact that both wt-ΔR and ΔR-wt channels exhibit similar PKA dependence and similar gating kinetics (Fig. 3, A and B) suggests that both halves of the CFTR dimer are properly expressed and inserted in the membrane of HEK 293 cells. The tandem linkage of CFTR apparently reduces the overall activity of the chloride channel due to physical constraints introduced at the junction of the two CFTR molecules, but does not affect the conduction property of the chloride channel, since cleavage of wt-e-wt with thrombin did not change the conductance state of the channel. Our data suggest that two CFTR molecules are required for the chloride channel to open to the 8-pS conductance state.
forms of CFTR and concluded that CFTR exists predominantly in a monomeric state. It may be that the intermolecular interactions between the CFTR molecules are weak and do not survive strong detergent solubilization (i.e. SDS) or that the CFTR dimers only represent a small percentage of the total CFTR proteins that could not be detected by the co-immunoprecipitation procedure. A recent study by Eskandari et al. (22) established structural evidence for a dimeric complex with the CFTR proteins. These investigators used freeze fracture electron microscopy to investigate the oligomeric assembly of membrane proteins expressed in Xenopus oocytes, and they concluded that the intramembrane structure of CFTR was consistent with a dimeric assembly of 12-transmembrane helix of the CFTR monomers.

Besides being of fundamental importance in understanding the mechanism by which the CFTR channel works, the concept that CFTR functions as a dimer may have implication for persons who are compound heterozygotes for CFTR mutations. Some mutant pairs may be able to complement each other thereby increasing the overall CFTR function, but other mutant pairs may not. This may explain some of the phenotypic variations in patients with CFTR mutations, especially those with some residual function. It will be important to know at what stage in the biosynthetic pathway of CFTR the dimers are formed, i.e. before leaving the endoplasmic reticulum or after trafficking to the apical membrane. More specifically, which portions of the CFTR molecule are involved in the contact interaction or constitute the binding site(s) for accessory proteins that could interact with CFTR?

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REFERENCES

1. Riordan, J. R., Rommens, J. M., Kerem, B.-S., Alon, N., Rozmahel, R., Grzelczak, Z., Zielenski, J., Lok, S., Plavsic, N., Chou, J.-L., Drumm, M. L., Iannuzzi, M. C., Collins, F. S. & Tsui, L.-C. (1989) Science 245, 1066–1073
2. Bear, C. E., Li, C., Kortner, N., Bridges, R. J., Jensen, T. J., Ramjeesingh, M. & Riordan, J. R. (1992) Cell 68, 809–818
3. Smit, L. S., Wilkinson, D. J., Mansoura, M. K., Collins, F. S. & Dawson, D. C. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 9963–9967
4. Tabcharani, J. A., Rommens, J. M., Hou, Y. X., Chang, X. B., Tsui, L. C., Riordan, J. R. & Hanrahan, J. W. (1993) Nature 366, 79–82
5. Gunderson, K. L., and Kopito, R. R. (1994) J. Biol. Chem. 269, 19349–19353
6. Egan, M., Platte, T., Aifone, S., Solow, R., Zeitlin, P. L., Carter, B. J. & Guggino, W. B. (1992) Nature 358, 581–584
7. Stutta, M. J., Canessa, C. M., Olsen, J. C., Hamrick, M., Cohn, J. A., Rossier, B. C. & Boucher, R. C. (1995) Science 269, 847–850
8. Quinton, P. M. (1986) Am. J. Physiol. 251, C649–C652
9. Welsh, M. J. & Smith, A. E. (1993) Cell 73, 1251–1254
10. Tsui, L. C. (1995) Am. J. Respir. Crit. Care Med. 151, 847–853
11. Zielenski, J. & Tsui, L. C. (1995) Annu. Rev. Genet. 29, 777–807
12. Gadsby, D. C. & Nairn, A. C. (1994) Trends Biochem. Sci. 19, 513–518
13. Cheung, M. & Akabas, M. H. (1997) J. Gen. Physiol. 109, 289–299
14. Xie, J., Drumm, M. L., Ma, J. & Davis, P. B. (1995) J. Biol. Chem. 270, 28084–28096
15. Ma, J., Tasch, J., Tao, T., Zhao, J., Xie, J., Drumm, M. L. & Davis, P. B. (1995) J. Biol. Chem. 271, 7351–7356
16. Rich, D. P., Gregory, R. J., Anderson, M. P., Manavalan, P., Smith, A. E. & Welsh, M. J. (1991) Science 253, 205–207
17. Ma, J., Zhao, J., Drumm, M. L., Xie, J. & Davis, P. B. (1997) J. Biol. Chem. 273, 28133–28141
18. Winter, M. C. & Welsh, M. J. (1997) Nature 399, 294–296
19. Loo, M. A. et al. (1997) Pediatr. Pulmonol. Suppl. 14, 212
20. Ware, A. P. et al. (1998) Pediatr. Pulmonol. Suppl. 17, 94
21. Marshall, J., Fang, S., Ostergaard, L. S., O’Riordan, C. R., Ferrara, D., Amara, J. F., Hoppe, H., Scheule, R. K., Welsh, M. J., Smith, A. E. & Cheng, S. H. (1994) J. Biol. Chem. 269, 2987–2995
22. Eskandari, S., Wright, E. M., Kremen, M., Starace, D. M. & Zampighi, G. A. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 11235–11240