The Interaction between Syntaxin 1A and Cystic Fibrosis Transmembrane Conductance Regulator Cl− Channels Is Mechanistically Distinct from Syntaxin 1A-SNARE Interactions*

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Radhika Ganeshan‡, Anke Diš, Deborah J. Nelson§, Michael W. Quick¶, and Kevin L. Kirk†

From the ‡Department of Physiology and Biophysics, Gregory Fleming James Cystic Fibrosis Research Center, University of Alabama at Birmingham, Birmingham, Alabama 35294, the §Department of Neurobiology, Pharmacology, and Physiology, University of Chicago, Chicago, Illinois 60637, and the ¶Department of Biological Sciences, University of Southern California, HNB 228, Los Angeles, California 90089

Syntaxin 1A binds to and inhibits epithelial cystic fibrosis transmembrane conductance regulator (CFTR) Cl− channels and synaptic Ca2+ channels in addition to participating in SNARE complex assembly and membrane fusion. We exploited the isoform-specific nature of the interaction between syntaxin 1A and CFTR to identify residues in the H3 domain of this SNARE (SNARE motif) that influence CFTR binding and regulation. Mutating isoform-specific residues that map to the surface of syntaxin 1A in the SNARE complex led to the identification of two sets of hydrophilic residues that are important for binding to and regulating CFTR channels or for binding to the syntaxin regulatory protein Munc-18a. None of these mutations affected syntaxin 1A binding to other SNAREs or the assembly and stability of SNARE complexes in vitro. Conversely, the syntaxin 1A-CFTR interaction was unaffected by mutating hydrophobic residues in the H3 domain that influence SNARE complex stability and Ca2+ channel regulation. Thus, CFTR channel regulation by syntaxin 1A involves hydrophilic interactions that are mechanistically distinct from the hydrophobic interactions that mediate SNARE complex formation and Ca2+ channel regulation by this t-SNARE.

The cystic fibrosis transmembrane conductance regulator (CFTR) is a cyclic AMP-activated anion channel that mediates salt and fluid transport across epithelial cells (1). The CFTR protein consists of a symmetric arrangement of two membrane-spanning regions, two nucleotide binding domains (nucleotide binding domains 1 and 2), and a central regulatory domain with multiple phosphorylation sites (2). The amino-terminal cytoplasmic tail binds to syntaxin 1A, a component of the membrane traffic machinery, and this interaction is blocked by Munc-18, a high affinity, syntaxin-binding protein (3). Syntaxin 1A inhibits CFTR-mediated chloride currents in a variety of cell types and expression systems. This effect of syntaxin 1A on CFTR channel activity may be due to the fact that the amino-terminal cytoplasmic tail of CFTR, to which syntaxin 1A binds, regulates channel gating apparently by interacting with the regulatory domain and/or nucleotide binding domain 1 (4). Syntaxin 1A is a t-SNARE that is highly expressed in neurons and, to a lesser extent, in a variety of epithelial cells (5). Together with SNAP-25 (another t-SNARE) and VAMP-2/synaptobrevin (a v-SNARE), syntaxin 1A assembles into core SNARE complexes that regulate membrane fusion at the presynaptic membrane in neurons. The ternary SNARE complex consists of a parallel four-helix bundle containing one coiled-coil domain each from syntaxin and VAMP-2/synaptobrevin and two from SNAP-25 (6). The four associating α-helices contain hydrophobic residues that are grouped into “layers” that zipper together the four-helix bundle, resulting directly or indirectly in bilayer mixing. This paradigm applies to nonneuronal as well as to neuronal fusion events; in the former case, other SNARE isoforms are involved. The interactions among v-SNAREs and t-SNAREs are typically nonselective, at least in vitro (7), presumably because hydrophobic residues that are common to multiple isoforms mediate these interactions.

The helical region of syntaxin 1A that participates in SNARE complex assembly, termed the SNARE motif or the H3 domain (8), is proximal to the COOH-terminal membrane anchor region and lies downstream of another helical domain referred to as Hbc. The SNARE motif, which forms an amphipathic helix, is composed of residues that are arranged in a heptad repeat manner designated a–g. The residues that map to heptad positions a and d are typically hydrophobic, well conserved across various syntaxin isoforms, and usually buried in the hydrophobic core of the SNARE complex. Conversely, residues at the b, c, and f heptad positions are more variable and are exposed on the surface of the ternary complex. The cytoplasmic domain of syntaxin 1A, encompassing the Hbc and H3 domains, also interacts with Munc-18a with high affinity, and this interaction prevents syntaxin 1A from participating in SNARE complex assembly in vitro (9).

In addition to its role in the assembly of SNARE complexes, syntaxin 1A also has been reported to modulate multiple types of ion channels and transporters (3, 10–12). For example, syntaxin 1A inhibits the activities of several types of voltage-gated Ca2+ channels in a variety of expression systems and cell types. It has been proposed that the simultaneous association of syntaxin 1A with synaptic vesicle proteins and voltage-gated Ca2+ channels may help couple SNARE complex formation/dissociation to the influx of Ca2+ at neurotransmitter release sites (13). With respect to CFTR-syntaxin interactions, syntaxin 1A, but not any other isoform that has been tested (i.e. syntaxins 2–5),
bends to the CFTR amino-terminal tail (N-tail) and inhibits channel activity (5). In addition, CFTR binds to the H3 domain of syntaxin 1A, and soluble syntaxin 1A peptides that lack the transmembrane region but include the H3 region can rescue CFTR-mediated currents from inhibition by membrane-anchored syntaxin 1A (3, 5). As argued for the syntaxin 1A-Ca2+ channel interaction, the interaction between this t-SNARE and CFTR channels may help synchronize the activity of this channel with protein traffic in epithelial cells. Thus, the ability of syntaxin 1A to influence the function of ion channels and to couple their activity to membrane traffic may be a general phenomenon. However, it is not clear if the structural basis of the interactions between syntaxin 1A and different ion channels is similar. In this regard, Bezprozvanny et al. (14) have reported that the regulation of voltage-gated Ca2+ channels by syntaxin 1A is disrupted by point mutations in specific hydrophobic residues in the H3 domain that are also implicated in SNARE complex stability.

In the present study, we exploited the isoform specificity of the syntaxin 1A interaction with CFTR to identify unique residues in the H3 domain of syntaxin 1A that participate in CFTR binding and channel regulation. These residues are hydrophilic and are located in the outer shell of the SNARE complex structure (i.e., distinct from those in the hydrophobic layers that stabilize the SNARE complex). Mutating these residues diminished both the physical and functional interactions of syntaxin 1A with CFTR but had no effect on the biochemical association of syntaxin 1A with other SNARE proteins or with Munc-18. Conversely, the CFTR-syntaxin 1A interaction was not affected by mutations in specific hydrophobic residues of the H3 domain that disrupt Ca2+ channel regulation and compromise SNARE stability. Our results indicate that syntaxin 1A regulates CFTR channels by a protein-protein interaction that is mechanistically distinct from how syntaxin 1A participates in SNARE complex assembly or regulates voltage-gated calcium channels.

EXPERIMENTAL PROCEDURES

Antibodies—A mouse monoclonal antibody to syntaxin 1A (Cl 78.3) was obtained from Synaptic Systems (Germany). An affinity-purified polyclonal antibody raised against human SNAP-23 and a mouse monoclonal antibody raised against the COOH-terminal tail of human CFTR have been described earlier (15). A mouse monoclonal antibody raised against Munc-18a was purchased from Transduction Laboratories.

cDNA Constructs and Purification of GST Fusion Proteins—The cDNA encoding human syntaxin 1A was obtained from a T84 cDNA library (3). Single and double alanine or leucine substitutions in the H3 domain of syntaxin 1A were generated by PCR mutagenesis. An appropriate mutagenic oligonucleotide was incorporated in the PCR reaction using cDNA encoding human syntaxin 1A as template. The upstream primer contained an EcoRI site, and the downstream primer included a XhoI site. After digestion with EcoRI and XhoI, the PCR product was ligated back into the appropriate sites in the pCDNA3 eukaryotic expression vector (Invitrogen). The cytosolic region (residues 1–266) of syntaxin 1A was amplified by PCR and subcloned into pGEX bacterial GST fusion expression vector (Amersham Biosciences). All point mutations were confirmed by DNA sequencing of the entire coding expression vector (Invitrogen). The cytosolic region (residues 1–266) of this syntaxin 1A mutant and the full-length coding region were amplified by PCR and subcloned into pGEX-2X-1 and pCDSN, respectively. All GST-syntaxin 1A fusion proteins (GST-syn1A, ΔC refers to deletion of the COOH-terminal membrane domain) and GST alone were expressed and purified from bacterial cells and dialyzed against phosphate-buffered saline as described (16). GST fusion constructs for mouse SNAP25 (residues 1–206) and rat VAMP2/synaptobrevin (residues 1–94) were gifts of Drs. J. Pevsner and M. Bennett, respectively, and have been described previously (17, 18). In experiments where GST-free proteins were used, fusion proteins were desalted with thrombin as described (19). Protein concentrations were estimated by standard micro-BCA protein assay (Pierce) or by Coomassie Blue staining of protein bands after SDS-PAGE using bovine serum albumin as a standard.

Cell Culture and Lysate Preparation—BHK cells that were stably transfected with full-length wild-type human CFTR were a gift from J. W. Hanrahan and were cultured as described (20). HT29/Cl19A human colonic epithelial cells expressing native CFTR, SNAP25, and Munc18 were propagated as described previously (3). Mouse L-fibroblasts stably transfected with full-length human CFTR and full-length human syntaxin 1A have been described previously (15). Cells grown in 10-cm dishes were lysed in situ on ice with chilled lysis buffer (phos- phatidylserine-containing 1% (v/v) Triton X-100, 1 mM EGTA, 1 mM dithiothreitol, and a protease inhibitor mixture of aprotinin, pepstatin A, and leupeptin at 2 μg/ml each and phenylmethylsulfonyl fluoride at 1 mM). Lysates were centrifuged at 10,000 × g (4°C) to remove cell debris before being used for binding assays.

Binding Assays—The indicated amounts of the GST-syn1AΔC proteins were added to clarified cell lysates (1-mL final reaction volume) and incubated overnight (12–16 h) at 4°C on a rotator. For titration binding experiments, the lysates were diluted in lysis buffer before adding fusion protein. The complexes were captured by mixing with excess glutathione-Sepharose for 2 h, and the beads were washed in several volumes of lysis buffer minus the protease inhibitors. The bound proteins were eluted with Laemmli buffer and separated by SDS-PAGE on 4–20% gradient gels (Bio-Rad) followed by immunoblotting on polyvinyldene difluoride membranes (PerkinElmer Life Sciences) using conditions described (5). CFTR immunoprecipitations were carried out to monitor CFTR protein levels in the lysates as described previously (15). The immunoreactive protein bands were visualized by enhanced chemiluminescence (Pierce). Signals were quantified by densitometric scanning followed by analysis with Scion Image (release beta 4.0.2) (Scion Corp.).

Analysis of SDS-resistant SNARE Complexes—Binary or ternary combinations of GST-free wild-type or mutant syntaxin 1A (residues 1–266), SNAP25, and VAMP2/synaptobrevin (residues 1–94) (2 μM) were mixed overnight at 4°C as described (5). After the addition of Laemmli buffer, samples were divided into two aliquots. One aliquot was incubated at 37°C for 3 min and the other at 100°C for 3 min. To generate the thermal melting profiles of the ternary complexes, the samples were divided into 14 aliquots after adding Laemmli buffer containing 2% SDS and incubated at temperatures between 25 and 100°C (in increments of 6°C) for 3 min in a programmable thermal cycler (MJ Research). The samples were resolved by SDS-PAGE on 4–20% gradient gels (Bio-Rad) and immunoblotted for syntaxin 1A. To quantitate the dissociation of the complexes, the signal intensities of the 100- and 200-kDa complexes were measured by densitometric scanning for each point on the temperature axis and normalized to the 100% standard. For SNARE titration experiments, various concentrations (0.05–1 μM) of recombinant SNAP25 were added to a binary mixture containing 1 μM each of recombinant syntaxin 1A (wild-type or mutant) and VAMP2/synapto- brevin and mixed overnight (16 h) at 4°C as above. After the addition of Laemmli buffer, samples were incubated at 97°C for 3 min and immunoblotted for syntaxin 1A.

Electrophysiology—The methods and solutions used for the Xenopus oocyte expression studies are described in detail elsewhere (16). The methods for the whole-cell patch clamp experiments performed on L-fibroblasts stably transfected with CFTR and syntaxin 1A are similar to those described earlier (15). The pipette solution contained 140 mM N-methyl-D-glucamine, 40 mM HCl, 100 mM l-glutam酸, 0.2 mM CaCl2, 2 mM MgCl2, 1 mM EGTA, 10 mM HEPES, and 5 mM ATP-Mg, pH 7.2. The bath solution contained 140 mM NMDG, 140 mM HCl, 2 mM CaCl2, 1 mM MgCl2, and 10 mM HEPES, pH 7.4. CFTR-mediated Cl− current was induced within 10–15 min of breaking into the cell by superfusing with a mixture containing 10 μM forskolin, 10 μM ethylxanthine, and 400 μM cAMP. cAMP-activated currents were recorded at +110 mV from a holding potential of −40 mV. All experiments were performed at 22–24°C.

RESULTS

Mutating Syntaxin 1A at Isoform-specific Residues Inhibits CFTR Binding or Munc-18 Binding—Our previous findings indicated that CFTR channels physically and functionally interact with human or mouse syntaxin 1A but not with syntaxins 2–5 (5). Therefore, we mutated four residues in the H3

A. P. Naren and K. L. Kirk, unpublished observations.
domain of syntaxin 1A that are unique to this isoform and that are well conserved across species (namely Ser$^{225}$, Tyr$^{235}$, Glu$^{238}$, and Val$^{248}$; see Fig. 1). These residues were replaced with alanine singly or in combination. In addition, we replaced Glu$^{238}$ with leucine, since human syntaxins 4 and 5 have leucine at the corresponding position. The abilities of these syntaxin 1A mutants to bind to CFTR as well as to SNAP-23 (a t-SNARE) and to Munc-18 were tested using the indicated GST-syn1A fusion proteins (see Fig. 2A). Introducing the alanine substitutions at residues Ser$^{225}$ and Glu$^{238}$ ( singly or in combination) as well as the leucine substitution at Glu$^{238}$ of syntaxin 1A resulted in a substantial loss of binding to CFTR as compared with the wild-type protein (upper panel). The E238A and E238L mutations had a more pronounced effect on CFTR binding than the S225A mutation (see also Fig. 3A). Notably, none of the syntaxin 1A mutants that showed comparable binding to SNAP-23 showed a marked decrease in its binding to Munc-18 (bottom panel). As seen from the upper panel of Fig. 2C, the CFTR binding signal was substantially lower for the E238A mutant as compared with wild-type protein at all lysate dilutions. However, the SNAP-23 binding signals were comparable for both syntaxin 1A fusion constructs at all lysate dilutions (lower panel). GST and GST-syn3ΔC were used as controls, and, as expected, GST-syn3ΔC could bind SNAP-23 but not CFTR, whereas GST alone could bind neither.

In summary, the data obtained from multiple binding assays performed using either native or recombinant CFTR indicate that the E238A syntaxin 1A mutant is specifically inhibited for CFTR binding.

Similar titration experiments were performed to compare the binding of Y235A, E238A, and wild-type syntaxin 1A to Munc-18 and SNAP-23 using fixed amounts of GST-syn1AΔC fusion proteins and varying dilutions of HT29-C119A epithelial cell lysates. As shown in the upper panel of Fig. 2D, the Y235A mutant showed considerably reduced Munc-18 binding as compared with wild-type or E238A syntaxin 1A even at the highest concentration of lysate. All three syntaxin 1A fusion proteins showed comparable binding to SNAP-23. In summary, we have generated point mutations in the H3 domain of syntaxin 1A that differentially inhibit binding to CFTR (S225A and E238A,L) and to Munc-18 (Y235A). Both types of mutations map to residues that are not implicated directly in SNARE complex formation (i.e. heptad positions b, c, and f), and, as expected, neither inhibited SNAP-23 binding.

**CFTR Binding Is Not Inhibited by Mutating Hydrophobic Residues Implicated in SNARE Complex Formation and Ca$^{2+}$-Channel Regulation (A240V/V244A)**—Since the preceding results indicate that two hydrophilic residues (Ser$^{225}$ and Glu$^{238}$) of syntaxin 1A are important for its interaction with CFTR, we next examined the effect of mutating residues buried in the hydrophobic layers of the SNARE complex on CFTR binding. For this purpose, we selected a syntaxin 1A double mutant, A240V/V244A, in which the mutated residues map to the a and d heptad positions predicted to be in the hydrophobic core of the SNARE complex. Another important feature of this double

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**Fig. 1.** Domain organization (bottom) and sequence alignment of the SNARE motif of human syntaxin isoforms (top) and of the corresponding regions in syntaxin 1A of human (hum), mouse (mou), and Drosophila (drome) (middle). Heptad positions a–g are indicated below the sequences. Hydrophobic residues at the a and d heptad positions are boxed. Residues unique to the syntaxin 1A isoform are in boldface type, and the corresponding residues in all isoforms are shaded.
mutant is that it was reported to be ineffective for regulating N-type Ca\(^{2+}\) channels in functional assays (14). We compared the CFTR binding of this double mutant with that exhibited by another double mutant in which the two hydrophilic residues that were implicated in CFTR binding based on the preceding data (Figs. 2 and 3A) were converted to alanine (S225A/E238A). As seen in Fig. 3B (upper panel), the A240V/V244A double mutant is not inhibited for binding CFTR unlike the S225A/E238A double mutant. Although residues Ala\(^{240}\) and Val\(^{244}\) are implicated in mediating SNARE-SNARE interactions, due to their location in the hydrophobic layers, the double mutation at these positions did not inhibit binding of syntaxin 1A to SNAP-23 in these pull-down experiments (see Fig. 3, lower panel). However, in studies described below, the A240V/V244A double mutation was found to destabilize core SNARE complexes assembled in vivo.

The E238A Mutation Does Not Inhibit the Formation of SDS-resistant SNARE Complexes in Vitro—A hallmark feature of syntaxin 1A is its ability to assemble into SDS-resistant complexes with SNAP-25 and VAMP-2 in vivo that mimic the properties of native fusion complexes isolated from brain vesicles (21). Accordingly we tested the abilities of the syntaxin 1A mutants to form SDS-resistant complexes with recombinant SNAP-25 and VAMP-2 proteins. As shown in Fig. 4A, at least three prominent high molecular weight complexes, presumably representing monomers (50–70 kDa) and multimers (>100 kDa) of the core SNARE complexes, could be detected when either wild-type or E238A syntaxin 1A was mixed with SNAP-25 and VAMP-2. These complexes were resistant to SDS at 37 °C but could be melted by heating to 100 °C. As expected, SDS-resistant complex formation required all three proteins. To rule out a more subtle effect of the E238A mutation on SNARE complex assembly, we also performed a titration experiment in which we monitored SDS-resistant complex formation induced by mixing increasing amounts of SNAP-25 with fixed amounts of VAMP-2 and syntaxin 1A (wild-type or E238A). As seen from Fig. 4B, titrating in increasing amounts of SNAP-25 resulted in an increased appearance of SDS-resistant complexes to the same extent for either wild-type or E238A syntaxin 1A. Thus, a mutation that profoundly affects the binding of syntaxin 1A to CFTR produced no discernible effect on its ability to form SDS-resistant SNARE complexes with VAMP-2 and SNAP-25.

Mutating Hydrophobic Residues (A240V/V244A) Destabilizes SNARE Complexes, but Mutating Hydrophilic Residues That Participate in CFTR Binding (Ser\(^{225}\) and Glu\(^{238}\)) Does Not—The steady-state formation of SNARE complexes at 37 °C may not be a sensitive indicator of subtle effects of syntaxin 1A mutations on the stability of these complexes. Thus, in order to examine whether point mutations in syntaxin 1A altered the

![Figure 2](image-url)
formation or stability in vitro. On the other hand, mutations that do destabilize SNARE complexes and inhibit Ca\(^{2+}\) channel regulation (A240V/V244A) do not appreciably affect CFTR binding (Fig. 3B).

**Syntaxin 1A Mutants That Are Inhibited for Binding to CFTR Are Also Inhibited for Regulating CFTR-mediated Cl\(^{-}\) Currents**—We next tested the abilities of these syntaxin 1A mutants to regulate CFTR-mediated chloride currents in order to determine (i) whether syntaxin 1A mutations that disrupt CFTR binding also inhibit CFTR channel regulation by this t-SNARE and (ii) whether syntaxin 1A mutations that destabilize SNARE complexes and inhibit regulation of voltage-gated Ca\(^{2+}\) channels (A240V/V244A) are disrupted for CFTR regulation. CFTR-mediated currents were measured in *Xenopus* oocytes co-expressing CFTR and full-length syntaxin 1A proteins by two-electrode voltage clamp analysis. As documented in Fig. 6A, CFTR-mediated currents in oocytes were inhibited in a dose-dependent manner when these oocytes were co-injected with increasing amounts of wild-type syntaxin 1A cRNA, as previously observed (3). Similar degrees of inhibition were observed when CFTR was co-expressed with the V248A and Y235A syntaxin 1A mutants that displayed wild-type levels of binding to CFTR (Fig. 2A). The A240V/V244A double mutant also inhibited CFTR-mediated currents like wild-type syntaxin 1A. On the other hand, the S225A mutation, which moderately compromised CFTR binding, and the E238A and S225A/E238A mutations, which severely inhibited CFTR binding (Fig. 3A), were moderately and severely inhibited for regulating CFTR currents. The attenuation of CFTR-mediated currents by wild-type or mutant syntaxin 1A could be reversed in all cases by first microinjecting into the oocytes botulinum neurotoxin C1 (BoNT/C1), a protease that cleaves syntaxin 1A (Fig. 6B). In order to verify that the relative ineffectiveness of certain syntaxin 1A mutants to inhibit CFTR currents was not due to poor protein expression, we immunoblotted oocyte membranes to confirm the expression of the various syntaxin 1A constructs (Fig. 6C). In combination with our binding data, the results of Fig. 6 indicate that (i) there is a strong correlation between CFTR binding strength and CFTR channel regulation for the various syntaxin 1A mutants that were tested and (ii) a syntaxin 1A mutation (A240V/V244A) that destabilizes SNARE complexes and that disrupts Ca\(^{2+}\) channel regulation in oocytes has no discernible effect on CFTR regulation in this expression system.

In a complementary set of functional experiments, wild-type and mutant GST-syn1A\(\Delta\)C proteins were screened for their abilities to stimulate CFTR-mediated Cl\(^{-}\) currents in L-fibroblasts that were stably transfected with recombinant CFTR and full-length wild-type syntaxin 1A. Previously, we had shown that a GST fusion protein containing the soluble cytosolic domain of syntaxin 1A (GST-syn1A\(\Delta\)C), when introduced into these cells via whole cell patch pipettes, could stimulate CFTR-mediated currents presumably by competing with the membrane-anchored syntaxin 1A for CFTR binding (15). This was also true for GST-syn1A\(\Delta\)C containing the Y235A or A240/V244A mutations, both of which bind CFTR (Fig. 7). Conversely, the E238A mutant and to a lesser extent the S225A mutant (both of which showed reduced binding to CFTR) were compromised in their abilities to stimulate currents in this functional assay. Unexpectedly, the V248A mutant fusion protein, which does bind to CFTR, was also inactive when tested in this assay, despite the fact that the full-length V248A syntaxin 1A did inhibit CFTR-mediated currents in the oocyte expression system (Fig. 6). At present, we have no explanation for this disparity other than the possibility that the peptide rescue experiments may not provide a direct measure of the strength
of the CFTR-syntaxin 1A interaction. Given that this assay may require that the GST-syn1A ΔC peptide disrupt CFTR interactions with multiple SNARE proteins (see “Discussion”), the binding assays and the oocyte expression experiments may provide more direct measures of the strength of the interaction between CFTR and syntaxin 1A.

**Munc-18 Is Unable to Rescue CFTR-mediated Currents from Inhibition by Y235A Syntaxin 1A**—Munc-18 blocks the inhibitory effect of syntaxin 1A on CFTR-mediator currents, due presumably to its ability to block the binding of syntaxin 1A to CFTR (4). In the present study, we identified a novel syntaxin 1A mutation (Y235A) that was inhibited for Munc-18 binding (Fig. 2, A and D). Furthermore, this mutant was able to interact with CFTR as detected in our in vitro binding experiments (Fig. 2A) and electrophysiological assays (Figs. 6A and 7). Accordingly, we examined the functional effect of this mutation in the context of the reciprocal regulation of CFTR by syntaxin 1A and Munc-18 by recording currents in *Xenopus* oocytes coexpressing the three proteins (Fig. 8A). Co-expression of Munc-18 could rescue CFTR currents from inhibition by wild-type syntaxin 1A in oocytes but not from inhibition by Y235A syntaxin 1A. The studies were next extended to L-fibroblasts expressing recombinant CFTR and syntaxin 1A, where CFTR-mediated currents were not detectably affected by wild-type syntaxin 1A or noncovalent Munc-18-1A-D interactions. We identified two hydrophilic amino acids (Glu<sup>238</sup> and Ser<sup>225</sup>) that are specific to the syntaxin 1A isoform and that are necessary for binding to CFTR and modulate CFTR-mediated currents in the oocyte expression system. Mutations did not, however, inhibit syntaxin 1A binding to SNAP-25 or Munc-18; nor did they affect the ability of syntaxin 1A to assemble into SNARE complexes with SNAP-25 and VAMP-2 or the stability of these complexes. This is consistent with the positions of residues Glu<sup>238</sup> and Ser<sup>225</sup> on the outer surface (heptad positions b and c) of the ternary SNARE complex structure. Conversely, mutations in neighboring hydrophobic amino acids (A240V/V244A) that are located in the inner layers (heptad positions a and d) of the core SNARE complex, did not detectably affect either the binding of syntaxin 1A to CFTR or its ability to regulate its channel activity. Thus, the nature of the syntaxin 1A-CFTR interaction appears to be mechanistically distinct from syntaxin 1A-SNARE interactions. The former involves hydrophilic surface residues that are specific to this isoform (Glu<sup>238</sup> and Ser<sup>225</sup>) whereas the latter is mediated by hydrophobic residues present in other syntaxin isoforms and other SNAREs as well. This probably explains why CFTR regulation is specific to the syntaxin 1A isoform, whereas SNARE-SNARE interactions are promiscuous at least in vitro.

**DISCUSSION**

The Syntaxin 1A-CFTR Interaction Involves Hydrophilic Residues That Are Not Involved in SNARE-SNARE Interactions—The goals of this project were to characterize the molecular basis of the interactions between syntaxin 1A and CFTR and to compare the nature of these interactions with that of SNARE-SNARE interactions. We identified two hydrophilic amino acids (Glu<sup>238</sup> and Ser<sup>225</sup>) that are specific to the syntaxin 1A isoform and that are necessary for binding to CFTR and regulating its channel activity. Mutating these residues (E238A and S225A) disrupted the biochemical association of syntaxin 1A with CFTR and the ability of syntaxin 1A to modulate CFTR-mediated currents in the oocyte expression system. The mutations did not, however, inhibit syntaxin 1A binding to SNAP-25 or Munc-18; nor did they affect the ability of syntaxin 1A to assemble into SNARE complexes with SNAP-25 and VAMP-2 or the stability of these complexes. This is consistent with the positions of residues Glu<sup>238</sup> and Ser<sup>225</sup> on the outer surface (heptad positions b and c) of the ternary SNARE complex structure. Conversely, mutations in neighboring hydrophobic amino acids (A240V/V244A) that are located in the inner layers (heptad positions a and d) of the core SNARE complex, did not detectably affect either the binding of syntaxin 1A to CFTR or its ability to regulate this channel. Thus, the nature of the syntaxin 1A-CFTR interaction appears to be mechanistically distinct from syntaxin 1A-SNARE interactions. The former involves hydrophilic surface residues that are specific to this isoform (Glu<sup>238</sup> and Ser<sup>225</sup>) whereas the latter is mediated by hydrophobic residues present in other syntaxin isoforms and other SNAREs as well. This probably explains why CFTR regulation is specific to the syntaxin 1A isoform, whereas SNARE-SNARE interactions are promiscuous at least in vitro.
The E238A and S225A Mutations Do Not Produce Equivalent Effects on CFTR Binding—The results of the titration binding assays indicated that mutating residue Ser225 alone produced an intermediate effect on CFTR binding as compared with mutating residue Glu238 alone or mutating both residues (S225A/E238A). Conceivably, the negative charge on the Glu238 residue may render it more important for mediating the CFTR-syntaxin 1A interaction. In this regard, the binding interaction between CFTR and syntaxin 1A is salt-sensitive, which implies that electrostatic interactions contribute to the binding between these two proteins. In our titration experiments, the CFTR binding signal for the E238A mutant was considerably lower as compared with the wild-type protein even at saturating concentrations. An explanation for this observation could be that CFTR binding at higher concentrations of syntaxin 1A is limited because of oligomerization of this t-SNARE. It has been shown that syntaxin 1A oligomerizes at protein concentrations >2 μM and that the homo-oligomers are not random aggregates but form parallel helices similar to SNARE complexes (22). Preliminary gel filtration analysis revealed that there was an increase in the oligomeric forms of both the wild-type and E238A syntaxin 1A fusion proteins as the protein concentration was increased from 0.5 to 2 μM. The E238A mutation per se had no apparent effect on oligomerization as analyzed in this manner (data not shown). Thus, although one might expect that the E238A mutant should be able to achieve wild-type levels of CFTR binding at higher protein concentrations (>2 μM), the decrease in the concentration of the form competent to bind CFTR might prevent this. It is also possible that the E238A mutation has an indirect effect on CFTR binding by altering multimerization (although see above) or by producing a conformational change in syntaxin 1A, which, in effect, would shield other residues in the H3 domain that mediate CFTR binding. On the other hand, it is noteworthy that the E238A mutation had a very specific effect on CFTR binding (namely this mutation did not inhibit binding to other proteins that interact with the SNARE motif such as SNAP23 and Munc18). In addition, the E238A mutant, but not the A240V/V244A mutant, was able to assemble into stable SNARE complexes like wild-type syntaxin 1A. Thus, although we cannot completely exclude the possibility that the E238A mutation affects CFTR binding as a consequence of altering the oligomerization or conformation of syntaxin 1A, it clearly has a very specific effect on the CFTR-syntaxin 1A interaction.

The CFTR-Syntaxin 1A Interaction Appears to Be Different from the Syntaxin 1A-Ca\(^{2+}\) Channel Interaction—There is increasing evidence that syntaxin 1A modulates the activities of multiple ion channels at the plasma membrane. Such interactions may link the functions of certain ion channels to the exocytotic machinery. Catterall and colleagues (23) reported that syntaxin 1A biochemically interacts with the II/III cytosolic loop (“synprint loop”) of the α1B subunit of the N-type Ca\(^{2+}\) channel. However, functional studies using the oocyte expression system revealed that mutating hydrophobic residues Ala\(^{240}\) and Val\(^{244}\) of syntaxin 1A, located in the SNARE-stabilizing layers, resulted in appreciable loss of Ca\(^{2+}\) channel reg-

\[^{3}\] A. P. Naren and K. L. Kirk, unpublished results.
ulation but did not disrupt binding to the synprint motif. Furthermore, deletions within the II/III loop region of α1H that eliminated the synprint site did not altogether abolish calcium channel modulation by syntaxin 1A (14). This suggests that the binding and modulatory functions of syntaxin 1A can be disconnected in the case of the Ca\(^{2+}\) channel and/or that syntaxin 1A binds to multiple regions of this channel. In this respect, the regulation of CFTR by syntaxin 1A appears to be different. Point mutations in syntaxin 1A that disrupted CFTR binding also disrupted CFTR regulation. Also, the A240V/V244A double mutation did not affect the regulation of CFTR by syntaxin 1A but did destabilize core SNARE complexes, consistent with the predicted roles of these two hydrophobic residues. The latter results are in agreement with the findings of a related study in which mutations of the corresponding residues of Drosophila syntaxin 1 (A243V/V247A) severely compromised SNARE complex stability and disrupted synaptic transmission in flies (24). Thus, residues in the SNARE motif that facilitate the inhibition of N-type Ca\(^{2+}\) channels also govern SNARE complex formation and stability. In contrast, our results obtained with the S225A/E238A double mutant indicate that these hydrophilic residues are not involved in forming or stabilizing SNARE complexes, although mutating these residues had a pronounced effect on CFTR binding and regulation. This is the first report of mutations in the SNARE motif of syntaxin 1A that affect ion channel regulation without affecting SNARE complex assembly or stability.

The strong correlation between the binding of the various syntaxin 1A mutants to CFTR and their abilities to regulate CFTR channels in oocytes supports a simple model in which syntaxin 1A regulates CFTR via a specific protein-protein interaction. Whether this interaction affects the gating of the CFTR channel, its intracellular traffic, or both is a matter of some debate (25, 26), but the results of this and other studies (16) favor the view that syntaxin 1A must bind to CFTR in order to regulate its function. On this basis, one might conclude that syntaxin 1A by itself is sufficient to modulate CFTR activity \textit{in vivo}. However, two observations indicate that there may be more than one SNARE protein involved in regulating CFTR at least in mammalian cells. First, we reported recently...
that CFTR channels preferentially associate with a t-SNARE heterodimer in mammalian cells that includes both SNAP-23 and syntaxin 1A. These two t-SNAREs appear to have additive or possibly cooperative effects on CFTR channel function (15). In support of these findings, the present results indicate that syntaxin 1A residues that mediate CFTR binding do not participate in SNARE-SNARE interactions, which, therefore, could allow syntaxin 1A to simultaneously complex with a t-SNARE partner via hydrophobic interactions as well as interact with CFTR via hydrophilic surface residues. Second, in the present study, we observed that the V248A syntaxin 1A peptide (unlike the wild-type syntaxin 1A peptide) was unable to rescue CFTR-mediated currents from inhibition by membrane-anchored syntaxin 1A in mouse L-fibroblasts. This contrasts with the inhibitory effect of full-length V248A syntaxin 1A on CFTR-mediated currents in oocytes. Conceivably, the V248A mutation inhibits the ability of the syntaxin 1A peptide to disrupt the interactions between CFTR and the t-SNARE complex in mammalian cells.

The Syntaxin 1A-CFTR Interaction Can Be Uncoupled from the Syntaxin 1A-Munc-18 Interaction—Munc-18a inhibits the syntaxin 1A-CFTR interaction due presumably to its ability to block the binding of syntaxin 1A to the CFTR channel (4). Unlike other proteins that interact with only the H3 region of syntaxin 1A, Munc-18 requires the entire NH2-terminal region of syntaxin 1A for high affinity binding (18). Moreover, Munc-18 is believed to bind to a closed syntaxin conformation, and mutations that destabilize this closed conformation compromise binding (9). In the present study, we identified a novel syntaxin 1A mutant (Y235A) that is inhibited for binding Munc-18 but is able to physically and functionally interact with CFTR. The Y235A mutation eliminates the ability of Munc-18 to neutralize the inhibitory effect of syntaxin 1A on CFTR activity presumably by disrupting the Munc-18-syntaxin 1A interaction. In the crystal structure of the syntaxin 1A-Munc-18a complex, Tyr235 of syntaxin 1A does not directly contact any residue in Munc-18a; rather, it is flanked on either side by residues that do directly interact with Munc-18a (27). Presumably, this mutation alters the local conformation of syntaxin 1A to a sufficient degree to inhibit Munc-18 binding.

In conclusion, our results indicate that syntaxin 1A biochemically and functionally interacts with the CFTR channel by a mechanism that is different from how this t-SNARE interacts with other SNAREs or apparently with voltage-gated calcium channels. The CFTR interaction involves surface-exposed hydrophobic residues that are unique to this isoform rather than hydrophobic residues that mediate SNARE complex assembly. The strong correlation between the binding and functional regulation of CFTR channels by the syntaxin 1A mutants that were tested favors the hypothesis that this t-SNARE regulates CFTR channels via a specific protein-protein interaction.

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