Regulation of CFTR chloride channels by syntaxin and Munc18 isoforms

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The cystic fibrosis gene encodes a cyclic AMP-gated chloride channel (CFTR) that mediates electrolyte transport across the luminal surfaces of a variety of epithelial cells[1–4]. The molecular mechanisms that modulate CFTR activity in epithelial tissues are poorly understood. Here we show that CFTR is regulated by an epithelially expressed syntaxin (syntaxin 1A), a membrane protein that also modulates neurosecretion[5–7] and calcium-channel gating[8–11] in brain. Syntaxin 1A physically interacts with CFTR chloride channels and regulates CFTR-mediated currents both in *Xenopus* oocytes and in epithelial cells that normally express these proteins. The physical and functional interactions between syntaxin 1A and CFTR are blocked by a syntaxin-binding protein of the Munc18 protein family (also called n-Sec1; refs 12–14). Our results indicate that CFTR function in epithelial cells is regulated by an interplay between syntaxin and Munc18 isoforms.

Figure 1 shows that syntaxin 1A and a Munc18 isoform are detectably expressed in human colonic epithelial cells. In initial immunoblotting experiments, we detected a 35K (M_r 35,000) membrane protein in T84 and HT29-CL19A human colonic epithelial cell lines that was recognized by a panel of syntaxin 1-specific antibodies (Fig. 1a, b). We confirmed that colonic epithelial cells express the complementary DNA encoding human syntaxin 1A by screening a T84 cDNA library at low stringency using a rat syntaxin 1A cDNA probe (results not shown). During the screening of this epithelial library, we also isolated the cDNA encoding the human isoform of another member of this protein family, syntaxin 3 (Genbank accession number U32315). By quantitative immunoblotting, we determined that syntaxin 3, like syntaxin 1A, is expressed in human colonic epithelial cell lines, as well as in native colon and trachea, and at 5–10 times higher levels than syntaxin 1A (results not shown). Multiple antibodies to each isoform labelled the apical poles of T84 and HT29-CL19A colonic epithelial cells in immunofluorescence experiments (results not shown). We also affinity-purified a syntaxin 1A-binding protein from T84 cells that was determined to be a Munc18 isoform on the basis of microsequencing (Fig. 1c) and immunoblotting (data not shown). Thus, colonic epithelial cells express syntaxins 1A and 3 and a high-affinity syntaxin-binding protein of the Munc18 family[12–14].

CFTR chloride channels interact with syntaxin 1A in an isoform-specific manner (Fig. 2). CFTR could be precipitated from colonic epithelial cell lysates with a glutathione-*S*-transferase (GST) fusion protein containing the cytosolic domain of syntaxin 1A (GST–syn1AΔC, where ΔC refers to deletion of the C-terminal membrane domain[5]). No such binding was observed for six other proteins that are more abundant in epithelial cells than CFTR, including the

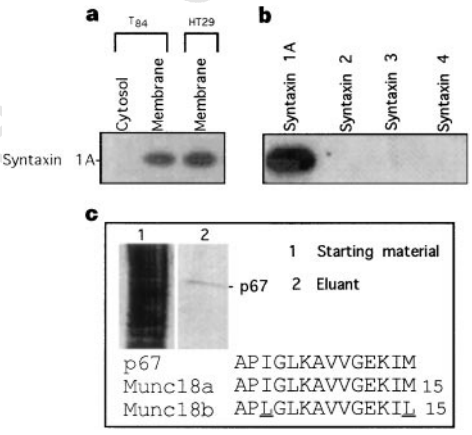

Figure 1 Expression of syntaxin and Munc18 isoforms in colonic epithelial cells. **a**, Immunoblot detection of syntaxin 1 in crude membranes prepared from T_{84} and HT29-CL19A colonic epithelial cell lines (50 μg each lane). Membranes and cytosol were prepared as described[26]. **b**, Isoform specificity of syntaxin 1 polyclonal antibody verified by immunoblotting recombinant cytosolic domains of the indicated syntaxins (50 ng each lane). The 35K membrane protein in colonic cells was also detected using two additional syntaxin 1-specific polyclonal antibodies and a commercially available syntaxin 1-specific monoclonal antibody (HPC-1; Sigma; results not shown). **c**, Purification and microsequence of Munc18 isoform from T84 colonic epithelial cells. Shown are Coomassie-blue-stained starting material (crude membrane extract; 50 μg) and 67K protein eluted from a GST–syn1AΔC affinity column in 1M NaCl. Purification method has been described[12].

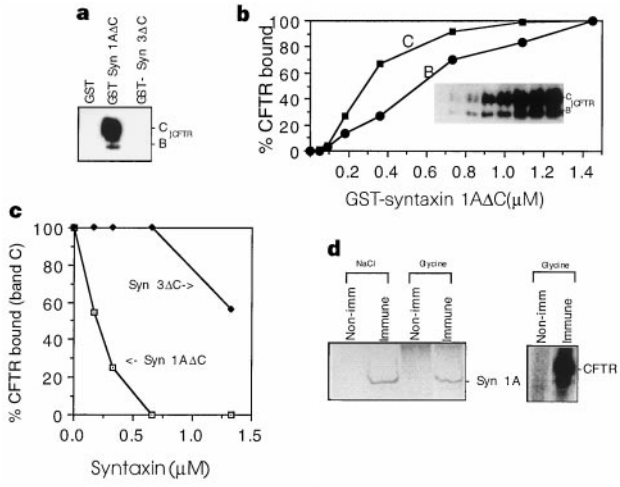

Figure 2 CFTR physically interacts with syntaxin 1A. **a**, *In vitro* binding of CFTR in HT29-CL19A cell lysates to recombinant cytosolic domain of syntaxin 1A fused to GST (GST-syn1AΔC) and immobilized on glutathione–agarose beads (50 μg each fusion protein). Bound CFTR was detected by immunoblotting. Bands B and C represent immature and mature CFTR, respectively. **b**, CFTR binding saturates at submicromolar concentrations of soluble GST–syn1AΔC. Immunoblot of CFTR binding at each GST–syn1AΔC concentration is shown in inset (repeated three times with similar results). **c**, Competition for CFTR binding between GST–syn1AΔC (10 μg throughout) and increasing concentrations of syntaxin cytosolic domain cleaved free of GST by thrombin (repeated 3 times with similar results). **d**, Syntaxin 1 co-immunoprecipitates with CFTR from HT29-CL19A cell lysates. Proteins immunoprecipitated with CFTR antibody were eluted in 1.0 M NaCl followed by 0.1 M glycine (pH 2.5) and probed for syntaxin 1A by immunoblotting and for CFTR by immunoprecipitation followed by *in vitro* phosphorylation as described[20].

apical membrane marker aminopeptidase (results not shown). Both the mature form (band 'C') and immature form (band 'B') of CFTR[2] exhibited syntaxin 1A-binding activity, although the affinity of this interaction is higher for the mature form (Fig. 2b). In solution binding assays, we determined that the binding of CFTR to syntaxin 1A was saturable with half-maximal binding of band 'C' at a GST–syn1AΔC concentration of ~300 nM. The binding of CFTR to GST–syn1AΔC could be competitively inhibited by submicromolar concentrations of soluble syntaxin 1A that had been cleaved free of GST (Fig. 2c). Soluble syntaxin 3 cytoplasmic domain was less effective at inhibiting the interaction between CFTR and GST–syn1AΔC (Fig. 2c), which confirms the isoform specificity of this interaction (see also Fig. 2a). We were also able to co-immunoprecipitate syntaxin 1A with CFTR from colonic epithelial cell lysates (Fig. 2d) and from COS-7 fibroblasts expressing recombinant CFTR and syntaxin 1A (results not shown) using multiple antibodies raised against each protein. However, the efficiency with which these two proteins could be co-immunoprecipitated from detergent lysates was low, consistent with the dynamic nature of the functional interactions between these proteins (see below).

We examined the ability of recombinant syntaxin 1A to regulate CFTR-mediated chloride currents in two-electrode voltage-clamp studies of *Xenopus* oocytes (Fig. 3). CFTR currents in cRNA-injected oocytes exhibited linear current–voltage behaviour and were blocked by diphenylamine carboxylate (results not shown), as described[15,16]. Full-length syntaxin 1A inhibited CFTR-mediated currents (Fig. 3a) in a dose-dependent manner (Fig. 3b) without changing their linear I–V behaviour or reversal potential (not shown). The syntaxin 1A inhibition of CFTR currents was acutely reversed by injection of botulinum neurotoxin C1 (BONT/C1; Fig. 3c), an endoprotease that cleaves the cytosolic domain of syntaxin 1A from its membrane anchor[6]. BONT/C1 had no effect on CFTR currents in the absence of syntaxin 1A (Fig. 3c). In contrast to syntaxin 1A, syntaxin 3 failed to inhibit CFTR currents and

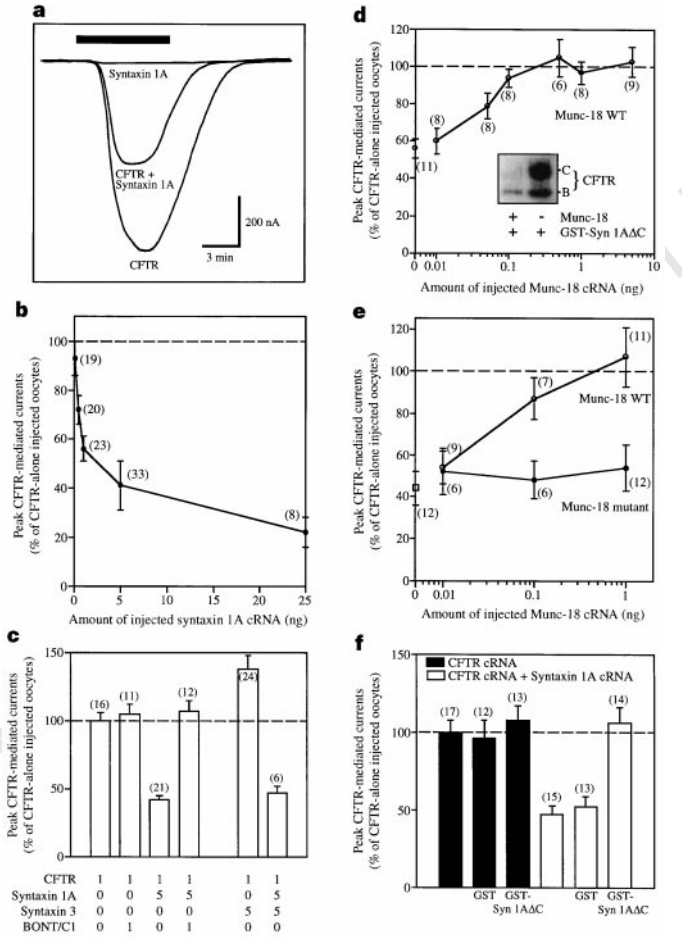

Figure 3 Syntaxin 1A and Munc18 regulate CFTR-mediated chloride currents in *Xenopus* oocytes. **a**, CFTR-mediated chloride currents in oocytes injected with 1 ng CFTR cRNA, 5 ng syntaxin 1A cRNA, or both. The bar above the traces indicates the duration of application of cAMP cocktail (see Methods). **b**, Dose-dependent inhibition of CFTR currents by syntaxin 1A. **c**, Injection of botulinum neurotoxin C1 (1 ng per oocyte) 30–60 min before current recording reverses the syntaxin inhibition of CFTR, whereas syntaxin 3 does not inhibit CFTR currents. **d**, Munc18a rescues CFTR from syntaxin 1A inhibition in oocytes and blocks the *in vitro* binding of CFTR to GST–syn1AΔC (inset). Bands B and C represent immature and mature CFTR, respectively. **e**, Co-expression of a Munc18a mutant (D34N; M38V) that lacks syntaxin-binding activity does not prevent syntaxin 1A inhibition of CFTR currents. **f**, Injection of GST–syn1AΔC (estimated final concentration, 50 μg ml^{-1}) but not GST (50 μg ml^{-1}) 40–60 min before current recording reverses the inhibition of CFTR currents by full-length syntaxin 1A. Numbers of oocytes assayed are shown in parentheses.

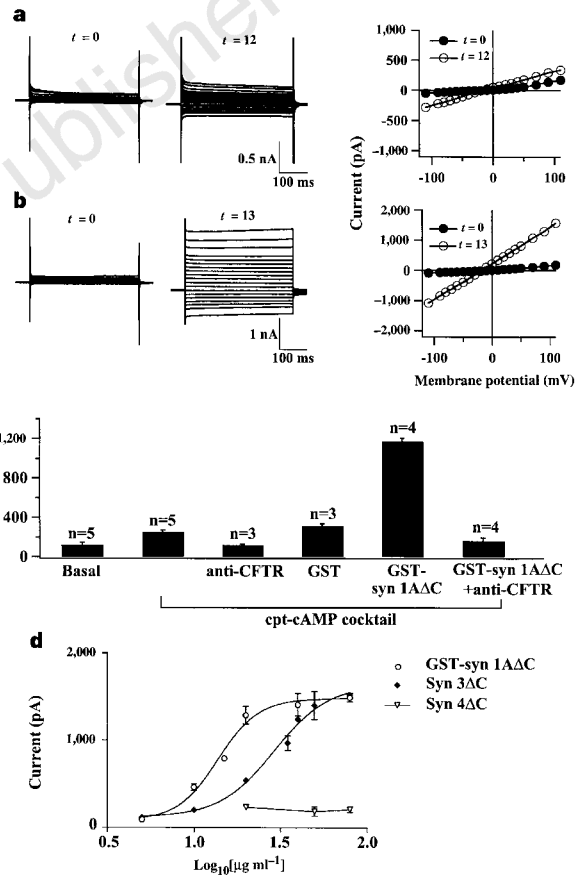

Figure 4 Soluble syntaxin 1A cytosolic domain potentiates CFTR-mediated chloride currents in T_{84} colonic epithelial cells that endogenously express CFTR and membrane-anchored syntaxin 1A. **a**, Left: basal whole cell currents recorded immediately after establishing whole-cell configuration in the presence of 500 μM cpt-cAMP in the pipette solution; centre, currents recorded 12 min later; right, associated current–voltage relationship (*I–V*) at *t* = 0 min (black circles) and 12 min (white circles). **b**, Whole-cell currents from a cell voltage-clamped as in **a**, with the addition of 350 nM GST–syn1AΔC (20 μg ml^{-1}) to the pipette solution. **c**, Summary of mean peak current amplitude for data obtained from T84 cells at a holding potential of +110 mV following a 40-min equilibration with a pipette solution that contained GST (750 nM), GST–syn1AΔC (350 nM), and/or CFTR antibody (0.1 μg ml^{-1}). Currents were activated with a cAMP cocktail added to the extracellular bath (see Methods). Data are expressed as mean ± s.e.m. **d**, Isoform specificity of the potentiation of epithelial CFTR chloride currents by syntaxin cytosolic domains. The syntaxin-dependent increases in Cl^- current measured at +110 mV in the presence of the cAMP cocktail are shown (n = 3–7 cells for each data point).

instead had a moderate stimulatory effect which was overcome by co-expression with syntaxin 1A. Inhibition of CFTR currents by syntaxin 1A was CFTR-specific; that is, syntaxin 1A did not inhibit the activities of either the alpha-7 homomeric nicotinic acetylcholine receptor or the GAT1 GABA transporter when these molecules were expressed in oocytes (results not shown).

CFTR currents in oocytes could be rescued from syntaxin 1A inhibition by two additional manoeuvres: co-expressing recombinant Munc18a or by injecting the soluble cytosolic domain of syntaxin 1A (Fig. 3d, e, f). Figure 3d shows that Munc18a inhibited the *in vitro* binding of syntaxin 1A to CFTR (inset) and prevented the inhibition of CFTR currents by syntaxin 1A in a dose-dependent manner when these molecules were co-expressed in oocytes. A Munc18a mutant that lacks syntaxin-binding activity (binding data not shown) was unable to rescue CFTR currents from syntaxin inhibition (Fig. 3d). Figure 3f shows that the cytosolic domain of syntaxin 1A (GST–syn1AΔC), like BONT/C1, also acutely reversed the inhibition of CFTR currents by full-length syntaxin 1A when injected into co-expressing oocytes. GST–syn1AΔC had no effect on CFTR currents in the absence of full-length syntaxin 1A, so this soluble fragment stimulated CFTR currents by disrupting the functional interaction between CFTR and membrane-anchored syntaxin 1A. Together with our BONT/C1 data, these results indicate that: (1) syntaxin 1A must be membrane-anchored to inhibit CFTR currents, as also suggested for the regulation of calcium channels by syntaxin 1A (ref. 9), and (2) the interaction between CFTR and membrane-anchored syntaxin 1A is dynamic (that is, acutely reversible).

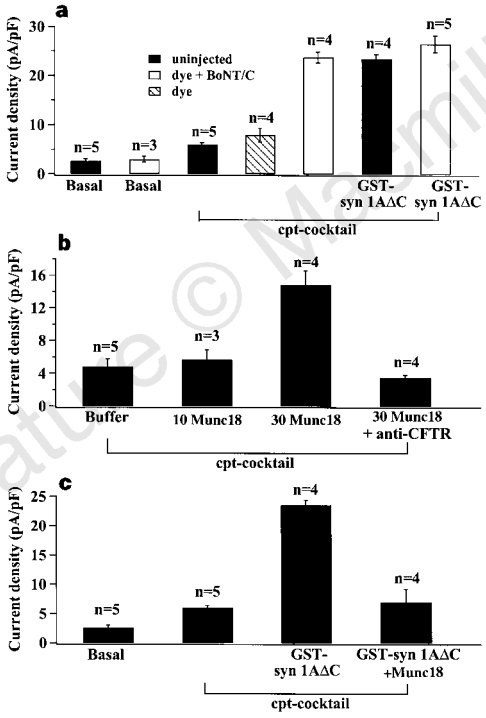

Figure 5 Botulinum neurotoxin C1 and Munc18a also potentiate CFTR-mediated currents in epithelial cells. **a**, BONT/C1 mimics the effects of soluble GST-syn1AΔC on cAMP-activated Cl^- currents in epithelial cells. T_{84} cells were microinjected with 0.5% Texas red for later identification, or with dye plus 1 ng μl^{-1} BONT/C1 6–24 h before patch clamping. Cl^- currents were activated with the cAMP cocktail added to the bath (see Methods) in the presence or absence of 350 nM GST-syn1AΔC in the pipette. **b**, GST-Munc18a fusion protein augments CFTR-mediated currents in T_{84} cells in a concentration-dependent manner. Comparison of mean peak Cl^- currents under control conditions and in the presence of 10 $\mu g\ ml^{-1}$ GST-Munc18a, 30 $\mu g\ ml^{-1}$ GST-Munc18a (~300 nM) or 30 $\mu g\ ml^{-1}$ GST-Munc18a plus CFTR-blocking antibody. in the pipette. **c**, Munc18 negates the effects of GST-syn1AΔC on CFTR currents in T_{84} cells. Comparison of mean peak Cl^- currents under control conditions and in the presence of 20 $\mu g\ ml^{-1}$ GST-syn1AΔC with or without 10 $\mu g\ ml^{-1}$ GST-Munc18a in the pipette.

We next examined the biological relevance of the interactions among CFTR, syntaxin 1A and Munc18 by performing whole-cell patch-clamp studies of epithelial cells that endogenously express these three proteins. Figure 4 shows that the cytoplasmic domain of syntaxin 1A (GST–syn1AΔC) augments CFTR currents in colonic epithelial cells, as it should if endogenous membrane-anchored syntaxin 1A normally attenuates the activity of native CFTR in these cells. Inclusion of 350 nM GST–syn1AΔC in the patch pipette resulted in a 2–3-fold stimulation of whole-cell chloride currents activated by a maximal dose of cAMP in T84 cells (Fig. 4) or Caco-2 colonic epithelial cells (data not shown). These large currents appeared within 2–5 min of membrane rupture by the patch pipette and were CFTR-mediated on the basis of three criteria: voltage-independent with a chloride-selective reversal potential (Fig. 4a, b; refs 15, 16); insensitive to 1 mM DIDS in the extracellular bath but inhibited 90 ± 1% ($n = 3$) by 1 mM diphenylamine carboxylate[17,18]; and inhibited by a CFTR-blocking antibody[19,20] (Fig. 4c). GST alone had no effect on cAMP-activated chloride currents (Fig. 4c). In addition, GST–syn1AΔC had no significant effect on currents that were measured in the absence of cAMP or on currents that were activated by calmodulin-dependent kinase II (results not shown), which modulates a non-CFTR-mediated chloride conductance in these cells[21,22]. The potentiation of epithelial CFTR currents by soluble syntaxin was isoform-specific, with a rank order of syntaxin 1A ≫ 3 ≫ 4 (Fig. 4d). The half-maximal effective concentration (EC_{50}) for CFTR current activation by GST–syn1AΔC (~250 nM) agreed well with the EC_{50} for *in vitro* binding (~300 nM; Fig. 2b). The ability of GST–syn3AΔC to activate CFTR currents at higher concentrations is presumably due in part to its ability to compete weakly with endogenous syntaxin 1A for CFTR binding (Fig. 2c), although this construct may influence CFTR activity in other ways as well (by altering apical membrane traffic, for example).

Figure 5 shows that epithelial CFTR currents are also potentiated by two other reagents that rescued CFTR from inhibition by membrane-anchored syntaxin 1A in oocytes: BONT/C1 and Munc18a. Microinjection of BONT/C1 into T84 colonic epithelial cells potentiated cAMP-activated whole-cell currents to the same degree as a maximal dose of GST–syn1AΔC (Fig. 5a). The neurotoxin had no significant effect on basal chloride currents nor did the toxin have an additive effect on cAMP-induced currents when combined with GST–syn1AΔC, as would be expected if these reagents activate CFTR currents by the same mechanism. Recombinant Munc18a also stimulated CFTR-mediated chloride currents in a dose-dependent fashion when introduced through the patch pipette (Fig. 5b), which indicates that this soluble syntaxin-binding protein is a limiting factor in the activation of 'whole cell' CFTR currents in these epithelial cells. In addition, at very low concentrations, Munc18a blocked the effect of GST–syn1AΔC on CFTR currents, presumably by inhibiting the binding of soluble syntaxin 1A to CFTR. Thus, Munc18 also modulates CFTR function in epithelial cells probably by controlling the availability of syntaxin 1A for CFTR.

In summary, our results indicate that CFTR physically and functionally interacts with an epithelially expressed syntaxin 1 isoform that is a negative modulator of CFTR-mediated chloride currents. This negative modulation of CFTR function by syntaxin 1A occurs in epithelial cells as well as in heterologous expression systems, because epithelial CFTR currents were markedly potentiated by three different reagents that rescue CFTR from inhibition by membrane-anchored syntaxin 1A in oocytes (that is, BONT/C1, Munc18 and syntaxin 1A cytosolic domain). The mechanism by which syntaxin 1A modulates CFTR currents could involve regulation of CFTR traffic to or from the cell surface or direct regulation of CFTR channel activation through protein–protein interactions. This latter mechanism is consistent with the emerging view that

membrane-anchored syntaxin 1A directly modulates N-type calcium-channel activity in brain[9–11]. It will be important to determine whether syntaxin 1A directly binds to CFTR and if it regulates epithelial CFTR chloride channels and neuronal calcium channels by similar mechanisms. We speculate that the CFTR–syntaxin–Munc18 interactions play a role in fine-tuning CFTR activity in response to certain physiological cues, such as the activation of second messenger pathways that regulate the physical interactions between these proteins[23,24]. Understanding the mechanisms by which these molecules regulate CFTR activity may be relevant to the design of strategies for augmenting epithelial CFTR function in cystic fibrosis. □

Methods

***In vitro* binding and co-immunoprecipitation.** Qualitative 'pull down' assays were done by adding immobilized GST, GST–syn1AΔC or GST–syn3ΔC (50 μg) to a 1% NP-40 lysate of HT29-CL19A cells for 12 h at 4 °C. Beads were pelleted, washed extensively in lysis buffer and analysed for CFTR by immunoblotting. Inhibition of CFTR-syntaxin 1A binding by Munc18a (Fig. 3d) was verified by preincubating GST–syn1AΔC (15 μg) with or without excess Munc18a before assaying CFTR binding. Quantitative solution binding assays were done by adding soluble eluted GST fusion protein to an HT29-CL19A cell lysate for 3 h at 4 °C. GST fusion protein was then precipitated with excess glutathione–agarose and CFTR binding quantified by immunoblotting and densitometry. Co-immunoprecipitation was performed by passing HT29-CL19A cell lysates (0.8% Triton X-100 in HEPES-buffered saline (pH 7.4)) through a hydrazide-derivatized disc (Actidisc, FMC Corp) to which anti-CFTR IgG (anti NBD1; residues 426–588; ref. 20) or non-immune IgG was covalently bound. Bound proteins were eluted and analysed (Fig. 2 legend).

Electrophysiology. *Xenopus* oocytes were injected with the cRNAs encoding CFTR (1 ng), full-length syntaxin 1A (5 ng, or as indicated in Fig. 3) and Munc18a (as indicated in Fig. 3) and assayed 48–72 h later for CFTR currents[25]. Currents were typically activated with a cocktail containing 20 μM forskolin, 100 μM dibutryl cyclic AMP and 100 μM IBMX. A similar inhibition of CFTR currents by syntaxin 1A was also observed using a supermaximal CFTR activation cocktail (20 μM forskolin, 200 μM dibutryl cAMP and 5 mM IBMX; data not shown). Whole-cell patch-clamping of T84 cells was done as described[19,22], except that 5 mM instead of 1 mM Mg-ATP was used in the pipette solution. Cell capacitance was measured by integrating the current during a 10-mV voltage step and subtracting a baseline established ~15 ms after the step. Currents were activated with 500 μM cpt-cAMP in the pipette or with an extracellular cocktail (100 μM cpt-cAMP, 50 μM forskolin and 50 μM IBMX) for those experiments involving pre-equilibration of the cell interior with CFTR-blocking antibody[19].

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Caenorhabditis elegans CED-9 protein is a bifunctional cell-death inhibitor

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The *Caenorhabditis elegans* gene *ced-9* prevents cells from undergoing programmed cell death and encodes a protein similar to the mammalian cell-death inhibitor Bcl-2 (refs 1–7). We show here that the CED-9 protein is a substrate for the *C. elegans* cell-death protease CED-3 (refs 8, 9), which is a member of a family of cysteine proteases first defined by CED-3 and human interleukin-1β converting enzyme (ICE)[10–12]. CED-9 can be cleaved by CED-3 at two sites near its amino terminus, and the presence of at least one of these sites is important for complete protection by CED-9 against cell death. Cleavage of CED-9 by CED-3 generates a carboxy-terminal product that resembles Bcl-2 in sequence and in function. Bcl-2 and the baculovirus protein p35, which inhibits cell death in different species through a mechanism that depends on the presence of its cleavage site for the CED-3/ICE family of proteases[9,13–17], inhibit cell death additively in *C. elegans*. Our results indicate that CED-9 prevents programmed cell death in *C. elegans* through two distinct mechanisms: first, CED-9 may, by analogy with p35 (refs 9, 17), directly inhibit the CED-3 protease by an interaction involving the CED-3 cleavage sites in CED-9; second, CED-9 may directly or indirectly inhibit CED-3 by means of a protective mechanism similar to that used by mammalian Bcl-2.

Baculovirus p35 protein, a general inhibitor of programmed cell death[9,13–17], is a substrate for the *C. elegans* cell-death protease CED-3 and may act as a competitive inhibitor of CED-3 (ref. 9). We tested whether CED-9 protein, an endogenous *C. elegans* cell-death inhibitor[1,2], might act similarly. We found that ^{35}S-methionine-labelled CED-9 synthesized *in vitro* was cleaved by purified CED-3 protease[18] to generate two products of relative molecular masses

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