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cAMP-regulated trafficking of epitope-tagged CFTR

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cAMP-regulated trafficking of epitope-tagged CFTR. The cystic fibrosis transmembrane conductance regulator (CFTR) is a phosphorylation-activated chloride channel responsible for cAMP-induced Cl secretion across the apical membranes of epithelial cells. To optimize its detection in membrane localization studies, we tagged CFTR with epitope sequences at the carboxy terminus or in the fourth external loop. The function of six different tagged-CFTRs was tested in two different physiological assays. CFTRs containing the M2 epitope responded to cAMP, whereas cells expressing CFTR with the hemagglutinin HA tag showed little or no cAMP response. Using CFTR tagged in the fourth external loop, we demonstrate that cAMP activation using forskolin results in an increase in CFTR in the plasma membrane of HeLa cells. Forskolin inhibited CFTR endocytosis, and this contributes to the increase in cell surface CFTR expression. Our results indicate that regulation of cell surface CFTR contributes to the increase in plasma membrane Cl conductance evoked by cAMP stimulation.

Cystic fibrosis transmembrane conductance regulator (CFTR) is involved in the cAMP-dependent regulation of membrane trafficking. Epithelia that express wild-type (wt) CFTR show cAMP-induced activation of exocytosis and inhibition of endocytosis [1]. These cAMP-induced changes in membrane recycling cause a proliferation of membrane surface area detected as an increase in membrane capacitance measurements [2]. Cystic fibrosis (CF) cells lack the cAMP-dependent regulation of these membrane trafficking events, which can be restored by expression of wt CFTR in CF cells [1]. Prince and Marchase [3] used a surface labeling strategy to measure directly the effect of cAMP-activation on CFTR endocytosis. Forskolin inhibited endocytosis of 50% of plasma membrane CFTR in T84 cells, detected from immunoprecipitation of the surface-labeled protein.

cAMP-regulation of membrane trafficking events could contribute to the expression of active CFTR Cl channels in the apical membranes of secretory epithelial cells, providing an ancillary means of increasing apical Cl conductance, in addition to the direct activation of membrane-resident CFTR. Of critical importance is the question of whether CFTR itself is a passenger in this cAMP-regulated apical membrane traffic or whether it regulates these events from another location. If it is the former, then it becomes important to define the membrane turnover properties of various CFTR mutants. Their residency in the plasma membrane may correlate with impairments described for regulation of their Cl conductance, and this would support the idea that conduction and trafficking are causally related. Moreover, enhancing the plasma membrane residence time of mutant CFTRs may provide a means of enhancing apical Cl conductance.

Our understanding of wild-type (wt) and mutant CFTR localization, trafficking and membrane insertion is incomplete. These issues could be addressed at greater resolution with more sensitive CFTR detection assays. We chose to epitope-tag the CFTR protein on one of the external loops and at the C-terminus as a means to detect the protein when it is expressed in various cell lines and tissues. The advantage of this approach is the use of a small highly hydrophillic epitope, engineered into the protein at a specific site, and the availability of a specific monoclonal antibody that can be used to detect the epitope. This results in increased sensitivity and specificity of protein detection. Epitope-tagging has been used successfully with a variety of proteins to determine their cellular locations [4], subcellular targeting [5] and protein-protein interactions [6].

In this paper, we briefly review the procedures used for tagging and functional analysis of six CFTR constructs, tagged with two different epitopes [7]. Using one of these constructs, we demonstrate that cAMP-activation results in (1) an increase in the number of CFTR molecules in the plasma membrane under steady-state conditions and (2) inhibition of CFTR endocytosis.

Methods

Virus, plasmid and cells

Recombinant vaccinia virus expressing the T7 bacteriophage RNA polymerase (vTF7-3) and the pTM1 plasmid were provided by Dr. B. Moss of the National Institutes of Health [8]. Stocks of vTF7-3 were prepared in HeLa cells, and infectivity titers were determined by plaque assay on HeLa cells. HeLa cells were maintained as monolayer cultures in Delbecco's minimal essential medium (DMEM) containing 10% heat-inactivated calf serum.

Epitope-tagged CFTR constructs

Epitope tags were introduced into the CFTR coding sequence using a PCR-based technique, an approach that resulted in 50 to 70% of clones positive for insertion of the epitope tag (data not shown). Sequences encoding either the Influenza hemagglutinin (HA) epitope, YPYDVPDYA, [9] or the M2 epitope, DYKDDDDK (Kodak) were inserted in frame with the coding sequence of CFTR after nucleotide positions 2820 (HA and M2 epitopes), 2835 (M2 epitope) or 4572 (HA and M2 epitopes). The corresponding M2 or HA-tagged CFTRs are designated by the site of epitope addition (that is, the amino acid immediately preceding the epitope sequence). The protocol was performed as described

Table 1. SPQ analysis of tagged CFTR proteins expressed in HeLa cells

	wt	M2-901/dg	M2-901	wt/dg	M2-C	vv
Control	1.5 ± 0.1	1.8 ± 0.1	0.81 ± 0.1	1.2 ± 0.1	1.6 ± 0.2	1.2 ± 0.1
cAMP	5.9 ± 0.6[a]	9.5 ± 1.1[a]	7.9 ± 0.98[a]	8.6 ± 1.1[a]	5.6 ± 1.0[a]	1.0 ± 0.1

The values represent the slopes associated with dequenching of SPQ fluorescence in nitrate media in the presence (cAMP) and absence (control) of 400 μM cpt-cAMP, 5 μM forskolin and 100 μM IBMX.
[a] $P < 0.0001$

by Howard et al [7]. Site-directed mutagenesis was carried out using the method of Deng and Nickoloff [10].

RNA synthesis

Epitope-tagged CFTR constructs (pBluescript vector) were linearized and RNA was synthesized from the DNA templates using the Ribomax™ kit (Promega) and the bacteriophage T7 RNA polymerase as described by Howard et al [7]. Each preparation of RNA was analyzed by denaturing gel electrophoresis for full-length CFTR RNA.

Expression of CFTR using the vaccinia/T7 system

HeLa cells were plated on collagen-coated coverslips in 35 mm dishes at a density of 5×10^5. Cells were infected with vTF7-3 at a multiplicity of infection (MOI) of 7. Following adsorption, the monolayers were washed twice in serum-free media and transfected with pTM1⁻CFTR (± tag sequences) using lipofectin (BRL) at a concentration of 10 μg/ml according to the manufacturer's procedures. Following five hours of incubation, the monolayers were washed twice with DMEM containing 10% calf serum and incubated in media containing serum prior to analysis.

Expression of CFTR using Xenopus laevis oocytes

Isolation of oocytes and injection of CFTR RNA were performed as described previously [11]. Oocytes were injected with 50 ng of RNA for either wt CFTR or tag CFTR in 50 nl water, or with water alone. Membrane currents were evaluated two days after injection by double-electrode voltage clamp as described [11]. Current-voltage relations were determined before and 15 to 25 minutes after perfusion with a cAMP cocktail consisting of 10 μM forskolin and 1 mM 3-isobutyl-1-methylxanthine (IBMX). To determine whether current responses following cAMP cocktail perfusion were significantly different from baseline, paired *t*-tests were performed to compare the membrane conductances (slope of the I-V relationship) before and after cAMP treatment.

SPQ fluorescence microscopy

HeLa cells infected with vTF7-3 and transfected with plasmid DNA were monitored for plasma membrane Cl permeability by measuring the fluorescence dequenching of the halide-sensitive fluorophore, SPQ (Molecular Probes) as described [12]. The cells were exposed to 400 μM cpt-cAMP, 5 μM forskolin, and 100 μM IBMX to determine the effect of increasing cellular cAMP on halide permeability during bath anion substitution. In cells expressing wt CFTR, this cocktail has been shown previously to produce a maximal rate of SPQ fluorescence dequenching in response to cAMP in cells expressing wt CFTR [12].

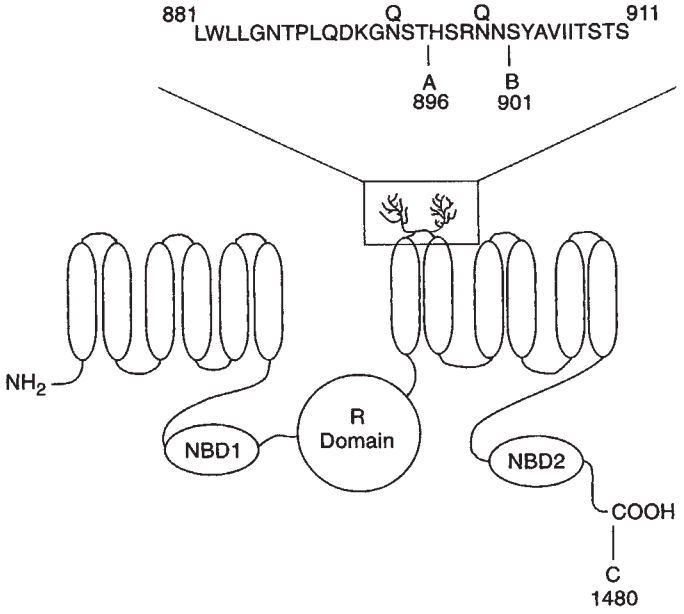

Fig. 1. *Schematic of CFTR protein.* Letters represent single amino acid code. Arrows represent place of insertion of the epitope tag.

Immunofluorescence

Cell surface CFTR under steady-state conditions. For cell surface staining, HeLa cells were infected with vTF7-3 and transfected with plasmid DNA as described above. At eight hours post-infection, cells were incubated with culture media with or without 10 μM forskolin for ten minutes at 37°C. The media were then removed and the cells rapidly cooled to 4°C by washing twice with ice cold PBS. Then they were incubated in 50 μl of M2 antibody (7 μg/ml, Kodak) for 15 minutes at 4°C. The coverslips were again washed twice in PBS and incubated in 50 μl of a 1:10 dilution of a fluorescein-conjugated goat anti-mouse antibody (Boehringer Mannheim, Mannheim, Germany) for 15 minutes at 20°C. The coverslips were washed twice in PBS, the cells were then fixed by incubation in 1% formalin for 15 minutes at 20°C and mounted on glass slides in 0.1% paraphenyldiamine, 90% glycerol, 0.1 M PBS, pH 7.4.

Quantitation of cell surface fluorescence was performed by collecting images with a cooled CCD camera mounted on a Zeiss IM-35 microscope equipped with a 16XPlan-Neofluar objective and appropriate filter sets to separate the fluorescence of FITC from that of the nuclear stain, Hoescht 33342. Images of five

Table 2. Whole-cell chloride conductance from *Xenopus* oocytes injected with 50 ng *in vitro* transcribed mRNA

	Water	M2-C	wtCFTR	M2-901*	wtCFTR*
Control	3.7 ± 0.55	1.77 ± 0.44	4.11 ± 0.61	2.65 ± 0.44	3.38 ± 1.25
cAMP	4.29 ± 0.83	63.44 ± 15.8[a]	60.47 ± 17.6	18.63 ± 6.54[b]	31.65 ± 7.94

Values represent the slopes associated with the whole cell conductance (nS) before cAMP stimulation (control) and after stimulation with cAMP (10 μM forskolin and 1 mM 3-isobutyl-1-methylxanthine, IBMX). Compare M2-901* and wtCFTR* which were assayed on the same day in a separate experiment from the other constructs.
[a] $P < 0.05$; [b] $P < 0.05$

randomly selected fields were collected, relayed to a Macintosh computer and analyzed as follows. A threshold fluorescence intensity value differentiating between fluorescently stained and unstained cells was determined based on the intensity profiles of unstained cells. The criteria for determining the threshold was that less than 5% of autofluorescence may fall above the threshold. The same threshold was applied to all images taken and intensity values above the threshold were integrated for each set of five images for a given preparation. The number of cell nuclei of each field was determined based on the staining of DNA with Hoescht 33342. The integrated FITC fluorescence of each set of images was normalized to the number of nuclei observed on the corresponding Hoescht images to obtain relative cell fluorescence.

Endocytosis

vTF7-3 infected HeLa cells expressing M2-901/dgCFTR were incubated in the presence or absence of forskolin for 30 minutes at 37°C and then stained with M2 antibody at 4°C for 15 minutes. The cells were either stained immediately with FITC-labeled secondary antibody at 4°C (zero time point) or warmed to 37°C for one, two or five minutes in the presence or absence of forskolin prior to secondary antibody staining. Other procedures were as described above.

Results

The criteria to be met in tagging CFTR were to produce a protein that: (i) was readily detectable by immunofluorescence using a commercially available antibody; (ii) could be detected at the surface of non-permeabilized cells; and (iii) would demonstrate a cAMP-inducible chloride current. It was essential that tagged CFTR perform as a cAMP-activated Cl conductance in various physiological assays (discussed below). Therefore, the proposed physical structure of the protein was considered with regards to placement of the epitope.

As discussed previously [7], the 1480 amino acid CFTR protein is predicted to have twelve membrane spanning domains, with only 4% of the protein sequence external to the plasma membrane [13]. Both the amino and carboxy termini of the protein are predicted to be intracellular. The overall amino acid homology among CFTRs of different species is greater than 70% identity and 85% similarity. Other proteins have been shown to retain function when epitope tags are fused to their carboxy terminus [14], and therefore we tagged CFTR at this site. We also tagged CFTR at two different locations in the fourth extracellular loop. A tag on an extracellular loop would allow immunofluorescent localization of CFTR in the plasma membrane without fixation or permeabilization.

Briefly, the rationale for tagging CFTR at the fourth extracellular loop was as follows. Of the amino acids predicted to be extracellular, the fourth loop is the largest. This loop shows structural divergence among species (51% homology), as compared to 93% similarity for the first external loop, 94% similarity for the first nucleotide binding domain and 85% overall similarity for the CFTR protein. We reasoned that the fourth loop might tolerate addition of amino acids without disruption of function. However, the fourth extracellular loop contains two asparagines (amino acids 894 and 900) that are glycosylated [15], and this glycosylation is substantial, resulting in an apparent shift in molecular weight of 15,000 to 30,000 daltons. To determine whether CFTR glycosylation would interfere with its detection by antibody, we evaluated the influence of mutagenizing the asparagines to glutamines on CFTR function and immunofluorescence. Prior studies [15] suggested that function was preserved by this procedure, but the assay employed (SPQ fluorescence) is relatively insensitive from a quantitative viewpoint. Our results reproduced these findings. When the M2 epitope was added to either deglycosylated (dg) or wt CFTR, the cAMP stimulatory cocktail increased SPQ fluorescence in a manner similar to that observed for wt CFTR. There were no apparent differences in stimulation of wt and dg CFTR and these proteins were functional whether the tags were added after amino acid 901 (4th extracellular loop) or at the C-terminus. However, tag constructs utilizing the hemagglutinin epitope were not functional in this assay (see Howard et al [7]).

Tagged CFTR function was analyzed with greater sensitivity by injecting *Xenopus* oocytes with RNAs obtained from the tag constructs (Table 2) [7]. As with SPQ, the wild-type and M2-tagged constructs generated a whole-cell Cl conductance response to cAMP. However, in this assay, the deglycosylated CFTRs (both tagged and untagged) showed conductance responses that were only 20% of the response of wt CFTR with the glycosylation sites intact (data not shown) [7]. These results show that it is the absence of CFTR glycosylation and not the addition of the epitope tag that results in the reduced whole-cell Cl conductance response of dgCFTRs. M2-C/CFTR retains the glycosylation sites and yields a whole-cell conductance comparable to wild-type CFTR (Table 2). The SPQ assay did not detect these glycosylation-associated differences in CFTR function, presumably due to its lower sensitivity.

The M2-901/CFTR functioned like wild-type CFTR and was readily detected at the cell surface in live, non-permeabilized cells (see below and [7]). To further analyze the effect of the tag on function and trafficking of CFTR, five mutations were produced in combination with this tagged construct, and their function and surface expression were analyzed. The five mutations studied

Table 3. SPQ analysis of tagged mutant CFTR proteins

	ΔF508	G551D	K1250M	N1303K	G1349D
Control	0.95 ± 0.14	1.06 ± 0.09	1.22 ± 0.08	1.33 ± 0.20	0.84 ± 0.18
cAMP	1.17 ± 0.16	1.15 ± 0.11	8.73 ± 0.86[a]	1.59 ± 0.24	2.98 ± 0.36[a]

The values represent the slopes associated with dequenching of SPQ fluorescence in nitrate media in the presence (cAMP) and absence (control) of 400 μM cpt-cAMP, 5μM forskolin and 100 μM IBMX.
[a] $P < 0.001$

were: ΔF508, G551D, K1250M, N1303K, and G1349D. These mutations have been previously characterized for their processing to form glycosylated proteins (that is, the molecular weight shift associated with carbohydrate addition to form a "C band," suggesting transit through the Golgi). They were also analyzed for function, using the SPQ assay. As shown in Table 3, the M2/K1250M and M2/G1349D mutants showed significant responses to cAMP stimulation in the SPQ assay when expressed in HeLa cells. The M2/ΔF508, M2/G551D, and M2/N1303K mutants did not show significant cAMP-induced changes in Cl permeability. These findings are in agreement with the previously published functional analyses of these CFTR mutants [15]. The results suggest that addition of the M2 epitope after amino acid 901 does not alter the pattern of processing and functional expression of these structural CFTR variants.

The mutants G551D, K1250M, and G1349D have been shown previously to be processed to glycosylated proteins; they are therefore thought to traffic to the cell surface. ΔF508 and N1303K are not glycosylated and thus are not expected to be expressed in the plasma membrane. The cell surface expression of these mutants was determined in HeLa cells after vaccinia/T7 expression by immunofluorescence [7]. M2/ΔF508 and M2/N1303K were not detected at the cell surface by immunofluorescence, whereas the other three M2/CFTR mutants (G551D, K1250M and G1349D) showed cell surface expression. Thus, these results also correlate with the biochemical data regarding mutant CFTR processing (formation of the C band) [15].

The development of an extracellular epitope-tagged CFTR construct that both traffics and functions like wild-type CFTR enables us to study the cell biology of this protein in greater detail. Results from previously published studies suggest that CFTR confers cAMP-regulated plasma membrane recycling (endocytosis and exocytosis) in a manner that would be expected to increase the amount of plasma membrane CFTR when cells are stimulated by cAMP, and this would provide one means of increasing their Cl conductance [1]. This proposal was tested directly by assessing cell surface expression of M2-901/CFTR in HeLa cells in the presence and absence of cAMP. Live, non-permeabilized cells were incubated in media in the absence or presence of 10 μM forskolin for ten minutes prior to staining with FITC-labeled secondary antibody at 4°C. After staining, the cells were fixed and mounted for microscopic examination and quantitation. Table 4 shows the fluorescence intensity from three independent experiments of this type. As a control, the same assays were performed in HeLa cells expressing vesicular stomatitis virus (VSV) glycoprotein G using methods identical to those for CFTR. There is no evidence to indicate that VSV-G protein undergoes cAMP-regulated membrane trafficking, and these studies did not show a change in cell surface VSV-G protein labeling upon forskolin stimulation (data now shown).

Table 4. Quantitation of cell surface fluorescence intensity of HeLa cells expressing M2-901/CFTR in the absence or presence of 10 μM forskolin

	Experiment 1	Experiment 2	Experiment 3
(−) Forskolin	11,497 (354)	13,335 (210)	13,737 (378)
(+) Forskolin	33,217 (366)	27,212 (187)	19,810 (307)

Number in parentheses are number of cells assayed in each experiment. Quantitation was determined as outlined in the Methods section.

Similar experiments were carried out using an MDCK cell-line that is stably transfected with the pMEP4 vector subcloned with M2-901/CFTR. In this construct, M2-901/CFTR is expressed from the inducible metallothionein promoter. Preliminary data show that after 20 minutes exposure to 10 μM forskolin at 37°C, a twofold increase in cell surface fluorescence was detected using the M2 antibody (data not shown).

These results indicate that forskolin stimulates steady-state expression of M2-901/CFTR at the cell surface. This increase in plasma membrane CFTR could result from an inhibition of CFTR endocytosis, a stimulation of CFTR exocytosis or both. Results obtained prior to the availability of the M2-901/CFTR construct address this issue. Experiments were performed to study the effect of cAMP on the endocytosis of the M2-901 tagged CFTR. vTF7-3 infected HeLa cells expressing M2-901/dgCFTR were incubated in the presence or absence of forskolin for 30 minutes at 37°C and then stained with M2 antibody at 4°C for 15 minutes. The cells were either stained immediately with FITC-labeled secondary antibody at 4°C (zero time point) or warmed to 37°C for one, two or five minutes in the presence or absence of forskolin prior to secondary antibody staining. As shown in Figure 2, most of the M2/CFTR was endocytosed from the cell surface within two minutes of warming at 37°C. However, in the presence of forskolin, CFTR endocytosis was significantly inhibited.

The rapid endocytosis of M2/CFTR and its inhibition by forskolin is in sharp contrast to the lack of endocytosis of the VSV-G protein under similar conditions (data not shown). There is no evidence to suggest that VSV-G protein undergoes plasma membrane recycling. Its surface expression did not change during five minutes incubation at 37°C in the presence or absence of forskolin. This provides further evidence that CFTR endocytosis is not evoked non-specifically by the experimental conditions employed (such as induced by antibody binding). Quantitation of

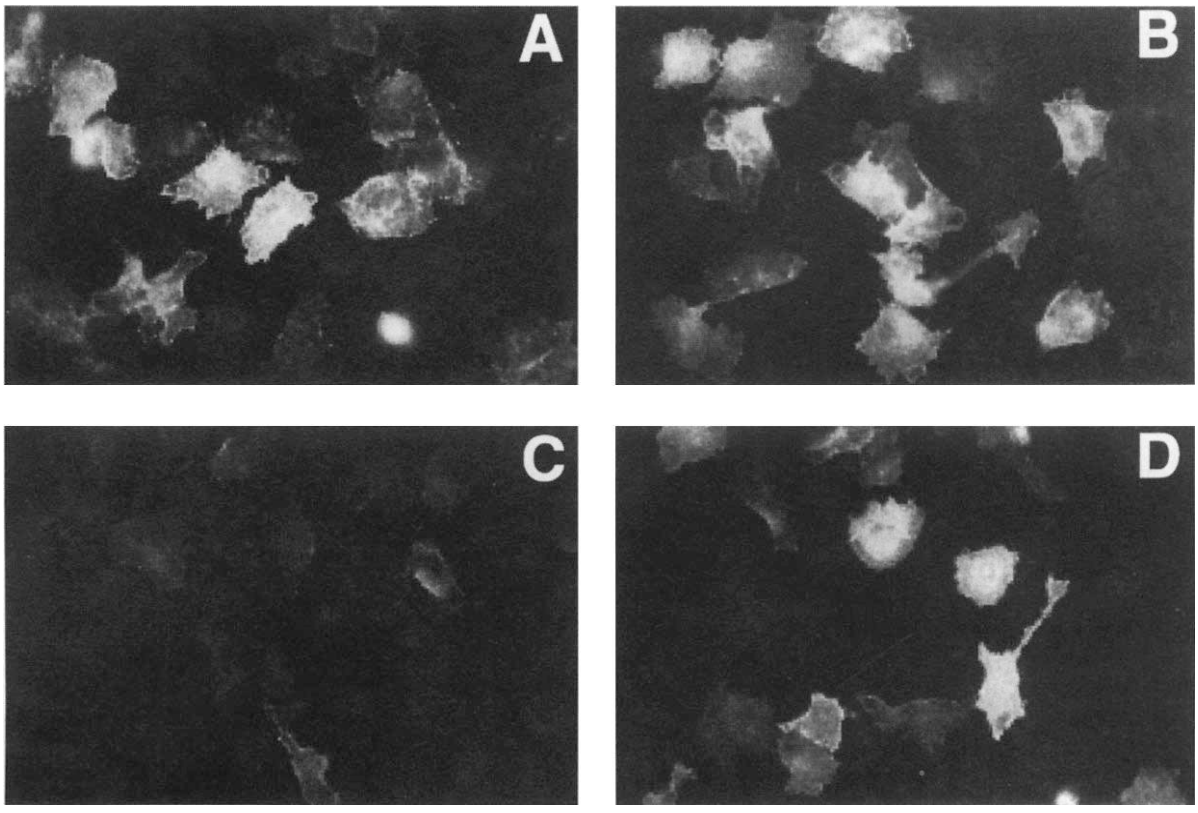

Fig. 2. *The effect of forskolin on M2-901/dgCFTR endocytosis.* Cell surface CFTR at zero-time (**A, B**) or two minutes. (**C, D**) after rewarming to 37°C in the absence (A,C) or presence (B,D) of 10 μM forskolin.

these experiments can yield a rate coefficient for CFTR endocytosis that can be compared among different experimental conditions (stimulation) and between different CFTR mutants.

Discussion

This study was undertaken to access the cellular location of CFTR when it is expressed in the plasma membrane domain as an epitope-tagged protein. This required the insertion of an epitope within an extracellular loop of CFTR. Most epitope tagging studies have added epitope sequences to either the C-terminus or N-terminus of expressed proteins. Of critical importance in establishing this as a useful technique for CFTR localization was retention of CFTR function. Adding charged amino acid sequences to an internal site raises the possibility of disrupting tertiary structure and impairing the cAMP-regulated Cl channel function of the protein. This could result from an impairment in channel conductance, gating or regulation, or from disruption of its cellular trafficking properties. Indeed, in prior studies we found that certain epitope sequences interfere with the ability of tagged CFTRs to traffic to the plasma membrane domain [7].

The utility of the extracellular epitope tag is demonstrated by our studies of the regulation of cell surface expression of CFTR. The adequacy of the M2-901/CFTR construct in this respect is shown by its plasma membrane distribution. In addition, its function as a cAMP stimulated Cl conductance is not different from wild type CFTR, as assessed in two functional assays. The function of M2-901/CFTR as a regulated chloride conductance was demonstrated utilizing both fluorescence and Cl conductance assays. In HeLa cells transiently expressing the tagged construct, increases in fluorescence upon cAMP stimulation were not different from those observed with wild type CFTR. In addition, when this construct was expressed in *Xenopus* oocytes, the chloride conductance increase observed on stimulation by cAMP agonists was also similar to the observed for wild-type CFTR. Other studies not reported here have suggested that the single channel properties of M2-901/CFTR are also indistinguishable from wt CFTR.

We performed a number of studies to determine whether carbohydrate addition to the fourth extracellular loop would interfere with the ability of M2 antibodies to stain the surface of non-permeabilized cells. Our fear was that steric hindrance produced by the addition of carbohydrate at these sites would prevent antibody binding. In anticipation of this problem, we produced a deglycosylated version of the M2-CFTR construct (dg CFTR), but the experimental results showed these concerns were unfounded. In fact, the deglycosylated CFTR yielded a lower level of Cl conductance stimulation regardless of whether it contained the tag sequence. The mechanism whereby deglycosylation compromises the cAMP-dependent Cl conductance is under further

study, and may involve changes in its plasma membrane expression. Nevertheless, we identified a site for insertion of the tag sequence, following amino acid 901, that yielded a fully functional tagged protein. This M2-901 construct appears to be optimal for the detection of cell surface CFTR and for further analysis of its regulation.

Assessment of several M2-tagged CFTR mutants confirmed its utility. The expression of a number of disease mutations with the M2-901 tag showed that their cell surface expression correlated with their ability to undergo carbohydrate addition, a measure of whether they traffic through the protein secretory pathway. Particularly interesting in this respect were the ΔF508 and G551D tag constructs; both of these mutations produce severe CF. They exemplify differences in the way CFTR mutants are processed by cells, and differences in the molecular mechanisms whereby the defect in cAMP-stimulated Cl conductance is produced. In the case of ΔF508 CFTR, the lack of cell surface expression correlated with the absence of carbohydrate processing. In the case of G551D CFTR, a glycosylated protein is produced. Previous studies using permeabilized cells have suggested that this protein co-localizes with the Na,K-ATPase and is thus plasma membrane associated [12]. However, at the level of resolution provided by indirect immunofluorescence, we could not rule out the possibility that the G551D protein was present at a submembrane site. The external epitope tag clearly shows that G551D CFTR resides within the plasma membrane. Thus, the defect in cAMP-stimulated Cl conductance of G551D CFTR arises from alterations in its ATP-dependent gating and not from defects in its cellular location. The correlation of surface expression with the glycosylation patterns of various mutants confirms the utility of the M2-901 tag for further studies of the cell biology of mutant CFTR proteins.

An extracellular CFTR tag permits direct assessment of the role of CFTR in acute regulation of membrane trafficking and whether there is cAMP-dependent exocytosis and endocytosis of CFTR itself. The results from the trafficking assays using the M2-901 construct indicate that CFTR participates in this process. Steady-state labeling by the M2 antibody showed a two- to threefold increase in cell surface CFTR on stimulation by forskolin. The timing of the assay (5 to 10 min stimulation) correlates with the time for maximal stimulation of Cl conductance in whole-cell patch clamp recordings or for transepithelial Cl secretion across epithelial monolayers. Thus, these results indicate that insertion of CFTR into the plasma membrane is one mechanism whereby stimulation of protein kinase A leads to an increase in apical membrane Cl conductance.

Finally, M2-901 CFTR was utilized to assess the effect of cAMP on CFTR endocytosis. Prince and Marchase [3] found a 50% decrease in the rate of internalization of surface CFTR when T84 cells were stimulated with cAMP-dependent agonists. Although they have not been quantitated, our studies support these findings and indicate that a decrease in CFTR endocytosis is one mechanism whereby cAMP stimulation enhances its cell surface expression during steady-state stimulation of Cl secretion. One caveat is whether the *de novo* proteins synthesized by vaccinia virus, used as the expression vector in these experiments, could contribute to the cAMP effect seen with CFTR. To answer that question, a polarized, stable cell line expressing CFTR was developed. Preliminary data using this cell line shows the same cAMP effect on CFTR as that seen in HeLa cells.

The epitope tagged CFTR should be useful for a variety of cell biological studies in which high resolution detection of cellular location of this protein is desirable. Antibodies raised to various domains of CFTR have lacked the necessary sensitivity to detect low levels of protein expression. The added antigenic epitope is distinct from endogenous peptide sequences, and provides for a low background. This is particularly true for the CFTR construct tagged at an extracellular locus since cell permeabilization is not required in the assay. Finally, the use of an epitope-tagged CFTR can provide for its experimental detection during exogenous expression, such as in gene transfer experiments, where it may be necessary to identify the protein in a background of endogenous wt CFTR.

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