Constitutive internalization of cystic fibrosis transmembrane conductance regulator occurs via clathrin-dependent endocytosis and is regulated by protein phosphorylation

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Although the cystic fibrosis transmembrane conductance regulator (CFTR) is primarily implicated in the regulation of plasma-membrane chloride permeability, immunolocalization and functional studies indicate the presence of CFTR in the endosomal compartment. The mechanism of CFTR delivery from the cell surface to endosomes is not understood. To delineate the internalization pathway, both the rate and extent of CFTR accumulation in endosomes were monitored in stably transfected Chinese hamster ovary (CHO) cells. The role of clathrin-dependent endocytosis was assessed in cells exposed to hypertonic medium, potassium depletion or intracellular acid-load. These treatments inhibited clathrin-dependent endocytosis by > 90 %, as verified by measurements of ¹²⁵I-transferrin uptake. Functional association of CFTR with newly formed endosomes was determined by an endosomal pH dissipation protocol [Lukacs, Chang, Kartner, Rotstein, Riordan and Grinstein (1992) *J. Biol. Chem.* **267**, 14568–14572]. As a second approach, endocytosis of CFTR was determined after cell-surface biotinylation with the

cleavable sulphasuccinimidyl-2-(biotinamido)ethyl-1,3-dithiopropionate. Both the biochemical and the functional assays indicated that arresting the formation of clathrin-coated vesicles inhibited the retrieval of the CFTR from the plasma membrane to endosomes. An overall arrest of membrane traffic cannot account for the inhibition of CFTR internalization, since the fluid-phase endocytosis was not effected by the treatments used. Thus the efficient, constitutive internalization of surface CFTR (5 % per min) occurs, predominantly by clathrin-dependent endocytosis. Stimulation of protein phosphorylation by cAMP-dependent protein kinase A and by protein kinase C decreased the rate of internalization of cell-surface biotinylated CFTR, and contributed to a substantial diminution of the internal CFTR pool compared with that of unstimulated cells. These results suggest that the rate of CFTR internalization may participate in the determination of the CFTR channel density, and consequently, of the cAMP-stimulated chloride conductance of the plasma membrane.

INTRODUCTION

The cystic fibrosis transmembrane conductance regulator (CFTR), the cystic fibrosis (CF) gene product, is a chloride-selective ion channel. CFTR is regulated at the single-channel level by cAMP-dependent protein kinase A (PKA)- and protein kinase C (PKC)-mediated phosphorylation, ATP binding and ATP hydrolysis (for reviews, see [1–4]). A growing body of evidence has corroborated the notion that translocation of CFTR from an internal vesicular pool may contribute to the cAMP-stimulated plasma-membrane chloride conductance in certain cells. First, in addition to its plasma membrane localization, wild-type CFTR was detected in organelles along the endocytotic pathway by immunocytochemical analysis at the light- and electron-microscopy levels in secretory epithelia [5–9]. Secondly, CFTR conferred PKA-stimulated anion conductance to endosomal membranes both in non-polarized Chinese hamster ovary (CHO) cells transfected with CFTR cDNA and in T-84 gut-epithelial cells expressing CFTR endogenously [10,11]. Thirdly, CFTR appears to be rapidly internalized from the apical plasma

membrane of T-84 cells [12]. In addition, insertion of CFTR seems to occur from a subapical vesicular pool into the plasma membrane in T-84 epithelia [13] and in non-polarized cells transiently expressing an epitope-tagged construct of CFTR [14].

In principle, distribution of membrane transporters such as CFTR, glucose transporter or water channels between the plasma membrane and intracellular compartments is determined by the rate constants of their exocytosis and retrieval from the plasma membrane [15,16]. Evidence is accumulating in support of the co-existence of several different internalization pathways [17,18], such as clathrin-dependent and clathrin-independent endocytosis, potocytosis and macropinocytosis in immortalized cell lines and primary cells. However, the internalization route of CFTR had not been identified.

Integral membrane proteins undergoing clathrin-dependent endocytosis concentrate in clathrin-coated pits. This process is thought to be driven by the association of the plasma membrane adaptor protein (AP-2) with tyrosine or dileucine-based endocytotic targeting signal(s) on the cytosolic domain of membrane proteins [19–21]. Endocytotic targeting signals can be identified

Abbreviations used: CCV, clathrin-coated vesicle; CF(TR), cystic fibrosis (transmembrane conductance regulator); pH_{en}, pH in the endosomal compartment; RPMI-H medium, bicarbonate-free RPMI 1640 medium buffered with 10 mM Hepes/NaOH at pH 7.4; CHO, Chinese hamster ovary; E_m, membrane potential; NMG, N-methyl-D-glucamine; PKA, cAMP-dependent protein kinase A; PKC, protein kinase C; CPT-cAMP, 8-(4-chlorophenylthio)adenosine 3',5'-cyclic monophosphate; NHS-SS-biotin, sulphasuccinimidyl-2-(biotinamido)ethyl-1,3-dithiopropionate; MESNA, 2-mercaptoethanesulphonic acid; SF6847, 3,5-di-tert-butyl-4-hydroxybenzylidenemalononitrile.

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at the C- and N-terminal cytoplasmic domains of CFTR [22]. In addition, the presence of CFTR was indicated by biochemical and functional studies in clathrin-coated vesicles derived from the Golgi and plasma membrane fraction of T-84 epithelia [23]. On the basis of these observations, we hypothesized that the clathrin-dependent endocytotic pathway plays a central role in CFTR recruitment from the plasma membrane. To test our hypothesis *in vivo*, clathrin-coated vesicle (CCV) formation was abrogated by three different means, and association of CFTR with endosomes was determined. The results demonstrate that assembly of clathrin-coated pits and subsequent budding of CCV are necessary for the delivery of CFTR to newly formed endosomes. In addition, the effect of activation of PKA- and PKC-mediated phosphorylation both on the rate of CFTR internalization and on the size of internal CFTR compartment was investigated.

EXPERIMENTAL

Cell lines

Two independently derived clones of CFTR-expressing CHO cells, designated as CHO-BQ1 and CHO-BQ2, were grown in monolayer or suspension culture in α -modified minimum essential medium supplemented with 7% (v/v) fetal bovine serum and either 10 μ M or 200 μ M methotrexate, respectively. According to Western-blot analysis, the expression level of CFTR in CHO-BQ1 cells was similar to that of CaCo-2 epitheloid colon-carcinoma cells, expressing CFTR endogenously [24], whereas CHO-BQ2 cells displayed a higher (> 20-fold) level of CFTR expression (results not shown). Δ F508CFTR-expressing cells were cultured under the same conditions as CHO-BQ2 cells [25].

Fluorimetric membrane-potential measurements to monitor CFTR activity in CHO-BQ1 cells

The anionic fluorescent probe bisoxonol (DiSBAC₂ [3]) was used to estimate the membrane potential (E_m) of cells in suspension at 37 °C, as described previously [24].

Determination of ¹²⁵I-transferrin uptake

¹²⁵I-Transferrin uptake was monitored as a measure of clathrin-dependent endocytosis. Human holo-transferrin (Sigma) was iodinated with the chloramine T method in the presence of 3 mCi/ml Na¹²⁵I (Amersham, Canada) to a specific radioactivity of 0.5–0.7 μ Ci/ μ g, according to the procedure described previously [26]. CHO-BQ1 monolayers on 6-well tissue-culture dishes were depleted of endogenous transferrin by incubation in serum-free medium for 30 min at 37 °C. ¹²⁵I-Transferrin uptake was measured in bicarbonate-free RPMI 1640 medium buffered with 10 mM Hepes/NaOH at pH 7.4 (RPMI-H medium) containing 2–4 μ g/ml ¹²⁵I-transferrin and 1 mg/ml ovalbumin after 2 and 9 min incubations at 37 °C. Extracellular transferrin was removed by extensive washing with acid (0.2 M NaCl and 0.2 M acetic acid) and medium {150 mM NaCl/20 mM Hepes/10 mM glucose/5 mM KCl/1 mM MgCl₂/1 mM CaCl₂ (pH 7.35) [26]}. Cells were solubilized in 0.1% (v/v) Triton X-100/0.1 M NaOH, and the intracellularly accumulated radioactivity was counted in an LKB γ -counter. The rate of transferrin endocytosis was calculated from the difference of ¹²⁵I-transferrin uptake between 2 and 9 min, and corrected for non-specific binding [26]. Experiments were performed in triplicate. When the effect of hypertonic exposure, K⁺-depletion or acidification of the cytosol was examined, ¹²⁵I-transferrin uptake was measured in hypertonic

RPMI-H medium, K⁺-free Na⁺ medium or Na⁺-free *N*-methyl-D-glucamin (NMG) medium, respectively (see below).

Inhibition of clathrin-dependent endocytosis

Three different approaches were employed to inhibit CCV formation in CHO-Q1 cells [27–29]. (1) Cells were incubated in hypertonic medium (RPMI-H medium supplemented with 0.1, 0.2 or 0.3 M sucrose) either for 15 min at 37 °C or for 30 min at 4 °C. (2) Depletion of cytosolic K⁺ was achieved by hypotonic swelling of the cells in 50% diluted RPMI-H medium containing ouabain (1 mM) for 5 min, and then incubating them in ice-cold K⁺-free medium [100 mM NaCl/50 mM Hepes/1 mM MgCl₂/0.1 mM CaCl₂/10 mM glucose (pH 7.3)] for 20 min. (3) Acidification of the cytosol was accomplished with the NH₄Cl pre-pulse method [30]. Following the incubation in RPMI-H medium supplemented with 40 mM NH₄Cl, cells were transferred to Na⁺-free medium [140 mM NMG-Cl/10 mM Hepes/1 mM MgCl₂/0.1 mM CaCl₂/10 mM glucose (pH 7.3)]. Cytosolic pH determinations [31] with the pH-sensitive fluorescent dye, 2',7'-biscarboxyethyl-5(6)-carboxyfluorescein (BCECF), indicated that the cytoplasmic pH was shifted from 7.2 to $\approx$ 6.4 (results not shown).

Measurements of the internalization of the fluid-phase tracer, fluorescein-dextran

Cells were resuspended in the medium specified, containing fluorescein-dextran (5 mg/ml, M_r 70000) and incubated for 12 min at 37 °C. After washes with ice-cold Hank's buffered salt solution, the amount of intracellular fluorescein-dextran was measured fluorimetrically in the presence of anti-fluorescein IgG (0.1 unit/ml) by clamping the pH in the endosomal department (pH_{en}) to 7.4 with monensin and nigericin [31].

Percoll-density-gradient fractionation

To examine whether the internalized fluorescein-dextran is restricted predominantly to early endosomes, the distributions of specific organellar markers and fluorescein-dextran were compared. Early endosomes (sorting and recycling endosomes) were labelled in the presence of 5 mg/ml fluorescein-dextran (12 min at 37 °C) and 5 μ g/ml ¹²⁵I-transferrin (30 min at 37 °C) respectively. Extracellular transferrin and dextran were removed by extensive washes with ice-cold medium (see above). CHO-BQ1 cells were homogenized by nitrogen cavitation [25] and the postnuclear supernatant was fractionated on a self-forming Percoll-density gradient [25% Percoll/0.25 M sucrose/10 mM Hepes/1 mM EDTA (pH 7.3)] for 100 min (28000 g at 4 °C). The gradient was downloaded with a fraction collector (Auto Densi-Flow; Labconco) from the bottom of the tubes (see Figure 1). The density profile of the Percoll-density gradient was optimized with density-marker beads (Sigma) in preliminary experiments.

Determinations of organellar marker distribution

Alkaline phosphatase and β -glucuronidase, specific markers of plasma membrane and lysosomes respectively, were measured as described previously [27]. The sedimentation profile of ¹²⁵I-transferrin was established by determining the radioactivity of each fraction. The fluorescein-dextran content of each fraction was measured as described above [10]. The Percoll-density-gradient profile of the CHO-BQ1 cell homogenate indicates that the distribution of internalized fluorescein-dextran overlaps almost completely with the ¹²⁵I-transferrin distribution, and is distinct from the late endosomes/lysosomes, marked by the

major peak of the β -glucuronidase activity (Figure 1) and by the distribution of fluorescein-dextran internalized overnight (results not shown).

Determination *in situ* of pH_{en} and assessment of CFTR contribution to the ionic conductance of endosomes

Continuous measurement of the *in situ* pH_{en} was performed as in [10]. At steady-state pH_{en}, dissipation of the pH gradient was initiated by the vacuolar H⁺-ATPase inhibitor, bafilomycin B (0.5 μ M) and the protonophore, 3,5-di-tert-butyl-4-hydroxybenzylidenemalononitrile (SF6847; 10 μ M). The initial rate of pH_{en} dissipation was determined in the absence and presence of forskolin (20 μ M) or CTP-cAMP (0.5 mM). The stimulatory effect of PKA activation on the rate of endosomal alkalinization, an indicator of CFTR activity ([10], and see below), was expressed as a percentage of the basal H⁺-efflux rate.

Biotinylation and internalization assay of CFTR

Reversible cell-surface biotinylation of metabolically labelled cells provides a tool to follow the internalization process of plasma membrane proteins [12,32]. To obtain detectable signals of endocytosis, the CHO-BQ2 cell line, overexpressing CFTR, was used in these experiments.

Radioactive labelling of CHO-BQ2 cells was carried out with [³⁵S]methionine and [³⁵S]cysteine (0.1–0.2 mCi/ml; Amersham) for 2–3 h and chased in the presence of excess cold methionine and cysteine [25]. Cell-surface proteins were biotinylated with sulphosuccinimidyl-2-(biotinamido)ethyl-1,3-dithiopropionate (NHS-SS-biotin) (1 mg/ml in PBS) twice for 15 min on ice. Following four washes with ice-cold PBS-CM medium (PBS supplemented with 0.1 mM CaCl₂ and 1 mM MgCl₂), non-reacted NHS-SS-biotin was quenched with 0.1 % BSA in PBS. Internalization of biotinylated CFTR was initiated by the addition of prewarmed RPMI-H medium and then transferring the dishes to 37 °C, followed by arrest of internalization after the chase period (1.5–7 min) with ice-cold medium. Biotin molecules remaining at the cell surface were removed by reducing their disulphide bonds with the reducing agent 2-mercaptoethanesulphonic acid (MESNA) [33]. Cells were treated three times for 30 min with 50 mM MESNA in 100 mM NaCl, 50 mM Tris/HCl, 1 mM MgCl₂ and 0.1 mM CaCl₂, pH 8.6, at 4 °C. The efficiency of biotin stripping (92.2 ± 2.3 %, $n = 5$) was evaluated by measuring the relative amount of biotinylated CFTR both before and after the reduction of the disulphide bonds on cells kept at 4 °C. Immunoprecipitation of the biotinylated CFTR was achieved with M3A7 and L12B4 monoclonal anti-CFTR antibodies [7] according to published protocol [25]. Immuno-isolated CFTR was eluted from protein G beads with 1 % (w/v) SDS. An aliquot (10–15 %) of the eluate was saved to monitor labelling efficiency of CFTR by fluorography (see lower panels of Figures 4B, 5 and 6). Biotinylated CFTR was separated with streptavidin–Sepharose (Sigma) and analysed by SDS/PAGE and fluorography [25]. Radioactivity incorporated in the biotinylated CFTR was quantified by phosphorimage analysis using ImageQuant software. To assess the size of the recycling CFTR compartment, in some of the experiments, biotinylation was performed at 37 °C (see Figure 6).

RESULTS

If continuous formation of CCV is required for the internalization of CFTR, inhibition of clathrin-dependent endocytosis will decrease the amount of CFTR associated with endosomes. To test this prediction, first we implemented various perturbations

Figure 1 Distribution of fluorescein-dextran and organelar markers of the postnuclear supernatant of CHO-BQ1 cells on Percoll-density gradient

The endosomal compartment was labelled with ¹²⁵I-transferrin (¹²⁵I-Tf) for 30 min at 37 °C and fluorescein-dextran (FD) for 12 min at 37 °C. ¹²⁵I-Tf associated with the cell surface was removed with acid washes. Cells were homogenized by nitrogen cavitation and the postnuclear supernatant was fractionated on a 25 % Percoll-density gradient. The Percoll-density gradient was downloaded from the bottom. The distribution of plasma membrane and lysosomal markers, alkaline phosphatase (□) and β -glucuronidase (○) respectively, FD and ¹²⁵I-Tf were determined as described in the Experimental section. The density profile of the Percoll-density gradient was with density-marker beads (Sigma).

that could arrest the internalization of clathrin-dependent endocytosis in CHO cells.

Arresting clathrin-dependent endocytosis in CFTR-expressing CHO cells

Clathrin-dependent endocytosis can be inhibited by exposing cells to hypertonic medium, by depleting their K⁺-content or by acidifying the cytosol [27–29]. Since the efficacy of these methods is highly dependent on the cell-type, the incubation conditions were first optimized for CFTR-expressing CHO cells.

The activity of clathrin-dependent endocytosis was measured

Table 1 Effect of perturbants of CCV formation on ^{125}I -transferrin and fluorescein-dextran uptake

Receptor-mediated endocytosis is inhibited by exposing the cells to hypertonic medium, K^+ -depletion or acidification of the cytosol. Exposure of cells to hypertonic medium (0.3 M sucrose; RPMI-H medium supplemented with 0.3 M sucrose), to K^+ -depletion and to cytosolic acidification was accomplished as described in the Experimental section. ^{125}I -Transferrin uptake was measured for 7 min in the appropriate medium (RPMI-H medium, hypertonic medium, K^+ -free or Na^+ -free), at 37 °C. Data are expressed as percentages of ^{125}I -transferrin uptake of CHO-BQ1 cells in RPMI-H medium (control). To measure the fluid-phase tracer uptake, cells were allowed to internalize fluorescein-dextran for 12 min at 37 °C and the amount of accumulated fluorescein-dextran was expressed as the percentage of dextran taken up by untreated cells. Values are means $\pm$ S.E.M., $n = 3-9$.

Treatment	^{125}I -Transferrin uptake (%)	Fluorescein-dextran uptake (%)
Control	100.0	100.0
Hypertonic	5.2 ± 3.0	141.1 ± 8.3
K^+ -depletion	9.2 ± 4.1	138.9 ± 5.6
Acidification	2.8 ± 2.5	47.8 ± 5.5

by ^{125}I -transferrin uptake. Incubation of CHO-Q1 cells for 15 min in medium with an osmotic concentration of 0.45 osmol/litre (osM) inhibited ^{125}I -transferrin uptake by 50 % (results not shown). Maximal inhibition (95 ± 3 %, mean $\pm$ S.E.M., $n = 3$) was observed at 0.6 osM (Table 1). Under optimal conditions the two other perturbants caused an almost complete arrest of CCV formation. Depletion of the K^+ content or acidification of the cytosol decreased ^{125}I -transferrin uptake by 91 ± 4 % ($n = 6$) and 98 ± 3 % ($n = 6$) respectively (Table 1).

To assess whether the above manipulations were selectively inhibiting clathrin-dependent endocytosis, the uptake of fluorescein-dextran, a fluid-phase tracer, was determined. None of the three perturbants of CCV formation arrested membrane traffic, as determined by fluorescein-dextran accumulation (Table 1). Similar observations were reported previously in rat fetal fibroblasts and in HeLa cells overexpressing a mutant form of dynamin [34,35].

Inhibition of clathrin-dependent endocytosis prevents the recruitment of functional CFTR to endosomes

CFTR recruitment to endosomes upon inhibition of CCV formation was evaluated with a functional assay, as described previously [10]. The principle of the method is based on the observation that rapid pH_{en} dissipation, induced by the vacuolar H^+ -ATPase inhibitor bafilomycin and the protonophore SF6847, is rate-limited by the counter-ion conductance of the endosomal membrane in CHO cells. If CFTR residing in endosomes becomes phosphorylated, it will behave as a chloride ionophore. This additional counter-ion conductance accelerates H^+ -efflux from endosomes. The pH_{en} dissipation protocol is illustrated in Figure 2 and discussed in [10]. Addition of bafilomycin and SF6847 induced a rapid dissipation of the acidic pH_{en} (Figure 2). When the cytoplasmic cAMP concentration was raised with forskolin [or 8-(4-chlorophenylthio)adenosine 3',5'-cyclic monophosphate (CTP-cAMP)], the rate of endosomal alkalinization was accelerated by 70–80 % in CFTR-expressing CHO cells, but remained unaltered in the parental CHO cells (see Table 2 and Figure 2 in [10]). Meanwhile, M3A7, a monoclonal anti-CFTR antibody, completely prevented the PKA-stimulated pH dissipation of endosomes isolated from CFTR-expressing cells [10]. These and several additional *in vivo* and *in vitro* experiments provided unequivocal evidence that the cAMP-stimulated component of the rapid H^+ -efflux is attributed to CFTR activation in endosomes

Figure 2 *In situ* measurements of pH_{en} to detect wild-type and $\Delta\text{F508CFTR}$ in endosomes of stably transfected CHO cells

The traces are typical pH_{en} recordings in CHO-BQ1 cells. Endosomes were labelled with pH-sensitive fluorescein-dextran and the cells were resuspended in Na^+ medium containing 0.1 unit/ml anti-fluorescein IgG at 37 °C. Endosomal acidification was dissipated by sequential addition of 0.5 μM bafilomycin B (BAF) and 5 μM SF6847 (SF). At the end of the incubation, 20 mM NH_4Cl was added to complete dissipation. Where indicated, 2.5 μM valinomycin (VAL) was added. The rate of dissipation was calculated from the pH_{en} changes recorded during the first 20 s after addition of SF, on graphs replotted at a higher time resolution.

both in non-polarized CHO cells and in T-84 epithelia [10,11]. Notably, valinomycin, a potassium ionophore, by elevating the counter-ion conductance of the endosomal membrane could accelerate also the pH_{en} dissipation rate by more than two-fold (Figure 2 and [10]).

Having established three different means to abrogate clathrin-dependent endocytosis, and an assay to detect CFTR in endosomes, we asked whether CCV formation is necessary for CFTR targeting to endosomes. First, clathrin-dependent endocytosis was arrested in hypertonic medium, and subsequently the forskolin-stimulated pH_{en} dissipation was assessed. Whereas functional CFTR was associated with endosomes of control cells, as indicated by the 70 % (71.0 ± 6.0 %, $n = 5$) stimulation of H^+ -efflux, acceleration of H^+ -efflux was abolished in cells pre-incubated in 0.3 M sucrose-containing medium for 15 min at 37 °C (Table 2). Partial loss of the forskolin-stimulated H^+ -efflux was detected in 0.1 M sucrose-containing medium (Table 2), in agreement with the osmotic sensitivity of receptor-mediated internalization [28]. Delivery of CFTR to the endosomal compartment was resumed following the re-introduction of the cells into iso-osmotic medium, implying that clathrin-coated endocytosis was inhibited reversibly (Table 2).

Next, the effects of the two other perturbants of CCV formation on CFTR endocytosis was examined. Both depletion of cellular K^+ -content and acidification of the cytosol diminished the forskolin-stimulated H^+ -efflux by more than 90 %, consistent with our hypothesis that CCVs play a central role in the delivery of CFTR from the plasma membrane to endosomes (Table 2). Alternatively, these treatments interfere with the activation process of CFTR or diminish the sensitivity of the H^+ -efflux assay (e.g. by attenuating the basal counter-ion conductance of endosomes); however, the following experiments indicate that these possibilities are unlikely.

First, we ruled out that activation of CFTR is impaired by monitoring the plasma-membrane chloride conductance with the potential-sensitive dye bisoxonol. The rapid and substantial depolarization of the plasma E_m upon addition of forskolin (or CTP-cAMP, results not shown) indicates that the PKA sensitivity

Table 2 Inhibition of clathrin-dependent endocytosis prevents the recruitment of CFTR to endosomes

CFTR association with endosomes was measured by the rapid H^+ -efflux assay as in Figure 2. After arresting CCV formation of CHO-BQ1 cells in hypertonic medium (RPMI-H medium supplemented with 0.1 M or 0.3 M sucrose), with depletion of the cellular K^+ content or by cytosolic acidification, endosomes were labelled with fluorescein-dextran in the appropriate medium (12 min, 37 °C) and the rate of pH_{en} dissipation was determined in the presence or absence of forskolin (20 μ M). The forskolin-induced H^+ -efflux was expressed as the percentage of basal rate of H^+ release. Where indicated, H^+ -efflux was stimulated with 2.5 μ M valinomycin (+ valinomycin) instead of forskolin. The reversibility of the hypertonic exposure was assessed by re-introducing the cells into iso-osmotic medium for 30 min and repeating the H^+ -efflux assay (sucrose + wash-out).

Treatment	Rate of H^+ -efflux from endosomes (%)
Control	170.0 $\pm$ 6.0
Sucrose (0.1 M)	133.5 $\pm$ 3.5
Sucrose (0.3 M)	102.0 $\pm$ 1.2
Sucrose + valinomycin	215.0 $\pm$ 22.0
Sucrose + wash-out	155.0 $\pm$ 4.1
K^+ -depletion	96.2 $\pm$ 2.0
Acidification	99.6 $\pm$ 2.0
Acidification + valinomycin	197.3 $\pm$ 10.7

Figure 3 Effect of hypertonic exposure and acidification of the cytosol on the cAMP-stimulated plasma membrane depolarization in CFTR-expressing CHO-BQ1 cells

The plasma membrane cAMP-sensitive Cl^- permeability was determined by membrane potential (E_m) measurement in CHO-BQ1 cells fluorimetrically using bisoxonol (DiSBAC₂ [3]; 200 nM). Clathrin-dependent endocytosis of CHO-BQ1 cells was abrogated with hypertonic treatment (hypertonic) for 27 min or acidification of the cytosol (acidified) as described in the Experimental section. The plasma-membrane cAMP-stimulated Cl^- permeability was monitored in NMG medium by subjecting the cells with forskolin (FOR; 20 μ M) at 37 °C. Similar results were obtained with CPT-cAMP (0.5 mM, results not shown). The cationophore gramicidin (GRA; 2 μ M) repolarizes the E_m , indicating that the depolarization induced by FOR is reversible.

of CFTR was retained after hypertonic exposure or acid-load of CHO-BQ1 cells (Figure 3). It should be noted that the effect of K^+ -depletion could not be assessed owing to the resulting depolarization of the resting E_m .

The absolute sensitivity of the H^+ -efflux assay was estimated by utilizing the temperature-sensitive mutant $\Delta F508CFTR$. $\Delta F508CFTR$ is subjected to intracellular retention and degradation at 37 °C [36]. At reduced temperatures, a small fraction of the newly synthesized protein is rescued and accumulates at the plasma membrane [37]. Accordingly, a modest stimulation (35–37%) of the pH_{en} dissipation rate by PKA activation could be detected on cells kept at 22 °C but not at 37 °C, whereas valinomycin produced an effect similar to that found in CFTR-

Table 3 Detection of $\Delta F508CFTR$ in endosomes at reduced temperature with the pH_{en} dissipation assay

The rate of endosomal H^+ -efflux in $\Delta F508CFTR$ -expressing CHO cells grown at 37 °C or at 22 °C (for 24–36 h) was measured as described in the legend to Table 2. The effect of forskolin (20 μ M), CPT-cAMP (0.5 mM) or valinomycin (2.5 μ M) was expressed as a percentage of the basal H^+ -efflux, measured after the addition of bafilomycin B and SF6847. Data are means $\pm$ S.E.M. of 3 or 4 separate determinations.

Treatment	Rate of H^+ -efflux (%)	
	37 °C	22 °C
Forskolin	100.0 $\pm$ 2.0	136.8 $\pm$ 6.0
CPT-cAMP	100.0 $\pm$ 2.0	134.7 $\pm$ 6.1
Valinomycin	255.4 $\pm$ 15.6	260.0 $\pm$ 12.0

expressing CHO cells (Table 3). Metabolic pulse-chase experiments and whole-cell chloride-current measurements suggest that the expression level of $\Delta F508CFTR$ in cells grown at reduced temperatures is significantly lower (< 15%) than that of its wild-type counterpart (results not shown). These observations imply that the H^+ -efflux assay is able to detect small quantities of CFTR in endosomes, a prerequisite for the appropriate interpretation of the experiments reported above.

Finally, exposure of the cells to hypertonic medium or acidification of the cytosol did not interfere with the valinomycin-induced pH_{en} dissipation, suggesting that these treatments did not decrease the sensitivity of the assay (Table 2 and Figure 1 in [10]). Thus the failure to detect CFTR association with endosomes in cells lacking CCV formation reflects the requirement of a functional clathrin-dependent endocytotic pathway for targeting of CFTR to endosomes.

The effect of hypertonic incubation on the internalization of biotinylated CFTR

To study the internalization pathway of CFTR by biochemical methods, and provide additional proof that CFTR is routed from the plasma membrane to endosomes, a cell-surface biotinylation assay was established in CHO-BQ2 cells overexpressing CFTR.

NHS-SS-biotin, a cleavable derivative of NHS-biotin [32], was used to covalently modify the ϵ -amino-groups of lysine residues on the extracellular surface of CFTR in metabolically labelled CHO-BQ2 cells. Following cell-surface biotinylation of monolayers at 4 °C, the temperature of the medium was shifted to 37 °C for a period of 1.5 to 7 min to allow the internalization of plasma-membrane constituents. Biotin molecules remaining at the cell surface were cleaved with the membrane-impermeant reducing agent MESNA. Biotinylated CFTR was immunoprecipitated sequentially with anti-CFTR antibody (M3A7 and L12B4 [7]) and streptavidin-Sepharose, and visualized by fluorography. Approximately 15% (14.4 $\pm$ 2.2%, n = 3) of the cell-surface-labelled CFTR became endocytosed during the first three minutes of internalization (Figure 4), which was followed by a gradual loss of intracellular CFTR. When cells were preincubated in hypertonic medium for 30 min on ice, the internalization of CFTR was inhibited by 46.0 $\pm$ 9.4% (n = 4; Figure 5 and Table 4). The incomplete inhibition of CFTR endocytosis could be attributed to two factors. First, hypertonic exposure of CHO-BQ2 cells was performed at low temperature, under conditions where CCV formation, and consequently CFTR internalization, is inhibited partially. Secondly, CHO-BQ2 cells

Figure 4 Kinetics of the internalization of biotinylated CFTR

(A) Outline of the experimental protocol. (B) CHO-BQ2 cells were metabolically labelled with [³⁵S]methionine and [³⁵S]cysteine for 2 h and chased for 2.5 h. Cell-surface proteins were biotinylated with NHS-SS-biotin (1 mg/ml) on ice or at 37 °C. Internalization of cell-surface proteins was achieved by shifting the temperature to 37 °C for the indicated periods (1.5–7 min). Biotin molecules remaining at the cell surface were stripped with 50 mM MESNA. Biotinylated CFTR was immunoprecipitated with anti-CFTR antibodies (M3A7 and L12B4), and subsequently with streptavidin–Sepharose and visualized by fluorography. Lower half of the fluorograph represents 10% of the total CFTR immunoprecipitate, to verify the reproducibility of CFTR labelling in the different samples. The stripping efficiency of biotin was measured on cells kept at 4 °C. (C) Quantification of the radioactivity associated with the internalized CFTR from (B). Each value was corrected with the stripping efficiency and expressed as the percentage of radioactivity associated with biotinylated CFTR at the cell surface. The data are representative results of two independent experiments.

express at least 20-fold more CFTR than CHO-BQ1 cells, used for the functional detection of CFTR in endosomes. As in the case of other membrane proteins ([38] and references therein), saturation of clathrin-coated pits may lead to the internalization of CFTR via non-clathrin-coated endocytosis, which is resistant to hypertonic treatment. However, it was impossible to conduct the biotinylation experiments in cells other than CHO-BQ2 cells due to the low signal-to-noise ratio detected in CHO-BQ1 cells. Despite the fact that the biotinylation experiments were carried out under suboptimal conditions, the inhibitory effect of hypertonic medium is consistent with the notion that clathrin-mediated endocytosis plays a role in CFTR delivery to endosomes.

Table 4 Modulation of CFTR internalization by both inhibition of CCV formation and activation of protein phosphorylation

Plasma-membrane-resident CFTR was biotinylated at 4 °C and the amount of internalized molecules was determined after 3 min of endocytosis at 37 °C, as described in the legend to Figure 4. PKA and PKC were stimulated with forskolin and PMA respectively during endocytosis. CCV formation was inhibited by preincubating the cells in medium supplemented with 0.3 M sucrose for 30 min at 4 °C (hypertonic). Quantification of internalized CFTR was done by phosphorimage analysis and expressed as the percentage of the amount endocytosed in untreated cells (control). Data are means ± S.E.M. of 3 separate determinations.

Treatment	Internalization of CFTR (%)
Control	100.0
Forskolin (20 µM)	72.8 ± 5.9
PMA (100 nM)	50.3 ± 6.6
Sucrose (0.3 M)	53.9 ± 9.4

Figure 5 The effect of hypertonic medium and stimulation of protein phosphorylation on the initial rate of CFTR internalization

Plasma-membrane-resident CFTR was biotinylated at 4 °C and the amount of internalized molecules was determined after 3 min of endocytosis at 37 °C, as described in the legend to Figure 4. Where indicated, PKA and PKC were stimulated with forskolin (20 µM) and PMA (100 nM) respectively during endocytosis. CCV formation was inhibited by preincubating the cells in medium supplemented with 0.3 M sucrose for 30 min at 4 °C (hypertonic). Stripping efficiency was evaluated on cells kept at 4 °C (stripped). Upper fluorograph is representative of three similar experiments. The lower fluorograph contains 10% of the total CFTR immunoprecipitate from corresponding samples of the upper gel.

Regulation of CFTR turnover by PKA- and PKC-mediated phosphorylation

Although a correlation has been established between the phosphorylation state and the open probability of the CFTR channel (e.g. [2,3]), the effect of phosphorylation on the turnover of CFTR at the plasma membrane has not been resolved. The impact of stimulation of protein phosphorylation on the rate of biotinylated CFTR internalization was evaluated. Activation of PKA and PKC by forskolin and PMA, respectively, inhibited the rate of CFTR endocytosis by 27.2 ± 5.9% and 49.8 ± 6.6% (n = 3) respectively (Figure 5 and Table 4).

Figure 6 Biotinylation time course (A) and the effect of PKA and PKC stimulation on the size (B) of the internal CFTR compartment

(A) Biotinylation of CHO-BQ2 cells was performed as described in the legend to Figure 5, but the temperature was kept at 37 °C to allow continuous membrane turnover. At the indicated time points, cells were chilled and biotin remaining at the cell surface was stripped with MESNA (compare lanes 3 and 4). Biotinylated CFTR molecules were isolated and visualized as described in the legend to Figure 4. Stripping efficiency was evaluated on cells kept at 4 °C (lane 1 and 2). (B) The exchangeable intracellular CFTR compartment was biotinylated at 37 °C as in A, in the presence or absence of the indicated agonist. Cell-surface-associated biotin was removed with MESNA at 4 °C. PMA; 100 nM, forskolin (FOR); 20 μ M, or control (DMSO). The lower fluorographs on each panel contain 10% of the total CFTR immunoprecipitate from corresponding samples of the upper gels.

To analyse the effect of PKA and PKC on CFTR distribution between the plasma membrane and the internal compartment, a new experimental approach was implemented. Biotinylation was performed at 37 °C, which allowed the detection of CFTR molecules that are inserted into or retrieved from the plasma membrane during a certain period of time. Subsequent removal of biotin from the cell surface revealed the amount of biotinylated and internally translocated CFTR. Biotinylation of this intracellular CFTR compartment reaches saturation between 20 and 30 min of labelling (Figure 6A). The size of this internal compartment is approximately 30% of the cell-surface-labelled CFTR pool (compare lanes 1 and 6 in Figure 6A). When PKA activity was stimulated with forskolin during biotinylation, the intracellular CFTR pool was diminished by 40% (Figure 6B).

The internal CFTR pool was decreased similarly (44%) upon activation of PKC with PMA (Figure 6B). Thus protein phosphorylation not only inhibits CFTR retrieval from the plasma membrane, but also appears to decrease the size of the intracellular exchangeable CFTR pool. However, these experiments cannot rule out the possibility that protein phosphorylation alters the rate of recycling of CFTR.

Collectively, the results of the biotinylation experiments indicate that CFTR undergoes constitutive endocytosis, and suggest that stimulation of both PKA and PKC may facilitate CFTR translocation from an intracellular compartment to the cell surface by inhibiting the internalization of CFTR.

DISCUSSION

Accumulating evidence suggests that a fraction of CFTR channels resides in the early endosomal compartment, but its functional significance remains elusive [5–11]. Early endosomes comprise at least two distinct, but interconnected, populations of tubulovesicular structures, the sorting and recycling endosomes [39]. Sorting endosomes have the potential to modulate CFTR-channel density at the plasma membrane by directing incoming CFTR molecules for either degradation in lysosomes, or back to the plasma membrane via the recycling compartment. To better understand the regulation of CFTR density at the cell surface, we attempted to delineate the internalization pathway of CFTR from the plasma membrane and its regulation by PKA- and PKC-mediated phosphorylation.

A number of considerations favoured the use of stably transfected CHO cells as a model to investigate the mechanism of CFTR internalization. First, CFTR was successfully identified in the endosomal compartments in both stably transfected CHO cells and T-84 epithelia, with approximately the same abundance [10,11]. Secondly, the fluid-phase and clathrin-dependent endocytotic pathways have been well-characterized functionally and pharmacologically in fibroblasts [39]. Finally, but most importantly, the molecular machinery required for targeting plasma membrane proteins to coated pits and CCV, to our present knowledge, is indistinguishable in non-polarized cells and in epithelia [40,41].

On the basis of putative endocytotic targeting motifs identified at the C- and N-terminal cytoplasmic domains of CFTR [22], both by immunocytochemical localization studies of CFTR at the electron microscopic level [5,9] and by subcellular fractionation studies of T-84 cells [23], we hypothesized that CFTR is targeted to clathrin-coated pits and, subsequently, to CCV during internalization. Almost complete inhibition of clathrin-dependent endocytosis, verified by 125 I-transferrin-uptake measurements, was achieved by three different means. All three perturbants of clathrin-dependent endocytosis prevented delivery of CFTR to newly forming endosomes. Control experiments ruled out the possibility that the inhibitors interfered either with the assay or with the activation of CFTR by PKA, or that they caused irreversible deterioration of the cells. From these results, we concluded that continuous formation of CCV is required to deliver CFTR to early endosomes. Consistently with this notion, the internalization of cell-surface-biotinylated CFTR was also inhibited in hypertonic medium (Table 4).

Although the kinetics of accumulation of biotinylated CFTR in T-84 and CHO cells were similar, the efficiency of internalization appears to be different. Approximately 16% of the cell-surface-associated CFTR was endocytosed in one min in T-84 cells [12], and 5% was internalized in CHO cells (Figure 4). These numbers are in agreement with the internalization efficiency of membrane proteins containing an endocytotic targeting se-

quence [19,42], and support our hypothesis that CFTR is recruited by clathrin-dependent endocytosis. Meanwhile, integral membrane proteins that are not preferentially recruited in clathrin-coated pits are internalized at a rate that is 5- to 10-fold slower [19].

In-depth morphological analysis at the electron-microscopic level indicated that membrane flow via the clathrin-dependent and clathrin-independent endocytotic pathways converges [43,44]. If a similar paradigm pertains to fluid-phase markers, fluorescein-dextran should accumulate in the same compartment, irrespective of CCV formation. Preventing the budding of CCV from the plasma membrane for 15 min has led to the depletion of the CFTR content of early endosomes, implying that CFTR departs rapidly from this compartment. However, the destiny of CFTR from sorting endosomes is at present not known. Taking into account its long metabolic half-life ($t_{1/2} = 12-16$ h [24,36,45,46]) and rapid internalization rate (5-16% per min of surface CFTR), it is conceivable that CFTR is routed constitutively to the plasma membrane by bulk membrane flow, and avoids premature lysosomal degradation [47]. Several lines of evidence support this hypothesis. First, gradual decrease of the internal CFTR pool is detectable after 3 min of internalization in cell-surface-biotinylated T-84 [12]. Secondly, transferrin receptors and Rab4 were found to associate with CFTR in subapical endosomes by immunogold electron microscopy in ductal epithelium of submandibular gland [5]. Whereas transferrin receptor is acknowledged as one of the most specific markers of recycling endosomes, rab4, a low-molecular-mass G-protein, has been proposed to regulate membrane traffic from early endosomes back to the plasma membrane [48]. Thirdly, if CFTRs were sorted to the late endosomes/lysosomes as opposed to recycling endosomes, taking into account the slow kinetics of transfer between early and late endosomes ($t_{1/2} = 17$ min [47]), significant CFTR activity should have been detected with the functional assay upon inhibition of clathrin-dependent endocytosis. However, this was not the case. Collectively, these observations suggest that a significant fraction of internalized CFTR recycles back to the cell surface from the endosomal compartment.

Although it has been postulated that CFTR expression confers cAMP regulation of endocytotic and exocytotic membrane traffic in epithelia (e.g. [49]), very little is known about the dynamic participation of CFTR in this process. Immunolocalization assessment, reflecting the steady-state distribution of CFTR, is hampered both by the lack of highly specific anti-CFTR antibody and the low copy-number of CFTR. Accordingly, cAMP-dependent redistribution of CFTR to the plasma membrane could not be detected with immunocytochemical and cell-surface biotinylation techniques [50,51]. On the other hand, activation of PKA inhibited CFTR internalization in T-84 cells [12,13] and stimulated the translocation of an epitope-tagged CFTR in HeLa cells [14]. To overcome the technical difficulties described above, we developed a reversible cell-surface biotinylation method to monitor CFTR internalization. This technique was utilized to demonstrate that activation of PKA inhibits the rate of CFTR endocytosis and evokes a significant decrease in the extent of CFTR association with the early endosomal compartment in CHO cells. These observations are consistent with the hypothesis that PKA-stimulated translocation of CFTR may provide an ancillary mechanism to strengthen the activation of the plasma-membrane chloride conductance upon elevation of the cytosolic cAMP concentration. Additional experiments are required to assess the physiological significance of CFTR translocation, considering that substantial variations may occur in the ratio of the cell surface and internal CFTR pool. For example, in T-84 epithelia, the amount of CFTR residing internally was

approximately equivalent to the amount associated with the plasma membrane [51].

Activation of PKC, the effector of another major second-messenger cascade, inhibited CFTR endocytosis and significantly decreased the internal CFTR pool (Table 4). As with PKA, PKC activation may play a role in amplifying chloride secretion during CFTR expression by promoting CFTR accumulation at the cell surface. In this context, it is noteworthy that PKC activation potentiated both the cAMP-mediated CFTR chloride current in inside-out patches on CHO cells and the whole-cell chloride current of pancreatic-duct cells [52,53].

The molecular target of protein phosphorylation in the process of CFTR trafficking is currently unknown. Potential candidates are CFTR itself, which contains multiple PKA/PKC consensus phosphorylation sites [2,3], and components of the clathrin-dependent endocytotic machinery, including dynamin and subunits of the adaptor-2 complex, which have been invoked in the regulation of CCV formation and disassembly in a phosphorylation-dependent manner [54]. Delineation of the regulatory mechanism of CFTR internalization could facilitate the design of pharmacological approaches to increase the amount of mutant, but at least partially functional, CFTR such as $\Delta F508$ CFTR [37] at the plasma membrane, and thus ameliorate the CF phenotype. Further studies are required to delineate the complexity and specificity of the regulation of CFTR trafficking in epithelia.

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