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Cystic fibrosis transmembrane conductance regulator activation stimulates endosome fusion *in vivo*

(endocytosis/fluorescence/Bodipy-avidin/ratio imaging/vesicle transport)

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ABSTRACT **Previous studies have suggested a role for cystic fibrosis transmembrane conductance regulator (CFTR) in the regulation of intracellular vesicular trafficking. A quantitative fluorescence method was used to test the hypothesis that CFTR expression and activation affects endosome–endosome fusion in intact cells. Endosomes from CFTR-expressing and control (vector-transfected) Swiss 3T3 fibroblasts were labeled by internalization with 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene (Bodipy)-avidin, a fluid-phase marker whose fluorescence increases ≈8-fold upon biotin binding. Cells were washed, chased, and then labeled with biotin-albumin or biotin-transferrin. The fraction of Bodipy-avidin-labeled endosomes that fused with biotin-containing endosomes (f_{fusion}) was quantified by ratio imaging microfluorimetry. Endosome fusion in unstimulated CFTR-expressing cells was similar to that in control cells. However, in CFTR-expressing cells activated by forskolin, f_{fusion} was increased by 1.30 ± 0.18- and 2.65 ± 0.17-fold for a 0 and 10 min chase time between avidin and biotin-albumin pulses; f_{fusion} also increased (1.32 ± 0.11-fold) when biotin-transferrin replaced biotin-albumin. The stimulation of endosome fusion was not due to differences in rates of endocytosis or endosomal acidification. Endosome fusion was not stimulated by forskolin in Cl^--depleted CFTR-expressing cells, suggesting that the increase in endosome fusion is due to the CFTR chloride channel activity. These results provide evidence that CFTR is involved in the regulation of endosome fusion and, thus, a possible basis for the cellular defects associated with cystic fibrosis.**

Cystic fibrosis (CF) is a genetic disease caused by mutations in the gene encoding the CF transmembrane conductance regulator (CFTR) protein (1). CFTR is a 180-kDa glycoprotein that functions as an adenosine 3′,5′-cyclic monophosphate (cAMP)-regulated Cl^- channel at the apical plasma membrane of epithelial cells (2–5). Although the function of CFTR as a Cl^- channel has been studied extensively, the cellular abnormalities that link the CF genotype to clinical disease are not completely understood. In addition to its well-established role as a plasma membrane Cl^- channel, a number of other cellular sites and functions have been reported for CFTR (reviewed in refs. 6 and 7). CFTR has been proposed to function as a transporter for ATP (8), water (9), and HCO_3^- (10), as well as a regulator of Na^+ channels (11) and the outwardly rectifying Cl^- channel (12). Pier *et al.* (13) recently reported that CFTR expression at the cell surface may be involved in the internalization of *Pseudomonas aeruginosa*.

Two possible functions of CFTR within cells have been proposed. Barasch *et al.* (14) reported evidence for defective acidification of the endosomal and secretory pathways in CF cells. They proposed that vesicular CFTR functioned as a Cl^- channel, which regulated acidification by shunting the interior positive diffusion potential generated by the electrogenic proton pump. However, subsequent studies showed no effects of CFTR expression and activation on acidification of the endosomal (15–17) or trans-Golgi (18) compartments.

Bradbury *et al.* (19) reported that cAMP stimulation by forskolin decreased the rate of endocytosis and increased exocytosis in epithelial cells that express wild-type CFTR, but not in cells expressing the most common mutant ΔF508 CFTR. They proposed that CFTR may be a general regulator of vesicular trafficking and that the pleiotropic nature of the defects found in CF might be due to abnormal vesicular trafficking in CF cells. Similar effects of CFTR on the rates of exocytosis and/or endocytosis were found in CFTR-expressing vs. CF epithelial cell lines (20–22); however, in other studies, stimulation of cAMP increased exocytosis in both CFTR-expressing and CF pancreatic cells (17) or had no effect on the rate of exocytosis (23). Prince *et al.* (21) identified CFTR in a rapidly recycling endocytic compartment in T84 cells and found that ≈50% of cell-surface CFTR was internalized within minutes, a rate much faster than that for other apical membrane glycoproteins. Subsequent studies indicated that CFTR is present (24, 25) and functional (15, 16) in endosomes of CFTR-expressing cells. Although several of the cited studies report apparently conflicting results, they together provide provocative evidence for a role of CFTR in vesicular trafficking.

The purpose of this study was to investigate whether CFTR expression and activation affects a key cellular process associated with vesicular trafficking–endosome fusion. Endosome–endosome fusion was compared in stably transfected 3T3 fibroblasts (vector control vs. CFTR expressing). This system was chosen because a large body of information was already available on endocytosis, including data that CFTR is expressed and functional at the plasma membrane and in endosomes (16). In addition, these transfected cells permitted direct comparison of cells not expressing CFTR with those expressing relatively high levels of CFTR. Endosome fusion was measured *in vivo* using a quantitative ratio imaging assay based on an ≈8-fold increase in 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene (Bodipy)-avidin fluorescence upon binding of biotinylated substrates (26). The fluorescence increase is very fast, irreversible, and pH independent. Endosomes were labeled by internalization in intact cells with Bodipy-avidin and a reference indicator (rhodamine B), chased for specified times with marker-free medium, and then labeled with biotin-albumin or transferrin. Fusion between Bodipy-avidin- and biotin-labeled endosomes was quantified

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Abbreviations: CF, cystic fibrosis; CFTR, CF transmembrane conductance regulator; Bodipy, 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene; TMR, tetramethylrhodamine; Tf, transferrin; HRP, horseradish peroxidase; Cl_2Cf, 5(and 6)-carboxy-2′,6′-dichlorocarboxyfluorescein; B/R, Bodipy-to-rhodamine B.

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by ratio-imaging fluorescence microscopy. A significant increase in endosome fusion efficiency was observed only in CFTR-expressing cells after cAMP stimulation. The increase in endosome fusion was not related to rates of endocytic uptake or to endosomal acidification, but required chloride. The results provide evidence for a novel intracellular role for CFTR in endosome–endosome fusion and thus a possible basis for the cellular defects associated with CF.

MATERIALS AND METHODS

Reagents. Bodipy-avidin, biotinamido caproate-*N*-hydroxy succinimidyl ester, rhodamine B-dextran (M_r, 40,000), 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-propionic acid succinimidylester (Bodipy FL C_3-SE) and tetramethylrhodamine-transferrin (TMR-Tf) were purchased from Molecular Probes. Neutravidin was obtained from Pierce. Defatted bovine serum albumin (BSA), horseradish peroxidase (HRP, type II), human transferrin, biotinylated human transferrin, forskolin, and 1,9-dideoxyforskolin were purchased from Sigma. 3-(6-Methoxyquinolino)propanesulfonate (SPQ), biotinylated BSA, and 5(and 6)-carboxy-2',6'-dichlorofluorescein–5(and 6)-carboxytetramethylrhodamine-dextran (M_r, 40,000; Cl_2Cf-TMR-dextran) were synthesized as described (26, 27). Bodipy-neutravidin was prepared by reaction of 7 mg of neutravidin with 230 μg of Bodipy FL C_3-SE in 4 ml of bicarbonate buffer (100 mM) for 1 h. The product was dialyzed against water and lyophilized.

Cell Culture. Control (vector transfected) and stably transfected Swiss 3T3 fibroblasts expressing CFTR were provided by Michael Welsh (University of Iowa). Phenotype analysis was performed by the SPQ assay (16). Only CFTR-expressing cells showed forskolin-stimulated plasma membrane Cl^- conductance. Cells were cultured at 37°C in 95% air/5% CO_2 in DME-21 medium supplemented with 10% heat inactivated fetal bovine serum (20 min at 56°C) and 1% penicillin–streptomycin. Cells were grown on autoclaved 18-mm diameter round glass coverslips and used when nearly confluent at 12–16 h after plating. For assay of HRP and TMR-Tf uptake, cells were grown in polystyrene 12-well tissue culture plates and used when nearly confluent.

Endosome Fusion Assay. Coverslips were washed twice in internalization medium (IM; 40 mM Na_2CO_3/70 mM NaCl/5 mM KCl/10 mM Hepes/10 mM D-glucose, pH 7.4), incubated with IM containing Bodipy-avidin (1 mg/ml) plus rhodamine B-dextran (1 mg/ml) at 37°C for 10 min, and then washed twice in IM. After a specified chase time in nonfluorescent serum-free, phenol red-free DME H21 at 37°C, cells were labeled with biotin-transferrin (180 μg/ml) or biotin-BSA (16 mg/ml) for 10 min at 37°C. After labeling, cells were washed briefly and perfused with IM at 15°C in a 200-μl perfusion chamber (16). Some experiments were performed in Cl^--free buffer (135 mM sodium isethionate/3 mM potassium isethionate/1 mM magnesium acetate/1 mM calcium acetate/1.5 mM KH_2PO_4/8 mM Na_2HPO_4/5 mM glucose) after 1 h incubation in Cl^--free buffer using Bodipy-neutravidin (1 mg/ml) instead of Bodipy-avidin. In some experiments forskolin (50 μM) was present throughout the labeling period.

Fluorescence Microscopy. The perfusion chamber was positioned on the stage of a noninverted epifluorescence microscope. Cells were illuminated by a stabilized Hg–Xe arc lamp using standard fluorescein isothiocyanate (for Bodipy) and rhodamine (for rhodamine B) filter sets. Fluorescence was collected by a 60× oil immersion objective (Nikon Plan Apo, numerical aperture 1.4) and imaged by a 14-bit, 512 × 512 pixel, cooled CCD camera (AT200 series, Photometrics, Tucson, AZ) containing a high-sensitivity back-thinned detector (TK512CB, Tektronix). In a typical experiment, an image of endosomes was focused manually using the rhodamine filter set. A rhodamine image was acquired over 1 s, the rhodamine filter set was exchanged for the fluorescein isothiocyanate filter set (≈2 s), and a Bodipy image was acquired over 2 s. Less than 2% photobleaching occurred under these conditions.

Image Analysis. Quantitative ratio imaging of Bodipy-to-rhodamine fluorescence in individual endosomes was carried out using customized image processing software (27). Well-demarcated, fluorescently labeled endosomes were identified in the rhodamine image; endosome area, generally 12–30 pixels, was defined based on an algorithm using maximum pixel intensity at the centroid of the endosome and background pixel intensity. Area-integrated pixel intensities of individual endosomes were then computed using as local background the median intensity in a single pixel layer surrounding each endosome. The same set of endosome areas (centers repositioned, if necessary) were used to compute area-integrated pixel intensities in the Bodipy image. After area-integration and background subtraction, Bodipy-to-rhodamine B intensity ratios (B/R) were computed for each endosome. Data were displayed as histograms of the number of endosomes vs. B/R ratio histograms. The fraction of avidin-labeled endosomes that fused with biotin-labeled endosomes was determined by linear regression of B/R histograms to a combination of distributions measured for unfused and 100% fused endosomes (26).

Endosome pH Measurements. The pH of individual fluorescently labeled endosomes was measured by quantitative ratio imaging as described above (27). Endosomes were pulse-labeled by a 10-min incubation with 5 mg/ml Cl_2Cf-TMR-dextran in IM at 37°C, and then maintained at 37°C for 15 min. Image pairs were acquired using fluorescein and rhodamine filter sets. At the end of each experiment, cells were perfused with high K^+ buffers containing 5 μM of nigericin titrated to pH 4 and pH 7.5. The Cl_2Cf/TMR fluorescence ratio was computed in individual endosomes as described above, and endosome pH was determined from the Cl_2Cf/TMR ratio using an *in vivo* calibration curve (27). Results were expressed as average endosome pH or histograms of percentage of labeled endosomes in 0.25 pH unit intervals.

HRP and TMR-Tf Uptake. For HRP uptake, cells were washed 3 times with PBS containing 10 mM D(+)-mannose and 2 mg/ml BSA and incubated for 15 min at 37°C with 50 μg/ml HRP in DME H21. In some experiments, 50 μM of forskolin was present for 10 min before and during the HRP incubation. In control studies, cells were incubated with HRP at 4°C. After uptake, the cells were washed 5 times with ice-cold PBS containing 10 mM D(+)-mannose and 2 mg/ml BSA and then 5 times with ice-cold PBS. Cells were homogenized with a Dounce-type homogenizer and centrifuged (1500 × *g* for 5 min) to pellet nuclei and unbroken cells. Triton X-100 (final concentration 0.2%) was added to one-half of the samples to release the endocytosed HRP for determination of internal (latent) HRP activity. Protein concentration was determined by the Peterson assay (28) and HRP content was measured using the substrate *o*-dianisidine. For TMR-Tf uptake, cells were incubated for 5 min at 37°C with TMR-Tf (180 μg/ml) in PBS in the presence or absence of excess unlabeled Tf (18 mg/ml). Cells were then washed 5 times with ice-cold BSA containing 1 mg/ml BSA and 5 times with PBS. Cells were homogenized and Triton X-100 was added to a final concentration of 0.2% for measurement of TMR fluorescence by cuvette fluorimetry.

RESULTS

To test whether endosome fusion could be detected in the transfected 3T3 fibroblasts *in vivo*, the fluorescence of internalized Bodipy-avidin was quantified in individual endosomes. Cells were labeled by internalization with Bodipy-avidin and rhodamine B-dextran for 10 min at 37°C in the absence or presence of biotin. The Bodipy-to-rhodamine fluorescence

signal ratio (B/R) was computed from background-subtracted, area-integrated pixel intensities of individual endosomes as described in *Materials and Methods*. Fig. 1*A* shows the data as a histogram of the percentage of endosomes vs. B/R ratio; geometrically increasing B/R ratio intervals (factor = 1.5) were chosen to visualize the distributions. There was a clear separation of B/R values for endosomes labeled in the absence (hatched bars, equivalent to "unfused" endosomes) and presence (stippled bars, equivalent to "fused" endosomes) of biotin. It was thus possible to distinguish between fused and unfused endosomes with a specificity of >90%. Expression of CFTR and cAMP stimulation by forskolin had no effect on B/R ratios measured with zero and maximum biotin (data not shown).

The effect of CFTR expression and activation on endosome fusion in control (vector transfected) and CFTR-expressing 3T3 fibroblasts was investigated. Cells were labeled by internalization with Bodipy-avidin for 10 min at 37°C, chased in marker free medium for 0 or 10 min, and then labeled with biotin-albumin for 10 min. In some experiments, forskolin was present during the incubations to activate CFTR Cl^- channels. The histograms of percentage of endosomes vs. B/R ratio in Fig. 1 *B* and *C* are wider than that in Fig. 1*A*, indicating that both fused and unfused endosomes were present. As expected, a greater fraction of endosomes had high B/R ratios (indicating more endosome fusion) for the 0 min (Fig. 1*B*) vs. 10 min chase time (Fig. 1*C*). There was no significant difference in endosome fusion in unstimulated control (solid bars) and CFTR-expressing (open bars) fibroblasts as shown by the near identical histograms (Fig. 1 *B Left* and *C Left*) [f_{fusion} = 0.54 and 0.58 (no chase), 0.26 and 0.25 (10 min chase)]; (see Table 1). However, in forskolin-stimulated cells, there was a significant increase in endosome fusion in the CFTR-expressing cells [f_{fusion} = 0.69 (no chase) and 0.53 (10 min chase)], which is seen by the right shift of the histogram to higher B/R ratios (Fig. 1 *B Right* and *C Right*). The increase in fusion was very pronounced in cells chased for 10 min between the avidin and biotin-albumin pulses (Fig. 1*C Right*).

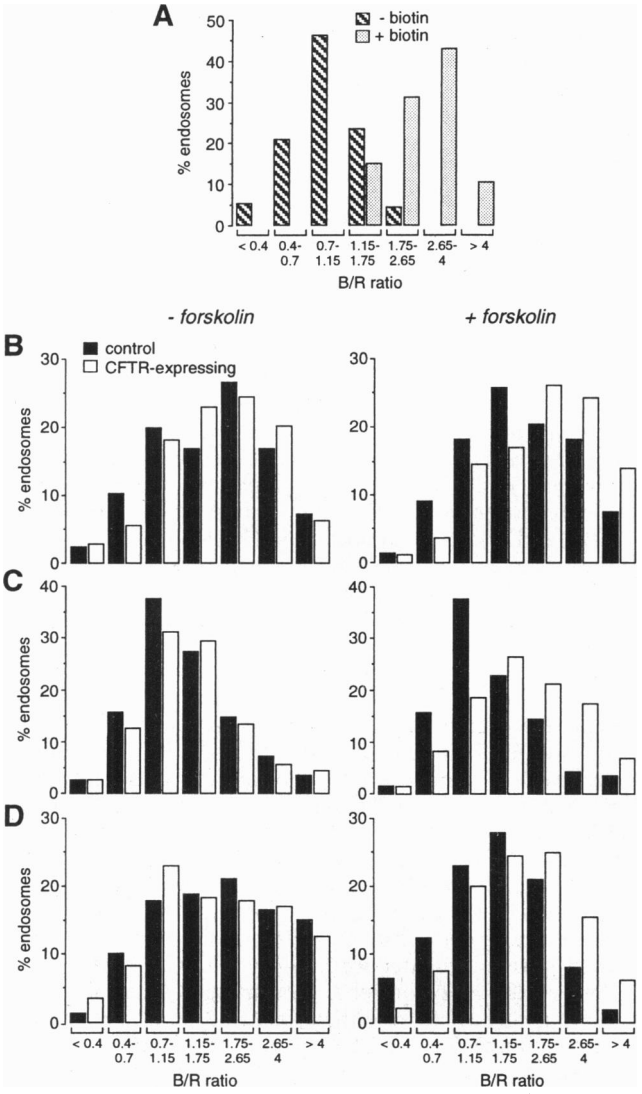

FIG. 1. Histogram distribution of the percentage of endosomes vs. B/R fluorescence signal ratio. (*A*) Endosomes were labeled with Bodipy-avidin without (hatched bars) and with (stippled bars) prebinding of biotin (1.5 mM). (*B–D*) Histograms of B/R ratios for Bodipy-avidin-labeled endosomes in control (solid bars) and CFTR-expressing (open bars) 3T3 fibroblasts without (*Left*) and with (*Right*) forskolin stimulation (50 μM). Endosomes were pulse-labeled for 10 min with Bodipy-avidin (1 mg/ml) at 37°C, chased for 0 min (*B*) or 10 min (*C*) with marker-free medium, and then labeled with biotin-albumin (16 mg/ml). (*D*) The experiment was done as in *B* except biotin-albumin was replaced by biotin-transferrin (180 μg/ml).

To test whether the forskolin effect was due to a stimulation of intracellular cAMP, a forskolin analog, which does not stimulate cAMP (1,9-dideoxyforskolin; ref. 29) was tested. There was no significant difference in endosome fusion in CFTR-expressing cells without vs. with the addition of dideoxyforskolin (Table 1).

To determine whether CFTR expression and activation also increased endosome fusion in the receptor-mediated pathway, experiments were carried out in which the fluid phase marker biotin-albumin was replaced by biotin-transferrin, a marker of the early/recycling endosomal compartment. There was no significant difference in endosome fusion in unstimulated cells (Fig. 1*D Left*). Forskolin addition caused inhibition of endosome fusion in both control and CFTR-expressing cells, but resulted in a net increase in f_{fusion} in CFTR-expressing cells (Fig. 1*D Right*) [f_{fusion} = 0.37 (control) vs. 0.49 (CFTR-expressing cells)].

There are several possible explanations for the increase in endosome fusion observed only in CFTR-expressing cells after cAMP stimulation. Activation of CFTR could affect the rate of endocytosis, the kinetics of endosome acidification, and/or cellular ion concentrations or membrane potentials. Alternatively, the activated CFTR protein might itself act as a fusogenic factor.

The rates of fluid-phase and receptor-mediated endocytosis were measured in control and CFTR-expressing cells without and with forskolin stimulation. For fluid-phase endocytosis, endosomes were labeled with HRP for 15 min at 37°C and latent (intraendosomal) HRP activity was measured. For receptor-mediated endocytosis, TMR-transferrin uptake was measured from the difference in fluorescence in endosomes formed in the absence vs. the presence of excess unlabeled transferrin. There was no significant uptake of HRP or TMR-Tf when the incubation was performed at 4°C. Fig. 2 shows that expression and stimulation of CFTR had no significant effect on the endocytic uptake of HRP or TMR-Tf. These results indicate that the rate of endocytosis cannot account for the increase in endosome fusion.

Maturation of endosomes was assessed from the appearance of fluorescently labeled endosomes at various incubation times and by measurement of endosomal acidification. Fig. 3 shows representative photomicrographs of endosomes from unstimulated and forskolin-stimulated CFTR-expressing fibroblasts at 10 and 30 min after labeling with rhodamine B-dextran. There was no apparent difference in endosome appearance with forskolin, nor was there a difference in endosomes from control vs. CFTR-expressing cells (not shown). Endosome pH was measured by pulse-labeling with the pH-sensitive fluorophore Cl_2Cf-TMR-dextran for 10 min, followed by incubation

Table 1. Summary of endosome fusion experiments

Cell type	Condition	Chase time, min	f_{fusion}	*n*
	Internalization of Bodipy-avidin and biotin-albumin			
Control	−forskolin	0	0.54 ± 0.04	166
	+forskolin	0	0.53 ± 0.05	133
CFTR	−forskolin	0	0.58 ± 0.04	144
	+forskolin	0	0.69 ± 0.06*	166
Control	−forskolin	10	0.26 ± 0.03	281
	+forskolin	10	0.20 ± 0.03	255
CFTR	−forskolin	10	0.25 ± 0.03	376
	+forskolin	10	0.53 ± 0.03*	437
CFTR	−dideoxyforskolin	10	0.29 ± 0.02	547
	+dideoxyforskolin	10	0.31 ± 0.03	668
	Internalization of Bodipy-avidin and biotin-Tf			
Control	−forskolin	0	0.54 ± 0.05	220
	+forskolin	0	0.37 ± 0.02	389
CFTR	−forskolin	0	0.52 ± 0.05	231
	+forskolin	0	0.49 ± 0.03**	242
	Internalization of Bodipy-neutravidin and biotin-albumin			
CFTR	−forskolin	10	0.28 ± 0.04	164
	+forskolin	10	0.49 ± 0.06***	134
CFTR-	−forskolin	10	0.37 ± 0.03	183
(Cl^- depleted)	+forskolin	10	0.34 ± 0.03	193

The value of f_{fusion} was computed from the B/R distribution by linear regression analysis of data from *n* endosomes. Experiments were performed with specified chase times at 37°C. Where indicated, forskolin (50 μM) or dideoxyforskolin (50 μM) was present throughout the experiment.
*, $P < 0.01$ compared to control cells and unstimulated CFTR-expressing cells (unpaired *t* test).
**, $P < 0.01$ compared to forskolin stimulated control cells (unpaired *t* test).
***, $P < 0.01$ compared to unstimulated CFTR-expressing cells (unpaired *t* test).

at 37°C and ratio imaging of Cl_2Cf (pH sensitive) to TMR (pH insensitive) fluorescence in individual endosomes. Fig. 4*A* shows no significant difference in the averaged endosome pH for control vs. CFTR-expressing cells (without and with forskolin simulation) at 10, 25, and 30 min. To test whether a subpopulation of endosomes with altered pH might be responsible for the increase in endosome fusion, the endosome pH distribution was determined. Fig. 4*B* shows the pH distribution at 25 min after labeling as the percentage of endosomes in 0.2 pH unit intervals. There was a unimodal pH distribution for control and CFTR-expressing cells (without vs. with forskolin) without evidence for subpopulations of endosomes; similar unimodal distributions without apparent differences were obtained for 10- and 30-min incubation times (not shown). These results indicate that the increases in endosome fusion are not due to an effect of CFTR activation on endosome maturation.

To test whether the Cl^--transporting function of CFTR is responsible for the increase in the rate of endosome fusion, experiments were carried out in cells depleted of Cl^- by incuba-

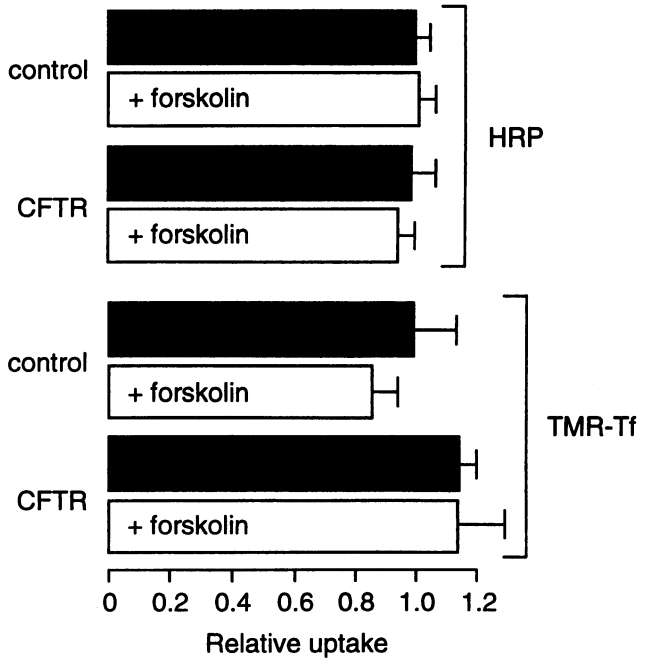

FIG. 2. Effect of CFTR expression and activation on HRP and TMR-Tf uptake. Endosomes were labeled by incubation of cells with HRP (50 μg/ml) for 15 min or TMR-Tf (180 μg/ml) for 5 min at 37°C in the absence (solid bars) or presence (open bars) of forskolin (50 μM). Data are the mean ± SEM for 4–6 experiments.

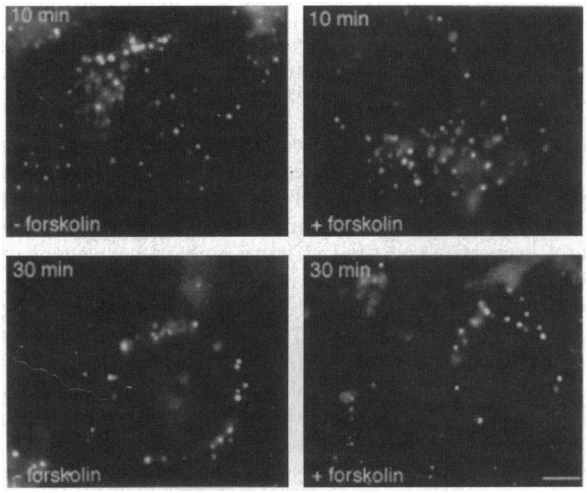

FIG. 3. Effect of forskolin on endosome morphology. Photomicrographs of rhodamine B-dextran-labeled endosomes of CFTR-expressing 3T3 fibroblasts without (*Left*) and with (*Right*) stimulation by forskolin (50 μM). Fibroblasts were labeled for 10 min with rhodamine B-dextran (1 mg/ml), and then incubated for 0 (*Upper*) or 20 (*Lower*) min at 37°C. Illumination, imaging, and display conditions were identical for all micrographs. (Bar = 5 μm.)

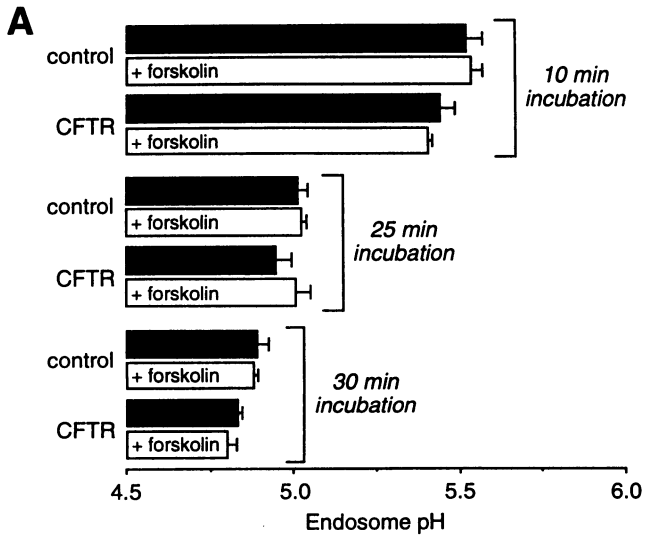

FIG. 4. Effect of forskolin on endosome pH in transfected fibroblasts. (*A*) Averaged endosome pH in vector-transfected and CFTR-expressing fibroblasts at 10, 25, and 30 min after pulse-labeling with 5 mg/ml Cl_2Cf-TMR-dextran. Data for 10 and 30 min were taken from ref. 18. Values are the mean ± SE of the averaged data obtained from three separate sets of experiments. The total number of endosomes analyzed without and with forskolin stimulation was 303 and 337 (control) and 812 and 511 (CFTR expressing). (*B*) Distribution of endosome pH values at 25 min after pulse-labeling as the percentage of endosomes in 0.2 pH unit intervals.

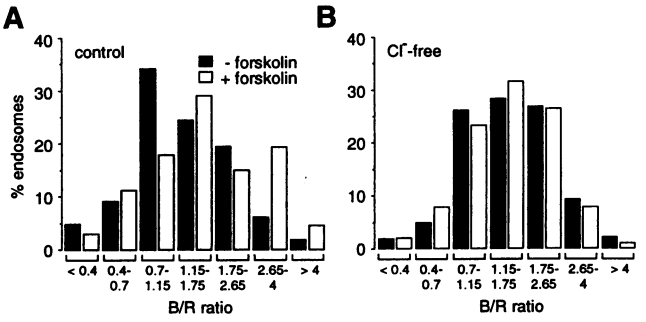

FIG. 5. Effect of Cl^- depletion on endosome fusion. Histogram distribution of the percentage of endosomes vs. B/R fluorescence signal ratio without (solid bars) and with (open bars) forskolin stimulation (50 μM). (*A*) Endosomes were labeled with Bodipy-neutravidin (1 mg/ml) for 10 min at 37°C, chased for 10 min in marker-free medium, and then labeled with biotin-albumin (16 mg/ml) for 10 min in Cl^--containing buffer. (*B*) Experiments were done as in *A* except that labeling was performed in Cl^--free buffer after 1-h incubation in Cl^--free buffer.

tion in isethionate buffer. Because of nonspecific binding of Bodipy-avidin to cells in Cl^--free buffer, Bodipy was conjugated to neutravidin, a deglycosylated avidin having very low nonspecific binding. Bodipy-neutravidin and Bodipy-avidin had the same optical properties and similar fluorescence enhancement upon biotin binding. In control experiments in Cl^--containing buffer, a similar increase in the rate of endosome fusion upon forskolin stimulation was observed in CFTR-expressing cells using Bodipy-neutravidin or Bodipy-avidin (Fig. 5*A*, Table 1). Endocytosis in Cl^--depleted cells was comparable to that in the Cl^--containing buffer. However, there was no significant effect of forskolin stimulation on the rate of endosome fusion (Fig. 5*B*, Table 1), suggesting that the increased endosome fusion in forskolin-stimulated CFTR-expressing cells is related to the Cl^--transporting function of CFTR.

DISCUSSION

Previous reports that CFTR activation is associated with decreased endocytosis and increased exocytosis have suggested that CFTR is a regulator of vesicular trafficking (19–22). Our study used a quantitative fluorescence approach (26) to test the hypothesis that endosome fusion is regulated by CFTR expression and activation. A transfected fibroblast system was chosen for the reasons mentioned in the Introduction, and because the well-demarcated endosomes in fibroblasts permitted fluorescence ratio imaging of individual endosomes without the technical difficulties encountered in several epithelial cell systems tested (T84, Calu-3, CFPAC; data not shown). In addition, the decrease in the rate of endocytosis seen in epithelial cells upon cAMP stimulation (19–22) would confound the quantitative comparison of endosome fusion data. A concern in any transfected cell model is that the findings on cell phenotype may not apply to native cells. In the case of CFTR, transfected cell models have reproduced the native phenotype very well for studies of CFTR plasma membrane function (4), CFTR expression and function in endosomes (15–17, 25), and CFTR processing and degradation (30, 31). We feel that although further studies of regulation of endosome fusion by CFTR are needed in native epithelia, the novel observation that CFTR activation can increase endosome fusion is independent of cell type.

The principal observation was that CFTR expression and activation was associated with a significant increase in endosome–endosome fusion. Increased fusion was found with either a fluid-phase marker or a receptor-bound ligand (transferrin), which was localized to early/recycling endosomes. The stimulation of fusion was not caused by increased net endocytic uptake or by altered endosomal acidification, but was abolished when cellular chloride was replaced by the relatively impermeant anion isethionate. Therefore, the stimulation of endosome fusion is probably related to the Cl^--transporting function of CFTR. Interestingly, Prince *et al.* (21) have reported that CFTR Cl^--channel function was also responsible for the observed decrease in the rate of endocytosis upon cAMP stimulation; replacement of chloride by gluconate abolished the cAMP effect. The mechanism of the chloride effect is not defined by these studies, but may involve plasma membrane CFTR activation resulting in increased cytosolic $[Cl^-]$ and increased cytosolic $[K^+]$, and/or changes in endosome membrane potential and ionic content. For example, it has been shown that $[Cl^-]$ is important for the activation of G-proteins (32), which may be involved in endosome fusion. A new fluorescence cell-free assay of endosome fusion (33) should permit direct examination of the influence of these factors on endosome fusion, as well as testing whether CFTR has intrinsic fusogenic activity. Regarding the latter possibility, we note that the isolated first nucleotide binding domain of

CFTR promotes liposome fusion *in vitro* (34), and that the two nucleotide binding domains have considerable homology with G-proteins, including the ADP ribosylating factor family (35, 36).

Although the results here suggest that CFTR is involved in endosome fusion, they do not address the mechanism(s) by which altered endosome fusion in CF results in various cellular abnormalities. Abnormal vesicle fusion might affect several important cellular processes, such as the steady-state distribution of intravesiclar enzymes and the processing of internalized and secreted proteins. The altered distribution of two sialyltransferences reported in CF epithelial cells (37) might account for the decreased sialylation of secreted proteins in CF (38). Sniders and Rogers (39) and Duncan and Kornfeld (40) reported that internalized proteins are exposed to a sialyltransferase in the trans-Golgi network ≈1 out of 10 internalization cycles. Defective vesicle fusion in the secretory pathway of CF cells might thus alter post-translational modification of proteins by changing the time and environment for exposure of proteins to Golgi enzymes. The mechanisms suggested by our results will require direct experimental verification.

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1. Riordan, J. R., Rommens, J. M., Kerem, B., Alon, N., Rozmahel, R., Grzelczak, Z., Zielenski, J., Lok, S., Plavsic, N., Chou, J. L., Drum, M. L., Januzzi, M. C., Collins, F. S. & Tsui, L.-C. (1989) *Science* **245,** 1066–1073.
2. Rich, D. P., Anderson, M. P., Gregory, R. J., Cheng, S. H., Paul, S., Jefferson, D. M., McCann, J. D., Klinger, K. W., Smith, A. E. & Welsh, M. J. (1990) *Nature (London)* **347,** 358–363.
3. Anderson, M. P., Gregory, R. J., Thompson, S., Souza, D. W., Paul, S., Mulligan, R. C., Smith, A. E. & Welsh, M. J. (1991) *Science* **253,** 202–205.
4. Anderson, M. P., Rich, D. P., Gregory, R. J., Smith, A. E. & Welsh, M. J. (1991) *Science* **251,** 679–682.
5. Bear, C. E., Li, C. H., Kartner, N., Bridges, R. J., Jensen, T. J., Ramjeesingh, M. & Riordan, J. R. (1992) *Cell* **68,** 809–818.
6. Wine, J. J. (1995) *Curr. Biol.* **5,** 1357–1359.
7. Biwersi, J. & Verkman, A. S. (1994) in *Current Topics in Cystic Fibrosis*, eds. Dodge, J. A., Brock, D. J. H. & Widdicombe, J. H. (Wiley, Chichester, U.K.), pp. 151–171.
8. Reisin, I. L., Prat, A. G., Abraham, E. H., Amara, J. F., Gregory, R. J., Ausiello, D. A. & Cantiello, H. F. (1994) *J. Biol. Chem.* **269,** 20584–20591.
9. Hasegawa, H., Skach, W., Baker, O., Calayag, M. C., Lingappa, V. & Verkman, A. S. (1992) *Science* **258,** 1477–1479.
10. Poulsen, J. H., Fischer, H., Illek, B. & Machen, T. E. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 5340–5344.
11. Stutts, M. J., Canessa, C. M., Olsen, J. C., Hamrick, M., Cohn, J. A., Rossier, B. C. & Boucher, R. C. (1995) *Science* **269,** 847–850.
12. Schwiebert, E. M., Egan, M. E., Hwang, T. H., Fulmer, S. B., Allen, S. S., Cutting, G. R. & Guggino, W. B. (1995) *Cell* **81,** 1063–1073.
13. Pier, G. B., Grout, M., Zaidi, T. S., Olsen, J. C., Johnson, L. G., Yankaskas, J. R. & Goldberg, J. B. (1996) *Science* **271,** 64–67.
14. Barasch, J., Kiss, B., Prince, A., Saiman, L., Gruenert, D. & Al-Awqati, Q. (1991) *Nature (London)* **352,** 70–73.
15. Lukacs, G. L., Chang, X. B., Kartner, N., Rotstein, O. D., Riordan, J. R. & Grinstein, S. (1992) *J. Biol. Chem.* **267,** 14568–14572.
16. Biwersi, J. & Verkman, A. S. (1994) *Am. J. Physiol.* **266,** C149–C156.
17. Dunn, K. W., Park, J., Semrad, C. E., Gelman, D. L., Shevell, T. & McGraw, T. E. (1994) *J. Biol. Chem.* **269,** 5336–5345.
18. Seksek, O., Biwersi, J. & Verkman, A. S. (1996) *J. Biol. Chem.* **271,** 15542–15548.
19. Bradbury, N. A., Jilling, T., Berta, G., Sorscher, E. J., Bridges, R. J. & Kirk, K. L. (1992) *Science* **256,** 530–532.
20. Santos, G. F. & Reenstra, W. W. (1994) *Biochim. Biophys. Acta* **1195,** 96–102.
21. Prince, L. S., Workman, R. B., Jr., & Marchase, R. B. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 5192–5196.
22. Schwiebert, E. M., Gesek, F., Ercolani, L., Wjasow, C., Gruenert, D. C., Karlson, K. & Stanton, B. A. (1994) *Am. J. Physiol.* **267,** C272–C281.
23. Dho, S., Grinstein, S. & Foskett, J. K. (1993) *Biochim. Biophys. Acta* **1225,** 78–82.
24. Webster, P., Vanacore, L., Nairn, A. C. & Marino, C. R. (1994) *Am. J. Physiol.* **267,** C340–C348.
25. Bradbury, N. A., Cohn, J. A., Venglarik, C. J. & Bridges, R. J. (1994) *J. Biol. Chem.* **269,** 8296–8302.
26. Emans, N., Biwersi, J. & Verkman, A. S. (1995) *Biophys. J.* **69,** 716–728.
27. Zen, K., Biwersi, J., Periasamy, N. & Verkman, A. S. (1992) *J. Cell Biol.* **119,** 99–110.
28. Peterson, G. L. (1977) *Anal. Biochem.* **83,** 346–356.
29. Seamon, K. B., Daly, J. W., Metzger, H., de Souza, N. J. & Reden, J. (1983) *J. Med. Chem.* **26,** 486–489.
30. Ward, C. L. & Kopito, R. R. (1994) *J. Biol. Chem.* **269,** 25710–25718.
31. Lukacs, G. L., Mohamed, A., Kartner, N., Chang, X. B., Riordan, J. R. & Grinstein, S. (1994) *EMBO J.* **13,** 6076–6086.
32. Nakajima, T., Sugimoto, T. & Kurachi, Y. (1992) *J. Gen. Physiol.* **99,** 665–682.
33. Emans, N. & Verkman, A. S. (1996) *Biophys. J.* **71,** 487–494.
34. Lee, G., Huang, Z., Wang, Y., Sorscher, E., Jacobson K. A., BarNoy, S. & Pollard, H. B. (1995) *Pediatr. Pulmonol.*, Suppl. 12, 185.
35. Manavalan, P., Dearborn, D. G., McPherson, J. M. & Smith, A. E. (1995) *FEBS Lett.* **366,** 87–91.
36. Carson, M. R. & Welsh, M. J. (1995) *Biophys. J.* **69,** 2443–2448.
37. Weyer, P., Barasch, J., Al Awqati, Q., Ausiello, D. A. & Brown, D. (1995) *Pediatr. Pulmonol.*, Suppl. 12, 238.
38. Cheng, P. W., Boucher, R. C., Yankaskas, J. M. & Boat, T. F. (1988) in *Cellular and Molecular Basis of Cystic Fibrosis*, eds. Mastella, G. & Quinton, P. M. (San Francisco Press, San Francisco), pp. 233–238.
39. Snider, M. D. & Rogers, O. C. (1985) *J. Cell Biol.* **100,** 826–834.
40. Duncan, J. R. & Kornfeld, S. (1989) *J. Cell Biol.* **106,** 617–628.