Cholesterol Depletion and Genistein as Tools to Promote F508delCFTR Retention at the Plasma Membrane

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Key Words

CFTR • Chloride channel • Cholesterol • Cystic Fibrosis • Endocytosis • Genistein • Niemann Pick type C

Abstract

Background/aims: F508delCFTR-, but not wtCFTR-, expressing fibroblasts resemble Niemann Pick type C cells in the massive intracellular accumulation of free cholesterol. The recruitment and activation of F508delCFTR by cholesterol depletion was studied. **Methods:** Filipin staining, forskolin-stimulated anion efflux and FITC-dextran uptake were studied in control cells and fibroblasts treated with 2-hydroxypropyl β -cyclodextrin phosphatidylcholine large unilamellar vesicles to deplete cellular free cholesterol. **Results:** Treatment of F508delCFTR-, but not wtCFTR-, expressing fibroblasts with 2-hydroxypropyl β -cyclodextrin resulted in a reduction in cellular cholesterol and a potentiation of the forskolin-induced anion efflux. In addition, forskolin also promoted a massive increase in the rate of endocytosis in F508delCFTR fibroblasts, which was absent in genistein- or cyclodextrin-treated cultures. **Conclusion:**

The results not only suggest that reducing cellular cholesterol may serve as pharmacotherapeutic tool in the treatment of cystic fibrosis but also reveal a novel mechanism for genistein regulation of F508delCFTR, i.e. retention by inhibition of endocytosis.

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Introduction

Cystic fibrosis (CF), the most common fatal hereditary disease among Caucasians, is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) [1]. CFTR is a cAMP/protein kinase A- and cGMP/protein kinase G-II-regulated Cl⁻ channel [2, 3] crucially involved in transepithelial salt and water transport. In the Western population more than 70% of the mutations in CFTR appear to be a deletion of a phenylalanine residue at amino-acid position 508 (F508delCFTR) [1, 4], resulting in an incompletely processed protein, defective in its ability to traffic to the plasma membrane [5, 6]. Although a small fraction of F508delCFTR protein may reach the plasma

membrane and retain its function as cAMP-activated chloride channel [7-9], the reduced number of channels at the cell surface leads to dramatically impaired transepithelial transport of chloride [8]. In addition, although glycosylation of CFTR is not required for CFTR function [10], F508delCFTR exhibits a significant decrease in the open probability [11, 12] and a strongly reduced cell surface half-life as compared to wild type [8, 13, 14], which also contribute to the diminished Cl⁻ conductance and, consequently, water flux.

In a previous study, we investigated the role of cholesterol-containing micro-domains of the plasma membrane in the activation of Volume-Regulated Anion Channels (VRACs). Intestine 407 cells were treated with 2-hydroxypropyl β -cyclodextrin in the presence of Phosphatidylcholine Large Unilamellar Vesicles (CD/PC-LUVs). CD/PC-LUVs depletes plasma membrane cholesterol. Consequently, the endosomal recycling rate was accelerated to replenish plasma membrane cholesterol. As a result, the endocytosis was inhibited [15-17]. When Intestine 407 cells were depleted of cholesterol, the cell swelling-provoked anion conductance was increased approximately 2 fold [18]. From these studies, we proposed a model of VRAC regulation involving recruitment of channels from cholesterol containing intracellular compartments [18]. This model is supported by our observation that in Niemann Pick type C (NPC) fibroblasts lacking the functional NPC1 protein involved in cellular cholesterol transport, as well as in cells treated with an inhibitor of intracellular cholesterol transport, the cell swelling-induced anion efflux was strongly impaired [18]. In addition, experiments with Niemann Pick type C skin fibroblasts revealed that the volume-sensitive anion efflux could be restored by CD/LUVs-induced cholesterol depletion. (C.H.Lim and B.C.Tilly, unpublished results).

Recently, White et al. showed that F508delCFTR-expressing epithelial cells develop an NPC-like phenotype [19, 20]. To investigate whether recruitment and activation of F508delCFTR could be (partially) restored by cholesterol depletion, analogous to the model proposed for VRAC regulation in Intestine 407 cells [18], the forskolin-stimulated anion efflux was studied in wtCFTR- and F508delCFTR-expressing murine 3T3 fibroblasts. The results clearly demonstrate that a reduction of cellular cholesterol promotes an increase in the forskolin-stimulated anion efflux from F508delCFTR-containing fibroblasts whereas the efflux from wtCFTR-expressing cells was not affected. In addition, the results suggest inhibition of endocytosis as a novel mechanism by which genistein is able to enhance CFTR activity in intact cells.

Material and Methods

Materials

Radioisotope was obtained from Amersham Netherlands B.V. ('s Hertogenbosch, the Netherlands). FITC-conjugated dextran (MW=10,000 Da) and FM1-43 were from Invitrogen Molecular Probes (Leiden, the Netherlands). Other chemicals were purchased from Sigma (St. Louis, MO, USA).

Cell culture

Swiss 3T3 fibroblasts stably expressing human wtCFTR or F508delCFTR were a generous gift of Dr. Michael J. Welsh [21]. The cells were grown routinely as monolayers in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum, 40 mg/l penicillin and 90 mg/l streptomycin under a humidified atmosphere of 95% O₂ and 5% CO₂ at 37°C. All experiments were carried out at 37°C.

Filipin staining

Cultures of cells were fixated using 3% paraformaldehyde in phosphate-buffered saline (PBS) for 60 min. Thereafter, the cells were washed three times (phosphate-buffered saline), treated with 50 mM glycine in PBS (30 min) to quench paraformaldehyde and stained with filipin (40 μ g/ml, 30 min). Epifluorescence was examined using an inverted Olympus IX50 microscope and images were acquired and analysed using AnalySiS imaging software (Soft Imaging Systems, Münster, Germany).

FM1.43 staining

Cultures of cells were fixated using 3% paraformaldehyde in phosphate-buffered saline (PBS) for 60 min. Thereafter, the cells were washed three times (phosphate-buffered saline), treated with 50 mM glycine in PBS (30 min) to quench paraformaldehyde and stained with FM1.43 (1 μ M, 30 min). Epifluorescence was examined using an inverted Olympus IX50 microscope and images were acquired and analysed using AnalySiS imaging software (Soft Imaging Systems, Münster, Germany).

Determination of cellular total free cholesterol

Cells were sonicated in PBS and cholesterol in the cell lysate was determined enzymatically using a commercially available kit (Wako Chemicals GmbH, Neuss, Germany) according to the manufacturer's instructions.

Efflux assay

Monolayers of cells were loaded for 2 h with 5 μ Ci ¹²⁵I- in the absence or presence of 2% 2-hydroxypropyl- β -cyclodextrin (CD) and 0.5 mM phosphatidylcholine large unilamellar vesicles (PC-LUVs). Prior to the assay, the cells were washed 3 times in isotonic buffer (130 mM NaCl, 5 mM KCl, 1.3 mM CaCl₂, 1 mM MgCl₂, 10 mM glucose and 20 mM HEPES, pH 7.4). Isotope efflux was determined at 37°C by replacing the medium at 1 – 2 minute intervals. Radioactivity in the medium was determined by gamma- radiation counting and expressed as fractional efflux per minute as described previously [22].

Fig. 1. Increased intracellular accumulation of cholesterol in fibroblasts expressing human F508delCFTR. Cellular distribution of cholesterol in filipin-stained parental (A, a), wtCFTR- (B, b) and F508delCFTR- (C, c) expressing fibroblasts. Each image is representative of a total of 15 images taken from 5 individual experiments using 5 different batches of cells. A-C: phase contrast images; a-c: fluorescent images.

FITC-dextran uptake

Cells on coverslips at 80-90% confluency were incubated for 10 min in a medium containing 0.5 mg/ml FITC-dextran (10,000 Da) in the absence or presence of forskolin (10 μ M) and/or genistein (100 μ M). After the incubation, cells were washed three times with ice-cold phosphate-buffered saline and fixed with 2% paraformaldehyde for 20 min. Using a 40X oil immersion objective (Axiovert 135M, Zeiss, Oberkochen, Germany), confocal images of 512 X 512 pixels (0.22 μ m /pixel) were constructed by summation of 15 optical sections parallel to the substratum, each 1 μ m apart. Endocytosis was quantified using KS400 software (Zeiss Oberkochen, Germany) by counting the number of fluorescent spots sized between 3 and 75 pixels, after subtraction of background fluorescence. Background fluorescence was determined by measuring the mean pixel intensity and adding five times its standard deviation of a circle drawn in the background.

Results

Intracellular accumulation of cholesterol in murine 3T3 fibroblasts expressing F508delCFTR

As compared to the parental and wtCFTR-expressing cells, F508delCFTR fibroblasts contain an enlarged intracellular area of vesicular structures, localized predominantly around the nucleus (Fig. 1 A-C). Because White et al. recently showed that F508delCFTR-expressing epithelial cells develop a NPC-like phenotype, we studied the amount and cellular distribution of cholesterol in these fibroblast cell lines. Using filipin-treated cell cultures, accumulation of free cholesterol was

found around the nucleus of F508delCFTR-expressing fibroblasts (Fig. 1c), a phenomenon characteristic for NPC1-deficient cells [23-26]. In contrast, little if any filipin staining was observed in parental or wtCFTR expressing cells (Fig. 1a, b). Determination of the total amount of free cellular cholesterol confirmed the increased accumulation of cholesterol in F508delCFTR cells (cholesterol content of parental, wtCFTR- and F508delCFTR-expressing 3T3 fibroblasts: 17, 18 and 24 μ g/mg protein, respectively).

CD/PC-LUVs treatment promotes rescue of F508delCFTR

Recently, we demonstrated that the osmotic cell swelling-induced activation of VRAC (Volume Regulated Anion Conductance) was impaired in both NPC-1 fibroblasts as well as in cholesterol-loaded Intestine 407 epithelial cells [18]. In addition we found that treatment of these cells with CD/PC-LUVs markedly potentiated the ionic response, through a mechanism which most likely involved recruitment of additional channels by promoting vesicular transport [18]. To investigate the effect(s) of CD/PC-LUVs treatment on the anion efflux of the different clones of 3T3 fibroblasts, we first tested the effects of forskolin and genistein on wtCFTR fibroblasts. In line with previous studies, forskolin and genistein by themselves were able to promote the efflux of iodide from wtCFTR fibroblasts, simultaneous addition of both agonists, however, elicited the largest response (Fig. 2A). As expected, genistein and forskolin did not provoke a

Fig. 2. CD/PC-LUVs treatment potentiate the forskolin- and genistein-induced anion efflux from F508delCFTR but not from parental or wtCFTR-expressing cells. A) Fractional ^{125}I efflux from wtCFTR-expressing fibroblasts (A) stimulated with either forskolin (10 μM ; open circles), genistein (100 μM ; closed triangles) or both (closed circles). B - D) ^{125}I efflux from control (open circles) and CD/PC-LUVs-treated (closed circles) cultures of parental (B), wtCFTR- (C) and F508delCFTR- (D) expressing fibroblasts stimulated with forskolin (10 μM) and genistein (100 μM). Closed triangles represents the unstimulated control. Data are expressed as mean $\pm$ S.E.M for $n=3$. Arrow marks time point of forskolin and/or genistein addition. No error bar is given when the SEM is smaller than the size of the symbol used. Asterisk indicates a significant difference from the control ($p < 0.05$).

^{125}I -efflux from parental 3T3 fibroblasts lacking CFTR (Fig. 2B). In contrast, a slight increase in the anion efflux was observed after stimulating F508delCFTR fibroblasts with genistein and forskolin (Fig. 2D). This response was enhanced by at least two-fold in F508delCFTR cells treated with CD/PC-LUVs. As compared to low temperature rescue of F508delCFTR function, a 2 h period of CD/LUVs treatment enhanced F508delCFTR activity to a level of approx 50% of an overnight incubation at 26°C (results not shown). Notably, the response to genistein and forskolin was not increased in CD/PC-LUVs-treated wtCFTR cells (Fig. 2C). These results suggest that CD/PC-LUVs treatment, analogous to its effect on the recruitment of VRAC, is able to increase surface expression of F508delCFTR.

Cholesterol accumulation is reduced in CD/PC-LUVs-treated F50delCFTR fibroblasts

To evaluate whether cholesterol depletion is able to promote vesicle movement to the plasma membrane, the effects of CD/PC-LUVs on the intracellular distribution of cholesterol was studied in the different cell models. As shown in Fig. 3A, accumulation of cholesterol was largely reduced in F508delCFTR-expressing cells treated with CD/PC-LUVs, but not in the parental cell line or in wtCFTR fibroblasts. Notably, incubation of the three different cell types with the fluorescent membrane probe FM-1.43 did not show any difference in the amount or distribution of intracellular vesicular compartments nor did it reveal a decrease in staining after CD/PC-LUVs treatment (Fig. 3B). Taken together, these results suggest

Fig. 3. Reduction of intracellular cholesterol in F508delCFTR fibroblasts after CD/PC-LUVs treatment. Filipin staining (A) was compared with FM-1.43 staining (B). Images of parental (A, a), wtCFTR (B, b) and F508delCFTR (C, c) fibroblasts were taken before (A - C) and after (a - c) CD/PC-LUVs treatment. Each image is representative of a total of 9 images taken from 3 individual experiments.

that a distinct intracellular compartment exists in F508delCFTR-expressing cells, containing free cholesterol and at least some F508delCFTR, that can be accumulated at the plasma membrane by reducing plasma membrane cholesterol.

Regulation of endocytosis in 3T3fibroblasts by forskolin and genistein.

To further pursue a putative role for vesicle cycling in the regulation of plasma membrane expression of F508delCFTR, the magnitude of endocytosis was studied by quantitating the cellular uptake of fluorescently labelled dextran. In F508delCFTR-expressing fibroblasts, the

uptake of FITC-dextran was robustly increased by stimulating the cells with forskolin (Fig. 4 and Table 1). In contrast, forskolin appears to reduce endocytosis in both parental and wtCFTR cells (Table 1). Surprisingly, the uptake of FITC-dextran was almost completely abolished in both control and forskolin-stimulated F508delCFTR cells treated with genistein (Fig. 4 and Table 1). A comparable effect of genistein was also observed in parental and wtCFTR fibroblasts (Table 1). Similar to genistein treatment, incubation with CD/PC-LUVs largely prevented the uptake of dextran in both parental, wt-CFTR expressing and F508delCFTR-expressing cells (Fig. 4 and Table 1).

Fig. 4. Modulation of the rate of endocytosis by forskolin and genistein in F508delCFTR cells. Endocytosis was determined by the uptake of FITC-conjugated dextran. Control cells (A,a) were compared with treated cells. Cultures were treated with 10 μ M forskolin (B, b), 100 μ M genistein (C, c), forskolin and genistein (D, d) or CD/PC-LUVs (E, e) as described under “Materials and Methods”. A–E: fluorescence images. a–e: fluorescence merged with phase contrast images. Each image is representative of a total of 9 images taken from 3 individual experiments.

Table 1. Modulation of endocytosis in parental, wtCFTR- and F508delCFTR-expressing fibroblasts. Endocytosis was determined by the uptake of FITC-conjugated dextran and quantitated as described under “Materials and Methods”. Data are expressed as the number of fluorescent spots (mean $\pm$ S.E.M for n=3). Concentrations used: forskolin, 10 μ M; genistein, 100 μ M. Asterisk indicates a significant difference from the untreated control ($p < 0.01$).

	Treatment	Parental	wtCFTR	F508delCFTR
Control	-	50 $\pm$ 1	64 $\pm$ 4	22 $\pm$ 1
	Forskolin	24 $\pm$ 5*	20 $\pm$ 4*	113 $\pm$ 5*
	Genistein	10 $\pm$ 2*	18 $\pm$ 5*	4.7 $\pm$ 0.3*
	Forsk. + Gen.	9 $\pm$ 2*	13 $\pm$ 2*	7 $\pm$ 3*
CD/PC-LUVs	-	7 $\pm$ 5*	11 $\pm$ 1*	2.0 $\pm$ 0.3*
	Forskolin	1.3 $\pm$ 0.2*	11 $\pm$ 2*	4 $\pm$ 1*
	Genistein	1.3 $\pm$ 0.9*	0.7 $\pm$ 0.1*	0.7 $\pm$ 0.2*
	Forsk. + Gen.	9 $\pm$ 2*	1.0 $\pm$ 0.2*	1.0 $\pm$ 0.3*

Discussion

In this study, we have demonstrated that a CD/PC-LUVs-mediated reduction in cellular cholesterol boosted the forskolin-provoked anion efflux from F508delCFTR-expressing cells approximately 2 fold. Modulation of ionic conductances by alterations in the lipid composition of membranes is not unprecedented and has been reported

for VRAC as well as for several cation channels [15-17, 27, 28]. At least two different mechanisms can underlie the observed increase in the forskolin-stimulated efflux: (1) a modulation of the activity of the channel protein itself, altering the channel open and closed state [27, 29], and (2) an increase in the number of channels in the plasma membrane by recruitment from intracellular compartments and/or by a prolonged retention in the

membrane [15-17]. It is now well established that cholesterol-containing microdomains play an important role during the process of endocytosis. Indeed, depletion of plasma membrane cholesterol using CD/PC-LUVs largely inhibits endocytosis [15, 16] and, *vice versa*, increasing membrane cholesterol is found to promote endocytosis [30]. Although we do not entirely exclude the possibility that the open channel probability of F508delCFTR may also be affected by CD/PC-LUVs treatment, both the reduction of intracellular filipin staining and the inhibition of FITC-dextran uptake upon CD/PC-LUVs treatment indicate that additional channels are incorporated and/or retained into the plasma membrane in F508delCFTR-expressing fibroblasts. A similar mechanism has recently been proposed for the activation of a compensatory Cl⁻ current in osmotically stimulated Intestine 407 cells [18].

Both wild-type and mutant CFTR are internalised by clathrin-dependent endocytosis [31-33], although it seems that the rate of internalisation is higher for mutant CFTR [14, 34]. Using dominant-negative mutants of Rme-1, a protein involved in endosomal cycling, it has been demonstrated that at least part of the CFTR escapes lysosomal degradation and recycles back to the plasma membrane [35]. Endocytotic trafficking of CFTR is regulated by several members of the Rab family of GTPases, including Rab 4 and 11, leading to recycling of the channels to the plasma membrane, and Rab 5, which promotes channel endocytosis [14]. Transport towards the membrane is inhibited in F508delCFTR-expressing cells but can be rescued by reduced temperature [6, 36], chemicals chaperones like DMSO, glycerol [37, 38] and proteasome inhibitors [14], most likely by promoting proper folding or stabilization of the mutant protein.

In this study, we showed that F508delCFTR-expressing fibroblasts, like several other F508delCFTR expressing cell models [19, 20], have a NPC-like phenotype, i.e. a marked intracellular accumulation of cholesterol. Although cholesterol-containing vesicles are present throughout the cell, a marked accumulation of cholesterol was observed around the nucleus. This predominant perinuclear staining likely reflects the most important cellular area of CFTR synthesis, maturation and/or degradation, but may be additionally enhanced by the increased cell thickness at this region. The direct relation between F508delCFTR expression and cholesterol accumulation has not been fully established yet, however, White et al. reported that alterations in the regulation of NOS2, STAT1, SMAD3, and Rho A protein expression, which occur in NPC-1 cells, are also observed in these

CF models, suggesting that similar regulatory mechanism are involved [19]. Notably, cholesterol accumulation and an increase in Rho A expression was also observed in cells defective in ATP-binding cassette protein A1 (ABCA1), a cholesterol transporting ABC-transporter protein structurally related to CFTR [39]. Recently, the NPC-1 protein has been proposed to function as a lipid flippase of late endosomes and to promote the generation of transport vesicles by membrane deformation. The normal function of NPC-1 is affected by the absence of NPC-2, a cholesterol binding protein, in such way that the cytosolic factors necessary for vesicle budding were inefficiently recruited, thereby disrupting vesicular formation and finally leading to the accumulation of many lipids [40]. In addition, increased cholesterol accumulation by itself can impair F508delCFTR trafficking by a direct inhibition of Rab 4, one of the Rab G-proteins involved in CFTR recycling [25].

CFTR is activated by cAMP-dependent activation however, this process poorly activates F508delCFTR. The isoflavone genistein is able to potentiate CFTR by a direct binding to NBD-2 [41-43]. Our efflux assay demonstrated that genistein could augment the cAMP-dependent activation in CFTR-expressing fibroblasts. In F508delCFTR-expressing cells, neither genistein nor forskolin alone could induce a significant increase in the conductance after the CD/PC-LUVs treatment. However, the combination of both stabilized and potentiated the anion conductance. The cAMP-dependent activation is known to stimulate trafficking of CFTR to the plasma membrane in control cells [32, 33, 44, 45] but not in CF cells [45]. Our dextran experiment demonstrates that forskolin stimulates endocytosis in F508delCFTR-expressing fibroblasts but not in parental and wtCFTR-expressing cells. This is in line with previous observations that the half-life of F508delCFTR in the plasma membrane is reduced [13, 14, 34] and with the findings of Bradbury et al. that elevated levels of cAMP fail to inhibit fluid phase endocytosis in F508delCFTR expressing cells [46]. This study also demonstrates for the first time that the genistein, which has previously been found to inhibit endocytosis in some model systems [47, 48], can fully counter act the forskolin-induced FITC dextran uptake (Fig. 4). This novel finding suggest that the beneficial effect of genistein on CFTR-mediated Cl⁻ secretion may result from a dual action: potentiation of the CFTR Cl⁻ channel by binding to CFTR and stabilizing the channel in its open state [41-43] and promotion of its retention in the plasma membrane through inhibition of endocytosis.

In this study we showed that treatment of

F508delCFTR-expressing fibroblasts with CD/PC-LUVs potentiated the forskolin-induced anion efflux approximately 2 fold. We were however, unable to detect an increase in the amount of fully glycosylated mature CFTR (C-band) in CD/PC-LUVs-treated cells by Western blotting (results not shown). This indicates that CD/PC-LUVs treatment does not promote maturation of F508delCFTR but instead stimulates F508delCFTR trafficking to the membrane or, more likely, promotes channel retention.

Using mouse models, Dorin et al. demonstrated that 5% mature CFTR is sufficient to restore intestinal chloride transport to 50% of normal level and to reach a 100% survival [49]. Furthermore, 8% of mature protein was found to correspond with normal cAMP-mediated chloride secretion in caecum and 40% residual cAMP-stimulated jejunal chloride secretion [50, 51]. Because the forskolin-

induced efflux in cells treated with CD-LUVs is approx. 10-15 % of the maximum response observed in CFTR expressing cells (Fig 2D) it would be of interest to further investigate the potential beneficial effects of a drug-mediated decrease in cellular cholesterol, for example by using atorvastatin, on the rescue of F508delCFTR in a more physiological model.

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References

- 1 Kerem B, Rommens JM, Buchanan JA, Markiewicz D, Cox TK, Chakravarti A, Buchwald M, Tsui LC: Identification of the cystic fibrosis gene: genetic analysis. *Science* 1989;245:1073-1080.
- 2 Anderson MP, Gregory RJ, Thompson S, Souza DW, Paul S, Mulligan RC, Smith AE, Welsh MJ: Demonstration that CFTR is a chloride channel by alteration of its anion selectivity. *Science* 1991;253:202-205.
- 3 Golin-Bisello F, Bradbury N, Ameen N: STa and cGMP stimulate CFTR translocation to the surface of villus enterocytes in rat jejunum and is regulated by protein kinase G. *Am J Physiol Cell Physiol* 2005;289:708-716.
- 4 Bobadilla JL, Macek Jr M, Jason P, Fine JP, Farrell PM: Cystic fibrosis: a worldwide analysis of CFTR mutations-correlation with incidence data and application to screening. *Hum Mutat* 2002;19:575-606.
- 5 Cheng SH, Gregory RJ, Marshall J, Paul S, Souza DW, White GA, O'Riordan CR, Smith AE: Defective intracellular transport and processing of CFTR is the molecular basis of most cystic fibrosis. *Cell* 1990;63:827-834.
- 6 Denning GM, Ostedgaard LS, Welsh MJ: Abnormal localization of cystic fibrosis transmembrane conductance regulator in primary cultures of cystic fibrosis airway epithelia. *J Cell Biol* 1992;118:551-559.
- 7 Haws CM, Nepomuceno IB, Krouse ME, Wakelee H, Law T, Xia Y, Nguyen H, Wine JJ: Delta F508-CFTR channels: kinetics, activation by forskolin, and potentiation by xanthines. *Am J Physiol* 1996;270:1544-1555.
- 8 Lukacs GL, Chang XB, Bear C, Kartner N, Mohamed A, Riordan JR, Grinstein S: The delta F508 mutation decreases the stability of cystic fibrosis transmembrane conductance regulator in the plasma membrane. Determination of functional half-lives on transfected cells. *J Biol Chem* 1993;268:21592-21598.
- 9 Li C, Ramjeesingh M, Reyes E, Jensen T, Chang X, Rommens JM, Bear CE: The cystic fibrosis mutation (delta F508) does not influence the chloride channel activity of CFTR. *Nat Genet* 1993;3:311-316.
- 10 Gregory RJ, Rich DP, Cheng SH, Souza DW, Paul S, Manavalan P, Anderson MP, Welsh MJ, Smith AE: Maturation and function of cystic fibrosis transmembrane conductance regulator variants bearing mutations in putative nucleotide-binding domains 1 and 2. *Mol Cell Biol* 1991;11:3886-3893.
- 11 Sheppard DN, Rich DP, Ostedgaard LS, Gregory RJ, Smith AE, Welsh MJ: Mutations in CFTR associated with mild-disease-form Cl⁻ channels with altered pore properties. *Nature* 1993;362:160-164.
- 12 Dalemans W, Barbry P, Champigny G, Jallat S, Dott K, Dreyer D, Crystal RG, Pavirani A, Lecocq JP, Lazdunski M: Altered chloride ion channel kinetics associated with the delta F508 cystic fibrosis mutation. *Nature* 1991;354:526-528.

- 13 Sharma M, Pampinella F, Nemes C, Benharouga M, So J, Du K, Bache KG, Papsin B, Zerangue N, Stenmark H, Lukacs GL: Misfolding diverts CFTR from recycling to degradation: quality control at early endosomes. *J Cell Biol* 2004;164:923-933.
- 14 Gentzsch M, Chang XB, Cui L, Wu Y, Ozols VV, Choudhury A, Pagano RE, Riordan JR: Endocytic trafficking routes of wild type and DeltaF508 cystic fibrosis transmembrane conductance regulator. *Mol Biol Cell* 2004;15:2684-2696.
- 15 Subtil A, Gaidarov I, Kobylarz K, Lampson MA, Keen JH, McGraw TE: Acute cholesterol depletion inhibits clathrin-coated pit budding. *Proc Natl Acad Sci USA* 1999;96:6775-6780.
- 16 Rodal SK, Skretting G, Garred O, Vilhardt F, van Deurs B, Sandvig K: Extraction of cholesterol with methyl-beta-cyclodextrin perturbs formation of clathrin-coated endocytic vesicles. *Mol Biol Cell* 1999;10:961-974.
- 17 Pike LJ, Casey L: Cholesterol levels modulate EGF receptor-mediated signaling by altering receptor function and trafficking. *Biochemistry* 2002;41:10315-10322.
- 18 Lim CH, Schoonderwoerd K, Kleijer WJ, de Jonge HR, Tilly BC: Regulation of the cell swelling-activated chloride conductance by cholesterol-rich membrane domains. *Acta Physiol (Oxf)* 2006;187:295-303.
- 19 White NM, Corey DA, Kelley TJ: Mechanistic similarities between cultured cell models of cystic fibrosis and Niemann-Pick type C. *Am J Respir Cell Mol Biol* 2004;31:538-543.
- 20 White NM, Jiang D, Burgess JD, Bederman IR, Previs SF, Kelley TJ: Altered cholesterol homeostasis in cultured and *in vivo* models of cystic fibrosis. *Am J Physiol Lung Cell Mol Physiol* 2007;292:476-486.
- 21 Berger HA, Anderson MP, Gregory RJ, Thompson S, Howard PW, Maurer RA, Mulligan R, Smith AE, Welsh MJ: Identification and regulation of the cystic fibrosis transmembrane conductance regulator-generated chloride channel. *J Clin Invest* 1991;88:1422-1431.
- 22 Vaandrager AB, Bajnath R, Groot JA, Bot AG, De Jonge HR: Ca²⁺ and cAMP activate different chloride efflux pathways in HT-29.cl19A colonic epithelial cell line. *Am J Physiol* 1991;261:958-965.
- 23 Ko DC, Gordon MD, Jin JY, Scott MP: Dynamic movements of organelles containing Niemann-Pick C1 protein: NPC1 involvement in late endocytic events. *Mol Biol Cell* 2001;12:601-614.
- 24 Neufeld EB, Wastney M, Patel S, Suresh S, Cooney AM, Dwyer NK, Roff CF, Ohno K, Morris JA, Carstea ED, Incardona JP, Strauss JF 3rd, Vanier MT, Patterson MC, Brady RO, Pentchev PG, Blanchette-Mackie EJ: The Niemann-Pick C1 protein resides in a vesicular compartment linked to retrograde transport of multiple lysosomal cargo. *J Biol Chem* 1999;274:9627-9635.
- 25 Choudhury A, Sharma DK, Marks DL, Pagano RE: Elevated endosomal cholesterol levels in Niemann-Pick cells inhibit rab4 and perturb membrane recycling. *Mol Biol Cell* 2004;15:4500-4511.
- 26 Wojtanik KM, Liscum L: The transport of low density lipoprotein-derived cholesterol to the plasma membrane is defective in NPC1 cells. *J Biol Chem* 2003;278:14850-14856.
- 27 Levitan I, Christian AE, Tulenko TN, Rothblat GH: Membrane cholesterol content modulates activation of volume-regulated anion current in bovine endothelial cells. *J Gen Physiol* 2000;115:405-416.
- 28 Romanenko VG, Rothblat GH, Levitan I: Modulation of endothelial inward-rectifier K⁺ current by optical isomers of cholesterol. *Biophys J* 2002;83:3211-3222.
- 29 Romanenko VG, Rothblat GH, Levitan I: Sensitivity of volume-regulated anion current to cholesterol structural analogues. *J Gen Physiol* 2004; 23:77-87.
- 30 Sharma DK, Brown JC, Choudhury A, Peterson TE, Holicky E, Marks DL, Simari R, Parton RG, Pagano RE: Selective stimulation of caveolar endocytosis by glycosphingolipids and cholesterol. *Mol Biol Cell* 2004;15:3114-3122.
- 31 Bradbury NA, Cohn JA, Venglarik CJ, Bridges RJ: Biochemical and biophysical identification of cystic fibrosis transmembrane conductance regulator chloride channels as components of endocytic clathrin-coated vesicles. *J Biol Chem* 1994;269:8296-302.
- 32 Lukacs GL, Segal G, Kartner N, Grinstein S, Zhang F: Constitutive internalization of cystic fibrosis transmembrane conductance regulator occurs via clathrin-dependent endocytosis and is regulated by protein phosphorylation. *Biochem J* 1997;328:353-361.
- 33 Prince LS, Workman RB Jr, Marchase RB: Rapid endocytosis of the cystic fibrosis transmembrane conductance regulator chloride channel. *Proc Natl Acad Sci USA* 1994;91:5192-5196.
- 34 Swiatecka-Urban A, Brown A, Moreau-Marquis S, Renuka J, Coutermarsh B, Barnaby R, Karlson KH, Flotte TR, Fukuda M, Langford GM, Stanton BA: The short apical membrane half-life of rescued {Delta}F508-cystic fibrosis transmembrane conductance regulator (CFTR) results from accelerated endocytosis of {Delta}F508-CFTR in polarized human airway epithelial cells. *J Biol Chem* 2005; 80:36762-36772.
- 35 Picciano JA, Ameen N, Grant BD, Bradbury NA: Rme-1 regulates the recycling of the cystic fibrosis transmembrane conductance regulator. *Am J Physiol Cell Physiol* 2003;5:1009-1018.
- 36 Egan ME, Schwiebert EM, Guggino WB: Differential expression of ORCC and CFTR induced by low temperature in CF airway epithelial cells. *Am J Physiol* 1995;268:243-251.
- 37 Brown CR, Hong-Brown LQ, Biwersi J, Verkman AS, Welch WJ: Chemical chaperones correct the mutant phenotype of the delta F508 cystic fibrosis transmembrane conductance regulator protein. *Cell Stress Chaperones* 1996;1:117-125.
- 38 Sato S, Ward CL, Krouse ME, Wine JJ, Kopito RR: Glycerol reverses the misfolding phenotype of the most common cystic fibrosis mutation. *J Biol Chem* 1996;271:635-638.
- 39 Schmitz G, Buechler C: ABCA1: regulation, trafficking and association with heteromeric proteins. *Ann Med* 2002;34:334-347.
- 40 Ioannou YA: Guilty until proven innocent: the case of NPC1 and cholesterol. *Trends Biochem Sci* 2005;30:498-505.
- 41 French PJ, Bijman J, Bot AG, Boomaars WE, Scholte BJ, de Jonge HR: Genistein activates CFTR Cl⁻ channels via a tyrosine kinase- and protein phosphatase-independent mechanism. *Am J Physiol* 1997;273:747-753.
- 42 Wang F, Zeltwanger S, Yang IC, Nairn AC, Hwang TC: Actions of genistein on cystic fibrosis transmembrane conductance regulator channel gating. Evidence for two binding sites with opposite effects. *J Gen Physiol* 1998;111:477-490.
- 43 Melin P, Thoreau V, Norez C, Bilan F, Kitzis A, Becq F: The cystic fibrosis mutation G1349D within the signature motif LSHGH of NBD2 abolishes the activation of CFTR chloride channels by genistein. *Biochem Pharmacol* 2004;67(12):2187-2196.
- 44 Schillers H, Danker T, Madeja M, Oberleithner H: Plasma membrane protein clusters appear in CFTR-expressing *Xenopus laevis* oocytes after cAMP stimulation. *J Membr Biol* 2001;180:205-12.

- 45 Lehrich RW, Aller SG, Webster P, Marino CR, Forrest JN Jr: Vasoactive intestinal peptide, forskolin, and genistein increase apical CFTR trafficking in the rectal gland of the spiny dogfish, *Squalus acanthias*. Acute regulation of CFTR trafficking in an intact epithelium. *J Clin Invest* 1998;101:737-745.
- 46 Bradbury NA, Jilling T, Berta G, Sorscher EJ, Bridges RJ, Kirk KL: Regulation of plasma membrane recycling by CFTR. *Science* 1992;256:530-532.
- 47 Salazar G, Gonzalez A: Novel mechanism for regulation of epidermal growth factor receptor endocytosis revealed by protein kinase A inhibition. *Mol Biol Cell* 2002;13:1677-1693.
- 48 Parker EM, Zaman MM, Freedman SD: GP2, a GPI-anchored protein in the apical plasma membrane of the pancreatic acinar cell, co-immunoprecipitates with src kinases and caveolin. *Pancreas* 2000;21:219-925.
- 49 Dorin JR, Farley R, Webb S, Smith SN, Farini E, Delaney SJ, Wainwright BJ, Alton E W, Porteous DJ: A demonstration using mouse models that successful gene therapy for cystic fibrosis requires only partial gene correction. *Gene Ther* 1996;3:797-801.
- 50 Dickinson P, Smith SN, Webb S, Kilanowski FM, Campbell IJ, Taylor MS, Porteous DJ, Willemsen R, de Jonge HR, Farley R, Alton EW, Dorin JR: The severe G480C cystic fibrosis mutation, when replicated in the mouse, demonstrates mistrafficking, normal survival and organ-specific bioelectrics. *Hum Mol Genet* 2002;11:243-251.
- 51 Charizopoulou N, Wilke M, Dorsch M, Bot A, Jorna H, Jansen S, Stanke F, Hedrich HJ, de Jonge HR, Tummeler B: Spontaneous rescue from cystic fibrosis in a mouse model. *BMC Genetics* 2006;7:18.