J. exp. Biol. 172, 245-266 (1992)
Printed in Great Britain © The Company of Biologists Limited 1992

CHLORIDE CHANNELS OF INTRACELLULAR ORGANELLES AND THEIR POTENTIAL ROLE IN CYSTIC FIBROSIS

BY QAIS AL-AWQATI, JONATHAN BARASCH AND DONALD LANDRY

Departments of Medicine and Physiology, College of Physicians and Surgeons of Columbia University, 630 W 168th Street, New York, NY 10032, USA

Summary

Chloride channels were previously purified from bovine kidney cortex membranes using a drug affinity column. Reconstitution of the purified proteins into artificial liposomes and planar bilayers yielded chloride channels. A $64\times10^3 M_r$ protein, p64, identified as a component of this chloride channel, was used to generate antibodies which depleted solubilized kidney membranes of all chloride channel activity. This antibody has now been used to identify a clone, H2B, from a kidney cDNA library. Antibodies, affinity-purified against the fusion protein of H2B, also depleted solubilized kidney cortex from all chloride channel activity. The predicted amino acid sequence of p64 shows that it contains two and possibly four putative transmembrane domains and potential phosphorylation sites by protein kinases A and C. There was no significant homology to other protein (or DNA) sequences in the data base including other anion channels or the cystic fibrosis transmembrane conductance regulator. The protein is expressed in all cells tested and probably represents the chloride channel of intracellular organelles.

Cystic fibrosis (CF) is associated with a defect in a cyclic-AMP-activated chloride channel in secretory epithelia which leads to decreased fluid secretion. In addition, many mucus glycoproteins show decreased sialylation but increased sulfation. We have recently shown that the pH of intracellular organelles is more alkaline in CF cells, an abnormality that is due to defective chloride conductance in the vesicle membranes. We postulate that the defect in the intracellular chloride channel, and hence the alkalization, could explain the glycosylation abnormalities since the pH optimum of Golgi sialyltransferase is acid while that of focusyl- and sulfotransferases is alkaline. Defects in sialyation of glycolipids might also generate receptors for *Pseudomonas*, which is known to colonize the respiratory tract of CF patients.

Introduction

A large number of chloride channels have been identified, frequently by expression of a chloride current on injection of total cellular RNA into *Xenopus* oocytes. After sib selection or other methods, a single message is identified which confers on the oocyte a new chloride current. A voltage-sensitive channel was isolated from *Torpedo* electric organ and its mammalian skeletal homologue was identified (Jentsch *et al.* 1990; Steinmayer *et al.* 1991*b*). Similar chloride currents are known to exist in the native cells, suggesting that these cDNAs encode proteins which mediate the currents. Further, a

Key words: chloride channel, cystic fibrosis, vacuolar ATPase, sialylation, *Pseudomonas*.

mouse mutant, *adr*, which has a low muscle chloride current, has an insertional mutation in this gene, further documenting the observation that the voltage-activated chloride current is coded for by these cDNAs (Steinmayer *et al.* 1991*a*). Another similar species was found to be present in many epithelial and non-epithelial cell types and coded for a hyperpolarization-sensitive chloride current. No such currents are typically found in these cells. Using the same methods, a chloride channel that is modulated by ATP was cloned from MDCK cells. The protein is curious in that it has no predicted transmembrane domains; however, it clearly conferred on oocytes a new (outwardly rectifying) chloride channel (Paulmichl *et al.* 1992). Porin, the anion channel of the outer membrane of mitochondria and bacteria, also has no transmembrane domains, but is clearly a channel as shown by its crystal structure. The multidrug resistance gene (*mdr*), which confers on cells the ability to transport a large number of chemically unrelated hydrophobic molecules, was expressed in heterologous cells. These cells displayed a volume-activated chloride current that was not present previously (Valverde *et al.* 1992). The cystic fibrosis gene product, CFTR, which bears some resemblance to the mdr protein (Riordan *et al.* 1989), also confers on heterologous cells a cyclic-AMP-activated chloride channel (Anderson *et al.* 1991; Kartner *et al.* 1991). Further, the protein was expressed and purified from insect cells infected with the high-yield baculovirus vector. When the protein was reconstituted into planar lipid bilayers, it generated cyclic-AMP-activated chloride channels (Bear *et al.* 1992).

It is interesting that all of these expressed genes resulted in a chloride current across the plasma membrane of the cell, implying that these proteins contain targetting sequences for that membrane. Alternatively, since these experiments largely rely on over-expression of proteins, it is possible that they may code for vacuolar chloride channels, but over-expression results in some of the protein reaching the surface.

A variety of methods have shown that intracellular organelles contain chloride channels. Using either voltage-sensitive $^{36}Cl^-$ uptake or incorporation into planar lipid bilayers, anion-sensitive channels with different characteristics have been found. These experiments do not definitively localize the channel since they were performed in isolated vesicles with varying degrees of purification. In addition, the absence of standard criteria for identification of intracellular organelles limits the assignment of vesicles to one or another compartment. In the future it will be necessary to provide evidence for the presence of chloride channels using immunoelectron microscopy. This evidence is not yet available since the abundance of chloride channels in intracellular organelles is low. Most studies have emphasized the role that these channels might play in the regulation of the proton-motive force generated by the proton-translocating V-ATPase that is found in a variety of organelles. However, there is no need to assume that all intracellular chloride channels are associated with this H^+-ATPase nor is there reason to suspect that all vesicles that contain H^+-ATPases contain Cl^- channels.

Role of the chloride channel in the regulation of vacuolar pH

Bioenergetics

Chloride conductivity is found in many subcellular organelles that contain H^+-

translocating ATPases and is critical to vesicle physiology. This enzyme, the V-ATPase, not only acidifies the lumen of organelles, producing a transmembrane pH difference (ΔpH), but is also electrogenic, i.e. capable of generating a membrane potential, $\Delta\Psi$; Fig. 1. The size of the electrochemical H^+ gradient or 'proton-motive force' $\Delta\mu$ generated by the ATPase is given by:

$$\Delta\mu = \boldsymbol{R}T\,\Delta\text{pH} + zF\Delta\Psi, \tag{1}$$

where $\boldsymbol{R}$, T, z and F have their usual thermodynamic meanings. At steady state, when the net rate of H^+ transport (J) is zero, this proton-motive force is produced by:

$$(\Delta\mu)_{J=0} = \Delta G_{\text{ATP}} Z, \tag{2}$$

where Z is the H^+/ATP stoichiometry and ΔG_{ATP} is the free energy of ATP hydrolysis in the cytoplasm, which is given by:

$$\Delta G_{\text{ATP}} = \Delta G_0 + \boldsymbol{R}T \ln [\text{ATP}]/([\text{ADP}][P_i]), \tag{3}$$

where ΔG_0 is the standard free energy of ATP hydrolysis (7.6 kcal mol^{-1}), P_i is inorganic phosphate and the bracketed values are concentrations of the reactants in the cytoplasm. If the proton permeability of the organellar membrane is low, the ATPase will generate a gradient large enough to reach its own reversal potential and thereby stop further transport of protons (Al-Awqati, 1986). Leak currents to ions such as Cl^- or K^+ change neither the proton-motive force nor the efficiency of coupling ATP hydrolysis with proton transport, but instead vary the fractional contributions of pH and membrane potential gradients to a fixed total H^+ electrochemical gradient (Al-Awqati, 1986; van Dyke, 1988). If the conductance of the membrane is high, then no membrane potential can form, and the proton-motive force is expressed only as a pH gradient. In isolated vesicles, this can be achieved by adding an electrogenic ionophore, such as valinomycin, which increases the membrane conductance (to K^+), collapsing any membrane potential and magnifying the pH difference (Glickman *et al.* 1983). If an organelle has little conductance for 'counterions' such as Cl^- or K^+, then a membrane potential rapidly forms, limiting further proton pumping and thus inhibiting the generation of ΔpH. Organellar Cl^- channels are thus critical for the developement of ΔpH, since Cl^- is the

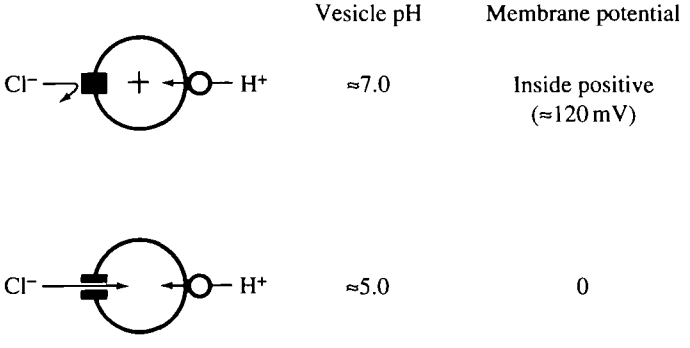

Fig. 1. A model for the generation of pH differences and membrane potential by intracellular organelles.

sole counterion of H^+ translocation in a number of organelles. For example, chromaffin granules generate pH gradients only in the presence of Cl^-, but not K^+, Na^+, Mg^{2+}, Ca^{2+}, SO_4^{2-} or isethionate (Johnson and Scarpa, 1979; Johnson *et al.* 1982) to which they are impermeant. An essential role for Cl^- conductance in ΔpH generation has also been found in clathrin-coated vesicles (Stone *et al.* 1983), neurohypophyseal granules (Russell and Holz, 1981), Golgi vesicles (Glickman *et al.* 1983), lysosomes (Schneider, 1981; Harikumar and Reeves, 1983) and multivesicular bodies (van Dyke, 1988) and the generation of ΔpH is apparently a conserved function of Cl^- channels, since Cl^-, but not K^+, supports ΔpH generation in corn root membrane vesicles (Bennett and Spanswick, 1983).

Measurement of vacuolar pH or ΔpH of a number of organelles of the endocytic (Tycko and Maxfield, 1982; Yamashiro *et al.* 1983, 1984) and secretory pathways (Barasch *et al.* 1988; Orci *et al.* 1987) reveals considerable variation. Different recycling endosomes have different pH values, as do β cell granules at different stages of maturation (Orci *et al.* 1987). Endosomes and Golgi vesicles are alkaline relative to lysosomes. Organelles that generate large pH gradients, such as lysosomes and chromaffin granules, are very permeable to Cl^-, whereas Golgi vesicles (Glickman *et al.* 1983) and isolated parafollicular cell granules (Barasch *et al.* 1988), which generate little or no ΔpH, even in the presence of 150 mmol l^{-1} Cl^-, must have a low membrane Cl^- conductance. Since these vesicles are acidified in the presence of valinomycin, the formation of a pH gradient must have been limited by the low vesicle counterion conductance. This result suggests that Cl^- channels in different organelles have different open probabilities and that the balance of ΔpH and $\Delta\Psi$ can be physiologically regulated by changes in the Cl^- conductance. Regulation of vacuolar pH is not likely to result from changes in the number of H^+-ATPase molecules in an organelle membrane, since a single molecule with a transport rate of 100 ions s^{-1} could maximally acidify a vesicle within 1 min.

It is likely that there are additional mechanisms that can control vacuolar pH besides Cl^- conductance. Investigators have found that some lysosomes and endocytic vesicles have K^+ conductances that can support submaximal ΔpH formation (Harikumar and Reeves, 1983; Galloway *et al.* 1983), allowing charge compensation for H^+ pumping. K^+ channels have not yet been characterized in intracellular vesicles. An additional mechanism that can regulate pH is the presence of electrogenic pumps other than H^+-ATPase in vacuoles that could generate a $\Delta\Psi$ opposing H^+ translocation. This has been described for 'early endosomes', which have Na^+/K^+-ATPases (Mellman *et al.* 1986; Cain *et al.* 1989).

An important assumption of the analysis presented above is that the proton-translocating V-ATPase has the same fundamental characteristics regardless of its organelle of origin. Based on equations 1–3, the maximum proton gradient generated by the V-ATPase is related to the H^+/ATP stoichiometry and the free energy of ATP hydrolysis in the cytoplasm. Hence, if ATPases generate different gradients it would be due to differences in the stoichiometry since the free energy of ATP hydrolysis is the same for all organelles. However, because the H^+/ATP stoichiometry is a fundamental property of the ATPase, it is likely, though not proven, that it is the same in all organelles.

Measurement of the pH difference and membrane potential *in situ* and in isolated vesicles is technically feasible. Qualitative studies showing that treatment which increases or decreases the pH or potential are relatively straightforward and highly informative. Absolute quantification, however, is still problematic. The actual value of the pH *in situ* or *in vitro* is a significant issue that needs to be addressed. We do not think that the technology is available to give absolute quantification with assurance.

A variety of organelles of the endocytic and secretory pathways contain a chloride conductance that is only now being characterized by direct measurement of channel properties. Endosomes from rat kidney cortex, fused by a dehydration/rehydration cycle and patched-clamped (Schmid *et al.* 1989), were found to contain an anion-selective conductance of 79 pS that is sensitive to anion channel blockers, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS), 4-acetamido-4′ isothiocyanostilbene-2,2′-disulfonic acid (SITS) and *N*-(propylphenyl)-*S*-nitroanthranilic acid (NPPB). The channel differs from those in the outer membrane of mitochondria (Colombini, 1983) and in endoplasmic reticulum (Schmid *et al.* 1988) by having a high selectivity for Cl^- over K^+. It has a linear current–voltage relationship and the kinetics of channel activity were not altered by Ca^{2+}, pH (7–7.8), ATP or the catalytic subunit of protein kinase A in this preparation.

Biochemical studies of a vacuolar chloride channel

We have used the indanyloxyacetic acids (IAAs) as inhibitory ligands for epithelial chloride channels (Landry *et al.* 1987) and identified one of them (IAA-94) as a ligand that had an inhibitory and binding potency in the micromolar range. Using an IAA affinity column, we purified four proteins from bovine kidney cortex which, when incorporated into liposomes, resulted in the appearance of voltage-sensitive chloride uptake (Landry *et al.* 1989). When these liposomes were fused with planar lipid bilayers, Cl^- channels were observed. One of these proteins, a 64×10^3 M_r protein (p64) elicited a monospecific antiserum which immunodepleted all reconstitutable chloride channel activity from solubilized bovine renal cortex membranes (Redhead *et al.* 1992). This antibody stained the apical membrane and intracellular organelles of epithelial cells. It also recognized proteins with similar apparent relative molecular masses in a variety of epithelial and non-epithelial cells from different species. The other proteins purified by the IAA affinity column were identified by their N-terminal sequence to be proteins that are known to be inhibited by ethacrynic acid, the parent structure of IAA. Hence, we concluded that they were drug-binding proteins rather than components of the channel. The sequence of p64 has now been completed and shows no homology to other known proteins. By Northern blot analysis it has also been shown to be present in a variety of epithelial and non-epithelial cell lines and tissues. Recent studies show that injection of the mRNA for p64 results in the formation of a new protein in *Xenopus* oocytes. Interestingly, the protein does not travel to the surface membrane, suggesting that it does not have targetting sequences for that membrane or that it has a dominant retention signal for intracellular organelles.

Recent studies have shown that brain clathrin-coated vesicles and kidney endosomes

respond to cyclic-AMP-dependent protein kinase by opening a Cl^- channel and phosphorylating a protein with a similar relative molecular mass ($66 \times 10^3 M_r$) (Bae and Verkman, 1990; Mulberg *et al.* 1991; Reenstra *et al.* 1992). These results suggest that the p64 that we purified might be the intracellular Cl^- channel. That we were able to show immunoreactivity in the apical membrane implies that one of the apical Cl^- channels is also related to this protein. An interesting question now is to relate p64 to CFTR. Since it is clear that tissues that do not express CFTR, e.g. the brain, have intracellular Cl^- channels, it follows that CFTR is not necessary for Golgi acidification. However, the situation may be different in cells that express CFTR, where this protein could be the major Cl^- conductance in intracellular organelles.

Regulation of the vacuolar chloride channel

Chloride channels are expected to have dramatic effects on regulation of the vacuolar pH because their conductance is so high. A single molecule of a channel can conduct 10^6–10^8 ions s^{-1}. The purified proton-translocating ATPase has a turnover number of not more than 100 ions s^{-1} $molecule^{-1}$. Hence, the membrane potential generated by 1000 ATPases per vesicle (clearly an upper limit) can be collapsed by a single molecule of a Cl^- channel that is open only 10 % of the time or even less. It is therefore likely that each vesicle contains a single chloride channel.

Recently we demonstrated that changes in the Cl^- conductance of a secretory granule can result in acidification of these organelles. We found that the parafollicular cells of the thyroid contain granules which did not generate a large pH difference (Barasch *et al.* 1988). These studies were performed *in situ*. When the granules were isolated and studied *in vitro*, we found that addition of ATP to the outside did not result in acidification of the granules. However, addition of valinomycin caused the vesicles to acidify, suggesting that the conductance of the membrane was limiting acidification. Treatment of parafollicular cells with secretogogues stimulated the acidification of granules *in situ*, as measured by weak base (DAMP) electron microscopic immunocytochemistry (Anderson *et al.* 1984; Anderson and Pathak, 1985). Also, granules isolated from treated cells were able to acidify and only marginally increased ΔpH when valinomycin was added, indicating that the stimulated vesicles had generated an increased ΔpH at the expense of $\Delta\Psi$. The stimulated acidification was due to an increased Cl^- conductance, since these granules did not acidify in gluconate buffers. Further, granules from stimulated cells displayed an increased flux of $^{36}Cl^-$ compared with granules from untreated cells, which had little if any Cl^- transport. These results demonstrate that physiological stimuli can change the Cl^- conductance and therefore pH in intracellular organelles, suggesting that such changes might play a role in the normal function of these organelles.

Bae and Verkman (1990) recently found that the Cl^- conductance of kidney endosomes controlled vacuolar pH. Phosphorylation with protein kinase A and ATP caused rapid acidification, whereas alkaline phosphatase treatment decreased ΔpH formation. These effects were not due to stimulation or inhibition of the V-ATPase, since acidification in the presence of valinomycin was not changed by prior treatment with kinases. Similar studies were performed with brain clathrin-coated vesicles (Mulberg *et*

al. 1991). Regulation of vesicle acidity by counterion conductance may be found in a number of organelles since a number of other investigators have also found increased intracellular vesicle acidification in response to secretogogues. These include intracellular canaliculi in parietal cells, which acidify in response to histamine by regulation of K^+ and Cl^- conductive pathways in parallel with H^+ pumping (Cupoletti and Sachs, 1985; Dibona *et al.* 1979), and islet cell β granules, which acidify in response to glucose (Pace and Sachs, 1982). There is also a report of secretogogue-induced changes in pancreatic granule conductance for Cl^-, although ion conductances were not measured directly (Gasser *et al.* 1986).

In the chloride channel of kidney cortex vesicles we found that addition of ATP caused a reduction in the chloride conductance. This effect was observed only at room temperature or higher. Indeed, simply raising the temperature increased the conductance, suggesting that the membranes contain phosphatases. To document this suggestion further, addition of ATP-γ-S was found to produce a larger inhibition. This nucleotide can be used by kinases to phosphorylate proteins, but protein phosphatases cannot readily remove the thiophosphate. These results suggest that, in this system, the vesicles contain a kinase and a phosphatase that can modulate the chloride conductance. The nature of this inhibitory kinase is not clear at present (Landry *et al.* 1987).

Role of vacuolar pH in cell biology

Why then is vacuolar pH tightly regulated by a counterion conductance or by other means (Mellman *et al.* 1986)? Functions ascribed to low vacuolar pH include the uptake of small molecules, such as biogenic amines in chromaffin granules (Johnson *et al.* 1982), processing of vacuolar constituents by proteolytic enzymes such as hormones (Hook *et al.* 1982; Orci *et al.* 1987) or protein degradation in lysosomes, and the interaction of ligands and receptors during routing of molecules within the vacuolar system (Klausner *et al.* 1983; Goldstein *et al.* 1985; Dautry-Varsat *et al.* 1983; Mellman *et al.* 1986). These functions have been identified using a variety of methods, many of which required collapse of the pH gradient in intracellular organelles by the use of permeant weak bases (e.g. NH_3 or chloroquine) or proton ionophores such as monensin, nigericin or carbonylcyanide-*p*-(trifluoromethoxy) phenylhydrazone (FCCP). However these drastic treatments may have effects that are independent of vacuolar alkalization. Such effects include the well-documented swelling of the terminal Golgi complex by monensin, which leads to a block in the transport of molecules through the secretory pathway. Swelling of organelles is also to be expected from treatment with large concentrations of weak bases since addition of a weak base to a cell results in the continuous accumulation of the protonated species, with consequent increased osmotic pressure, resulting in swelling. Hence, although there is no doubt that vacuolar pH plays a critical role in organellar function, there is a need for caution in interpreting these kinds of experiments as being due solely to alkalization.

It is interesting to compare the effects of treatments with ionophores and weak bases to mutations in the acidification mechanisms that have been recently identified. These mutations were generated by selection for resistance to toxins (e.g. diphtheria toxin) that

enter the cell by an acidification-dependent mechanism. When analysed in detail, they were found to have defects in endosomal acidification while lysosomal acidification was not affected (Roff *et al.* 1986). It is interesting that the defects in the organellar function of these cells were rather subtle. An important finding was that terminal sialylation, a trans-Golgi function, was found to be defective. This result suggests that a component mediating endosomal acidification also effects Golgi sialylation, perhaps by a similar defect in trans-Golgi acidification. Determination of the origin of the defect in each complementation group of mutants will elucidate the full range of mechanisms controlling vacuolar acidification. It is important to note that these cells did not exhibit the drastic changes seen in protein transport through the secretory pathway that is observed with monensin.

Reduced Cl^- conduction and its effect on vacuolar pH in cystic fibrosis

There is now convincing evidence that the fundamental defect in cystic fibrosis is the lack of activation of chloride channels by the cyclic-AMP-dependent protein kinase (Hwang *et al.* 1989; Frizzell *et al.* 1986; Welsh and Liedtke, 1986; Boucher *et al.* 1989; Li *et al.* 1988; Shoumacher *et al.* 1987. In secretory epithelia, Cl^- enters the cells across the basolateral membrane *via* a $Na^+,K^+/Cl^-$ cotransporter and accumulates in the cell above its electrochemical equilibrium concentration. Chloride channels in the apical membrane have a low open probability and hormones (and agents) that raise cyclic AMP levels increase this probability, leading to electrodiffusion of Cl^- into the luminal medium. This will lead to diffusion of Na^+, either through cells or between cells, and the consequent osmotic secretion of water. Cystic fibrosis (CF) is caused by defects in the cystic fibrosis transmembrane conduction regulator (CFTR) (Rommens *et al.* 1989; Riordan *et al.* 1989; Kerem *et al.* 1989), a transmembrane protein that mediates cyclic-AMP-regulated Cl^- conduction in secretory epithelia (Kartner *et al.* 1991; Anderson *et al.* 1991). The absence of regulated Cl^- transport across the apical membrane decreases transepithelial water secretion. In addition, the apical Na^+ channel of these epithelia is tonically open, which would exacerbate the decreased fluid secretion (Willumsen and Boucher, 1989). As a result, the layers of mucus (Rose, 1988; Boat and Cheng, 1980) that coat the respiratory tree, pancreatic ducts and intestine are dehydrated and difficult to clear. Dehydrated mucus blocks the pancreatic duct, the intestine and the bronchial tree and causes pancreatic insufficiency, 'meconium ileus' and devastating 'chronic obstructive pulmonary disease'. Defective transepithelial secretion of salt and water and the secretion of dehydrated mucus, however, do not suggest a mechanism for two other defining features of CF, chronic bronchitis with *Pseudomonas* species and altered terminal glycosylation of respiratory and gastrointestinal mucins. Changes in the structure of these mucins themselves are likely to be critical in the pathophysiology of CF by increasing the viscosity of secretions (Chace *et al.* 1983, 1985; Gupta *et al.* 1990; Litt *et al.* 1977; Mian *et al.* 1982).

CFTR has a 'mature' glycosylation pattern, suggestive of passage through at least part of the Golgi apparatus. Several recent studies have shown, using immunocytochemistry, that it is located on the apical plasma membrane of many secretory epithelia (see, for

example, Kartner *et al.* 1991). Whether CFTR is present in intracellular organelles remains to be discovered. We discuss here in detail a hypothesis that we recently proposed which argues for the centrality of a defect in an intracellular Cl^- channel in explaining many of the abnormalities in CF (Barasch *et al.* 1991). The mutant form of CFTR (ΔF508 and others) appears not to obtain mature glycosylation, but rather accumulates in the endoplasmic reticulum (ER), where it is degraded (Chen *et al.* 1990). However, recent physiological studies suggest that at least some mutant CFTR reaches the plasma membrane (Drumm *et al.* 1991; Daelmans *et al.* 1991).

If the Cl^- conductance in the vacuolar system is regulated by CFTR, then in CF the loss of counterion conductance would change the relative contributions of ΔpH and $\Delta\Psi$. We studied this question by incubating freshly isolated nasal polyps and immortalized respiratory epithelial cell lines with DAMP (Anderson *et al.* 1984), a weak base that distributes according to pH gradients and can be fixed and visualized by electron microscope (EM) immunocytochemistry. Identification of the organelles in this way posed a significant problem. The Golgi apparatus is easy to recognize because of the characterstic shape of stacks, and the trans-Golgi network was assumed to be the vesicles that were in close proximity to the stacks. Prelysosomes and endosomes (two acidic compartments), however, had to be distinguished from lysosomes. As a rule, lysosomes contained dense-cored material whereas prelysosomes usually have an internal membrane structure. However, prelysosomes or late endosomes have recently been found to be enriched in the mannose-6-phosphate receptor (man-6-PR) whereas the lysosomes are not (Griffiths *et al.* 1988). Fig. 2 shows an example of the problem where two vesicles that have dense cores are both labelled with DAMP but one is enriched for man-6-PR while the other is not.

Using both morphological criteria and simultaneous DAMP and man-6-PR labelling, we found reduced labelling of the Golgi region and prelysosome-like structures in CF, but the tissues had equivalent labelling to lysosomes. These results suggest that the pH of the trans-Golgi network and prelysosomes, but not of lysosomes, was more alkaline in CF.

To confirm these studies, we measured the rate and extent of acidification of isolated light vesicles obtained from respiratory epithelia. We found that acidification was reduced in CF. When valinomycin, an ionophore that is expected to collapse the $\Delta\Psi$, was added the rate of acidification in CF vesicles was stimulated. This result indicates that the V-ATPase in CF vesicles is functional, but that 'proton pumping' is limited by a $\Delta\Psi$. Since a $\Delta\Psi$ can form only in the absence of significant counterion conduction, these results further suggest that Cl^- conductance is diminished in CF vesicles. In fact, acidification of the normal light vesicle fraction was entirely dependent on Cl^- and was independent of K^+, since substitution of potassium gluconate for KCl abolished ΔpH formation. This finding contrasts with the kinetics of acidification in a heavy vesicle fraction, which contains lysosomes, where acidification is not stimulated by valinomycin and is not altered by substituting potassium gluconate for KCl, suggesting that lysosomes have K^+ channels. It is important to note that these assays were performed at high chloride concentrations, which would tend to increase the actual Cl^- conductance, thereby reducing any possible difference between CF and normal vesicles.

These data were supported by a third method of pH estimation in which CF cells were

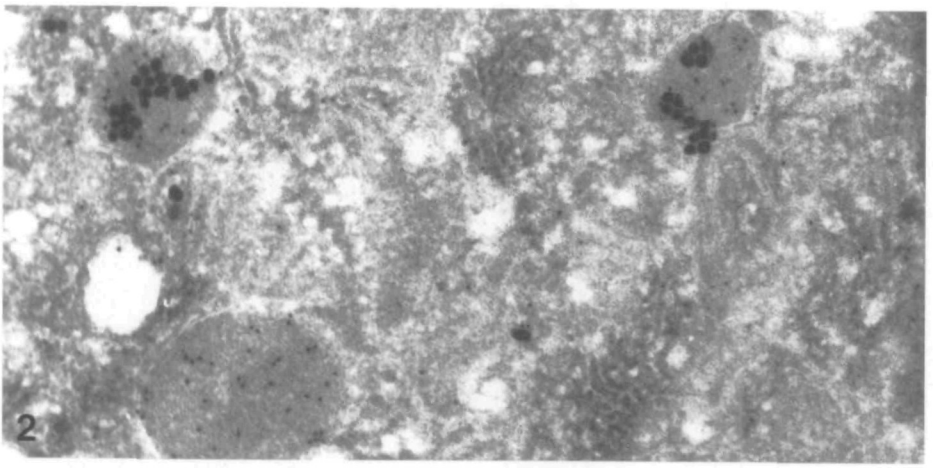

Fig. 2. Localization of the weak base DAMP (small gold particles) and the mannose-6-phosphate receptor (large gold particles) by immunoelectron microscopy in nasal polyp cells. Note that all three vesicles with dense cores accumulate DAMP but only one of them contains the receptor.

loaded with fluorescein-coupled transferrin and the pH of the transferrin-containing vacuoles was estimated. The endosomes were found to be mildly alkalized. Hence, using three independent methods of analysis, we have found that CF cells show a reduced rate of acidification in some vesicles. Recent unpublished studies were performed in CF-PAC cells, a pancreatic duct cell line derived from a patient with cystic fibrosis and cancer of the pancreas. These cells were transfected with a vector carrying the wild-type CFTR gene. We were able to confirm the defect in acidification in the CF-PAC cell compared to the those transfected with the wild-type gene. Hence, we now have evidence in three independent cell types, the primary culture of nasal polyps, the immortalized airway epithelial cell line and the CF-PAC cell lines.

We were unable to test the effect of cyclic AMP on the intravesicular pH since the addition of the second messenger resulted in a dramatic reorganization of the cytoplasm so that the identity of the vesicles could not be guaranteed. We do not know the actual concentration of cyclic AMP in these cells but, assuming that it is at a basal level, these data suggest that the mutant CFTR has a lower conductance than the wild-type molecule. This defect could be due to a decreased open probability of the unstimulated mutant CFTR. It should be emphasized that we do not know the open probability of the unstimulated wild-type or mutant CFTR. All we know is that protein kinase A increases it from a very low level to a higher level. Recent studies by Daelmans *et al.* (1991) show that the open probability of the stimulated mutated CFTR was much lower than that of the wild type. This observation is compatible with the idea that the unstimulated open probability of the mutant would also be lower, but no direct evidence for this exists at present. To function in the collapse of a membrane potential, unstimulated CFTR may have enough conductance. Using the calculation mentioned above, a channel needs to be

open for only a small percentage of the time to collapse the membrane potential generated by many proton pumps.

If CFTR is the chloride channel or a closely associated protein, then we would expect the trans Golgi and other vesicles to contain CFTR in normal cells and their acidification should be dependent on cyclic AMP. However, the sensitivity of detection by immunocytochemistry will probably be below the detection limit. As mentioned above, only one channel per vesicle can suffice to generate enough counterion conductance. These considerations raise a number of questions regarding the role of chloride channels in the regulation of Golgi pH in cells that do not express CFTR. It is well known that such cells can regulate the pH of endocytic vesicles by a chloride conductance; what is the nature of that chloride channel? And can one generate an acidification defect in these cells by transfection with the mutant CFTR? Are there additional, non-CFTR-dependent Cl^- conductances in epithelial organelles that may partially compensate for the CF defect? Does cyclic AMP increase the difference in Cl^- conduction between CF and control cells, manifest as increased vacuolar acidification in living cells? If so, what is the role of HCO_3^--permeability through the open chloride channel? This is an especially relevant question in cells that secrete base, such as the pancreatic duct, where the intracellular HCO_3^- concentration is expected to be as high as or higher than the Cl^- concentration. Do all the cells that have a demonstrable CF defect have defects in Golgi acidification? These are all important questions that will need to be answered by direct experiments.

Vacuolar pH, glycosylation and cystic fibrosis

Many investigators have found that CF mucins from the respiratory (Boat *et al.* 1974, 1976; Boat and Cheng, 1980; Chace *et al.* 1983; Frates *et al.* 1983; Koomans *et al.* 1986) and gastrointestinal (Clamp and Gough, 1979; Dische *et al.* 1959; Wesley *et al.* 1983) tracts and from epithelial explants have alterations in terminal glycosylation compared to mucins obtained from normal subjects or patients suffering from other types of chronic lung disease. A phenotype found by many, though not all, investigators includes a marked increase in sulfate content, a higher fucose content and less sialic acid in CF mucins and other glycoproteins (glycocalyx, Cheng *et al.* 1989). Respiratory mucins from CF patients have shown the most variable and minor changes, whereas secretions of explants of respiratory epithelium and particularly gastrointestinal mucins are consistent in showing alterations to these terminal capping reactions (see also Rose, 1988; Alhadeff, 1978; Scanlin *et al.* 1985). The variability in respiratory mucins is perhaps due to secondary effects of infection or problems of sampling (Houdret *et al.* 1989). As in studies of explants of respiratory tissues, we found that the CF cell line generated by Gruenert *et al.* (1990) used in the studies of vacuolar pH had decreased sialylation of secreted proteins but an identical pattern of protein secretion (Barasch *et al.* 1991).

Sialylation, sulfation and fucosylation of glycoproteins and mucins are late steps in the glycosylation pathway which grossly co-localize in the vacuolar system (Fig. 3). 2,6-Sialyltransferase was localized by EM immunocytochemistry (Taatjes *et al.* 1988) in the trans-Golgi network of intestinal epithelium and in distal structures, such as mucus droplets, 'multivesicular bodies', 'lysosome-like structures' and even plasma membrane

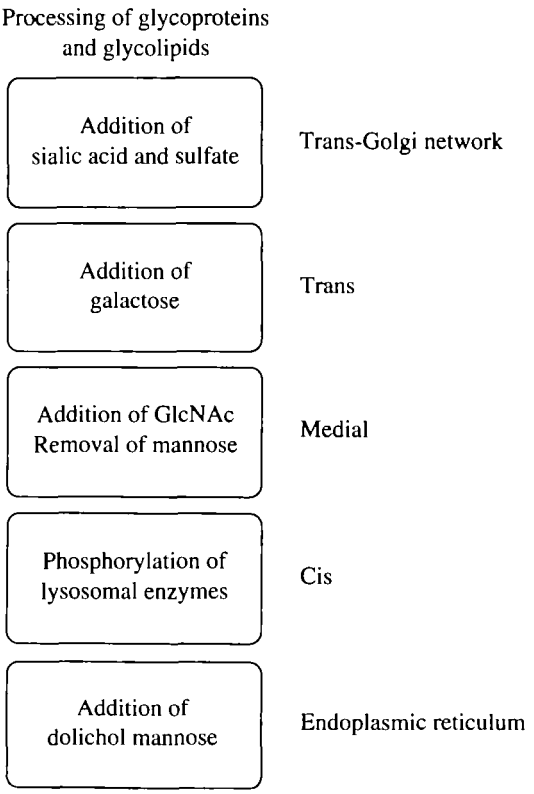

Fig. 3. Compartmentalization of Golgi functions in different stacks. Only the trans-Golgi network and in some cells the trans Golgi are acid.

of enterocytes and goblet cells. The post-Golgi localization may be due to release and secretion of inactive enzyme, since sialylation occurs in the trans Golgi, as determined by pulse–chase EM radioautography after injection of the sialic acid precursor *N*-acetylmannosamine (Bennett *et al.* 1981; Bennett and O'Shaughnessy, 1981). Fucosyltransferases may also be localized to vesicles containing sialyltransferase, since tritiated fucose distributes in the same way as sialic acid, principally to the Golgi apparatus and later throughout the vacuolar system (Bennett and Leblond, 1971; Bennett *et al.* 1974). In addition, a specific carrier for the fucose donor, GDP-fucose, is found in purified Golgi vesicles that also take up the sialic acid donor CMP-sialic acid (Capasso and Hirschberg, 1984*a,b*). The localization of sulfotransferase is also in the distal Golgi, since the sulfotransferase substrate, terminal galactose, is itself added late in glycosylation. In addition, sulfotransferase activity is concentrated in light vesicles from thyroid glands, consistent with its localization in the Golgi (Capasso and Hirschberg, 1984*a,b*; Kato and Spiro, 1989). Furthermore, a specific carrier for the sulfate donor adenosine 3′-phosphate-phosphosulfate (PAPS) is found in the same population of Golgi vesicles as sialyl and fucosyl transporters in a compartment containing the trans-Golgi marker thiaminepyrophosphatase (Capasso and Hirschberg, 1984*a,b*; Kato and Spiro, 1989).

There is much evidence to suggest that terminal glycosylation reactions are competitive for substrate. Oligosaccharides from clinical isolates (Roussel *et al.* 1975; Mawhinney *et al.* 1987; Lamblin *et al.* 1984) contain either sialic acid or sulfate, but generally not both, and model N-linked or O-linked oligosaccharides directly demonstrate exclusive sulfation or sialylation. For example, desialylated thyroglobulin or fetuin can be sulfated, whereas the sialylated forms are very poor substrates (Kato and Spiro, 1989). In fact, efficient sulfation is possible for disaccharides with exposed terminal galactose, suggesting that sulfation depends on available galactose rather than on the recognition of a peptide or mucin structure. Similarly, fucosyl- and sialyltransferases may compete for substrates. Desialylated transferrin, for example, can be resialylated by β-galactoside α-2,6-sialyltransferase or fucosylated by *N*-acetyl-glucosamine 1,3-fucosyltransferase, but the reactions were mutually exclusive (Beyer *et al.* 1979). Furthermore, lactoferrin, which is fucosylated, is a poor substrate for sialylation and, conversely, transferrin, which is sialylated, is a poor substrate for fucosylation. In fact, in nine attempts to fucosylate a sialylated molecule or sialylate a fucosylated molecule, eight reactions did not occur (Beyer *et al.* 1979; Paulson *et al.* 1978). Similar observations have been obtained from a wide range of tissues, including rat colonic mucins (Slomiany *et al.* 1990), pig liver α-acid glycoprotein (Jabbal and Schachter, 1971) and brain transferases (Baubichon-Cortay *et al.* 1983). If these mechanisms are generally applicable to mucins, then decreased sialylation may cause or be the result of increased fucosylation and might explain the predominance of a unique sialofucosylmucin in CF. These oligosaccharides contain a 'permitted' combination of internal fucose and terminal sialic acid (sialic acid 2,3-galβ1-4(fuc,3)glcNAc; Lamblin *et al.* 1984), whereas sialomucins not from CF contain sialic acid 2,6-galNAc or 2,6-gal linkages that are prohibited with increased internal fucosylation, which apparently occurs in CF.

We hypothesize that increased Golgi pH mediates the increased sulfation and fucosylation and decreased sialylation of CF mucins. Transferase enzymes have different pH optima (Fig. 4). Sialyltransferase enzymes have acid pH optima, particularly mucin sialyltransferase (2-6-galNAc linkage, pH 5.8) (Roseman *et al.* 1989; Carlson *et al.* 1973*a,b*; Baubichon-Cortay *et al.* 1983; Sherblom *et al.* 1986), whereas fucosyltransferases have activity at generally higher pH optima (7–8.5 for milk, mucin and liver fucosyltransferase; Jabbal and Schachter, 1971; Prieels *et al.* 1977; Bosman *et al.* 1968) as do sulfotransferases (pH 6.8–7; Kato and Spiro, 1989; Slomiany *et al.* 1987; Carter *et al.* 1988). Thus, as can be seen in Fig. 4, small changes in Golgi pH might alter the relative efficiency of the three terminal glycosylation enzymes and change the proportion of asialo/sialo and sulfated mucins.

Changes in ΔpH and $\Delta\Psi$ might also affect substrate availability, depending on the bioenergetics of the transport process for the sugar and sulfate donors. The donors are driven into Golgi vesicles by exchange of the nucleotide sugar for the uncoupled nucleoside monophosphate. These processes are thought to be energy-independent (Capasso and Hirschberg, 1984*b*); however, Golgi pH and membrane potential have not been rigorously manipulated, and sulfate transport may be electrogenic. Substrate availability would modulate enzyme activity since these reactions have high K_m values

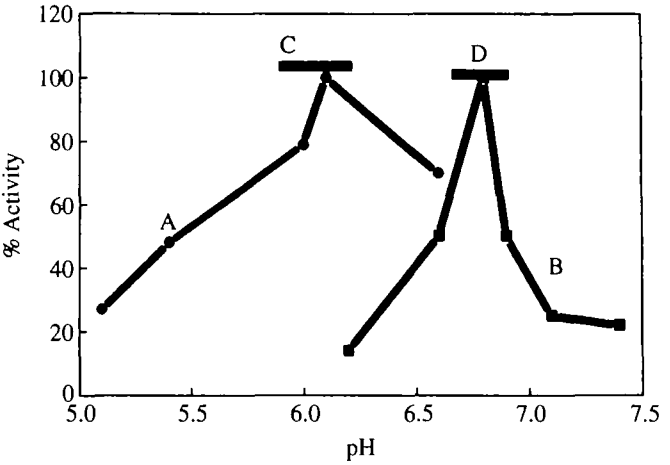

Fig. 4. The pH optima of glycoprotein transferases. A, 2-6 sialyltransferase; B, 6-galNAc sulfotransferase; C, 1-2Gal fucosyltransferase; D, 2-3 sialyltransferase.

[0.5 mmol l^{-1} for sialyltransferases (Roseman *et al.* 1989), 80 μmol l^{-1} for fucosyltransferase (Jabbal and Schachter, 1971), 5 μmol l^{-1} for sulfotransferase (Carter *et al.* 1988)]. In preliminary studies we found that the uptake of the sulfate donor PAPS was electrogenic. Vesicles loaded with KCl were passed through an anion exchange column to remove extravesicular chloride (Garty *et al.* 1983; Landry *et al.* 1987). This procedure generates a membrane potential (inside positive) only in vesicles that contain chloride channels. Addition of ^{35}S-labelled PAPS resulted in a time-dependent uptake of the nucleotide sulfate. However, addition of valinomycin, to collapse the membrane potential, resulted in inhibition of the uptake, indicating that a membrane potential can drive this process. Closure of the chloride channel in CF would be expected to polarize the Golgi apparatus so that its interior would be more positive. We suggest that this will contribute to enhanced sulfation by increasing the uptake of PAPS.

Potential generation of a Pseudomonas *receptor*

Alkalization of the Golgi might also provide an explanation for the universal occurrence of *Pseudomonas* infection in CF. Chronic colonization by bacteria in a tissue appears to require adherence of the organism to a cell surface receptor. The presence of a receptor for *Pseudomonas* in CF airways is suggested by a sevenfold increase in numbers of *Pseudomonas* (rough form) adhering to buccal cells of CF patients compared with control cells and a twofold increased adherence by mucoid species (Woods *et al.* 1980). Adherence was increased by treatment with neuraminidase, suggesting that the buccal receptor is an asialo glycoprotein or glycolipid. Two potential *Pseudomonas* receptors are asialo-GM_1 and asialo-GM_2, since multiple species of *Pseudomonas* and other respiratory pathogens such as *Haemophylus influenza* and *Staphylococcus aureus* (Krivan *et al.* 1988*a,b*) (the latter are found early in CF), but not non-respiratory pathogens, avidly bind these compounds. The binding was specific in that the pathogens

bound neither other model compounds nor GM_1 or GM_2. These data suggest that undersialylation uncovers a bacterial receptor and thus may encourage pulmonary infection. Fig. 5 shows an abbreviated diagram of ganglioside biosynthesis. Fig. 6 shows the pH optima of some of the relevant enzymes redrawn from the published literature. Increased synthesis of asialo gangliosides might occur by Golgi alkalization, because conversion of asialo-GM_3 to GM_3 requires a sialyltransferase with a pH optimum of 5.7 (Busant and Decker, 1986; Richardson *et al.* 1977) but the conversion of asialo-GM_3 to the asialo pathway (asialo-GM_2, asialo-GM_1) requires UDP-galNAc transferase with a pH optimum of 7.3 (Senn *et al.* 1981; Pohlentz *et al.* 1988). Using the immortalized cell lines, we found that that there was a larger amount of asialo-GM_1 in cells of CF patients compared to the normal controls (Barasch *et al.* 1991). However, ganglioside

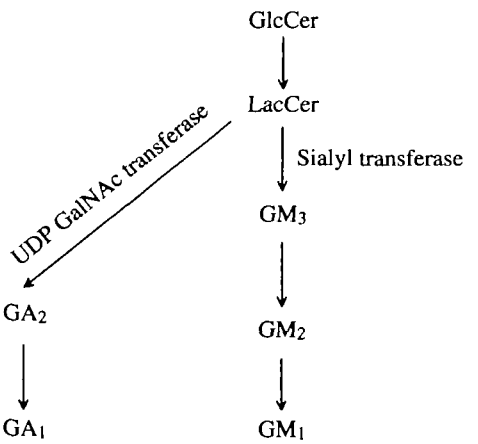

Fig. 5. An abbreviated map of ganglioside biosynthesis.

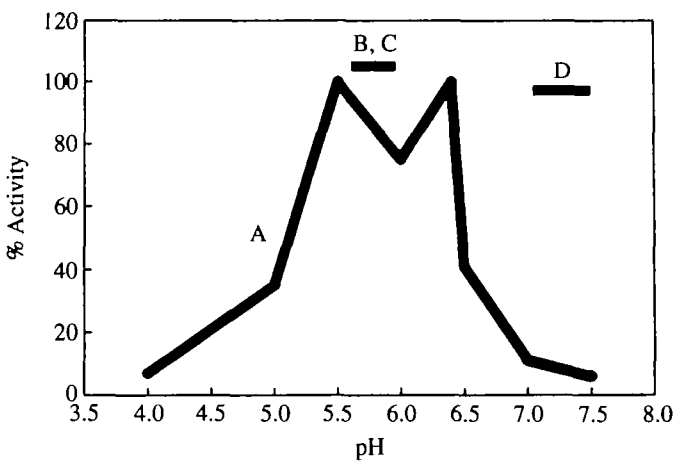

Fig. 6. The pH optima of the ganglioside biosynthetic enzymes. A, LacCer sialyltransferase; B, GD3 synthase; C, GM_3 sialyltransferase; D, LacCer UDP-GalNAc transferase.

biosynthesis is frequently altered in cells that have been immortalized; hence, a complete analysis of ganglioside biosynthesis in CF awaits a study of non-immortalized cell lines.

Further studies are needed to validate our hypothesis that changes in Golgi pH induced by low Cl^- conductance cause undersialylation and oversulfation. These studies would include the following. (1) To determine the precise oligosaccharide structure of CF mucin obtained from mucin-secreting cell lines. (2) To analyse the contribution of ΔpH and $\Delta\Psi$ to the uptake of sugar and sulfate donors in membranes capable of generating these gradients. (3) To analyse the effect of pH in modifying the interaction of sialyl, fucosyl and sulfur transferase enzymes by use of model oligosaccharides and Golgi fractions in competition reactions. (4) To analyse the effect of pH on ganglioside biosynthesis. (5) Most importantly, many of these studies should be performed in CF cells and compared with an appropriately matched control cell, particularly CF cells that have been 'cured' by the transfection of wild-type CFTR. Of course, one has to be aware of the problem that over-expression of CFTR might introduce its own changes in the biosynthesis of proteins.

Which functions will be affected by defective vacuolar acidification in cystic fibrosis?

The secretory pathway in cells subserves many important functions. Since the majority of functions in many organs seem to be normal, the defect in CF must be rather subtle. Hence, it is worth analyzing in some detail the functions that would be expected to change given that the pH change observed is not marked. To have an impact on vacuolar function, the pH-sensitive process must meet the following three criteria.

(1) *The pH dependence of the reaction has to be steep at the actual pH of the normal organelle.* Any process whose pH optimum is 'flat' or outside the range of changes induced by CF will not be affected. For instance, an increase in pH of endosomes may have no effect on iron unloading by transferrin. Such a process, although sensitive to pH, will require larger changes to have a significant effect.

Another type of argument might be applied to the case of intoxication by diphtheria toxin which is known to be pH-sensitive. Measurement of the effect of a potent toxin such as diphtheria toxin is performed by adding different concentrations of the toxin to cells, waiting for different periods and then measuring protein synthesis at the end of the experiment. Diphtheria toxin binds to the cell membrane *via* its B subunit, which is then internalized into an acidic compartment. At low pH, the A subunit penetrates into the cytoplasm. The A subunit of diphtheria toxin ADP-ribosylates an elongation factor, thereby stopping protein synthesis. Being an enzyme, one molecule of toxin is sufficient to kill a single cell. Because of this characteristic, the kinetics of the reaction resembles that of a 'dead end' reaction of a potent irreversible inhibitor. In irreversible inhibition, any concentration, however low, could lead to 100% inhibition. The only effect of lowering the concentration is to delay the appearance of the 100% inhibition (or any other measured effect). If CF cells are more resistant to diphtheria toxin, demonstration of resistance will depend on the dose of toxin added. At high doses, all cells will die quickly. At lower concentrations, CF cells will take a little longer to die because the number of

molecules entering the cell will be less than in the wild-type. Hence, as the concentration of toxin is reduced, the difference between wild-type and CF cells will increase.

(2) *An increased pH, by reducing the rate of the reaction, must allow a competing process to occur or the original reaction to remain incomplete.* A change in the rate of the reaction will produce an effect on the substrate only if the substrate rapidly traverses the compartment which contains the enzyme. Modification of resident proteins or slowly moving proteins will take longer to be completed. Since the transport of secreted proteins is generally rapid we expect that this criterion would be frequently met. We believe that this mechanism may explain why the sialylation of a large number of secreted proteins was found to be abnormal in CF cells. This also might provide the explanation for the increased sulfation.

(3) *Alteration in the described reaction, e.g. glycosylation, must change the characteristic function of the protein or uncover a new function for the affected protein.* Since it is known that removal of carbohydrates may have no effect on a variety of protein functions, it is not surprising that the functional consequences of the CF defect induced by Golgi alkalization are not widespread and severe. All secreted mucus glycoproteins may be oversulfated and undersialylated, but all it takes to produce the pathological consequences of CF is that the viscosity of one protein be changed by oversulfation and undersialylation.

References

AL-AWQATI, Q. (1986). Proton translocating ATPases. *A. Rev. Cell Biol.* **2**, 179–199.

ALHADEFF, J. H. (1978). Glycoproteins and cystic fibrosis: A review. *Clin. Genetics* **14**, 178–201.

ANDERSON, M. P., RICH, D. P., GREGORY, R. J., SMITH, A. E. AND WELSH, M. J. (1991). Generation of c-AMP activated chloride currents by expression of CFTR. *Science* **251**, 679–682.

ANDERSON, R. G. W., FALK, J. R., GOLDSTEIN, J. L. AND BROWN, M. S. (1984). Visualization of acidic organelles in intact cells by electron microscopy. *Proc. natn. Acad. Sci. U.S.A.* **81**, 4838–4842.

ANDERSON, R. G. W. AND PATHAK, R. K. (1985). Vesicles and cisternae in the trans-Golgi apparatus of human fibroblasts are acidic compartments. *Cell* **40**, 635–643.

BAE, H.-R. AND VERKMAN, A. S. (1990). Protein kinase A regulates chloride conductance in endocytic vesicles from proximal tubule. *Nature* **348**, 637–639.

BARASCH, J., GERSHON, M. D., NUNEZ, E. A., TAMIR, H. AND AL-AWQATI, Q. (1988). Thyrotropin induces the acidification of the secretory granules of parafollicular cells by increasing the chloride conductance of the granular membrane. *J. Cell Biol.* **107**, 2137–2147.

BARASCH, J., KISS, B., PRINCE, A., SALMAN, L., GRUENERT, D. AND AL-AWQATI, Q. (1991). Defective acidification of intracellular organelles in cystic fibrosis. *Nature* **352**, 70–73.

BAUBICHON-CORTAY, H., SERRES-GUILLAUMOND, M., LOUISOT, P. AND BROQUET, P. (1983). A brain sialyltransferase having a narrow specificity for *o*-glycosyl-linked oligosaccharide chains. *Carbohydrate Res.* **149**, 209–223.

BEAR, C. E., LI, C., KARTNER, N., BRIDGES, R. J., JENSEN, T. J., RAMJEESINGH, M. AND RIORDAN, J. R. (1992). Purification and functional reconstitution of the cystic fibrosis transmembrane conductance regulator. *Cell* **68**, 809–818.

BENNETT, A. B. AND SPANSWICK, R. M. (1983). Solubilization and reconstitution of an anion sensitive H-ATPase from corn root. *J. Membr. Biol.* **75**, 21–31.

BENNETT, G., KAN, F. W. K. AND O'SHAUGHNESSY, D. (1981). The site of incorporation of sialic acid residues into glycoproteins and the subsequent fates of these molecules in various rat and mouse cell types as shown by radioautography after injection of [^{3}H]*N*-acetylmannosamine. II. Observations in tissues other than liver. *J. Cell Biol.* **88**, 16–28.

BENNETT, G. AND LEBLOND, C. P. (1971). Passage of fucose-^{3}H label from the Golgi apparatus into dense

and multivesicular bodies in the duodenal columnar cells and hepatocytes of the rat. *J. Cell Biol.* **51**, 875–881.

BENNETT, G., LEBLOND, C. P. AND HADDAD, A. (1974). Migration of glycoprotein from the Golgi apparatus to the surface of various cell types as shown by radioautography after labeled fucose injection into rats. *J. Cell Biol.* **60**, 258–284.

BENNETT, G. AND O'SHAUGHNESSY, D. (1981). The site of incorporation of sialic acid residues into glycoproteins and the subsequent fates of these molecules in various rat and mouse cell types as shown by radioautography after injection of [^{3}H]*N*-acetylmannosamine. I. Observation in hepatocytes. *J. Cell Biol.* **88**, 1–15.

BEYER, T. A., REARICK, J. I., PAULSON, J. C., PRIEELS, J.-P., SADLER, J. E. AND HILL, R. L. (1979). Biosynthesis of mammalian glycoproteins. *J. biol. Chem.* **254**, 12531–12541.

BOAT, T. F. AND CHENG, P. W. (1980). Biochemistry of airway mucus secretions. *Fedn Proc. Fedn Am. Socs exp. Biol.* **39**, 3067–3074.

BOAT, T. F., CHENG, P. W., IYER, R. N., CARLSON, D. M. AND POLONY, I. (1976). Human respiratory tract secretions. Mucous glycoproteins of nonpurulent tracheobronchial secretions and sputum of patients with bronchitis and cystic fibrosis. *Archs Biochem. Biophys.* **177**, 95–104.

BOAT, T. F., KLEINERMAN, J. I., CARLSON, D. M., MALONEY, W. H. AND MATTHEWS, L. W. (1974). Human respiratory tract secretions. I. Mucous glycoproteins secreted by cultured nasal polyp epithelium from subjects with allergic rhinitis and with cystic fibrosis. *Am. Rev. resp. Disease.* **110**, 428–441.

BOSMAN, H. B., HAGOPIAN, A. AND EYLAR, E. H. (1968). Glycoprotein biosynthesis: Characterization of two glycoprotein fucosyl transferase in Hela cells. *Archs Biochem. Biophys.* **128**, 470–481.

BOUCHER, R. C., CHENG, E. H. C., PARADISO, A. M., STUTTS, M. J., KNOWLES, M. R. AND EARP, S. H. (1989). Chloride secretory response of cystic fibrosis human airway epithelia. Preservation of calcium but not protein kinase C and A dependent mechanisms. *J. clin. Invest.* **84**, 1424–1431.

BUSAM, K. AND DECKER, K. (1986). Ganglioside biosynthesis in rat liver. Characterization of three sialyltransferases. *Eur. J. Biochem.* **160**, 23–30.

CAIN, C. C., SIPE, D. M. AND MURPHY, R. F. (1989). Regulation of endocytic pH by the Na^+,K^+-ATPase in living cells. *Proc. natn. Acad. Sci. U.S. A.* **86**, 544–548.

CAPASSO, J. M. AND HIRSCHBERG, C. B. (1984*a*). Effect of a tractylosides, palmitoyl coenzyme A and anion transport inhibitors on translocation of nucleotide sugars and nucleotide sulfate into Golgi vesicles. *J. biol. Chem.* **289**, 4263–4266.

CAPASSO, J. M. AND HIRSCHBERG, C. B. (1984*b*). Mechanisms of glycosylation and sulfation in the Golgi apparatus: Evidence for nucleotide sugar/nucleoside monophosphate and nucleotide sulfate/nucleoside monophosphate antiports in the Golgi apparatus membrane. *Proc. natn. Acad. Sci. U.S.A.* **81**, 7051–7055.

CARLSON, D. M., JOURDIAN, G. W. AND ROSEMAN, S. (1973*a*). The sialic acids. XIV. Synthesis of sialyllactose by a sialyltransferase from rat mammary gland. *J. biol. Chem.* **218**, 5742–5749.

CARLSON, D. M., MCGUIRE, E. J., JOURDIAN, G. W. AND ROSEMAN, S. (1973*b*). The sialic acids. XVI. Isolation of a mucin sialyltransferase from sheep submaxillary gland. *J. biol. Chem.* **248**, 5763–5773.

CARTER, S. R., SLOMIANY, A., GWOZDZINSKI, K., LIAU, Y. H. AND SLOMIANY, B. L. (1988). Enzymatic sulfation of mucus glycoprotein in gastric mucosa. Effect of ethanol. *J. biol. Chem.* **263**, 11977–11984.

CHACE, K. V., FLUX, M. AND SACHDEV, G. P. (1985). Comparison of physicochemical properties of purified mucus glycoproteins isolated from respiratory secretions of cystic fibrosis and asthmatic patients. *Am. chem. Soc.* **24**, 7334–7341.

CHACE, K. V., LEAHY, D. S., MARTIN, R., CARUBELLI, R., FLUX, M. AND SACHDEV, G. P. (1983). Respiratory mucous secretions in patients with cystic fibrosis, relationship between levels of highly sulfated mucin component and severity of the disease. *Clin. chim. Acta* **132**, 143–155.

CHEN, S. H., GREGORY, R. J., MARSHALL, J., PAUL, S., SOUZA, D. W., WHITE, G. A., O'RIORDAN, C. R. AND SMITH, A. E. (1990). Defective intracellular transport and processing of CFTR is the molecular basis of most cystic fibrosis. *Cell* **63**, 827–834.

CHENG, P. W., BOAT, T. F., CRANFILL, K., YANKASKAS, J. R. AND BOUCHER, R. C. (1989). Increased sulfation of glycoconjugates by cultures of nasal epithelial cells from patients with cystic fibrosis. *J. clin. Invest.* **84**, 68–72.

CLAMP, J. R. AND GOUGH, M. (1979). Study of the oligosaccharide units from mucus glycoproteins of

meconium from normal infants and from cases of cystic fibrosis with meconium ileus. *Clin. Sci.* **57**, 445–451.

COLOMBINI, M. (1983). Purification of VDAC (voltage-dependent anion selective channel) from rat liver mitochondria. *J. Membr. Biol.* **74**, 115–121.

CUPOLETTI, J. AND SACHS, G. (1985). Regulation of gastric acid secretion *via* modulation of a chloride conductance. *J. biol. Chem.* **259**, 14952–14959.

DAELMANS, W., BABRY, P., CHAMPIGNY, G., JALLAT, S., DOTT, K., DREYER, D., CRYSTAL, R. G., PAVIRANI, A., LECOCQ, J.-P. AND LADZUNSKI, M. (1991). Altered chloride ion channel kinetics associated with the ΔF508 cystic fibrosis mutation. *Nature* **354**, 526–528.

DAUTRY-VARSAT, A., CIECHANOVER, A. AND LODISH, H. F. (1983). pH and the recycling of transferrin during receptor-mediated endocytosis. *Proc. natn. Acad. Sci. U.S.A.* **80**, 2258–2262.

DIBONA, D. R., ITO, S., BERGLINDH, T. AND SACHS, G. (1979). Cellular site of gastric acid secretion. *Proc. natn. Acad. Sci. U.S.A.* **76**, 6689–6693.

DISCHE, Z., DISANT'AGNESE, P. A., PALLAVICINI, C. AND YOULOS, J. (1959). Composition of mucoprotein fractions from duodenal fluid of patients with cystic fibrosis of the pancreas and from controls. *Pediatrics* **24**, 74–91.

DRUMM, M. L., WILKINSON, D. J., SMIT, L. S., WORRELL, R. T., STRONG, T. V., FRIZZELL, R. A., DAWSON, D. C. AND COLLINS, F. S. (1991). Chloride conductance expressed by the ΔF508 and other mutant CFTR's in *Xenopus* oocytes. *Science* **254**, 1797–1799.

FRATES, R. C., JR, KAIZU, T. T. AND LAST, J. J. (1983). Mucus glycoproteins secreted by respiratory epithelial tissue from cystic fibrosis patients. *Pediatr. Res.* **17**, 30–34.

FRIZZELL, R. A., HALM, D. R., RECHKEMMER, G. AND SHOEMAKER, R. L. (1986). Chloride channel regulation in secretory epithelia. *Fedn Proc. Fedn Am. Socs exp. Biol.* **45**, 2727–2731.

GALLOWAY, C., DEAN, G., RUDNICK, G. AND MELLMAN, I. (1983). Acidification of macrophage and fibroblast endocytic vesicles. *Proc. natn. Acad. Sci. U.S.A.* **80**, 3334–3338.

GARTY, H., RUDY, B. AND KARLISH, S. J. D. (1983). A simple and sensitive procedure for measuring isotope fluxes through ion specific channels in heterogeneous populations of membrane vesicles. *J. biol. Chem.* **258**, 13094–13099.

GASSER, K. W., DIDOMENICO, J. AND HOPFER, U. (1986). Secretogogues activate chloride transport pathways in pancreatic zymogen granules. *Am. J. Physiol.* **250**, G489–G496.

GLICKMAN, J., CROEN, K., KELLY, S. AND AL-AWQATI, Q. (1983). Golgi membranes contain an electrogenic H^+ pump in parallel to a chloride conductance. *J. Cell Biol.* **97**, 1303–1308.

GOLDSTEIN, J. L., BROWN, M. S., ANDERSON, R. G. W., RUSSELL, D. W. AND SCHNEIDER, W. J. (1985). Receptor mediated endocytosis. *A. Rev. Cell Biol.* **1**, 1–40.

GRIFFITHS, G., HOFLACK, B., SIMONS, K. AND MELLMAN, I. (1988). The mannose 6-phosphate receptor and the biogenesis of lysosomes. *Cell* **52**, 329–341.

GRUENERT, D. C., BASBAUM, C. B. AND WIDDICOMBE, J. H. (1990). Long-term culture of normal and cystic fibrosis epithelial cells grown under serum-free conditions. *In Vitro Cell Dev. Biol.* **26**, 411–418.

GUPTA, R., JENTOFT, N., JAMIESON, A. M. AND BLACKWELL, J. (1990). Structural analysis of purified human tracheobronchial mucins. *Biopolymers* **29**, 347–355.

HARIKUMAR, P. AND REEVES, J. P. (1983). The lysosomal proton pump is electrogenic. *J. biol. Chem.* **258**, 10403–10410.

HOOK, V. Y., EIDEN, L. E. AND BROWNSTEIN, M. J. (1982). A carboxypeptidase processing enzyme for enkephalin precursors. *Nature* **295**, 341–342.

HOUDRET, N., RAMPHAL, R., SCHARFMAN, A., PERINI, J.-M., FILLIAT, M., LAMBLIN, G. AND ROUSSEL, P. (1989). Evidence for the *in vivo* degradation of human respiratory mucins during *Pseudomonas aeruginosa* infection. *Biochim. biophys. Acta* **992**, 96–105.

HWANG, T. C., LU, L., ZEITLIN, P. L., GRUENERT, D. C., HUGANIR, R. AND GUGGINO, W. B. (1989). Cl channels in CF: Lack of activation by protein kinase C and cyclic AMP-dependent protein kinase. *Science* **244**, 1351–1353.

JABBAL, I. AND SCHACHTER, H. (1971). Pork liver guanosine diphosphate-L-fucose glycoprotein fucosyltransferases. *J. biol. Chem.* **246**, 5154–5161.

JENTSCH, T. J., STEINMAYER, K. AND SCHWARTZ, G. (1990). Cloning and expression of a voltage gated chloride channel from *Torpedo*. *Nature* **348**, 510–515.

JOHNSON, R. G., CARTY, S. AND SCARPA, A. (1982). A model of biogenic amine accumulation into

chromaffin granules and ghosts based on coupling to the electrochemical proton gradient. *Fedn Proc. Fedn Am. Socs exp. Biol.* **41**, 2746–2754.

JOHNSON, R. G. AND SCARPA, A. (1979). Proton motive force and catecholamine transport in isolated chromaffin granules. *J. biol. Chem.* **254**, 3750–3760.

KARTNER, N., HANRAHAN, J. W., JENSEN, T. J., NAISMITH, A. L., SUN, S., ACKERLEY, C. A., REYES, E. F., TSUI, L.-C., ROMMENS, J., BEAR, C. E. AND RIORDAN, J. R. (1991). Expression of cystic fibrosis gene in non-epithelial invertebrate cells produces a regulated anion conductance. *Cell* **64**, 681–691.

KATO, Y. AND SPIRO, R. G. (1989). Characterization of a thyroid sulfotransferase responsible for the 3-*O*-sulfation of terminal β-D-galactosyl residues in N-linked carbohydrate units. *J. biol. Chem.* **264**, 3364–3371.

KEREM, B. S., ROMMENS, J. M., BUCHANAN, J. A., MARKIEWICZ, D., COX, T. K., CHAKRAVARTI, A., BUCHWALD, M. AND TSUI, L.-C. (1989). Identification of the cystic fibrosis gene, genetic analysis. *Science* **245**, 1073–1080.

KLAUSNER, R. D., ASHWELL, G., VAN RENSWOUDE, J., HARFORD, J. B. AND BRIDGES, K. R. (1983). Binding of apotransferrin to K562 cells, explanation of the transferrin cycle. *Proc. natn. Acad. Sci. U.S.A.* **80**, 2263–2266.

KOOMANS, A. M., VON EULER MULLER, R. M. AND GILLIAM, H. (1986). X-ray microanalysis of goblet cells in bronchial epithelium of patients with cystic fibrosis. *J. submicrosc. Cytol* **18**, 613–615.

KRIVAN, H. C., GINSBURG, V. AND ROBERTS, D. D. (1988*a*). *Pseudomonas aeruginosa* and *Pseudomonas cepacia* isolated from cystic fibrosis patients bind specifically to gangliotertraosylceramide (Asialo GM1) and gangliotriaosylceramide (Asialo GM2). *Archs Biochem. Biophys.* **260**, 493–496.

KRIVAN, H. C., ROBERTS, D. D. AND GINSBURG, V. (1988*b*). Many pulmonary pathogenic bacteria bind specifically to the carbohydrate sequence GalNAcβ1-4Gal found in some glycolipids. *Proc. natn. Acad. Sci. U.S.A.* **85**, 6157–6161.

LAMBLIN, G., BOERSMA, A., KLEIN, A. AND ROUSSEL, P. (1984). Primary structure determination of five sialylated oligosaccharides derived from bronchial mucus glycoproteins of patients suffering from cystic fibrosis. *J. biol. Chem.* **259**, 9051–9058.

LANDRY, D. W., AKABAS, M. H., REDHEAD, C., EDELMAN, A., CRAGOE, E. J. AND AL-AWQATI, Q. (1989). Purification and reconstitution of chloride channels from kidney and trachea. *Science* **244**, 1469–1472.

LANDRY, D. W., REITMAN, M., CRAGOE, E. J., JR AND AL-AWQATI, Q. (1987). Epithelial chloride channel. Development of inhibitory ligands. *J. gen. Physiol.* **90**, 779–798.

LI, M., MCCANN, J. D., LIEDDTKE, C. M., NAIRN, N. C., GREENGARD, P. AND WELSH, M. J. (1988). Cyclic AMP dependent protein kinase opens chloride channels in normal but not cystic fibrosis airway epithelium. *Nature* **331**, 358–360.

LITT, M., KHAN, M. A. AND SHIH, C. K. (1977). The role of sialic acid in determining rheological and transport properties of mucus secretions. *Biorheology* **14**, 127–132.

MAWHINNEY, T. P., ADELSTEIN, E., MORRIS, D. A., MAWHINNEY, A. M. AND BARBERO, G. J. (1987). Structure determination of five sulfated oligosaccharides derived from tracheobronchial mucus glycoproteins. *J. biol. Chem.* **262**, 2994–3001.

MELLMAN, I., FUCHS, R. AND HELENIUM, A. (1986). Acidification of the endocytic and exocytic pathways. *A. Rev. Biochem.* **55**, 663–700.

MIAN, N., POPE, A. J., ANDERSON, C. E. AND KENT, P. W. (1982). Factors influencing the viscous properties of chicken tracheal mucins. *Biochim. biophys. Acta* **717**, 41–48.

MULBERG, A. E., TULK, B. M. AND FORGAC, M. (1991). Modulation of coated vesicle chloride channel activity and acidification by reversible protein kinase A-dependent phosphorylation. *J. biol. Chem.* **266**, 20590–20593.

ORCI, L., RAVAZZOLA, M., AMHERDR, M., MADSEN, O., PERRELET, A., VASSALLI, J. D. AND ANDERSON, R. G. W. (1987). Conversion of pro-insulin to insulin occurs coordinately with acidification of maturing secretory granules. *J. Cell Biol.* **103**, 2273–2281.

PACE, C. S. AND SACHS, G. (1982). Glucose induced proton uptake in secretory granules of β-cells in monolayer culture. *Am. J. Physiol.* **242**, 382–387.

PAULMICHL, M., LI, Y., WICKMAN, K., ACKERMAN, M., PERALTA, E. AND CLAPHAM, D. (1992). New mammalian chloride channel identified by expression cloning. *Nature* **356**, 238–241.

PAULSON, J. C., PRIEELS, J.-P., GLASGOW, L. R. AND HILL, R. L. (1978). Sialyl- and fucosyltransferases in the biosynthesis of asparaginyl-linked oligosaccharides in glycoproteins. Mutually exclusive

glycosylation by β-glactoside α2→6 sialyltransferase and *N*-acetylglucosaminide α1→6 3 fucosyltransferase. *J. biol. Chem.* **253**, 5617–5625.

POHLENTZ, G., KLEIN, D., SCHWARZMANN, G., SCHMITZ, D. AND SANDHOFF, K. (1988). Both GA2, GM2 and GD2 synthases and GM1b, GD1a and GT1b synthases are single enzymes in Golgi vesicles from rat liver. *Proc. natn. Acad. Sci. U.S.A.* **85**, 7044–7048.

PRIEELS, J.-P., BEYERS, T. AND HILL, R. L. (1977). Human milk fucosyltransferases. *Biochem. Soc. Trans.* **5**, 838–839.

REDHEAD, C. R., EDELMAN, A., BROWN, D., LANDRY, D. W. AND AL-AWQATI, Q. (1992). A ubiquitous 64 kDa protein is a component of a chloride channel of plasma and intracellular membranes. *Proc. natn. Acad. Sci. U.S.A.* **89**, 3716–3720.

REENSTRA, W. W., SABOLIC, I., BAE, H.-R. AND VERKMAN, A. S. (1992). Phosphorylation of endosome membrane proteins by protein kinase A. *Biochemistry, N. Y.* **31**, 175–181.

RICHARDSON, C. L., KEENAN, T. W. AND MOORE, D. J. (1977). Ganglioside biosynthesis. Characterization of cmp-*N*-acetylneuraminic acid: Lactosylceramide sialyltransferase in Golgi apparatus from rat liver. *Biochim. biophys. Acta* **488**, 88–96.

RIORDAN, J., ROMMENS, J. M., KEREM, B. S., ALON, N., ROZMAHEL, R., GRZELCZACK, Z., ZIELENSKI, J., LOK, S., PLAVSIC, N., CHOU, J.-L., DRUMM, M. L., IANNUZZI, M. L., COLLINS, F. S. AND TSUI, L.-C. (1989). Identification of cystic fibrosis gene, cloning and characterization of the complementary DNA. *Science* **245**, 1066–1073.

ROFF, C. F., FUCHS, R., MELLMAN, I. AND ROBBINS, A. R. (1986). Chinese hamster ovary cell mutants with temperature-sensitive defects in endocytosis. I. Loss of function on shifting to the nonpermissive temperature. *J. Cell Biol.* **103**, 2283–2297.

ROMMENS, J. H., IANNUZZI, M. C., KEREM, M. C., DRUMM, M. L., MEIMER, G., DEAN, M., ROZMAHEL, R., COLE, J. L., KENNEDY, D., HIDAKA, N., ZSIGA, M., BUCHWALD, M., RIORDAN, J. R., TSUI, L.-C. AND COLLINS, F. S. (1989). Identification of cystic fibrosis gene, chromosome walking and jumping. *Science* **245**, 1059–1065.

ROSE, M. C. (1988). Epithelial mucous glycoproteins and cystic fibrosis. *Horm. Metabol. Res.* **20**, 601–608.

ROSEMAN, S., CARLSON, D. M., JOURDIAN, G. W., MCGUIRE, E. J., KAUFMAN, B., BASU, S. AND BARTHOLOMEW, B. (1966). Animal sialic acid transferases (sialyl-transferases). In *Enzymes of Complex Saccharide Synthesis. Methods in Enzymology,* vol. **8** (ed. E. F. Neufeld and V. Ginsburg) pp. 354–372. New York: Academic Press.

ROUSSEL, P., LAMBLIN, G., DEGAND, P., WALKER-NASIR, E. AND JEANLOZ, R. W. (1975). Heterogeneity of carbohydrate chains of sulfated bronchial glycoproteins isolated from a patient suffering from cystic fibrosis. *J. biol. Chem.* **250**, 2114–2122.

RUSSELL, J. T. AND HOLZ, R. W. (1981). Measurement of ΔpH and membrane potential in isolated neurosecretory vesicles from bovine neurohypophyses. *J. biol. Chem.* **256**, 5950–5953.

SCANLIN, T. F., WANG, W.-M. AND GLICK, M. C. (1985). Altered fucosylation of membrane glycoproteins from cystic fibrosis fibroblasts. *Pediatr. Res.* **19**, 368–374.

SCHMID, A., BURCKHARDT, G. AND GOGELEIN, H. (1989). Single chloride channels in endosomal vesicle preparations from rat kidney cortex. *J. Membr. Biol.* **111**, 265–275.

SCHMID, A., GOGELEIN, H., KEMMER, T. P. AND SCHULZ, I. (1988). Anion channels in giant liposomes made of endoplasmic reticulum vesicles from rat exocrine pancreas. *J. Membr. Biol.* **104**, 275–282.

SCHNEIDER, D. L. (1981). ATP dependent acidification of intact and disrupted lysosomes. Evidence for an ATP driven proton pump. *J. biol. Chem.* **256**, 3858–3864.

SCHOUMACHER, R. A., SHOEMAKER, R. L., HALM, D. R., TALLANT, E. A., WALLACE, R. W. AND FRIZZELL, R. A. (1987). Phosphorylation fails to activate chloride channels from cystic fibrosis airway cells. *Nature* **330**, 752–754.

SENN, J., COOPER, C., WARNKE, P. C., WAGNER, M. AND DECKER, K. (1981). Ganglioside biosynthesis in rat liver. Characterization of UDP-*N*-acetylgalactosamine – GM_3acetylgalactosaminyltransferase. *Europ. J. Biochem.* **120**, 59–67.

SHERBLOM, A. P., SMAGULA, R. M., MOODY, C. E. AND ANDERSON, G. W. (1986). Sialyltransferase of bovine serum, age-and hormone-related changes. *Comp. Biochem. Physiol.* **84B**, 309–313.

SLOMIANY, B. L., LIAU, Y. H., CARTER, S. R., SAROSIEK, J., TSUKADA, H. AND SLOMIANY, A. (1987). Enzymatic sulfation of mucin in gastic mucosa, effect of sofalcone, sucralfate and aspirin. *Digestion* **38**, 178–186.

SLOMIANY, B. L., VARAHABHOTIA, L. N. AND SLOMIANY, A. (1990). Isolation and characterization of oligosaccharides from rat colonic mucus glycoprotein. *J. biol. Chem.* **255**, 9719–9723.

STEINMAYER, K., KLOCKE, R., ORTLAND, C., GRONEMEIER, M., JOCKUSCH, H., GRUNDER, S. AND JENTSCH, T. J. (1991*a*). Inactivation of muscle chloride channel by transposon insertion in myotonic mice. *Nature* **354**, 304–308.

STEINMAYER, K., ORTLAND, C. AND JENTSCH, T. J. (1991*b*). Primary structure and functional expression of a developmentally regulated skeletal muscle chloride channel. *Nature* **354**, 301–304.

STONE, D. K., XIE, X.-S. AND RACKER, R. (1983). An ATP-driven proton pump in clathrin-coated vesicles. *J. biol. Chem.* **258**, 4059–4062.

TAATJES, D. J., ROTH, J., WEINSTEIN, J. AND PAULSON, J. C. (1988). Post-Golgi apparatus localization and regional expression of rat intestinal sialyltransferase detected by immunoelectron microscopy with polypeptide epitope-purified antibody. *J. biol. Chem.* **263**, 6302–6309.

TYCKO, B. AND MAXFIELD, F. R. (1982). Rapid acidification of endocytic vesicles containing α_2-macroglobulin. *Cell* **28**, 643–651.

VALVERDE, M. A., DIAZ, M., SEPULVEDA, F., GILL, D. R., HYDE, S. C. AND HIGGINS, C. F. (1992). Volume-regulated chloride channels associated with the human multidrug-resistance P-glycoprotein. *Nature* **355**, 830–833.

VAN DYKE, R. W. (1988). Proton pump-generated electrochemical gradient in rat liver multivesicular bodies. *J. biol. Chem.* **263**, 2603–2611.

WELSH, M. J. AND LIEDTKE, C. M. (1986). Chloride and potassium channels in cystic fibrosis airway epithelia. *Nature* **322**, 467–470.

WESLEY, A., FORSTNER, J., QURESHI, R., MANTLE, M. AND FORSTNER, G. (1983). Human intestinal mucin in cystic fibrosis. *Pediatr. Res.* **17**, 65–69.

WILLUMSEN, N. J. AND BOUCHER, R. C. (1989). Shunt resistance and ion permeabilities in normal and cystic fibrosis airway epithelia. *Am. J. Physiol.* **256**, C1054–C1063.

WOODS, D. E., BASS, J. A., JOHANSON, W. G,.JR AND STRAUS, D. C. (1980). Role of adherence in the pathogenesis of *Pseudomonas aeruginosa* lung infection in cystic fibrosis patients. *Infection Immunity* **30**, 694–699.

YAMASHIRO, D. J., FLUSS, S. R. AND MAXFIELD, F. R. (1983). Acidification of endocytic vesicles by an ATP-dependent proton pump. *J. Cell Biol.* **97**, 929–934.

YAMASHIRO, D. J., TYCKO, B., FLUSS, S. R. AND MAXFIELD, F. R. (1984). Segregation of transferrin to a mildly acidic (pH 6.5) para-Golgi compartment in the recycling pathway. *Cell* **37**, 789–800.

CHAPTER 7. Physiology of eukaryote plasma membranes