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Defective acidification of the biosynthetic pathway in cystic fibrosis

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SUMMARY

Cystic fibrosis is associated with defective epithelial sodium chloride and fluid secretion in epithelia. In addition, there is widespread reductions in sialylation of secreted proteins and increases in the sulfation and fucosylation of mucus glycoproteins. The major morbidity in the disease is due to the colonization of respiratory epithelia by Pseudomonas. The cystic fibrosis gene (CFTR) is a cyclic AMP activated Cl channel, which when mutated is retained in the endoplasmic reticulum. We postulate that this Cl channel is responsible for effective acidification of the Golgi. In CF cells, we demonstrate the Golgi pH is higher than in normal cells and suggest that the abnormalities in glycoprotein biosynthesis is due to changes in the kinetics of sialyl transferase, a pH sensitive enzyme. Defects in sialylation also result in decreased sialylation of glycolipids and asialogangliosides are potential Pseudomonas receptors.

Key words: chloride channel, Golgi acidification, cystic fibrosis, sialyl transferase

INTRODUCTION

All eukaryotic cells tested have one or more type of chloride channel. In some cells they are present in the plasma membrane where they participate in the control of cell volume (probably in most cells), membrane potential (in muscle and nerve) or the secretion of NaCl and water (in epithelia). In addition, chloride channels have been found in intracellular organelles such as endosomes, lysosomes and Golgi where they are present in parallel to an electrogenic proton translocating ATPase and hence can control the pH gradient and membrane potential of these organelles. Based on single channel behavior, chloride channels exhibit marked diversity in conductance, current-voltage relation and regulation by modulators. Using these functional criteria, it was found that a single cell might contain as many as three types of plasma membrane Cl^- channels, in addition to the one or more types of intracellular channels.

Recent studies have identified a number of molecules that code for Cl^- channels that do not belong to a single family. The GABA and glycine receptors resembles the nicotinic receptor, a cation channel (Olsen and Tobin, 1990). The cystic fibrosis gene product (CFTR) and the multidrug resistance gene bear similarities to ATP-dependent solute transporters (Riordan, 1992; Welsh et al., 1992). A family of voltage-sensitive chloride channels found in *Torpedo* electric organ, mammalian muscle and epithelial cells do not resemble any known sequences (Steinmayer et al., 1991). Porins in outer membranes of bacteria and mitochondria do not even contain 'canonical' transmembrane hydrophobic helices (Weiss and Schulz, 1992). Neither does a channel recently cloned from MDCK epithelia (Paulmichl et al., 1992). This diversity suggests that there may be even more types of chloride channels to be discovered. Indeed, using the biochemical approach of solubilization and reconstitution, we (Landry et al., 1989, 1993) and others (Ran and Benos, 1991) have found two additional Cl^- channels in epithelial cells.

Most of the studies referred to above were directed towards the identification of plasma membrane Cl^- channels. We had discovered that Golgi, endosomes and secretory granules contain chloride channels, which are intimately involved in the control of the pH gradient generated by the H^+-ATPase (Glickman et al., 1983). That this Cl^- channel can control vacuolar pH in vivo was shown when we found that secretagogues induce acidification of secretory granules by opening Cl^- channels (Barasch et al., 1988). Since we had demonstrated that Golgi vesicles also contain Cl^- channels and they were not maximally acidified, we speculated that control of Golgi pH might profoundly affect the function of Golgi enzymes, many of which are pH-sensitive (Al-Awqati, 1986).

To provide molecular reagents for further analyses of these processes we decided to purify the Cl^- channel of intracellular organelles, which we term here a vacuolar chloride channel. We do not know whether there is a single molecular species that codes for vacuolar Cl^- channels or whether there are many. Given the level of molecular diversity in Cl^- channels already identified, it is probable that there are several kinds of intracellular Cl^- channels. We used an inhibitor derived from ethacrynic acid, IAA-94, to purify the drug binding proteins from bovine kidney cortex

intracellular vesicles and showed by reconstitution that the affinity-purified proteins contain the Cl^- channel (Landry et al., 1989, 1987). Two proteins were eliminated as drug-binding proteins and one of the remaining, a 64 kDa protein (p64), generated a specific antibody. This antibody was able to deplete all Cl^- channel activity from bovine kidney cortex, suggesting that it is a necessary component of the vacuolar channel (Redhead et al., 1992). We cloned and sequenced the cDNA for p64 and obtained a novel sequence of a membrane protein. Using antibodies we found that p64 is located in the perinuclear region (probably Golgi) and in the apical membrane of some epithelial cells (Landry et al., 1993).

CYSTIC FIBROSIS

The discovery that the phenotypic defect in cystic fibrosis is an abnormality of activation of an epithelial Cl^- channel has galvanized the field of study of Cl^- channels and awarded it a high visibility (Schoumacher et al., 1987; Welsh and Liedtke, 1986). The gene, CFTR, was rapidly identified (Welsh et al., 1986) and its function was analyzed in detail by transfection into heterologous cells where it causes the appearance of a new cyclic AMP- and ATP-activated small Cl^- channel with a linear I-V relationship (Riordan, 1992; Welsh et al., 1992). The major mutation, ΔF508 appears to be a temperature-sensitive mutation that causes an abnormality in proper folding of the protein (Chen et al., 1990; Denning et al., 1992). Such proteins get rapidly identified by a 'quality control' mechanism that leads to their degradation from the endoplasmic reticulum, preventing their progress through the Golgi and on to the apical plasma membrane (Hurtley and Helenius, 1989). Reduction of temperature results in the appearance of cyclic AMP-regulated chloride channels in the plasma membrane that have the same conductance and I-V relationship as the wild-type protein but with a lower open probability.

One puzzling finding is that cystic fibrosis (CF) cells demonstrate abnormalities in two other channels. The amiloride-sensitive Na channel is tonically open (Willumsen and Boucher, 1991). An outwardly rectifying Cl^- channel (ORCC) is present in CF cells but it cannot be regulated by protein kinase A (PKA) (Schoumacher et al., 1987; Welsh et al., 1986); transfection with the wild-type CFTR corrects this defect (Egan et al., 1992). Because the mutant and wild-type CFTR have essentially the same elctrophysiological characterstics (except for open probability), it is likely that the ORCC is a different protein. Since the outward rectifier exhibits defective regulation in CF cells, we can conclude that when CFTR does not leave the endoplasmic reticulum abnormalities in other proteins can result.

As far as we know, p64 is not directly involved in CF. The protein exists in CF epithelial cells and does not seem to have either an abnormal location or an abnormal molecular mass, in that none of these parameters are changed when the cells are transfected with the wild-type CFTR. However, it remains possible that p64 is the outward rectifier channel and we are now attempting to reconstitute the over-expressed protein in order to test this question.

DEFECTIVE ACIDIFICATION OF VACUOLES IN CYSTIC FIBROSIS

The clinical syndrome of cystic fibrosis is characterized by thick mucus in the gastrointestinal and respiratory tracts, and colonization and infection by *Pseudomonas* bacteria (Boat et al., 1989). Chemical analysis of the mucus has demonstrated that the structure of its glycoproteins is different from those of normal mucus, it is undersialyated, oversulfated and overfucosylated (Boat and Cheng, 1980; Cheng et al., 1989). While an abnormality in cyclic AMP-mediated NaCl secretion will lead to thick 'dehydrated' mucus it cannot by itself explain the other findings listed above. We recently provided evidence for a new hypothesis that can explain these manifestations of CF (Barasch et al., 1991). This is based on the fact that the Golgi H^+-ATPase is electrogenic; hence transport of protons results in hyperpolarization of the membrane potential such that continued H^+ transport becomes energetically unfavorable (Glickman et al., 1983). The pH of the Golgi will therefore depend on the number of such channels or on their open probability.

Evidence for acidification in the *trans*-Golgi network

A number of lines of evidence shows that some compartments of the Golgi are acidified. Isolation of vesicles from the *trans*-Golgi, i.e. vesicles enriched for galactosyl transferase, showed that these vesicles acidified their interiors by an electrogenic H^+-ATPase in parallel to a chloride channel (Glickman et al., 1983). Similar results were obtained by others and the ATPase has been purified from this source by two groups (Young et al., 1988; Moriyama and Nelson, 1990). That the enzyme was functional in generating pH gradients across the Golgi membranes was demonstrated by electron microscopy using the weak base DAMP (Anderson et al., 1984; Orci et al., 1987). It is interesting that the pH was not low in the medial and *cis* compartments.

Where estimates of Golgi pH exist, it is about one pH unit lower than the cytoplasm; i.e. around pH 6.4. It is interesting that the enzymes responsible for terminal sialylation of glycoproteins and glycolipids have their optimum function at pH 5.9. Further, mutants selected for a defect in acidification exhibit defective sialylation in the Golgi (Roff et al., 1987). These results demonstrate that the pH of the *trans*-Golgi network is acid and that the low pH plays a functional role in sialylation.

Evidence that opening of Cl^- channels acidifies granules

Parafollicular cells of the thyroid contain large granules, which store serotonin and calcitonin. These granules were isolated and found to contain an electrogenic H^+-ATPase. However, the pH of the granules could not be acidified in vitro unless the membrane potential generated by the ATPase was completely collapsed by the K ionophore valinomycin. These studies suggested that the conductance of the membrane to ions was very low. The pH of these granules was examined in vivo using the permeant weak base DAMP (Anderson et al., 1984; Orci et al., 1987). There was no acidification in resting cells. However, stimulation

of the cells by thyroid stimulating hormone (TSH) caused the appearance of acidification. Granules isolated from stimulated cells showed that they could now acidify in vitro and that their acidification was not dependent on valinomycin, suggesting that stimulation by TSH opened a conductance in the membrane. When the Cl^- permeability of the stimulated granules was examined it was found to be high while unstimulated granules had no Cl^- permeability. These studies demonstrated for the first time that a physiologically relevant change in the Cl^- conductance of an intracellular organelle could affect the vacuolar pH. Similar studies were later performed by Bae and Verkman (1990) using kidney cortex endosomes. They found that protein kinase A opened Cl^- channels and enhanced the acidification of these organelles.

Evidence for defective acidification of the Golgi in cystic fibrosis

We estimated the pH of the *trans*-Golgi network using the permeant weak base, DAMP. Organelles were identified by morphological and immunocytochemical criteria using the antibody to the mannose 6-phosphate receptor (M6PR). Compartments enriched in the M6PR were taken to represent the prelysosome (late endosome), (Griffiths et al., 1988). The *trans*-Golgi vesicles and the prelysosomes were found to have a higher pH in CF than in matched controls. However, lysosomes had the same pH. Hence, the defect was not simply an artifact of the accumulation or permeability of the cells to DAMP. We isolated light vesicles and heavy vesicles from immortalized respiratory epithelial cells and studied their acidification in vitro. We found that CF light vesicles (Golgi and endosomes) showed defective acidification, which was corrected if the membrane potential generated by the ATPase was nullified by a K ionophore, valinomycin. Light vesicles of normal cells did not require the addition of the ionophore to acidify maximally. Heavy vesicles (lysosomes) isolated from normal and CF cells acidified maximally regardless of the presence of valinomycin. These results confirm the acidification defect identified using the electron microscopic method.

The acidification defect was found in two sets of cells: primary cultures from nasal polyps of CF and normal children and a pair of immortalized respiratory epithelial cell lines, one from a patient with CF and the other from a normal person. Perhaps the most difficult question in these experiments is that of comparing CF cells with normal cells; the natural variation in the function of cells in culture could easily confound the results. To provide more convincing evidence for this hypothesis we have recently extended these observations to two sets of CF-PAC cells that have been 'rescued' by transfection with the wild-type CFTR gene. CF-PAC is a pancreatic adenocarcinoma cell line. In all four sets examined we have found the same defect in the acidification of Golgi and M6PR vesicles. These studies with DAMP are shown in Table 1. Hence, this defect has been observed in four pairs of cells.

Table 1. Accumulation of DAMP in identified compartments

Cell type	Mannose 6-P receptor	*n*	α-2,6-Sialyl transferase	*n*
CF-PAC	20.5±4.6	59	17.6±2.8	77
CF-PAC + CFTR	114.5±18	77	71.8±13	60

Values are number of gold dots/μm^2.

Consequences of the acidification defect on sialylation of proteins and lipids

Terminal glycosylation of secreted and membrane-spanning glycoproteins occurs in the *trans*-Golgi, the only region of this organelle that is acid. Some of the Golgi enzymes have steep pH optima; 2,6-sialyl transferase has a pH optimum of 5.9 while sulfotransferase has a pH optimum centered around pH 7.0. A large number of investigators have found that cystic fibrosis mucus is poor in terminal 2,6-sialic acid residues but enriched in sulfate. Since sialylation occurs on the same residue as sulfate and since it had been previously shown that the two processes are competitive with each other, reduced sialylation would be expected to cause increased sulfation.

We recently examined the sialylation of secreted proteins in a pair of airway epithelial cell lines. We found that there was a widespread reduction in the addition of sialic acid to these proteins (Barasch et al., 1991). We have repeated these studies now in the CF-PAC cell line and compared the sialylation with that occurring in the same cell line after transfection with the wild-type CFTR gene. We found that the sialylation is higher in the rescued cell compared to the CF cell. Hence, it seems that the defect in sialylation is not restricted to airway epithelial cells but is present in a pancreatic cells as well. It is likely therefore, that the sialylation defect is present in most or all cells that express CFTR. It had previously been demonstrated that gastric mucus isolated from patients with CF is undersialyated.

Expression of a potential *Pseudomonas* receptor

We have also provided evidence that gangliosides such as GM1 are undersialyated in CF cells. It is well known that the respiratory tract of young children with CF get colonized by *Pseudomonas* in the first few months of life, suggesting that a receptor has been exposed. Indeed, most of the morbidity and mortality of CF can be attributed to the consequences of respiratory infection by *Pseudomonas*. Krivan et al. (1988) demonstrated that *Pseudomonas* binds to asialo GM1 but not to (sialo) GM1, suggesting that asialo GM1 is a receptor for *Pseudomonas*. Since glycosphingolipids are targeted to the apical membranes in epithelia (van Meer and Simons, 1988), our hypothesis provides a potential explanation for the infection with *Pseudomonas* that occurs in CF. Recent studies by our colleague at Columbia have shown that CF epithelia bind more *Pseudomonas* organisms than normal respiratory epithelia in culture. Binding of the bacteria to the epithelium is reduced almost to the normal level if the organism is coated with excess asialo GM1 before binding (Saiman and Prince, 1993).

Sulfation in the Golgi and the role of the Cl^- channel

Proteins are sulfated on terminal galactose residues by reaction with the sulfate donor, phosphoadenosine monophosphate sulfate (PAPS) where the transfer of the sulfate leads

to the formation of 3′AMP and the sulfated protein. We found that the secreted mucus glycoproteins of CF-PAC are oversulfated when compared with the secretion from the same cells transfected with the wild-type CFTR gene.

The sulfate nucleotide is transported into the Golgi by a carrier that exchanges PAPS for 3′AMP. We had previously predicted that PAPS transport might be electrogenic, since PAPS has 4 negative charges while 3′AMP has 2.5. To test this hypothesis we loaded kidney Golgi vesicles with Cl^- and passed them down an anion exchange resin to generate a positive membrane potential inside only vesicles that have a Cl^- channel. When the vesicles were loaded with 3′AMP there was a large inhibition of the uptake of [^{35}S]PAPS by valinomycin or extravesicular Cl^-. When vesicles were loaded with PAPS, there was little effect of valinomycin. These results suggest that Golgi vesicles will accumulate larger amount of PAPS if their membrane potential is positive inside. Our hypothesis for CF is that the absent or closed Cl^- channel would result in a higher membrane potential in the Golgi. Hence, the increased sulfation is not only produced by the fact that the activity of sulfotransferase is independent of acidification, but also due to the possibility that there is a greater delivery of PAPS to the enzyme because of a possible hyperpolarization of the membrane. To test this directly, we are developing potential-sensitive probes that could measure the membrane potential in identified intracellular organelles.

The acidification defect is restricted to the biosynthetic pathway

We studied the sialylation of anonymous and known proteins in CF cells. Cells were labelled with [^{35}S]methionine to label amino acids and with the sialic acid precursor, *N*-[^{3}H]acetyl mannosamine. Secreted proteins were collected and analyzed by SDS-PAGE and bands were cut and counted to obtain $^{3}H/^{35}S$ ratios, which are measures of the specific activity of sialic acid addition. We found that all secreted proteins from the immortalized CF respiratory epithelial cell lines had reduced sialylation when compared with controls. In addition to these reported studies, we have now examined two pairs of CF-PAC cell lines, one member of each pair was rescued by the transfection of the wild-type CFTR gene. In these cell lines the defect in sialylation was corrected by transfection of the normal gene.

To gain more direct evidence we prepared antibodies to α-2,6-sialyl transferase and measured the pH in the environment of the enzyme using the DAMP method mentioned above. As seen in Table 1 the vesicles containing the transferase had a lower apparent pH (i.e. higher DAMP accumulation) in the rescued cells compared to the mutant cell line. As Table 1 shows, it appears that the pH defect is restricted to the biosynthetic pathway, since it was seen in sialyl transferase vesicles (*trans*-Golgi and beyond), and in M6PR vesicles, which are prelysosomes. The acidification of CF lysosomes was normal. These results are important, especially in reference to the studies of others who have studied the intravesicular pH of other cell lines. Lukacs et al. (1992) transfected CHO cell lines with CFTR and found that FITC-dextran was internalized into acid compartments that did not seem to be sensitive to cyclic AMP. Since the endocytosis of dextran and of transferin occurs into early endosomes, which do not intersect with the mannose 6-phosphate receptor compartment (Stoorvogel et al., 1989), it appears that the defective acidification is restricted to the biosynthetic compartment.

We recently started to examine the function of sialyl transferase in CF using our specific antibody to the α-2,6-sialyl transferase. We were startled to find that the trafficking of this enzyme was defective in CF. There was a decrease in the total amount of sialyl transferase in the cell, which was a consequence of increased degradation. Incubation of normal cells with NH_4Cl, an alkalinizing agent, reproduced this increased degradation. Further, addition of bafilomycin, a 'specific' inhibitor of the vacuolar ATPase, led to rapid degradation of sialyl transferase within five minutes of addition. These studies suggest that the acid pH of the Golgi is not only needed for the activity of this enzyme but is also required for correct trafficking. Defective acidification, hence, can cause 'secondary' changes in sialyl transferase, which can amplify the functional defect, thereby accentuating the sialylation defect.

Studies by others on the role of CFTR in vacuolar acidification

Lukacs et al. (1992) transfected CHO cells with wild-type and mutant CFTR and allowed them to internalize FITC-dextran. They found the acidification of the organelles labelled by this ligand to be the same regardless of the type of CFTR transfected. Dextran is internalized into early endosomes and then it is transferred to lysosomes. These compartments do not intersect the mannose 6-phosphate receptor compartment to any great extent. In addition, dextran labels a large number of vesicles, none of which was identified. Finally, it is not clear that transfection of wild-type or mutant CFTR into non-epithelial cells will shed any light on the sialylation and sulfation abnormalities in epithelial cells.

The complexity of vesicle trafficking in the cells and the number of independent pathways have to be taken into consideration when discussing the question of the role of CFTR in the acidifcation of intracellular organelles.

Is CFTR the only Golgi Cl^- channel?

Our results show that the absence of CFTR in the *trans*-Golgi of epithelial cells leads to abnormal acidification with consequent abnormalities in sialylation and sulfation. The question then arises of what is responsible for normal Golgi acidification in cells that do not express CFTR. There are at least two Cl^- channel proteins, which are expressed in all cells tested (Jensch et al., 1991; Landry et al., 1993).

The rate of transport of the H^+-ATPase is low, something of the order of 100 ions/second. The rate of transport of a Cl^- channel with a reasonable single channel conductance is at least 1,000,000 ions/second. Hence, to collapse the membrane potential generated by the few H^+-ATPases likely to be present in a single vesicle would require no more than a single Cl^- channel per vesicle, which needs to be open no more than 1% of the time. This calculation assumes that the conductance of the membrane to other ions is extremely low. We have no information regarding the state of membrane conductance of the *trans*-Golgi. While

the electrical conductance of artifical lipid bilayers is extremely low, it does vary with the lipid composition. Hence, it is possible that some cells would acidifiy their Golgi without requiring any Cl^- channel. The presence of Cl^- channels would accelerate the acidification.

Since the effect of acidification is expected to alter the sialylation and sulfation of some proteins and lipids, and since this process is known to be variable in different cells, it is possible that cells that do not express Golgi Cl^- channels could fail to maximally sialyate their proteins.

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