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Organelle Acidification and Disease

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A subset of cellular compartments maintain acidic interior environments that are critical for the specific functions of each organelle and for cell growth and survival in general. The pH of each organelle reflects the balance between proton pumping, counterion conductance, and proton leak. Alterations in steady-state organelle pH due to defects in either proton pumping activity or counterion conductance have been suggested to contribute to the pathology of several diseases; however, definitive evidence remains elusive. This review describes recent evidence for the misregulation of organelle pH in the progression of cancer, Dent's disease, and cystic fibrosis.

Key words: channel, endosome, Golgi, protein, V-ATPase

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The compartmentalization of cellular processes into distinct membrane-bound compartments with unique microenvironments has enabled cells to process numerous reactions simultaneously with precision and speed. In eukaryotes, these compartments include the organelles of the biosynthetic and endocytic pathways, which together form a highly differentiated membranous network that efficiently sorts and traffics newly synthesized and internalized molecules to their intended destinations. A key requirement for optimal function of many of these compartments is the maintenance of acidic pH lumens. Along the endocytic pathway, internalized molecules encounter successively decreasing pH in early endosomes, late endosomes, and lysosomes (1). Acidification plays multiple roles in this pathway: for example, the slightly acidic pH of early endosomes (pH ~6.2) facilitates receptor-ligand dissociation. Similarly, the uniquely low pH of lysosomes (pH ~4.8–5.2) enables the selective activation of lysosomal hydrolases once they reach this compartment. Acidification is also required for some functions of specialized cells, such as antigen processing by immune cells [reviewed in (2)]. In addition, many viruses and other pathogens require access to acidified endosomal compartments in order to efficiently infect cells (3–5).

In contrast to the endocytic pathway, most compartments that form the biosynthetic pathway are thought to have near-neutral pH. However, the *trans*-Golgi network (TGN), a major sorting site for newly synthesized molecules, has been demonstrated by several groups to be slightly acidified (pH ~6.0) [refs (6–8)]. Acidification of this compartment is important for the sorting of lysosomally destined enzymes, for the formation of regulated secretory granules, and for efficient constitutive cell surface delivery or secretion of some proteins (9,10).

Steady-state organelle pH is regulated and maintained by a balance between the rates of intralumenal proton pumping, counterion conductance, and intrinsic proton leak [Figure 1; see also (11,12) for recent reviews]. Protons are pumped into compartments against their concentration gradients by the vacuolar-type ATPase, a large multisubunit complex with a molecular mass of nearly 1×10^6 kDa. Assembly and activity

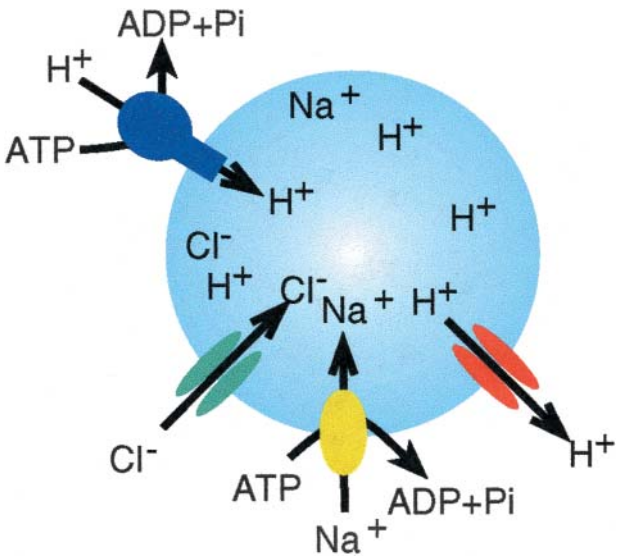

Figure 1: Regulation and maintenance of organelle pH. Acidified pH is generated and maintained by the balance between proton pump activity, which can be modulated by changes in membrane potential, and the rate of proton efflux. Protons are pumped into membranous compartments by the V-ATPase (blue). Pumping can be inhibited by the build-up of excess positive charge (membrane potential) which may be neutralized by passive influx of a counterion conductance (e.g. chloride entry, green), or by efflux of another cation (not shown). Alternatively, sodium entry (mediated by the Na^+/K^+-ATPase, yellow) could increase membrane potential to further inhibit V-ATPase activity and limit acidification. In addition, the rate of intrinsic or regulated hydrogen ion leakage from each compartment (red) contributes significantly to the steady-state concentration of protons in some organelles.

of this pump are regulated by numerous mechanisms [reviewed in (13)].

As the electrogenic V-ATPase pumps protons into a compartment, its ability to function further is theoretically limited by the accumulation of a positive membrane potential. In mammalian cells, passive anion flow through a chloride channel neutralizes this potential to allow acidification to continue (14–16). The importance of this counterion conductance in regulating organelle pH is not clear, and probably varies for different compartments [see (17) for review]. In some organelles, such as phagosomes, the intrinsic counterion permeation is considerably higher than proton pumping activity and is unlikely to contribute significantly to pH regulation (18). However, in other instances, it appears that counterion conductance can modulate pH. For example, maximal acidification of endosomes isolated from proximal tubules is dependent on chloride, and inhibitors of chloride channel activity reduce acidification (19). Moreover, acidification of secretory granules in parafollicular cells induced by the secretagogue thyrotropin was found to be mediated by an increase in their chloride permeability (20).

The third component of pH regulation is the rate at which protons exit a given compartment. All membranes are intrinsically permeable to protons at some level, and recent studies have suggested that the proton leak rate is a key regulator of pH in some organelles (8,12,21–23). Modeling studies by Wu et al. suggested that the decrease in pH between successive compartments along the secretory pathway was likely due to a corresponding decrease in proton efflux coupled with an increased V-ATPase pump density (21). The proton leak rate in the TGN has also been measured using other techniques and found to be quite high, but lower than that of earlier Golgi compartments, consistent with the greater acidity of the TGN (8). Interestingly, proton export from the TGN was voltage-sensitive and inhibited by Zn^{2+}, suggesting the involvement of a regulated channel (8).

Organelle acidification can be purposefully disrupted by various agents, including weak bases, V-ATPase inhibitors, and some ionophores. In addition, other conditions or treatments may indirectly influence pH by less direct means. For example, expression of mutant dynamin in HeLa cells results in a significantly elevated endosomal pH, perhaps by altering endosomal levels of ion channels or the V-ATPase (24). The dissipation of pH gradients in normally acidified compartments perturbs protein sorting and transport along both the biosynthetic and endocytic pathways, and also inhibits other functions such as lysosomal degradation (9,10). There are also several diseases in which proper acidification of some or all organelles has been suggested to be compromised. Recent examples have emerged in which pathogenesis correlates with a change in the function of various components that contribute to the maintenance of steady-state organelle pH. Alterations in both V-ATPase and counterion conductance function have been postulated to account for the pleiotropic manifestations in several diseases. A few examples are discussed below.

Altered pH in Cancer and Cell Transformation

Many transformed cells appear to have abnormal organelle and cytosolic pH, and there is some evidence that altered pH may be important in the transformation process. In 1990, Jiang et al. demonstrated that fibroblasts transformed by transfection with the *ras* oncogene had significantly higher intralysosomal pH compared with nontransformed parental cells (25). In addition, many tumor cell lines express V-ATPases at their plasma membranes, which may contribute to their altered cytosolic pH (26). Evidence that changes in cytosolic or organelle pH directly cause transformation has been elusive, but an interesting but complex picture is emerging from recent studies on cellular transformation by bovine and human papillomaviruses (BPV and HPV, respectively). These viruses, which cause squamous cell cancers, encode three transforming proteins: E5, E6, and E7. E5, which can transform cells even when expressed in the absence of E6 and E7, binds to growth factor receptors EGF and PDGF and modulates their phosphorylation and internalization. However, receptor binding can be dissociated from E5-mediated transformation, suggesting that transformation occurs via an unrelated mechanism (27).

Several lines of evidence suggest that E5-mediated inhibition of V-ATPase function causes cell transformation; however, the mechanism and site of E5 action are controversial. Expression of HPV E5 in keratinocytes slowed endosomal acidification, and caused the intracellular accumulation of undegraded growth factor receptors (28). Another study in fibroblasts also found that E5 expression blocked the delivery of fluorescent fluid phase markers to late endosomal compartments (29), consistent with the effects of pH perturbants on endocytic traffic observed by other laboratories (1,9). Together, these studies suggested that E5-mediated disruption of endosomal pH causes cell transformation by blocking down-regulation of mitogenic signals.

In contrast, two groups have demonstrated that both HPV and BPV E5 localize primarily to the Golgi complex rather than to endosomes (30,31). Expression of BPV E5 resulted in neutralization of Golgi pH by directly inhibiting V-ATPase function, and mutants of E5 that did not disrupt Golgi pH were unable to transform cells (30). Interestingly, mutations in the 16 kDa subunit that blocked V-ATPase function and disrupted Golgi acidification also caused cell transformation and induced anchorage-dependent growth of fibroblasts (30,32). Moreover, transformation of fibroblasts with E5 led to changes in cell morphology and motility similar to those induced by bafilomycin A_1 treatment (33). However, transformation of fibroblasts by *src* or *sis* oncogene expression did not affect Golgi pH, suggesting that alkalinization of the Golgi is not obligatory for some pathways of cell transformation (30). Thus, while it is likely that some transformation pathways require organelle pH perturbation, little is known about how this pathway operates.

Organelle Acidification and Drug Resistance

The altered pH observed in transformed cells may also determine their sensitivity to drugs. Many chemotherapeutics, including the anthracyclines (e.g. adriamycin and daunorubicin) and the vinca alkaloids (e.g. vinblastine and vincristine) are weak bases with pKs of 7–8, and are normally sequestered in acidified compartments. In untransformed cells, this limits their access to targets in the nucleus and cytoplasm where they cause cytotoxicity. Tumor cells, as noted above, typically have elevated organelle pH, and are thus sensitive to the cytotoxic effects of these drugs, which include the anthracycline-mediated inhibition of topoisomerase and protein kinase C activity and the disruption of microtubule organization by vinca alkaloids [see (34,35) for reviews]. Drug-resistant tumor cells typically have more alkaline cytoplasmic pH than drug-sensitive lines (36), and it has been proposed that this increased steady-state pH is responsible for the reduced accumulation of chemotherapeutics in the cytosol and nucleus of drug-resistant compared with drug-sensitive cells (37). Subsequent studies using MCF-7 breast cancer cells have refined this hypothesis to suggest that it is the pH gradient between the cytoplasm and organelles (rather than cytoplasmic pH per se) that regulates cytosolic levels of chemotherapeutics. SNAFL-calcein measurements by Schindler et al. demonstrated that intracellular organelles and vesicles in these cells do not acidify normally, resulting in a decreased pH gradient between the cytoplasm and the lumen (38,39) (Figure 2A,B). Moreover, the TGN and recycling endosomes are fragmented and dispersed throughout these cells (38). Interestingly, an adriamycin-resistant subclone of these cells demonstrated a normal acidification profile and organelle distribution pattern (Figure 2C). Disruption of vesicular acidification in these drug-resistant cells by the ionophores nigericin or monensin, or by inhibition of V-ATPase activity using bafilomycin A_1 or concanamycin A, led to resensitization of the cells to adriamycin (38,39). Furthermore, adriamycin was demonstrated to accumulate in acidic organelles in drug-resistant but not drug-sensitive cells, thus correlating organelle acidification with effective drug sequestration (39). Together, these studies suggest that drug sensitivity requires an acidification defect that blocks sequestration of chemotherapeutics into acidic organelles, and that restoration of normal acidification induces drug resistance.

The link between organelle acidification and drug sequestration was strengthened in recent experiments demonstrating that tamoxifen, which can resensitize drug-resistant tumor cells to other chemotherapeutic agents, selectively alkalinizes acidified organelles without altering cytoplasmic pH (40) (Figure 2D). As a result, chemotherapeutic drugs are redistributed to the cytoplasm and nucleus in cells treated with 0.5–10 μM tamoxifen, well below the 30-μM concentration attained when the drug is administered clinically (40). The mechanism by which tamoxifen disrupts acidification is complex, and appears to involve an increase in proton leak from acidified vesicles combined with a weak base effect (41). This effect on organelle pH is independent of estrogen receptor expression on the target cells and thus could account for some of the side-effects in nonreceptor-expressing cells that have been observed with this drug (40).

Counterion Conductance-Mediated Changes in Organelle Acidification

In addition to cell transformation and cancer therapy, where changes in organelle pH may be mediated by alterations in V-ATPase activity or proton leak, there are also examples in which changes in counterion conductance may cause defective organelle acidification. Two genetic conditions, Dent's disease and cystic fibrosis (CF), involve molecular defects in

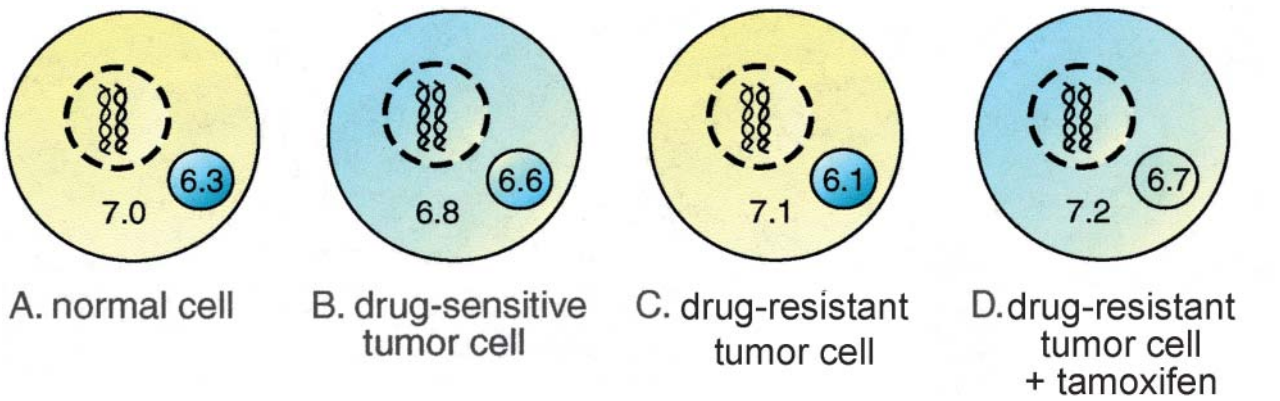

Figure 2: Model for the restoration of drug sensitivity in tumor cells by tamoxifen. (A) Chemotherapeutic agents such as anthracyclines and vinca alkaloids are weak bases and become sequestered in acidified compartments of normal cells (blue), thus diminishing their concentration in the cytoplasm and nucleus (yellow). (B) Drug-sensitive tumor cells have higher cytoplasmic and organelle pH compared with normal cells. In these cells, most chemotherapeutics are not efficiently sequestered and can access their cellular targets. (C) Drug-resistant tumor cells have restored organelle acidification, and like normal cells, can restrict the nuclear accumulation of weak base chemotherapeutics. (D) Tamoxifen disrupts the pH gradient across acidified compartments and thus blocks the sequestration of other chemotherapeutics to restore drug sensitivity. The pH values are from references (38–40).

chloride channels that may function to regulate organelle acidification in some cellular compartments. In the case of Dent's disease, a decrease in chloride permeability is thought to directly affect endosomal function, whereas recent data suggest that effects on sodium conductance are the primary cause of pH dysregulation in cystic fibrosis.

Dent's disease

Dent's disease is an X-linked renal disorder characterized by low molecular-weight proteinuria, and also frequently associated with excess urinary secretion of phosphate and calcium (hyperphosphaturia and hypercalciuria, respectively). While there is significant variability in the severity of this disease among patients, common clinical symptoms include kidney stones, rickets, and nephrocalcinosis (42). Several years ago, the gene mutated in Dent's disease was cloned and found to encode CLC5, a member of the CLC family of chloride channels (43,44). Mutations associated with Dent's disease tend to cluster in the transmembrane regions of CLC5, but mutations in other regions have also been found (24,45).

As predicted, CLC5 expression is highest in kidney, but the protein is also found in liver, brain, testes, and intestine. Within the kidney, the protein is most highly expressed in proximal tubule cells, and is also detected in intercalated cells of the cortical collecting duct and in the thick ascending limb of the loop of Henle (46–49). Within these cells, CLC5 localizes to subapical vesicles enriched in early endosomal markers and to the apical membrane (46–49). This distribution provides an intriguing clue to the clinical manifestations of Dent's disease: the proximal tubule of the kidney is responsible for endocytosis of low molecular-weight proteins that have passed through the glomerular filter. Interestingly, both receptor-mediated and fluid-phase endocytosis were markedly reduced in proximal tubules of CLC5 knockout mice compared with wild-type mice (50). Moreover, a direct correlation between Cy5-conjugated β-lactoglobulin uptake and CLC5 expression was observed in heterozygote females, which express CLC5 in random tubules (50).

How does a defect in CLC5 cause disease? The prevailing hypothesis is that CLC5 normally supplies the counterion conductance required for renal endosome acidification (49–51). In support of this, preliminary data cited in (50) but not yet published suggest that endosomes purified from CLC5-knockout mice are slower to acidify than wild-type endosomes. While the defects in membrane trafficking observed in CLC5-knockout mice are certainly consistent with those observed for cells in which organelle pH has been purposefully disrupted, a definitive role for CLC5 in endosome acidification has yet to be proven.

Defective acidification in cystic fibrosis

Cystic fibrosis is the most common fatal autosomal recessive disease affecting Caucasians, and occurs with an incidence of about 1 in 2000 live births. Hallmarks of the disease include pulmonary accumulation of viscous mucus, chronic airway infection, pancreatic dysfunction, high levels of sweat chloride, and infertility in males. Cystic fibrosis patients usually die of respiratory failure resulting from pulmonary obstruction and inflammation. The biochemical defect in CF has been traced to a defective chloride channel normally present at the apical surface of epithelial cells [see (52) for review]. This cAMP-regulated channel (CFTR, for cystic fibrosis transmembrane conductance regulator) is homologous to members of the ATP-binding cassette protein family of transporters. While mutations throughout the CFTR coding region cause CF, one particular mutation, deletion of the phenylalanine residue at position 508, is particularly frequent and comprises nearly 70% of CF alleles (52).

Some studies have suggested that CFTR-mediated chloride conductance regulates organelle pH in epithelial cells that normally express this channel. The 'acidification hypothesis' described by Barasch et al. (53,54) envisions a role for CFTR in dissipating the membrane potential that would accumulate during acidification of the TGN and endosomal compartments (similar to the postulated role of CLC5 in renal cells). A slight elevation in the pH of the *trans*-Golgi/TGN of CF cells might affect the glycosylation of newly synthesized glycoconjugates, which could explain the altered glycosylation of mucins isolated from CF patients that has been observed by many investigators [see (55) for review]. Intriguingly, a link between altered pH and glycosylation has already been established in tumor cells (56). In particular, glycoconjugates isolated from CF samples were found to be hyperfucosylated, hypersulfated, and hyposialylated compared with those analyzed in control samples. The accumulation of under-sialylated glycoconjugates at the cell surface of CF cells could also account for the predisposition of CF patients to infection by *Pseudomonas aeruginosa* bacteria, which have been demonstrated to bind to asialoglycolipids (57,58).

In favor of this elegant hypothesis, Barasch et al. estimated, using the membrane-permeant weak base DAMP, that TGN and endosomal pH in several CF cell lines were elevated by approximately 0.2 pH units compared to normal controls (53,54). The same investigators also found that a light vesicle fraction isolated from CF cells was slow to acidify compared to normal (53). Acidification of control cells required chloride as a counterion, as no acidification occurred in gluconate-substituted buffer. Moreover, treatment with valinomycin stimulated the acidification rate of CF but not of control cells, suggesting that acidification of CF cells was limited by a low chloride membrane conductance (53). Further support for this hypothesis comes from measured increases in cell surface levels of the asialoganglioside GA1 in immortalized CF compared with normal cells, and a concomitant decrease in the related monosialylated ganglioside GM1, raising the possibility that GA1 accumulates because sialylation is inefficient in CF cells (53,59). Finally, Barasch et al. found that degradation of endocytosed α_2-macroglobulin was delayed significantly in CF compared to normal cells, although lysosomal pH was unaltered, suggesting that defective endosomal acidification could account for the inefficient sorting of this protein to lysosomes (53).

By contrast, other groups have found no evidence that CFTR regulates organelle pH. No significant change in the pH of endosomes or the TGN was observed when CFTR was transfected into CHO, 3T3, and L cells (60–63). This is not necessarily unexpected, as endogenous acidification mechanisms presumably operate normally in these cells regardless of CFTR expression. In a more convincing study, however, Dunn et al. used fluorescence ratio imaging to carefully compare endosomal pH in the CF pancreatic adenocarcinoma cell line CFPAC-1 and a CFTR-rescued clone, and found no difference between the two (64).

In addition, while there is considerable evidence that CF-derived glycoconjugates differ from normal, it is not clear whether this is due to altered Golgi pH in CF cells. One complicating factor is that CF cells secrete large quantities of mucin compared to normal cells (65), and it is difficult to obtain large enough quantities of normal mucin for detailed structural and compositional studies (66,67). Furthermore, not all of the mucus-secreting cells in the airway express CFTR (68). In addition, airway infection and inflammation can affect the composition or structure of cell surface glycoconjugates and mucous secretions isolated from CF patients (67,69,70).

Comparisons of glycosylation patterns in CF and control cell lines have also yielded discrepant results. Decreased cell surface levels of the asialoglycolipid GM1 were observed in airway epithelial cells in which CFTR activity was down-regulated compared with their parental controls (71). Small differences in the sialic acid content of glycoconjugates were also measured in these cells (72). By contrast, expression of CFTR did not alter glycosylation of mucins in CFTR-expressing vs. nonexpressing matched colon carcinoma cells (73). Moreover, our laboratory showed that differences in terminal glycosylation between a CF cell line and its CFTR-rescued control were pH-independent and likely due to clonal drift (74). Finally, while disruption of pH clearly slows biosynthetic traffic, newly synthesized proteins were delivered to the cell surface with identical rates in CF cells and functionally rescued controls (75).

In a surprising twist, two recent reports have found that TGN pH in CF cells is *hyper*acidified compared with control cells. Chandy et al. performed fluorescence ratio imaging of pH-sensitive GFP-sialyltransferase chimeras to measure *trans*-Golgi pH in several cell lines (76). Interestingly, Golgi pH was 0.2 pH units lower in CF cells compared with genetically matched rescued cells (6.7 vs. 6.9, respectively). This difference was statistically significant, but considered unlikely to be physiologically important (76,77). Interestingly, Golgi pH measured in HeLa cells using the same method was considerably lower (pH 6.4), suggesting a differences in V-ATPase pumping activity between the two cell types (76). Using a similar approach, in which TGN pH was quantitated using a pH-sensitive GFP-TGN38 chimera, Poschet et al. found a more sizable acidification in CF cells relative to control (78). The TGN pH of CF cells in these experiments was 6.2 [compared with 6.7 measured in the same cells by Chandy et al. (76)], whereas rescued cells had an increased pH of 6.7 (78). In addition, this group also found that endosomal pH was also hyperacidified in CF cells, and that TfR recycling was altered (79). The TGN and endosomal hyperacidification observed in CF cells was Na^+ dependent, suggesting that CFTR regulation of Na^+/K^+-ATPase or epithelial sodium channel activity was responsible (78,79). Indeed, CFTR has been found to modulate the activity of numerous other ion transporters and channels (80). Thus, according to this model, the lack of CFTR in an acidified compartment might decrease Na^+/K^+-ATPase-mediated Na^+ influx or increase channel-mediated Na^+ efflux and thereby lower the membrane potential to enable hyperacidification.

The dramatic difference in TGN pH measured in the same cell lines by the two groups is likely not due to differential localization of the pH probes used, as the distribution of the GFP-TGN38 chimera overlapped nearly completely with co-expressed myc-tagged sialyltransferase (78). Moreover, lectin binding experiments suggested that CF cell glycoconjugates were hyposialylated compared with rescued controls, as predicted by the acidification hypothesis (78). Thus, while it is difficult to explain why airway cells (both wild-type and CFTR-corrected) appear to have markedly elevated TGN pH compared to other cell lines, it is possible that hyperacidification of the TGN could account for the altered glycosylation profile of CF cells previously observed by others. The exact mechanism by which CFTR regulates organelle pH, however, remains to be elucidated.

Concluding Remarks

It is clear that acidification of a subset of intracellular compartments is required for normal homeostasis. Multiple mechanisms contribute to the maintenance of organelle pH, and there is accumulating evidence that altered pH regulation is a hallmark of some disease states. For example, global effects on organelle acidification due to altered V-ATPase activity, levels, or localization have been shown to correlate with cell transformation. Furthermore, the restoration of regulated organelle pH in some tumor cells may contribute to their acquired resistance to chemotherapeutic drugs. Indeed, the efficacy of some chemotherapeutic agents such as tamoxifen that are particularly useful in treating drug-resistant tumors may be related to their ability to reverse such changes in organelle pH. In addition, changes in organelle pH may explain many of the symptoms observed in Dent's disease and cystic fibrosis.

Despite these tantalizing links, there is as yet no definitive example where a defect in acidification is the primary cause of disease pathogenesis. In part, this is because our understanding of how acidification contributes to cell homeostasis remains somewhat limited. For example, experimental data using different pH perturbants have yielded somewhat discrepant results regarding the role of acid pH in various membrane sorting and trafficking steps (10). In part, these discre-

pancies are due to our limited ability to selectively disrupt the pH of individual compartments (81); moreover, many pH perturbants are known to indirectly compromise other cellular functions (10). Finally, the components that regulate organelle pH have additional roles. For example, in addition to their functions as part of the V-ATPase holoenzyme, individual subunits and combinations of V-ATPase subunits have been suggested to participate in the regulation of homotypic membrane fusion, endocytosis, and other membrane trafficking events (82,83). Similarly, a regulatory role for CFTR in membrane traffic has been proposed that may involve pH-independent interactions with trafficking machinery (84). A better understanding of how these various components normally function together to regulate pH in different cell types and compartments will provide a stronger foundation with which to interpret the studies described above.

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