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A specific mutation abolishing Na^+/H^+ antiport activity in hamster fibroblasts precludes growth at neutral and acidic pH

(H^+-suicide selection/cytoplasmic pH/Na^+ influx/growth control/somatic cell genetics)

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ABSTRACT **A H^+-suicide technique based on the reversibility of Na^+/H^+ antiport was developed for the selection of mutants deficient in this membrane-bound activity. The strategy was to use the Na^+/H^+ antiporter as a H^+-vector killing device. Chinese hamster lung fibroblasts (CCL39) were loaded with LiCl and incubated in Na^+-, Li^+-free choline Cl saline solution (pH 5.5). Under these conditions, intracellular pH dropped in 5 min from 7.1 to 4.8, leading to a rapid loss of cell viability (<0.1% survival after 30 min). Cytoplasmic acidification and cell death were prevented by treatment with 5-*N*,*N*-dimethylamiloride, a potent inhibitor of Na^+/H^+ antiport. Of the H^+-suicide resistant clones that survived two cycles of selection, 90% were found deficient in Na^+/H^+ antiport activity. One class of mutants (PS10, PS12) fully resistant to the H^+-suicide test, does not acidify the cell interior in response to an outward-directed Li^+ gradient and has no detectable amiloride-sensitive Na^+ influx measured either in Li^+- or H^+-loaded cells. Growth of these fibroblast clones lacking Na^+/H^+ antiport was found to be pH conditional in HCO_3^--free medium. Whereas wild-type cells can grow over a wide range of external pHs (6.6–8.2), PS mutants cannot grow at neutral and acidic pHs (pH < 7.2); their optimal growth occurs at alkaline pH values (pH 8–8.3). These findings strongly suggest that the Na^+/H^+ antiport activity through regulation of intracellular pH plays a crucial role in growth control.**

The molecular events that control the reversible G_0/G_1 growth-arrest state of animal cells are largely unknown. A rather striking observation is that a variety of polypeptide growth factors (e.g., epidermal growth factor, platelet-derived growth factor, α-thrombin) acting on distinct membrane receptors (1–5), converge very early for the stimulation of ionic fluxes (6–10) and phosphorylation of a common set of proteins (11, 12). Therefore, a complete dissection and analysis of the early and ubiquitous biochemical changes linked to mitogenicity should help the understanding of growth control at a molecular level.

It is now well established that one of the earliest actions of growth factors on quiescent cells is the activation of a plasma membrane-bound Na^+/H^+ antiporter (13–15), leading to cytoplasmic alkalinization (13, 16–19). Intracellular pH (pH_i), therefore, has been postulated as a possible mitogenic signal, yet there is no direct proof favoring this hypothesis. To test this hypothesis and to analyze the role of Na^+/H^+ antiport in pH_i regulation, we developed a technique to select mutants deficient in Na^+/H^+-exchange activity. We applied this technique to Chinese hamster lung fibroblasts (CCL39), a cell line capable of entering a reversible G_0/G_1 growth-arrest state (20) and in which we have characterized a growth factor-activatable Na^+/H^+ antiporter (14, 21). In this report, we describe a highly specific H^+-suicide technique to isolate mutants lacking Na^+/H^+-exchange activity. Preliminary characterization of these mutants reveals that their growth is pH conditional in HCO_3^--free medium: at external alkaline pH, they grow at a normal rate, whereas their growth is totally prevented at neutral and acidic pH. This finding shows that the Na^+/H^+ antiporter, and therefore pH_i (16, 19), plays a determinant role in the control of growth.

MATERIALS AND METHODS

Materials. Materials used for these studies were obtained from the following sources: $^{22}NaCl$ (carrier-free) was from Radiochemical Centre (Amersham) and 7-[^{14}C]benzoic acid (29.4 mCi/mmol; 1 Ci = 37 GBq) was from New England Nuclear. 5-*N*,*N*-dimethylamiloride (DMA) was a gift from E. Cragoe, Jr. (Merck Sharp & Dohme). All other chemicals were of the highest purity commercially available.

Cells and Culture Conditions. The Chinese hamster lung fibroblast line CCL39 (American Type Culture Collection) and mutant derivatives PS6, PS10, PS12, PS20, and PS21 were maintained in Dulbecco's modified Eagle's medium (GIBCO) containing 25 mM $NaHCO_3$ and supplemented with 5% fetal calf serum/penicillin (50 units/ml)/streptomycin (50 μg/ml). Cells were maintained at 37°C in 5% CO_2/95% air.

Method of Selection for Mutants Lacking Na^+/H^+ Antiport Activity. CCL39 cells were mutagenized with ethyl methanesulfonate as reported (22). An exponentially growing culture, freshly trypsinized, was incubated for 2 hr at 37°C (1.5×10^6 cells per ml) in the saline medium: 130 mM LiCl/5 mM KCl/1 mM $MgSO_4$/2 mM $CaCl_2$/5 mM glucose/20 mM Hepes-Tris, pH 7.4. This solution, referred as LiCl saline solution, was removed by centrifugation and the cell pellet was washed once and then incubated at 37°C (2×10^6 cells per ml) in choline Cl acid saline solution (130 mM choline Cl/5 mM KCl/1 mM $MgSO_4$/2 mM $CaCl_2$/20 mM 2-(*N*-morpholino)ethanesulfonic acid-Tris, pH 5.5. After 60 min in this medium (including the centrifugation time) the cell pellet was immediately resuspended in regular HCO_3^-/CO_2-buffered culture medium, and the cells were plated at 2×10^6 cells per 100-mm dish. After 4 days of culture, resistant cells were trypsinized and subjected to an identical cycle. At the end of the second cycle, cells were plated at 10^5 cells per 60-mm dish, and 1 week later, resistant clones were picked up with cloning rings and recloned. The mutants PS6, PS10, PS12, PS20, and PS21 were selected from two independent mutagenized cell populations. This selection (one cycle) was also adapted with success to cells in monolayers by increasing the incubation time to 80 min in the choline Cl acid saline solution. The mutants arose after one cycle of selection at a frequency of $\approx 2 \times 10^{-6}$ and were stable over 4 months of continuous passage in the absence of selective pressure.

Cell Fusion and Selection of Hybrids. 023-61 cells (ouabain resistant and hypoxanthine phosphoribosyltransferase deficient) were obtained from CCL39 in two steps: resistance to

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Abbreviations: pH_i, intracellular pH; pH_o, extracellular pH; DMA, 5-*N*,*N*-dimethylamiloride.

3 mM ouabain (clone 023) and resistance to 50 μg of 8-azaguanine per ml (clone 023-61). PS120 and PS200, subclones of PS12 and PS20, respectively, were fused with 023-61 cells by the polyethylene glycol (PEG 1000) technique (23). Hybrids, which arose at a frequency of ≈10%, were selected by their ability to clone and grow continuously in the selective medium containing 3 mM ouabain and hypoxanthine/aminopterin/thymidine (HAT medium) (24).

Measurement of pH_i. pH_i was measured by the technique of distribution of the weak acid 7-[^{14}C]benzoic acid in intracellular and extracellular spaces (25). Confluent cultures in 16-mm wells were equilibrated at 37°C in HCO_3^--free saline medium at the extracellular pH (pH_o) indicated; 1 μCi of [^{14}C]benzoic acid per ml (34 μM) was added for 5 min and the wells were rapidly washed by aspiration 4 times with ice-cold phosphate-buffered saline (pH 7.4) (the 4 washes lasted 7 ± 1 sec). Cells were solubilized in 0.1 M NaOH and radioactivity was assayed by liquid scintillation spectrometry. A detailed analysis of this technique with critical evaluation of extracellular and intracellular water space, kinetics of equilibration, and pH_i calculation was reported elsewhere (19). Intracellular water space was calculated based on the amount of protein per well and the estimated value of 5 μl of cell water per mg of protein (19).

Measurements of Intracellular Cations. Cell monolayers were rinsed 4 times with ice-cold 0.1 M $MgCl_2$, and cells were lysed by incubation in toluene-saturated H_2O. Li^+, K^+, and Na^+ were measured by flame photometer.

$^{22}Na^+$ Uptake. Prior to Na^+ influx measurements, cells grown to confluency in 16-mm wells were preloaded either with Li^+ or H^+. Li^+ loading was carried out by incubating the cells at 37°C for 2 hr in the LiCl saline medium. H^+ loading was carried out as described by Roos and Boron (25). Cells were incubated for 30 min at 37°C in cultured medium buffered at pH 7.4 with 20 mM Hepes and containing 50 mM NH_4Cl. Cell monolayers were quickly washed with 135 mM choline chloride (pH 7.4) and incubated for 6 min in $^{22}Na^+$ uptake medium at 37°C: 135 mM choline Cl/1 mM $MgCl_2$/2 mM $CaCl_2$/1 mM ouabain/20 mM Hepes-Tris, pH 7.4/1 μCi of carrier-free $^{22}NaCl$ per ml. Influx of $^{22}Na^+$ was stopped by rinsing the cell monolayer 4 times with phosphate-buffered saline at 0°C (the 4 washes lasted 10 sec). The amiloride-sensitive $^{22}Na^+$ influx was determined as the difference in the initial rate of $^{22}Na^+$ uptake measured in both the absence and the presence of 100 μM DMA ($K_i = 0.2 \times 10^{-6}$ M (26).

RESULTS

The Na^+/H^+ exchanger has been identified in all vertebrate cells that have been examined (26, 27) and represents the primary mechanism of pH_i regulation (16, 18, 19, 27, 28). Consequently, a mutation deleting this essential function might be lethal. However, pH_i regulation might also be sustained by a separate Na^+-dependent Cl^-/HCO_3^- antiporter (unpublished results). Along this line, we observed that very potent analogs of amiloride [$K_i = 0.04 \times 10^{-6}$ M for inhibiting Na^+/H^+ antiport activity (26)] used at concentrations inhibiting >98% of Na^+/H^+ exchange activity could have opposite effects depending on the presence of bicarbonate in the culture medium. In HCO_3^-/CO_2-buffered medium, amiloride analogs have no effect on fibroblast growth, whereas growth is totally abolished in HCO_3^--free Hepes-buffered medium (data not shown). This finding is important, because it predicts that mutants devoid of Na^+/H^+ exchange activity should not be lethal if they are isolated and grown in HCO_3^--buffered medium.

Principle of the H^+-Suicide Selection Technique. The technique is based on the reversibility of Na^+/H^+ antiport and on the fact that Li^+ is as efficient as Na^+ for the trans-stimulation of H^+ movements (21). Under physiological conditions (Fig. 1*A*), the inward-directed Na^+ gradient maintained by the Na^+,K^+ ATPase drives H^+ generated by cell metabolism out of the cell. We postulated that this function, which is beneficial for the cell, would become deleterious if we reversed the functioning of the Na^+/H^+ antiporter by reversing the Na^+ gradient. The strategy chosen was to use the Na^+/H^+ antiporter as a H^+-vector killing device. Therefore,

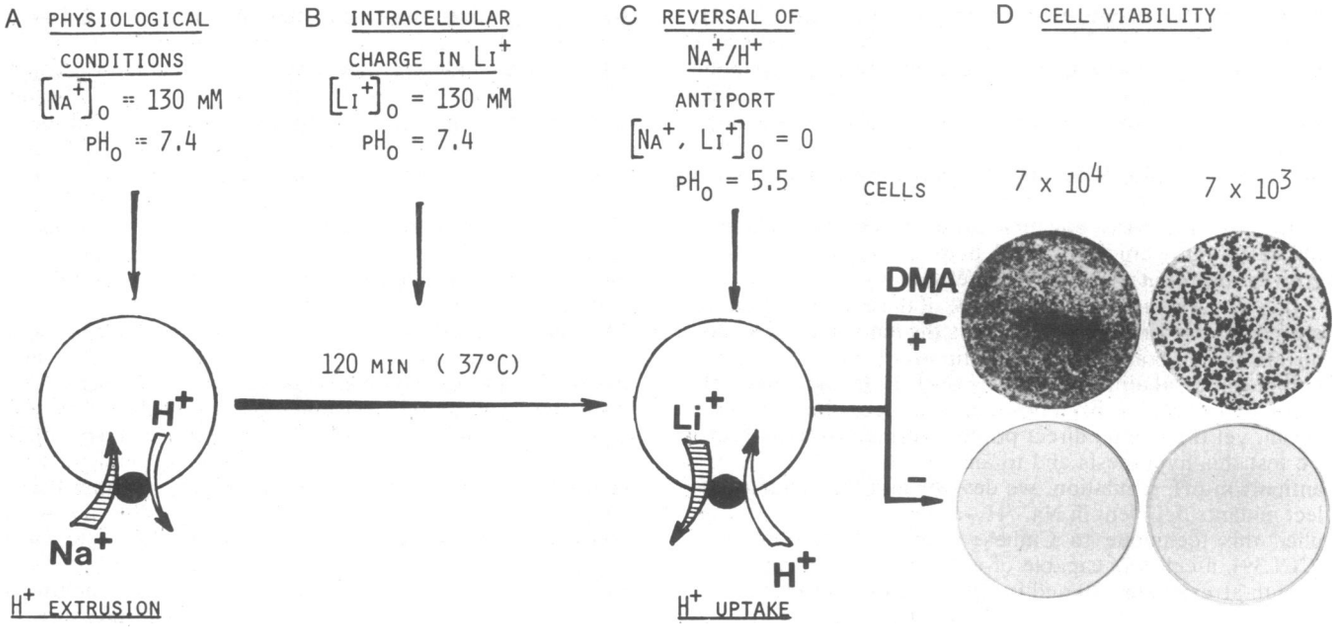

FIG. 1. Schematic representation of the proton-suicide technique and pictures showing H^+-induced cell killing via the Na^+/H^+ exchange. Trypsinized CCL39 cells were first incubated for 2 hr in suspension (8×10^6 cells per ml) in LiCl saline solution (pH 7.4). These Li^+-loaded cells were then rapidly centrifuged, washed once, and transferred at 37°C to the choline Cl acid saline solution (pH 5.5). One-half of the cell suspension received 100 μM amiloride analog (DMA) during the washing and the incubation at pH 5.5. After 60 min at pH 5.5 in the presence (+) or absence (−) of DMA, cells were diluted in growth medium and plated in 35-mm dishes at 7×10^4 and 7×10^3 cells. Colonies stained after 5 days of culture are shown in *D*.

mutants lacking Na^+/H^+ antiport activity were expected to survive the H^+ trap.

The first step consists of loading the cells with LiCl (Fig. 1*B*). After 2 hr in 130 mM LiCl (pH_o, 7.4), the intracellular Li^+ concentration reached 80–90 mM, and pH_i was 7.1. In the second step (Fig. 1*C*), we created two chemical gradients of opposite direction: an inward-directed H^+ gradient and an outward-directed Li^+ gradient, by replacing the external medium with a Na^+,Li^+-free choline Cl saline solution buffered at pH 5.5. We expected such conditions to cause a deleterious proton uptake, which should be prevented in the presence of the potent amiloride analog DMA. These predictions proved correct, because after a 60-min exposure to the choline Cl acid saline, cell viability dropped dramatically, and 100 μM DMA efficiently prevented cell death (Fig. 1*D*).

Fig. 2*A* shows a rapid and progressive loss of cell viability with time. A 15-min exposure to pH 5.5 is sufficient to kill 99% of the cells; 30 min decreases cell viability by more than 3 logarithms. Fig. 2*B* shows that the DMA protection is explained by its capacity to prevent acute intracellular acidification. After a 5-min exposure to pH_o 5.5, pH_i decreased from 7.1 to 4.8 and stayed below 4.8 for more than 30 min, whereas in the presence of DMA it decreased to 6.2 during the initial 5 min and thereafter declined slowly to reach a value close to pH_o 5.5. In the absence of DMA, H^+ rapidly accumulated in the cytoplasm against a concentration gradient, because addition of digitonin led to equilibration between pH_i and pH_o (Fig. 2*B*). Associated with the rapid proton uptake, we measured a significant efflux of Li^+, strongly inhibited by DMA. After 30 min of exposure to pH 5.5, the initial 90 mM intracellular Li^+ decreased to 20 mM in the absence and to 60 mM in the presence of DMA.

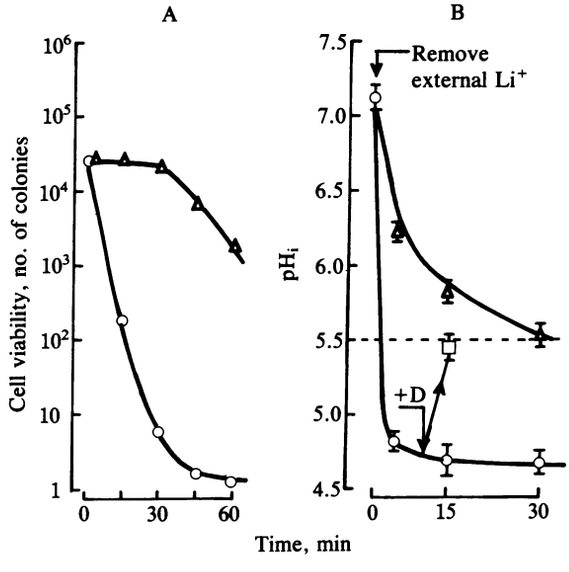

FIG. 2. Analysis of cell viability and intracellular pH after reversal of Na^+/H^+ antiport. (*A*) CCL39 cells loaded in Li^+ by a 2-hr incubation in LiCl saline solution were centrifuged, washed free of LiCl, and transferred for various periods of time at 37°C to the choline Cl acid saline solution (pH 5.5) in the absence (○) or presence (△) of 100 μM amiloride analog, DMA. At the times indicated, the cell suspension was diluted in growth medium and plated in 60-mm dishes. Cell viability was estimated after 1 week by counting colonies on duplicate dishes from serial dilutions of the initial cell suspension. (*B*) pH_i was estimated with the distribution of [^{14}C]benzoic acid. CCL39 cells grown to confluency in 16-mm wells were loaded with LiCl for 2 hr. The pH_i measured was 7.1. Cells were then washed twice free of Li^+ and incubated in the choline Cl acid saline solution (pH 5.5) in the absence (○) or presence (△) of 100 μM DMA. [^{14}C]Benzoic acid (1 μCi/ml) was added 5 min before each time point. Where indicated by the arrow (+D), 50 μM digitonin was added to parallel wells, and pH_i was measured (□). Values represent the mean ± SEM of three determinations.

The Proton Suicide-Resistant Clones (PS Mutants) Are Deficient in Na^+/H^+ Exchange Activity. The technique of selection outlined in Fig. 1 and detailed in *Materials and Methods* was applied to mutagenized CCL39 cells. The clones that survived two cycles of exposure to pH_o 5.5 were analyzed for (*i*) their capacity to resist a third proton suicide test, (*ii*) amiloride-sensitive Na^+ uptake influx rates, and (*iii*) intracellular acidification in response to the proton suicide test. Ninety percent of the clones that escaped the two cycles of selection are capable of resisting a third proton suicide test. These stable resistant clones fall into two classes. Cells of class 1 resist at almost 100% a 30-min proton suicide test, but they only resist partially when the test is applied for 60 min (10%–50% survival). These clones have <50% of normal Na^+/H^+ exchange activity. Class 2 cells (≈1/3 of the PS clones), represented by PS10, PS12, PS20, and PS21, are fully resistant to the 60-min proton suicide test. Amiloride-sensitive Na^+ influx was measured either in Li^+-loaded cells or in H^+-loaded cells (Fig. 3). Under these conditions of Na^+/H^+ antiport activation, amiloride-sensitive Na^+ influx represents, in wild-type cells, >95% of total Na^+ uptake. Interestingly, no detectable amiloride-sensitive Na^+ influx is observed in PS mutants of class 2 (PS10, PS12, PS20, and PS21); PS6, with ≈15% of Na^+/H^+ antiport residual activity, belongs to class 1. Accordingly, the amiloride-sensitive intracellular acidification (1 pH unit) induced by 30-min exposure to the proton suicide test, is abolished in PS12 and PS20 and is strongly decreased in PS6. Altogether, these results show that resistance to the proton suicide technique specifically selects for mutants impaired in Na^+/H^+ antiport activity.

Expression of Na^+/H^+ Antiport Activity in Hybrid Cells. To analyze the dominance/recessiveness of the character that endows proton suicide resistance, we crossed two PS mutants (PS120 and PS200, subclones of PS12 and PS20) with a ouabain resistant, hypoxanthine phosphoribosyltransferase deficient CCL39-derivative (023-61). Two hybrid clones derived from crosses between 023-61/PS120 and 023-61/PS200 (not shown) display intermediate levels of Na^+/H^+

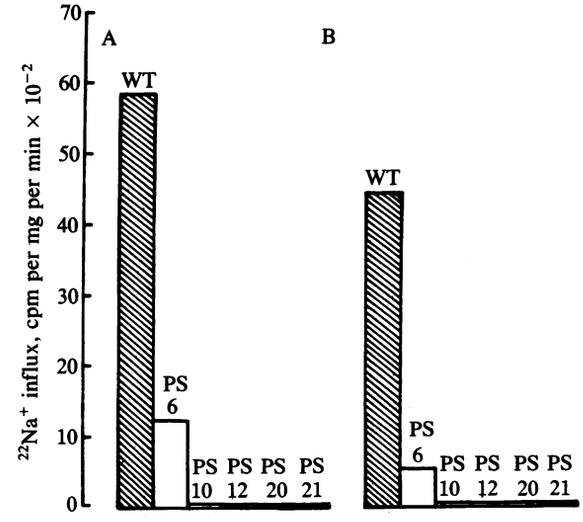

FIG. 3. Rates of amiloride-sensitive Na^+ influx in CCL39 and in H^+-suicide resistant cells (PS mutants). CCL39 and CCL39-derived mutant cells were loaded with either H^+ or LiCl prior to Na^+ uptake. Bars indicate the net amiloride-sensitive $^{22}Na^+$ influx in wild-type cells (WT) and in five H^+-suicide resistant clones (PS6, PS10, PS12, PS20, and PS21). (*A*) H^+-loaded cells; 50 mM extracellular NH_4^+ for 30 min at pH_o 7.4. (*B*) Li^+-loaded cells; 130 mM extracellular Li^+ for 120 min at pH_o 7.4.

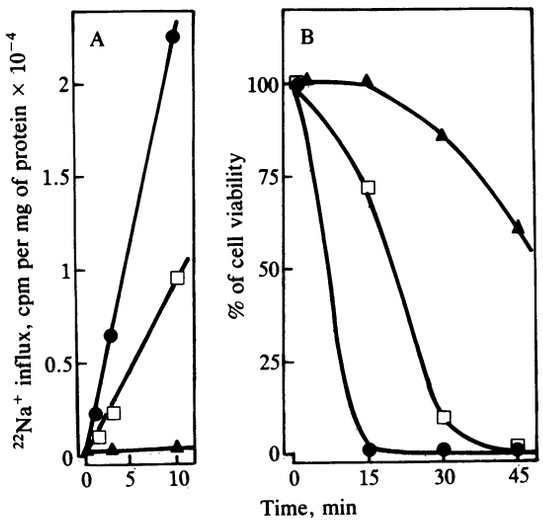

FIG. 4. Na^+/H^+ antiport activity and sensitivity to the proton-suicide test in parental cells, mutant PS120, and their hybrid derivatives. (*A*) Time-course of amiloride-sensitive Na^+ influx in wild-type 023-61 cells, ouabain resistant, hypoxanthine phosphoribosyltransferase deficient (●); mutant PS120 (▲); and hybrid cells 023-61 × PS120 (□). Before Na^+ uptake, cells were incubated for 30 min in 50 mM NH_4Cl to create an outward-directed H^+ gradient. (*B*) Cell viability as a function of time of reverse functioning of the Na^+/H^+ antiport for the mutant PS120 (▲), the CCL39-derivative 023-61 (●), and the hybrid cells 023-61 × PS120 (□). The conditions to induce H^+ uptake and to assay cell viability were the same as those described in legend of Fig. 2*A*.

antiport activity (42% ± 2% of the wild-type value; Fig. 4*A*), a result consistent with a co-dominant mutation. More than 90% of hybrid clones (tested directly on the dishes) have, as expected, an intermediate resistance phenotype in the proton suicide test, as shown in Fig. 4*B*.

Growth of Na^+/H^+ Antiport-Deficient Mutants Is pH Conditional. Because blockade of Na^+/H^+ antiport with potent amiloride analogs prevents reinitiation of DNA synthesis and growth in HCO_3^--free medium (26), we postulated that mutants lacking the Na^+/H^+ antiporter should be altered in their growth pattern. In HCO_3^--free medium buffered with either Pipes, Mops, or Hepes, clonal growth of wild-type CCL39 cells is observed in a pH range of 6.5 to 8.3, with an optimum value around pH 7.4. In contrast, clonal growth of PS10 and PS12 mutant cells is precluded at pH 7.2 and below. However, above this pH limit, clonal growth appears normal with an optimal pH for growth shifted to alkaline values 8–8.3. Fig. 5 shows exponential growth rates of wild-type cells and of 2 mutants lacking the Na^+/H^+ antiporter at various external pHs. Generation times at optimal pH are 12, 21, and 17 ± 1 hr for CCL39, PS10, and PS12 cells, respectively.

DISCUSSION

The proton-suicide method reported in this paper is highly efficient and specific for isolation of mammalian cells lacking Na^+/H^+ exchange activity. It is efficient because after two cycles of selection, 90% of the clones resistant to H^+ uptake were found to be defective in Na^+/H^+ antiport activity. Its specificity is demonstrated by the fact that the mutants analyzed (PS12 and PS20) are not impaired in other membrane transport systems such as the Na^+,K^+ ATPase, tetrodotoxin-sensitive Na^+ channels (29), amiloride-insensitive Na^+, or glucose and phosphate uptake. Therefore, this mutation does not result from a pleiotypic membrane defect but, rather, involves a specific alteration in the amiloride-sensitive Na^+/H^+ antiporter (14, 21). In three independent

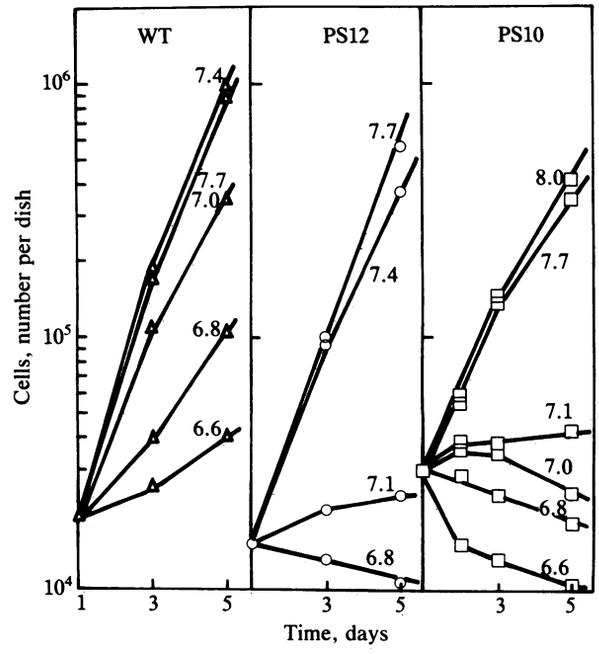

FIG. 5. Growth in HCO_3^--free medium of CCL39 cells and mutants lacking Na^+/H^+ antiport activity. Effect of external pH. Wild-type (WT) and mutant cells (PS12, PS10) were plated at ≈2 × 10^4 cells per 35-mm dish in regular medium buffered at pH 7.4 with CO_2/HCO_3^-. One day later (day 1), cells were washed with phosphate-buffered saline and incubated in HCO_3^--free growth medium buffered with either 30 mM 3-(*N*-morpholino)propanesulfonic acid for pH_o values of 6.6, 6.8, or 7.0 or with 30 mM *N*-2-hydroxyethylpiperazinepropanesulphonic acid for pH_o values of 7.4, 7.7, and 8.0 and supplemented with 100 μM hypoxantine/100 μM uridine/10% dialyzed fetal calf serum. Medium was changed on day 3. Values represent average cell number found in duplicate dishes.

selections, we analyzed 35 clones resistant to H^+ suicide. Although the pressure of selection was directed to specifically alter either the external H^+ site or the internal Na^+ (Li^+) site, all the mutants were found to be defective for both H^+ and Na^+ uptake. This result would favor the existence of either a common external site for H^+ and Na^+ or a close interdependence between these two sites, as already proposed (21). Alternatively, the simultaneous loss of both transport exchanges might simply reflect that a genetic event leading to a profound alteration of the Na^+/H^+ antiport protein occurs with a higher frequency than a discrete mutation altering one site of the antiport molecule and leaving the others intact.

Another point of interest is the emergence of a class of mutants (class 2) totally devoid of Na^+/H^+ antiport activity with a rather high frequency ($>10^{-6}$). This phenotype represents an additional example of high-frequency occurrence of recessive mutation in diploid cells (30–32). We observed that when the pressure of selection was lowered (incubation at pH_o 6.0 instead of 5.5; Fig. 1*C*) only mutants of class 1 with 30%–50% of Na^+/H^+ antiport residual activity were selected (data not shown). Therefore, in the light of a two-step model recently demonstrated for the *aprt* locus in Chinese hamster ovary cells (33), mutants of class 2 (PS10, PS12), with fully inactivated Na^+/H^+ antiport activity, might have resulted from two genetic events: one mutation occurring at one allele and some type of high-frequency inactivation event occurring at the homologous allele.

In the second part of this report, we analyzed the relationship between pH and cell proliferation. A large body of observations suggests that pH_i might play a determinant role in the control of growth (34). We show that removal of Na^+/H^+ antiport activity by genetic "surgery" abolishes fibroblast

growth at neutral and acidic pHs and that alkaline external pH (pH 8–8.3) is required to restore optimal exponential growth. Interestingly, and as expected, the pH-conditional growth characteristics of these mutants are completely reversed in bacterial mutants similarly defective in Na^+/H^+ antiport (35, 36). Physiological studies have clearly established that Na^+/H^+ antiport is the primary mechanism of pH_i regulation in most vertebrate cells. However, an entirely different transporter, which mediates the uptake of Na^+ and HCO_3^- and the loss of Cl^- and H^+, is responsible for pH_i regulation in all invertebrate animal cells examined (27). In this regard, comparison of PS mutant and parental cells was very instructive.

First, we showed that pH_i of PS12 mutant cells is 0.2–0.3 pH units more acidic than in the parent CCL39 cells and second, that growth factor-induced alkalinization in quiescent cells does not occur in Na^+/H^+ antiport mutant cells (19). Consequently, the failure of Na^+/H^+ antiport-deficient cells to grow in the range of neutral and acidic pHs (7.2–6.6) could be explained by their incapacity to reach a permissive pH_i value required for reinitiation of DNA synthesis (unpublished observations).

Two arguments favor this conclusion. First, growth is restored in PS10 and PS12 cells by increasing pH_o, a simple way to increase pH_i (19); second, the pH-conditional growth of PS mutants is abolished in CO_2/HCO_3^--buffered medium Consistent with the latter observation, we characterized ir CCL39 cells a Cl^-/HCO_3^- exchange involved in pH_i regulation (unpublished results; see refs. 28 and 37). The growth characteristics of these PS mutants are in close agreement with the conclusions drawn from our recent pharmacological studies (26). Indeed, using a series of amiloride analogs we established in HCO_3^--free medium, a tight correlation between blockade of the Na^+/H^+ antiport activity and inhibition of growth factor-induced DNA synthesis. We also found that inhibition of the Na^+/H^+ antiporter was no longer inhibitory for DNA synthesis at alkaline pH or in HCO_3^--buffered medium. Clearly, all these results suggest that the activity of the Na^+/H^+ antiporter regulates growth by regulating intracellular pH, a key step in growth control. At the same time, these results reveal that in HCO_3^--buffered medium Cl^-/HCO_3^- exchange can efficiently overcome a Na^+/H^+ antiport defect. The situation that prevails *in vivo* is unknown. Preliminary results indicate that tumor formation in athymic mice is strongly decreased with Na^+/H^+ antiport-deficient cells. More complete results in this direction should reveal the relative importance of the two pH_i-regulating systems for fibroblast growth *in vivo*.

Perhaps the most striking feature of the Na^+/H^+ antiporter is its powerful capacity to extrude H^+ out of the cell, a property linked to the inward-directed Na^+-gradient driving force and to the allosteric activation of the antiporter by intracellular protons (38, 39). A dramatic illustration of the protective action exerted by the Na^+/H^+ antiporter against acid overloading is provided by the following experiment. Induction of an acute acid load with NH_4^+ (25) in a mixed population of exponentially growing CCL39 and PS12 cells kills 100% of the Na^+/H^+ antiport-deficient cells with no effect on the wild-type cell population. These conditions, which exclusively favor the survival of cells endowed with a functional Na^+/H^+ antiporter, will serve as a basis for selection of revertants, transfectants, and (hopefully) cells with amplification of the Na^+/H^+ antiport system.

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