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The Na^+/H^+ Antiporter Potentiates Growth and Retinoic Acid-induced Differentiation of P19 Embryonal Carcinoma Cells*

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The Na^+/H^+ exchanger is a ubiquitous plasma membrane protein that is responsible for pH regulation and is activated by growth factors. We examined the role of the Na^+/H^+ exchanger in cell growth and differentiation. Treatment of P19 cells with the Na^+/H^+ exchanger inhibitor Hoe 694 eliminated retinoic acid-induced differentiation in this cell line. We developed a P19 embryonal carcinoma cell line that was deficient in the Na^+/H^+ antiporter. Na^+/H^+ exchanger-deficient cells were reduced in the rate of cell growth and this effect was enhanced by the removal of added HCO_3^- and by reducing extracellular pH. The antiporter-deficient cells were also markedly deficient in their ability to differentiate to neuronal-like cells and recovered this ability when the Na^+/H^+ antiporter was reintroduced. The results show that the absence of Na^+/H^+ antiport as a pH regulatory mechanism can result in deficiencies in both cell growth and differentiation in embryonal carcinoma cells.

The Na^+/H^+ exchanger is a plasma membrane protein that exchanges intracellular protons for extracellular sodium. In eukaryotes it is involved in pH regulation (1) and the control of cell volume and is stimulated by growth factors (2). Several isoforms of the protein have been identified (NHE1 to NHE5) in mammals, and the NHE1 isoform is the most ubiquitous isoform, being present in most, if not all, mammalian cells (3).

Studies have shown that NHE1 mRNA levels are increased due to a number of treatments including treatments that cause cellular differentiation and chronic acid loading (4–6). During differentiation of human leukemic cells (HL-60), there is an 18-fold increase in NHE1 transcription and a 7-fold increase in protein levels (4, 7). More recently we have shown that there is a transient increase in the level of NHE1 transcription during retinoic acid-induced differentiation of P19 cells and an increase in activity of the protein (8). We have also shown that NHE1 mRNA levels increase when L6 cells differentiate from myoblast to myotubes and the NHE1 promoter was also activated during L6 muscle cell differentiation (9). This data suggests that Na^+/H^+ antiporter plays an important role in cellular differentiation. Increased antiporter activity during differentiation has been suggested to be important for differentiation to occur, at least in some cell types (10, 11). However, this requirement has not been shown to occur universally and the role of the Na^+/H^+ exchanger may vary between cell types (12).

Another important related aspect of pH regulation is its role in cell growth and proliferation. It has long been suggested that the Na^+/H^+ antiporter may play a role in the onset and/or maintenance of cell proliferation (13). In HCO_3^- free media, some types of Na^+/H^+ exchanger-deficient cells cannot proliferate in media of low external pH (14). In addition, extracellular Na^+ is a limiting factor in growth factor dependent proliferation (15). The Na^+/H^+ exchanger inhibitor, amiloride and its analogs, block changes caused by growth factor stimulation especially in HCO_3^- free or low Na^+ medium (16). It is also clear that in some cell types the mechanism by which the Na^+/H^+ exchanger acts is to attain a permissive pH_i[1] that is critical to the development of mitogenic responses elicited by growth factors (17). In addition, Na^+/H^+ exchange is important in pH regulation in some tumor types and is a target for inhibition in tumor selective therapy (18). However, it should be noted that Na^+/H^+ antiport activation is not a prerequisite for cell proliferation in all cell types (19).

We recently showed that the NHE1 promoter is activated during the early stages of neuronal differentiation of P19 cells (8). This suggested that this protein may play an important role in differentiation in this cell type. To examine this possibility we studied the role of the NHE1 protein in cell growth and differentiation of P19 cells. The results show that the Na^+/H^+ exchanger plays an important role in cell proliferation and differentiation of P19 cells, an important and widely used model of cell differentiation. These findings are the first direct demonstration that deletion of the Na^+/H^+ exchanger can directly affect differentiation of mammalian cells.

EXPERIMENTAL PROCEDURES

Materials—Ethyl methanesulfonate (EtMes), all *trans*-retinoic acid and G418 were obtained from Sigma. 2′,7′-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM) was purchased from Molecular Probes (Eugene, OR). All other chemicals were of analytical or molecular biology grade and were purchased from Fisher Scientific, Sigma, BDH (Toronto, Ontario, Canada), or Life Technologies, Inc. The neuron specific class III β-tubulin monoclonal antibody was a gift from Dr. A. Frankfurter of the University of Virginia. Fluorescein-conjugated goat anti-rabbit IgG was from Bio/Can Scientific, Inc. (Mississaugua, Ontario, Canada). 5-(*N*,*N*-hexamethylene)-amiloride (HMA) was from Research Biochemicals International (Natick, MA). Hoe 694 was a gift from Drs. H. J. Lang and W. Scholz (Hoechst, Frankfurt).

Cell Culture—P19 mouse embryonal carcinoma cells were obtained

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[1] The abbreviations used are: pH_i, intracellular pH; BCECF-AM, 2′,7′-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester; EtMes, ethyl methanesulfonate; HMA, 5-(*N*,*N*-hexamethylene)-amiloride; α-MEM, α-minimum essential medium; PAGE, polyacrylamide gel electrophoresis.

from American Type Culture Collection. Wild-type and mutant P19 cells were maintained in α-minimum essential medium supplemented with 25 mM $NaHCO_3$, 2.5% fetal bovine serum, and 7.5% newborn calf serum as reported earlier (8). G20 cells were derived from mutated P19 cells (described below) and lacked Na^+/H^+ exchanger activity. Mutant G20 cells were grown in the above medium, which contained 20 mM HEPES pH 7.4 to ensure that the medium pH did not acidify with cell growth. Cell numbers were measured using a hemocytometer. To induce cell differentiation, wild-type and mutant P19 cells were grown in medium with 1 μM retinoic acid in nonadhesive plates for 48 h, and after aggregation they were plated on adhesive substrates without retinoic acid for another 48 h. In some experiments we examined the effect of Hoe 694 on differentiation of P19 cells. In these experiments, experimental cells had the same protocol except in the presence of 10 μM Hoe 694.

P19 cells were mutagenized with EtMes essentially as described elsewhere (20). P19 cells grown to 70% confluency were treated with 0.25 μl/ml EtMes in α-MEM overnight. With this treatment, 80–90% of the cells did not survive. Surviving cells were grown to confluency and then selected for lack of Na^+/H^+ antiport by the "proton suicide" technique. The technique is based on the reversibility of Na^+/H^+ antiport and the fact that Li^+ is transported by the exchanger. It results in reversal of activity and H^+ killing of cells with an active transporter (14). Trypsinized cells were incubated in LiCl buffer (130 mM LiCl, 5 mM KCl, 1 mM $MgSO_3$, 2 mM $CaCl_2$, 5 mM glucose, and 20 mM HEPES, pH 7.4) for 2 h at 37 °C. After centrifugation, the cell pellet was washed once with 1 × phosphate-buffered saline and then incubated with choline Cl buffer (130 mM choline chloride, 5 mM KCl, 1 mM $MgSO_3$, 2 mM $CaCl_2$, and 20 mM 2-(*N*-morpholino)ethanesulfonic acid, pH 5.5) for 1 h at 37 °C. The cells were pelleted again and resuspended and grown in α-MEM supplemented with 20 mM HEPES, pH 7.4. After 7–10 days, resistant colonies were formed and grown individually. The individual clones were selected two additional times using the proton suicide technique. The rate of recovery from an ammonium chloride-induced acid load was used to confirm that clones were devoid of Na^+/H^+ antiporter activity (21).

To reintroduce the Na^+/H^+ exchanger back into G20 cells, the expression system used was the pOPRSVicat mammalian expression system (Stratagene). The vector was digested with *Not*I and filled in with the enzyme Klenow, and a full-length insert coding for the human NHE1 isoform of the Na^+/H^+ exchanger (22) was blunt end ligated and screened for orientation. The resulting plasmid, $pYN4^+$, was used to transform G20 cells, using the calcium phosphate precipitation method as described earlier (8, 9). G20 cells were selected with 400 μg/ml G418. Resistant colonies (G20;pYN4) were identified, and individual colonies were repropagated until reaching a higher density.

Internal pH Measurement—Measurement of pH_i in P19 cells was essentially as described earlier (8). P19 and G20 cells were grown to 80% confluency on coverslips. Fluorescence measurements were made on a Shimadzu RF-5000 spectrofluorophotometer. Coverslips were incubated in a cuvette with constant stirring. The acetoxymethyl ester of BCECF-AM (0.1 μg/ml) was incubated with cells for 30–60 s in a solution containing 135 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgSO_4$, 5.5 mM glucose, 10 mM HEPES, pH 7.4, at 37 °C. Intracellular pH was measured using the dual excitation single emission ratio technique. Excitation wavelengths were at 452 and 500 nM with emission at 520 nM. This allows measurement of intracellular pH that is independent of cell concentration and dye loading (23). A calibration curve for intracellular dye was generated by the nigericin method (23). To measure initial rates of proton extrusion after an acid challenge the NH_4Cl prepulse method was used. The coverslip was transferred to a buffer containing 15 mM NH_4Cl for 5 min and then to a Na^+ free medium that contained 135 mM *N*-methyl-D-glucamine instead of NaCl. The buffer was then changed to one containing 135 mM NaCl, and the initial rate of rise of internal pH was measured during the first 40 s of recovery.

Polyacrylamide Gels and Immunostaining—P19 and G20 cells were grown in α-MEM supplemented with or without 25 mM $NaHCO_3$ for 2 days and collected in Buffer A (10 mM Tris, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride) using a rubber policeman. To characterize the extent of differentiation, P19 cells were treated with retinoic acid as described above and harvested 2 days after retinoic acid treatment. Cells were lysed by sonication for 45 s, and the protein concentration was determined by Bio-Rad protein assay. 25 μg of each cell lysate was separated on a 12% SDS-PAGE, and proteins were transferred electrophoretically to nitrocellulose membranes. Immunostaining of nitrocellulose was carried out in the presence of 1% skim milk powder using the neuronal specific class III β-tubulin monoclonal antibody (8). The antibody was detected using the Amersham enhanced chemiluminescence Western blotting and detection system as described by the manufacturer.

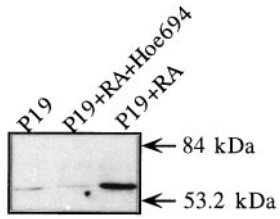

FIG. 1. **Effect of 10 μM Hoe 694 on the ability of P19 cells to differentiate to neuronal-like cells.** P19 cells were treated with 1 μM retinoic acid for 2 days in nontissue culture dishes with or without 10 μM Hoe 694. Cells were then transferred to tissue culture Petri dishes and grown for 2 more days in α-MEM in the presence or absence of Hoe 694 (pH 7.4). The cells were collected, and equal amounts of protein of each sample (25 μg) were separated by 12% SDS-PAGE and examined by Western blotting with neuronal specific class III anti-β-tubulin antibody. *Lane 1*, control cells not treated with retinoic acid (*P19*); *lane 2*, cells treated with 10 μM Hoe 694 throughout the differentiation procedure (*P19+RA+Hoe694*); *lane 3*, cells treated with retinoic acid in the absence of Hoe 694 (*P19+RA*).

In some experiments we examined the level of the Na^+/H^+ exchanger in membrane preparations from P19 and G20 cells. P19 and G20 cells were swollen in 10 volumes of lysis buffer (10 mM Tris, pH 8.0, 25 mM KCl, 2 mM $MgCl_2$, 2 mM EGTA, 2 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride) for 20 min at 4 °C. They were lysed by using 50 strokes with a tight fitting Dounce homogenizer on ice. The lysate was diluted to 20 volumes with lysis buffer containing sucrose added to make a final concentration of 250 mM. Unlysed cells and nuclei were removed by centrifugation at 1,000 × *g* for 10 min. The supernatant was centrifuged at 100,000 × *g* for 1 h to obtain a crude microsome fraction. The crude membrane fraction was solubilized with SDS-PAGE sample buffer and used for SDS-PAGE (9%) followed by Western transfer and immunoblotting. The antibody for immunoblotting was affinity purified anti-NHE1 antibody directed against the cytoplasmic domain of the Na^+/H^+ exchanger (24).

RESULTS

We initially examined whether the Na^+/H^+ exchanger could play a role in the differentiation of P19 cells. Cells were treated with retinoic acid in the presence of Hoe 694, a potent inhibitor of the Na^+/H^+ exchanger. Cell extracts were then examined for the presence of β-tubulin, a marker of neuronal differentiation (25). Typical results are shown in Fig. 1. Control cells contained residual amounts of β-tubulin, likely due to a small amount of spontaneous differentiation that usually occurs in these cells. P19 cells treated with retinoic acid and Hoe 694 showed only the same small amount of β-tubulin. In contrast P19 cells treated with retinoic acid in the absence of Hoe 694 showed a much larger amount of β-tubulin, which is typical of cells undergoing differentiation.

To establish a Na^+/H^+ exchanger-deficient cell line, P19 cells were treated with 0.25 μg/ml EtMes for 12 h. This treatment resulted in the death of approximately 90% of the cells. Following this treatment, cells were then subjected to the proton suicide selection technique for 3 h (20). The surviving cells formed colonies after 7–10 days of growth. We examined 22 colonies for Na^+/H^+ exchanger activity. Four out of 22 clones showed no, or greatly diminished, pH_i recovery when subjected to ammonium chloride-induced acid load. One of the mutant clones, G20, was reselected two more times by the proton suicide technique. This clone had the same morphology as the wild-type P19 cells. Since it was sensitive to external pH variation, the cells were maintained in medium containing HEPES, pH 7.4. We examined the G20 clone for Na^+/H^+ exchange activity using the NH_4Cl prepulse technique (Fig. 2*A*). The results showed that the rate of recovery from an acid load was greatly diminished in this cell line. There was no apparent difference in the degree of acidification induced by NH_4Cl sug-

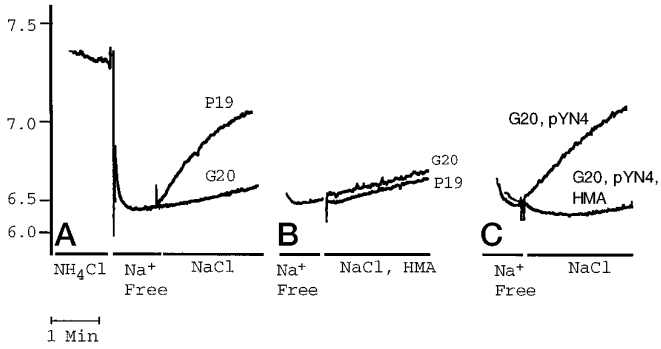

FIG. 2. **Recovery from an acute acid load by wild-type P19 cells and in mutant G20 cells.** Cells were prepared, and intracellular pH was measured as described under "Experimental Procedures." G20 cells were derived from mutated P19 cells and lacked Na^+/H^+ exchanger activity. The plasmid pYN4$^+$ contained full-length cDNA for the Na^+/H^+ exchanger and was used to transform G20 cells and make stable cell line (G20;pYN4) with Na^+/H^+ exchanger activity. Intracellular pH regulation of P19, G20, and G20;pYN4 cells was made on a Shimadzu RF-5000 spectrofluorophotometer as described earlier using the acetoxymethyl ester of 2′,7′-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein. Initial rates of proton extrusion were measured after an acid challenge using the NH_4Cl prepulse method (8). Results are typical of at least three experiments, and discontinuities in the tracings were caused by addition or removal of liquids. *A*, intracellular pH recovery of wild-type P19 and mutant G20 cells after ammonium chloride-induced acidosis. Only part of the NH_4Cl containing period is shown plus the time period in NaCl free buffer and the recovery in 135 mM NaCl. The recovery by P19 cells is superimposed on the results of the G20 cells. The degree of acidification by NH_4Cl was the same in all the cell types examined. *B*, intracellular pH recovery of wild-type P19 and mutant G20 cells in the presence of 10 μM HMA. Only part of the Na^+ free period is shown plus the recovery in the presence of 135 mM NaCl and 10 μM HMA. The tracing for P19 cells is superimposed on that of the G20 cells. *C*, recovery from acidosis by G20 cells transfected with full-length Na^+/H^+ exchanger cDNA (G20,pYN4). The Na^+ free period and the recovery period in 135 mM NaCl are shown. In the example indicated 10 μM HMA was added during the recovery period.

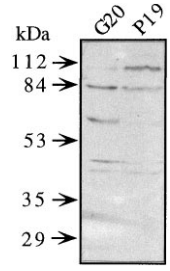

FIG. 3. **Analysis of Na^+/H^+ exchanger expression in P19 and G20 cells.** Western blot analysis of Na^+/H^+ exchanger expression in P19 and G20 cells. P19 and G20 cells were grown in α-MEM supplemented with 25 mM $NaHCO_3$. Cells were harvested, and crude microsomes were made as described in the "Experimental Procedures." Equal amounts of protein (25 μg) of each sample were separated by 9% SDS-PAGE and transferred to nitrocellulose for immunoblotting with affinity purified anti-Na^+/H^+ exchanger antibody.

gesting that the buffering capacity of G20 was unchanged in comparison with P19 cells. We noticed that there was still a very small and gradual recovery of pH_i in the G20 cells. To examine if this was due to Na^+/H^+ exchanger activity, we added 10 μM HMA to the recovery phase of both G20 and P19 cells. In the presence of HMA, the pH_i recovery was the same in both wild-type P19 and mutant G20 cells (Fig. 2*B*). This result indicates that NHE1 activity was excluded or very nearly absent in the slow pH_i recovery in mutant G20 cells. Some other exchanger-deficient clones had a tendency to "revert" to a cell line with an active Na^+/H^+ antiporter. We found that to prevent the reverting of the mutant clones, it was necessary to repeat the proton suicide technique routinely, especially after storing cells as a frozen stock (not shown). The steady state resting intracellular pH of P19 and G20 cells was determined in bicarbonate free medium. For P19 cells the resting pH_i was 7.2 ± 0.06, whereas for G20 cells it was 6.9 ± 0.07. There was no appreciable change in the resting pH_i in either case with the addition of 23 mM HCO_3^-.

To confirm that the effects on intracellular pH regulation were specifically due to deletion of the Na^+/H^+ antiporter, we reintroduced the exchanger back into G20 cells. G20 cells were stably transfected with cDNA coding for the complete coding region of the NHE1 isoform of the Na^+/H^+ exchanger using the calcium phosphate technique (22, 26). A stable cell line was selected using G418 resistance and then tested for antiporter activity. G20 cells stably expressing the transfected antiporter (G20;pYN4) recovered from an acid load in the same manner as control P19 cells (Fig. 2*C*). In addition, the recovery was inhibited by HMA, the inhibitor of the Na^+/H^+ antiporter.

To examine if the G20 cell line contained the Na^+/H^+ exchanger protein we used Western blotting with an antibody against the protein. The results (Fig. 3) showed that G20 cells had only a small amount of detectable immunoreactive protein of 100–105 kDa in size, whereas control cells contained much more immunoreactive protein. Both cells types contained an 80–90-kDa immunoreactive band, and G20 cells had a noticeable amount of smaller immunoreactive bands.

We also examined the growth rate of P19 and G20 cells in the presence of HCO_3^-. In this experiment cells were allowed to proliferate continuously in culture for 3 days. Under these conditions we found that P19 cells grew much better than G20 cells. When the Na^+/H^+ exchanger was introduced back into G20 cells, this effect was eliminated (Fig. 4). When the same experiment was repeated in the absence of HCO_3^-, growth was even more reduced in G20 cells in comparison with controls. For wild-type P19 cells the change to HCO_3^- free medium represented approximately a 34% drop in growth, whereas for G20 cells the same treatment caused approximately an 80% drop in the growth of these cells (not shown).

To examine if the absence of Na^+/H^+ exchange activity had an effect on differentiation in P19 cells we tested the ability of the cells to differentiate using neuronal specific class III β-tubulin as a marker. Under normal conditions, both wild-type P19 cells and mutant G20 cells contained low levels of β-tubulin (Fig. 5*A*) due to a small amount of cells that normally spontaneously differentiate. Control P19 cells were able to differentiate when treated with retinoic acid. G20 cells showed no apparent increase in the level of class III β-tubulin when treated with retinoic acid. In addition we could only detect a very low level of β-tubulin in G20 cells grown in medium without added HCO_3^-, suggesting that less spontaneous differentiation occurred (Fig. 5*B*). When cells were treated with retinoic acid in the absence of HCO_3^-, control cells maintained the ability to differentiate, whereas the levels of class III β-tubulin in G20 cells again did not increase with retinoic acid treatment. There also appeared to be a decrease in the amount of β-tubulin that is normally present in G20 cells that are not stimulated with retinoic acid.

We also examined the ability of G20 cells to differentiate when they had the Na^+/H^+ exchanger reintroduced (Fig. 5*C*). Control cells treated with retinoic acid once again showed much higher levels of class III β-tubulin than G20 cells. However G20 cells transformed with the Na^+/H^+ antiporter were equivalent to wild-type P19 cells. G20 cells transfected with the antiporter also showed the characteristic structural features of neuronal-like cells, similar to wild-type P19 cells but unlike G20 cells not containing the antiporter (not shown). These results show that reintroduction of the Na^+/H^+ antiporter into P19 exchanger-deficient cells facilitates the process of differentiation.

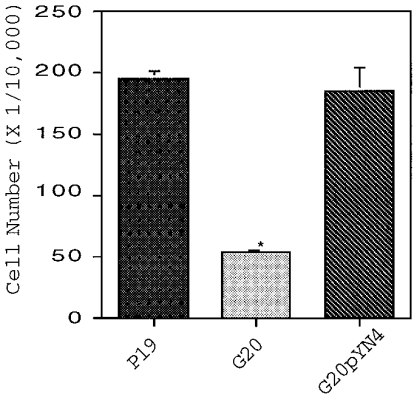

FIG. 4. **Continuous culture of P19, G20, and G20;pYN4 cells.** Cells were grown in α-MEM, 2.5% fetal bovine serum, 7.5% newborn calf serum, and 20 mM HEPES (pH 7.4) supplemented with 25 mM $NaHCO_3$. After 3 days growth, the cells were trypsinized and the cell number was estimated using a hemocytometer. Values represent the mean ± S.E. of three experiments. *, indicates significant difference from the value for P19 cells in the same medium at $p < 0.01$.

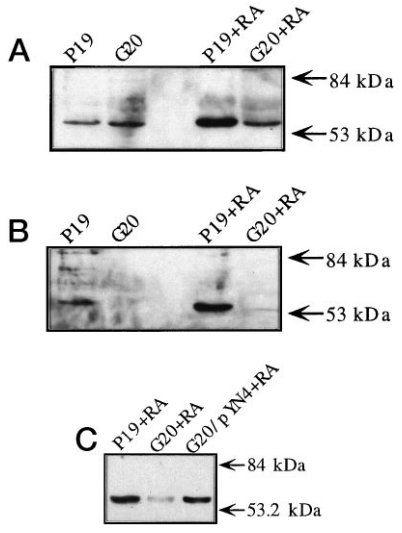

FIG. 5. **Effect of various treatments on retinoic acid-induced differentiation and retinoic acid-induced gene expression in P19, G20, and G20;pYN4 murine embryonal carcinoma cells.** Embryonal carcinoma cells were treated with 1 μM retinoic acid as described under "Experimental Procedures" and in the legend to Fig. 1. Cells of each type were collected and prepared for SDS-PAGE with equal amounts of protein used for each cell type (25 μg). Western blotting of transferred proteins was with neuronal specific class III β-tubulin antibody (8) as described under "Experimental Procedures." Results are typical of three experiments. *Panel A*, Western blot analysis of P19 and G20 cells induced by retinoic acid in the presence of added 25 mM $NaHCO_3$. The first two lanes are untreated P19 (*P19*) and untreated G20 (*G20*) cells as indicated. The next two lanes are retinoic acid-treated P19 cells (*P19+RA*) and retinoic acid-treated G20 cells (*G20+RA*). *Panel B*, the same cells were processed as in *panel A* except in medium without added $NaHCO_3$. *Panel C*, Western blot analysis of β-tubulin content of P19 cell lines after retinoic acid-induced differentiation as described in *panel A*. Samples were P19 (control) cells, G20 cells, and G20 transformed with the NHE1 isoform of the Na^+/H^+ exchanger (G20;pYN4).

DISCUSSION

P19 cells are a well known model for early embryonic determination and differentiation. Treatment of these cells with retinoic acid induces the development of neurons, astroglia and microglia cells normally derived from the neuroectoderm. Long term treatment of these cells with retinoic acid results in a high percentage of the cells expressing neuronal markers such as class III β-tubulin (27). Earlier, we showed that transcription and activity of the NHE1 protein are activated during neuronal differentiation of this cell type (8). In addition, in some other cell types such as HL60 cells, both the protein and mRNA for the NHE1 isoform of the protein are elevated dramatically when differentiation is induced (4, 28). This led us to suspect that the protein plays an important role in the differentiation process. In addition, a number of studies have suggested that the activity of the Na^+/H^+ antiporter either facilitates or is permissive in cell growth (13). Studies have also suggested that in some cell types the expression of the Na^+/H^+ antiporter is dramatically increased during stimulation of cell proliferation (29). For these reasons we examined the role of this protein in both cell proliferation and differentiation in P19 cells.

Initial experiments examined the effect of Hoe 694 on the differentiation of P19 cells. The results showed that this Na^+/H^+ exchanger inhibitor dramatically reduced the rate of differentiation in this cell type in comparison with controls (Fig. 1). Hoe 694 is a newly developed inhibitor of the Na^+/H^+ exchanger with a relatively high specificity for the NHE1 isoform (30). To confirm this result using a second independent technique, we chose to delete the Na^+/H^+ exchanger from P19 cells. Mutagenesis and the subsequent proton suicide technique (14) were used to select exchanger-deficient cells. We obtained several cell lines with similar characteristics that showed no significant Na^+/H^+ exchanger activity when measuring the recovery from an acutely induced acid load. One of these (G20 cells) was more stable for the mutation than the others and was examined for the presence and activity of the protein. It displayed no Na^+/H^+ exchange activity (Fig. 2), and resting pH_i declined in G20 cells. Western blotting suggested that the 100–105-kDa protein was either absent or greatly reduced in quantity (Fig. 3). In addition we noted the presence of a number of smaller immunoreactive proteins in G20 cells, which suggests that the defect may be related to targeting or degradation of the protein.

Therefore we examined the effects of deletion of the protein on the growth of G20 cells. When we grew cells continuously in culture for a period of 3 days, the effects on growth were pronounced (Fig. 4). We also found that an acidic external pH reduced cell growth in G20 cells much more than in controls (not shown). This was similar to a recent report that suggested that NHE1 expression is critical for inner medullary collecting duct cells to survive some types of acid stress (31). Earlier studies have shown that Na^+ is limiting in growth factor-dependent cell proliferation, and amiloride and amiloride analogs can block growth factor-induced stimulation of cell proliferation, especially in HCO_3^- free or low Na^+ medium (15, 16). The mechanism by which the Na^+/H^+ antiporter stimulates cell proliferation is not yet clear but may involve raising pH_i and facilitating entry into the S phase of the cell cycle (32, 33). The more acidic resting pH_i of G20 and the absence of the Na^+/H^+ antiporter to modulate pH_i might cause an inhibition of this normal progression. We noted that when HCO_3^- was removed from the medium of exchanger-deficient P19 cells, there was a dramatic enhancement of the effects on cell proliferation. The likely explanation is that without added HCO_3^- some bicarbonate-based pH regulatory proteins are not as effective in assisting with pH regulation in these cells. A candidate for this activity is the recently described Na^+-dependent HCO_3^-/Cl^- exchanger (34–36). This, or some other bicarbonate-based transporter, may contribute at least partially to pH_i regulation in P19 cells.

We examined the ability of G20 cells to differentiate and found that it was greatly impaired in comparison to wild-type P19 cells (Fig. 5). To our knowledge this is the first direct demonstration that the absence of a Na^+/H^+ antiporter affects the ability of cells to differentiate. This result confirms by an

independent method the results we observed by the addition of Hoe 694. In addition, reintroduction of the Na^+/H^+ exchanger back into G20 cells rescued the ability of these cells to differentiate. These results suggest that the activity of the Na^+/H^+ antiporter also facilitates differentiation in P19 cells. The results contrast with that observed in HL60 leukemic cells. Na^+/H^+ antiport was not required for HL60 differentiation (12). This observation was surprising because both protein and mRNA have been shown to be elevated during differentiation of this cell line (4, 28). It should be noted, however, that apart from the differences in cell origin, this conclusion was based on studies using dimethylamiloride (an amiloride analog) to inhibit Na^+/H^+ exchange. Under similar conditions using the same concentration of the more potent analog HMA we found only slightly reduced inhibition of differentiation in P19 cells (not shown). It may be that some residual Na^+/H^+ antiport remains in the presence of amiloride analogs that can contribute to cellular differentiation. Long term treatments of cells in culture with these analogs could also be affected by degradation or binding to components of serum. Alternatively, the differences observed between our study on P19 cells and HL60 cells may only be related to differences in cell types. Future studies may address this question.

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