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ABSTRACT

The Na\textsuperscript{+}/H\textsuperscript{+} exchanger is involved in a variety of cellular processes, including regulation of intracellular pH and possibly the control of cell growth and proliferation. To study the role of the Na\textsuperscript{+}/H\textsuperscript{+} exchanger in tumor growth, human sodium proton exchanger-deficient (HSPD) mutants were derived from the human bladder carcinoma cell line MGH-U1 (EJ) by the proton suicide selection technique (J. Pouyssegur et al., Proc. Natl. Acad. Sci. USA, 81: 4833-4837, 1984). The HSPD cells were ~40% larger and contained ~70% more DNA than the parental cells. They were unable to grow in vitro in the absence of bicarbonate at pH <7.0, whereas the parental cells grew well at pH >6.6. This difference in acid sensitivity was abolished in the presence of bicarbonate. In contrast to the parental MGH-U1 cells, the Na\textsuperscript{+}/H\textsuperscript{+}-deficient HSPD cells either failed to grow tumors, or showed severely retarded tumor growth when implanted into immune-deprived mice. This difference in tumor growth was not attributed to differences in cell size and DNA content, because Na\textsuperscript{+}/H\textsuperscript{+} exchange-competent large cells (HLC), derived during the same proton suicide selection process as the HSPD cells, grew tumors at a rate close to that of parental cells. Cells derived from the few tumors which grew after implantation of HSPD mutant cells were revertants which had regained Na\textsuperscript{+}/H\textsuperscript{+} activity. HSPD cells also failed to form spheroids in culture, and the only spheroid formed consisted of revertant cells which had regained both Na\textsuperscript{+}/H\textsuperscript{+} exchange activity and tumorigenic capacity. These results suggest that the Na\textsuperscript{+}/H\textsuperscript{+} exchanger is important for tumor growth.

INTRODUCTION

In many solid tumors, impaired growth of blood vessels leads to the development of hypoxic regions, due to limited diffusion of oxygen (1, 2). Increased lactate production and hydrolysis of ATP under hypoxic conditions leads to reduction of tumor pH; the average pH\textsubscript{e} measured with pH microelectrodes in a variety of tumors is approximately 0.5 pH unit lower than that of normal tissues (reviewed in Ref. 3). Typically, pH\textsubscript{e} ranges between 5.9 and 7.4 in tumors and between 7.3 and 7.8 in normal tissues.

Because of their internally negative membrane potential, together with their net metabolic acid production, mammalian cells must actively regulate their cytoplasmic pH. This need may be even greater in tumor cells situated in an acidic environment. Two types of system have been implicated in pH\textsubscript{e} regulation in mammalian cells: the amiloride-sensitive Na\textsuperscript{+}/H\textsuperscript{+} antipporter and the Cl\textsuperscript{-}/HCO\textsubscript{3}{-} exchanger (4) which can be coupled to the movement of Na\textsuperscript{+} (5). In cells surrounded by an acidic environment, in which the concentration of HCO\textsubscript{3}{-} is reduced, the role of the Na\textsuperscript{+}/H\textsuperscript{+} exchanger in controlling cytoplasmic pH may be particularly important.

The Na\textsuperscript{+}/H\textsuperscript{+} exchanger has been found to be involved not only in the regulation of pH\textsubscript{e}, intracellular Na\textsuperscript{+} concentration, and cell volume, but possibly also in initiation of cell growth and proliferation (reviewed in Refs. 6 and 7). A variety of mitogenic agents, including serum (8-11), specific growth factors such as epidermal growth factor (8, 9, 12) and platelet-derived growth factor (8, 10, 13), and the tumor-promoting phorbol esters (14, 15) have been found to activate the Na\textsuperscript{+}/H\textsuperscript{+} exchanger, resulting in an increase of cytoplasmic pH by 0.2-0.3 unit. This alkalinization is thought to be important for the initiation of cell proliferation. Activation of the antiport might be mediated via phosphorylation by protein kinase C and/or tyrosine kinase, which are known to be activated by a variety of Na\textsuperscript{+}/H\textsuperscript{+}-activating agents (6, 7, 16).

To study the importance of the Na\textsuperscript{+}/H\textsuperscript{+} exchanger for tumor growth, we have selected Na\textsuperscript{+}/H\textsuperscript{+} exchange-deficient mutants from a human tumor cell line. Here, we show that human tumor cells lacking the Na\textsuperscript{+}/H\textsuperscript{+} exchanger have either lost or severely reduced their capacity to grow tumors.

MATERIALS AND METHODS

Cells. The human bladder carcinoma cell line MGH-U1 (obtained from Dr. G. Prout, Urology Research Laboratory, Massachusetts General Hospital, Boston, MA), its subline (HLC) and its mutant (HSPD), as well as the CHO subline WT-5 and its mutant AP-1 (kindly provided by Dr. L. Siminovich, Mount Sinai Hospital Research Institute, Toronto, Ontario, Canada), were maintained in complete α-MEM supplemented with antibiotics and 10% FCS and buffered to pH 7.4 with 25 mM bicarbonate. Cultures, free of Mycoplasma, were reestablished from frozen stock approximately every 3 months.

Reagents. The tetraacetoxyethyl ester of BCECF was purchased from Molecular Probes (Eugene, OR), \textsuperscript{2}NaCl (200 μCi/ml) from Amersham (Oakville, Ontario, Canada) and DMA was generously provided by Dr. E. J. Cragoe, Jr. (Merck Sharp & Dohme, West Point, PA). Leishman’s stain and Triton X-100 were from BDH (Toronto, Ontario, Canada), and heterologous rabbit anti-mouse lymphocyte serum was from Johns Scientific (Toronto, Ontario, Canada). 1-β-D-Arabinofuranosylcytosine was obtained from The Upjohn Co. of Canada. Tris, MES, HEPES, and all other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Selection of Na\textsuperscript{+}/H\textsuperscript{+} Exchange-deficient Mutants. MGH-U1 cells were subjected to the proton suicide selection technique designed by Pouyssegur et al. (17). Exponentially growing cells were mutagenized with ethylmethanesulfonate (300 μg/ml) for 24 h, washed, and grown in α-MEM for 6-10 days. Cells were then trypsinized and incubated for 6 h at 37 °C in LiCl solution (130 mM LiCl, 5 mM KCl, 1 mM MgSO\textsubscript{4}, 2 mM CaCl\textsubscript{2}, 5 mM glucose, 20 mM HEPES-Tris, pH 7.4) followed by a 4-h incubation at 37 °C in choline chloride acid solution (130 mM choline chloride, 5 mM KCl, 1 mM MgSO\textsubscript{4}, 2 mM CaCl\textsubscript{2}, 5 mM glucose, 20 mM Tris-MES, pH 5.5). These incubation periods were longer than those recommended by Pouyssegur et al. (17), and were used here to make the selection conditions more stringent. Cells were then pelleted, resuspended in α-MEM, and seeded at 4 x 10\textsuperscript{5} cells/plate. Four weeks later, colonies from resistant cells (frequency ~2 x 10\textsuperscript{−4}) were picked with cloning rings and expanded. Eighteen of these clones were then subjected to a.
second cycle of selection identical to the first, but with a 4-h exposure to LiCl solution and a 2-h exposure to choline chloride acid solution. The second cycle of selection yielded one clone (frequency, 5 × 10−6) which was Na+/H+ exchange deficient. This clone was named HSPD (human sodium proton deficient). After serial passages of the HSPD cells in culture without selection pressure for 1 month in α-MEM, partial recovery of Na+/H+ exchange activity was observed. These cells were then subjected to a third cycle of selection which yielded 7 Na+/H+ exchange-deficient subclones. Two of these subclones (HSPD-1 and HSPD-2) were further characterized along with the original HSPD cells (before their partial reversion). These subclones were maintained in α-MEM and retained their Na+/H+ exchange-deficient phenotype for at least 5 months without selection pressure.

Not all the proton suicide-resistant cells were Na+/H+ exchange deficient. However, all the resistant cells tested were larger than the parental cells (see below). Two clones of these large cells, named HLC (human large cells), which were Na+/H+ exchange competent, were also chosen for further characterization.

Measurements of Intracellular pH. Intracellular pH was measured with the pH-sensitive, intracellularly trapped, fluorescent dye BCECF, as described previously (18). Cells (3 × 106) in suspension were loaded with the tetraacetoxymethyl ester of BCECF (2 μg/ml) for 30 min at 37°C in serum-free α-MEM, pelleted, and resuspended in fresh serum-free α-MEM (6 × 106 cells/ml). Aliquots (4.8 × 106 cells) were then added to a cuvet containing NMG+Cl− solution in a Perkin Elmer LS3 fluorescence spectrophotometer. Excitation and emission wavelengths were 495 and 525 nm, respectively. Cells were acid loaded with K+/H+ ionophore nigericin and the appearance of an amiloride-sensitive alkalinization, indicative of the presence of Na+/H+ exchange, was recorded following the addition of 100 mM NaCl. Calibration of pH versus fluorescence intensity was carried out in K+ solution (identical to NMG+Cl− solution but with isoosmotic replacement of KCl for NMG) with nigericin, as described previously (19).

22Na+ Uptake. 22Na+ uptake was measured as described previously (17). Cells were seeded in 35-mm multiwells in α-MEM. Two days later, the α-MEM was replaced with NH4Cl medium (α-MEM without NaHCO3, plus 50 mM NH4Cl, 0.1 mM uridine, 0.1 mM hypoxanthine, and 20 mM HEPES, pH 7.4) and cells were incubated for 30 min at 37°C. Cells were then washed twice with α-MEM, pelleted, and resuspended in fresh serum-free α-MEM (1 × 106 cells/ml). Aliquots (4.8 × 106 cells) were then added to a cuvet containing NMG+Cl− solution in a Perkin Elmer LS3 fluorescence spectrophotometer. Excitation and emission wavelengths were 495 and 525 nm, respectively. Cells were acid loaded with K+/H+ ionophore nigericin and the appearance of an amiloride-sensitive alkalinization, indicative of the presence of Na+/H+ exchange, was recorded following the addition of 100 mM NaCl. Calibration of pH versus fluorescence intensity was carried out in K+ solution (identical to NMG+Cl− solution but with isoosmotic replacement of KCl for NMG) with nigericin, as described previously (19).

RESULTS

Outcome of Selection of Na+/H+ Exchange-deficient Cells. MGH-U1 cells possess an amiloride-sensitive Na+/H+ exchanger, as demonstrated by the Na+−induced alkalinization of acid-loaded cells which could be inhibited (Fig. 1A) or prevented (data not shown) by amiloride. In contrast to the parental MGH-U1 cells (Fig. 1A), Na+−induced alkalinization was not seen in HSPD mutant cells (Fig. 1B) or in their subclones HSPD-1 and HSPD-2 (data not shown). This result, together with the observed ~10-fold reduction of amiloride-sensitive 22Na+ uptake in acid-loaded cells (Fig. 1D) relative to the parental cells, suggest that the HSPD, HSPD-1, and HSPD-2 cells are Na+/H+ exchange-deficient mutants.

In contrast to HSPD and its subclones, most of the resistant clones which survived the proton suicide selection were Na+/H+ exchange competent, as exemplified for HLC cells in Fig. 1C.

Cell Volume and DNA Content. All the proton suicide-resistant clones (whether Na+/H+ exchange deficient or Na+/H+ competent) originating from MGH-U1 cells were significantly larger than the parental cells (Table 1), with an average increase of median cell volume of 33−50%. To test whether this increased cell volume was cell line specific, cell volume of the unrelated Na+/H+−deficient AP-1 mutant derived from hamster ovary cells was compared to that of the wild type. The results revealed a similar (~40%) increase of cell volume relative to the parental WT-5 cells (Table 1).

Measurements of DNA content of PI-stained nuclei showed that the larger cells of both human and hamster origin contained more DNA than their respective parental cells. This was true regardless of whether the cells were Na+/H+ exchange deficient or competent (Fig. 2, A−C). From the measured G0/G1 peak position, it was calculated that the increase of DNA content

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Curves shown in A-C. Initial rates of alkalinization (\( \Delta p[H] \), min) summarized from cells in the presence or absence of bicarbonate at physiological exchange-deficient HSPD-1 cells did not grow at \( p[H] \) below 7.0. This difference in \( p[H] \) sensitivity was abolished in the parental MGH-U1 cells, which had 46/47 chromosomes. However, there was also a small population of aneuploid cells less than tetraploid, as is also suggested by the analysis of DNA marker chromosomes, of HLC and HSPD-1 cells relative to the parental cells.

**Table 1 Cell volumes**

<table>
<thead>
<tr>
<th>Original cell line</th>
<th>Clone</th>
<th>( Na^+ / H^+ ) exchanger</th>
<th>Median cell volume (( \mu m )) (mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MGH-U1 (human)</td>
<td>MGH-U1</td>
<td>+</td>
<td>916 ± 19 (( n = 7 ))</td>
</tr>
<tr>
<td></td>
<td>HSPD-1</td>
<td>-</td>
<td>1214 ± 65 (( n = 7 ))</td>
</tr>
<tr>
<td></td>
<td>HSPD-2</td>
<td>-</td>
<td>1157 ± 64 (( n = 8 ))</td>
</tr>
<tr>
<td></td>
<td>HLC</td>
<td>-</td>
<td>1373 ± 55 (( n = 4 ))</td>
</tr>
<tr>
<td></td>
<td>HSPD-2 spheroid (revertant)</td>
<td>+</td>
<td>912 ± 38 (( n = 6 ))</td>
</tr>
<tr>
<td>CHO (hamster)</td>
<td>WT-5</td>
<td>+</td>
<td>685 ± 15 (( n = 11 ))</td>
</tr>
<tr>
<td></td>
<td>AP-1</td>
<td>-</td>
<td>968 ± 30 (( n = 8 ))</td>
</tr>
</tbody>
</table>

*Values in parentheses indicate the number of independent measurements taken on different days.*

Relative to the parental cells was ~65% for the HSPD and HLC clones, and 75% for the AP-1 mutant. Analysis of karyotypes of MGH-U1, HSPD-1, and HLC cells (Fig. 2, D–F) revealed an increase in number of chromosomes (to 80–89), and of marker chromosomes, of HLC and HSPD-1 cells relative to the parental MGH-U1 cells, which had 46/47 chromosomes. However, there was also a small population of aneuploid cells within the parental (nonselected) cell population (data not shown). It appears that the proton suicide-selected cells were less than tetraploid, as is also suggested by the analysis of DNA content done with PI-stained cells (Fig. 2, A–C).

**Growth of Na\(^{+}/H\(^{+} \) Exchange-deficient Cells in Vitro** Fig. 3A illustrates the growth of mutant HSPD-1 and parental MGH-U1 cells in bicarbonate-free medium buffered to different \( p[H] \). Whereas the parental cells grew well at \( p[H] \geq 6.6 \), the \( Na^+ / H^+ \) exchange-deficient HSPD-1 cells did not grow at \( p[H] \) below 7.0. This difference in \( p[H] \) sensitivity was abolished in the presence of bicarbonate (Fig. 3B). Almost identical results (in the absence or presence of bicarbonate) were obtained with the subclone HSPD-2 (data not shown). Growth rate of the mutant cells in the presence or absence of bicarbonate at physiological \( p[H] \) (~7.4) was similar to that of the parental cells. To ensure that the difference in cell size and DNA content between the parental and the \( Na^+ / H^+ \) exchange-deficient cells were not responsible for the observed differences in growth rate at low \( p[H] \), growth characteristics of the large \( Na^+ / H^+ \) exchange-competent HLC cells were studied. The results show (Fig. 3C) that in bicarbonate-free medium there was no difference in growth between MGH-U1 and HLC cells at any of the \( p[H] \) values tested. Some variation in sensitivity of MGH-U1 cells to growth at \( p[H] \) 6.6 was found between separate experiments (compare Fig. 3A to 3C); it might have been caused by the small acid drift (~0.1 unit) of \( p[H] \) over time observed in the experiment described in Fig. 3C.

Survival curves of HSPD-1 and HSPD-2 cells exposed to different \( p[H] \) for 4 h in bicarbonate-free medium show that these cells were more sensitive to acute exposure to an acidic medium than MGH-U1 cells. In contrast, HLC cells were more acid resistant than the parental cells (Fig. 3D).

**Growth of Multicellular Spheroids** Multicellular spheroids are cell aggregates formed in culture by some tumor cell lines, which resemble tumor nodules morphologically (21). Because MGH-U1 cells are known to form multicellular spheroids in culture (24), the ability of the \( Na^+ / H^+ \) exchange-deficient HSPD-1 and HSPD-2 cells to form spheroids was compared to that of the parental MGH-U1 cells. The results show that the ability to form spheroids was reduced in \( Na^+ / H^+ \) exchange-deficient cells; the number of spheroids formed from 6.6 \( \times 10^4 \) cells (seeded in 12 multiwells) was ~50 for MGH-U1 cells (frequency ~8 \( \times 10^4 \)), 1 for HSPD-2 cells (frequency, 1.5 \( \times 10^3 \)), and 0 for HSPD-1 cells. Analysis of \( Na^+ / H^+ \) exchange activity of cells taken (and expanded) from the HSPD-2 spheroid showed recovery of the \( Na^+ / H^+ \) exchange activity (Fig. 5C), suggesting that these cells were revertants. These revertant cells had the same volume as the original MGH-U1 cells (Table 1).

**Tumor Growth in Vivo** To test the ability of \( Na^+ / H^+ \) exchange-deficient tumor cells to grow tumors in mice, HSPD, HSPD-1, and HSPD-2, or parental MGH-U1 cells, were injected into immune-deprived mice, and the growth rate of tumors was measured. Where tumors grew rapidly after implantation of the parental cells (100% tumor incidence within 1–2 weeks), the \( Na^+ / H^+ \) exchange-deficient cells either did not generate tumors at all, or showed severely retarded tumor growth (Fig. 4, A and B). The incidence of tumor growth (defined as tumors in legs with a mean diameter of >8.5 mm) of the \( Na^+ / H^+ \) exchange-deficient cells 40 days after injection of cells was 17% for HSPD cells, 0% for HSPD-2 cells, and 20% for HSPD-1 cells, compared with 88 and 100% for HLC and MGH-U1 cells, respectively. Fig. 4A also shows that the impaired capacity of the \( Na^+ / H^+ \)-deficient cells to grow tumors was not due to their larger size and greater DNA content, because the HLC cells grew tumors at a rate closer to that of MGH-U1 cells than to that of HSPD cells.

To test whether the delayed formation of tumors in mice implanted with HSPD cells was due to in vivo reversion of the mutation, small and large tumors were excised from mice bearing the HSPD tumors and their cells were reestablished in culture. These cells had regained \( Na^+ / H^+ \) exchange activity, and cells taken from a large tumor (1.13 g) had greater \( Na^+ / H^+ \) exchange activity than cells taken from a small tumor (0.27 g) (Fig. 5, A and B). This result suggests that revertant cells may be selected during tumor growth.

The revertant cells expanded from the HSPD-2 spheroid which had greater \( Na^+ / H^+ \) exchange activity than the parental
DISCUSSION

The present report describes the isolation of Na"/H" exchange-deficient human tumor cells which are acid sensitive in vitro in the absence of bicarbonate, and which have lost or reduced their normal capacity to grow tumors in vivo.

The proton suicide technique (17) led to the selection of a Na"/H" exchange-deficient mutant from MGH-U1 cells at a frequency of ~5 x 10^-8, approximately 40-fold lower than that reported for the Na"/H" exchange-deficient Chinese hamster lung fibroblasts (17). This selection procedure, however, resulted in the emergence of large Na"/H" exchange-competent cells with increased DNA content at a much higher frequency (~2 x 10^-6). Mutant cells lacking the exchanger were also larger than the parental cells. This observation was not restricted to MGH-U1 cells; the unrelated Na"/H" exchange-deficient AP-1 cells (Table 1; Fig. 2) and PKE5 cells derived from pig kidney epithelium (25) also showed increased volume relative to the parental cells. A possible explanation is that a
strong selection pressure mediated by entry of protons through the surface membrane leads to the emergence of resistant large cells due to their reduced surface to volume ratio. In MGH-U1 cells, two preexisting subpopulations have been reported (26), a major fraction of the cells with 46/47 chromosomes, and a minor subpopulation of larger cells with 90/92 chromosomes. It is therefore possible that in addition to selecting for Na+/H+ exchange-deficient mutants, the proton suicide technique preferentially selected for the subpopulation of large cells with reduced surface to volume ratio. The observation that the revertant (Na+/H+ exchange competent) HSPD-2 spheroid cells reverted in size back to that of the original MGH-U1 cells, but that HLC cells (also Na+/H+ exchange competent) were larger than MGH-U1 cells, suggests that the relationship (if any) between cell volume and the presence of the Na+/H+ antiporter is not simple. Although the number of chromosomes and the amount of DNA in G0/G1 stage of large cells (HSPD-1, HLC, AP-1) was less than double relative to the parental cells, the limited number of karyotypes available precludes determination of consistent chromosome abnormalities.

In the absence of bicarbonate, HSPD-1 (and HSPD-2) cells were unable to grow in culture at pH < 7.0, whereas the parental cells grew well at pH > 6.6. This observed acid sensitivity is in agreement with that reported for the Na+/H+ exchange-deficient Chinese hamster lung fibroblasts PS120 (17), for PKE5 cells (25), and for AP-1 cells. The different growth rates of MGH-U1 and HSPD-1 cells in acidic bicarbonate-free medium could not be attributed to differences in cell size and DNA content, because the HLC cells showed growth characteristics similar to those of MGH-U1 cells. The difference in acid sensitivity between the parental and mutant cells was abolished in bicarbonate-buffered medium, suggesting the existence of HCO₃⁻/Cl⁻-dependent pH regulatory or buffering systems, such as Na⁺/HCO₃⁻ exchange(s) demonstrated in other cell types (5, 9, 27, 28).

Colony-forming ability following acute exposure to pH 7.4–5.5 (in the absence of bicarbonate) showed that relative to the parental cells, Na+/H+ exchange-deficient cells were more acid sensitive, as expected, but that the Na+/H+ exchange-competent large cells were more acid resistant, possibly due to their decreased surface to volume ratio. Taken together, these results suggest that in acid pH, the Na+/H+ exchanger is necessary for tumor cell survival and proliferation in vitro when the HCO₃⁻/Cl⁻ exchanger(s) is largely inoperative. This result is in agreement with previous studies suggesting the requirement of a permissive pH, (achieved by the regulatory action of the Na⁺/H⁺ or Na⁺-coupled HCO₃⁻/Cl⁻ exchangers) for DNA synthesis (29).

The importance of the Na+/H+ exchanger for cell proliferation in vivo in an environment containing physiological concentration of bicarbonate is not clear. The results shown here, however, demonstrate that the Na+/H+ exchanger is important...
for tumor growth in vivo; HSPD (or HSPD-1 and HSPD-2) cells either lost, or severely reduced their capacity to form and grow tumors. This reduced tumorigenic capacity could not be attributed to their larger size and increased DNA content, or to the pretreatment with ethylmethane sulfonate, because the HLC cells grew tumors at a rate just slightly slower than that of the parental cells. Although Lagarde and Pouyssegur (30) have shown recently that tumor incidence of Na*/H* exchange-deficient PS120 cells in athymic nude mice was reduced relative to the pretreatment with ethylmethane sulfonate, because the HLC cells showed similar tumorigenic ability to the parental cells, strongly suggest that the molecular lesion responsible for reduced tumorigenicity is the absence of the Na*/H* antiporter and not other unrelated lesions that could have occurred during the proton suicide selection. Our results suggest, therefore, that the Na*/H* exchanger is important and possibly required for growth of tumors from MGH-U1 cells, although the universality of this observation in other tumor cell types remains to be demonstrated. At least two theoretical hypotheses could explain this requirement: (a) tumor pH (not measured in our experiments) and therefore bicarbonate concentrations might be reduced with tumor growth (3), leading to a greater dependency of cells on the Na*/H* exchanger for regulation of pH and survival in an acidic environment; and (b) in the absence of Na*/H* exchange (coupled with reduced bicarbonate concentration) tumor cells are unable to respond to mitogenic agents (30) known to activate the Na*/H* exchanger and elevate pH, in vitro (8, 14, 15, 31).

The suggestion that activation of the Na*/H* antiporter and elevation of pH may play a role in the stimulation of cell proliferation originated from the pioneering work of Johnson et al. (32) in the sea urchin egg, and from work of others (e.g., Ref. 33). It is now well established that numerous mitogens (and other stimuli) activate the antiporter and lead to increase of pH above its resting level (reviewed in Refs. 6 and 7). However, recent studies have demonstrated that this activation and the ensuing cytoplasmic alkalinization are not always required for mitogen-induced cell proliferation (34–36). These findings weakened the hypothesis that the Na*/H* antiporter is required for tumor growth due to its involvement in the mitogenic response.

Based on studies showing pH-dependent cytotoxicity of amiloride to acid-loaded MGH-U1 cells in vitro, we had suggested earlier that inhibitors of the Na*/H* exchanger might have potential use as anticancer agents (37). This suggestion is strengthened by the present experiments in vivo, where a central role of the Na*/H* exchanger for tumor formation was demonstrated.

**REFERENCES**


