Phosphoinositide 3-Kinase Is Involved in the Tumor-specific Activation of Human Breast Cancer Cell Na\(^+\)/H\(^+\) Exchange, Motility, and Invasion Induced by Serum Deprivation*

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Whereas the tumor acidic extracellular pH plays a crucial role in the invasive process, the mechanism(s) behind this acidification, especially in low nutrient conditions, are unclear. The regulation of the Na\(^+\)/H\(^+\) exchanger (NHE) and invasion by serum deprivation were studied in a series of breast epithelial cell lines representing progression from non-tumor to highly metastatic cells. Whereas serum deprivation reduced lactate production in all three cell lines, it inhibited NHE activity in the non-tumor cells and stimulated it in the tumor cells with a larger stimulation in the metastatic cells. The stimulation of NHE in the tumor cell lines was the result of an increased affinity of the internal H\(^+\) regulatory site of the NHE without changes in sodium kinetics or expression. Serum deprivation conferred increased cell motility and invasive ability that were abrogated by specific inhibition of the NHE. Inhibition of phosphoinositide 3-kinase by overexpression of a dominant-negative mutant or wortmannin incubation inhibited NHE activity and invasion in serum replete conditions while potentiating the serum deprivation-dependent activation of the NHE and invasion. These results indicate that the up-regulation of the NHE by a phosphoinositide 3-kinase-dependent mechanism plays an essential role in increased tumor cell invasion induced by serum deprivation.

Carcinogenesis is a dynamic process resulting in the accumulation of genetic alterations of varying character (1, 2). The progression of epithelial carcinoma has been broadly classified into normal, hyperplastic, dysplastic, in situ, and invasive carcinoma. This progression from healthy tissue to metastatic disease occurs via a graded series of genetic/biochemical alterations that are still not clearly understood. As metastatic spread of malignant cells via invasion to distant tissues is the primary cause of treatment failure and subsequent death in cancer patients, one of the fundamental problems in oncology is understanding the mechanisms driving neoplastic progression and controlling invasion (3, 4).

One of the principal in vivo tumor hallmarks is the presence of severe extracellular areas of low pH, low serum levels, and hypoxia that develop early in the development of the tumor (5). These factors are usually closely linked and define the tumor metabolic microenvironment (6–8). There is now considerable evidence indicating that exposure to these factors increases the metastatic potential of cancer cells by increasing such processes as invasion (9–11), the production of angiogenic factors (12–15), the selection of aggressive cells within a tumor (16–19), and even contributes to tumor genetic instability (8, 20). This appreciation of the critical importance of the response of a tumor cell to its own microenvironment as one of the main driving forces for progression has shifted emphasis to determining the role of the various components of the tumor microenvironment in the regulation of tumor pathophysiological processes and determining how this contributes to neoplastic progression.

There has been increasing evidence for the role of the acid component of the tumor microenvironment in controlling self-organized growth, invasive capacity, and subsequent malignant progression (10, 11, 21). However, the mechanisms by which a tumor cell acidifies its microenvironment and how these mechanisms are, in turn, regulated by the other components of the tumor microenvironment are still unclear. Whereas lactate production/release is commonly considered to be the primary tumor microenvironmental acidification mechanism (22), tumors in nude mice derived from cells lacking lactate dehydrogenase were fully able to acidify their microenvironment (23). Furthermore, recent work in vivo has shown that in serum replete conditions the major component of extracellular acidification is lactate release, whereas in serum deprived conditions, common to aggressive tumors, lactate production/release stopped while the microenvironment continued to acidify (24).

Clearly, there is another mechanism of cellular pH regulation that contributes to extracellular acidification particularly in nutrient deprived conditions. Intracellular pH (pHi) is stringently regulated at levels somewhat higher than stoichiometrically predicted by various mechanisms including several plasma membrane ion transporters and an intrinsic cytosolic buffering capacity (25). There are data demonstrating that the cell membrane Na\(^+\)/H\(^+\) exchanger (NHE) is the principal pH-regulating mechanism in tumor cells (26–28), suggesting that altered regulation of this transporter could constitute that mechanism. However, its altered regulatory response to serum deprivation, the role of the tumors neoplastic state in this

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§ The abbreviations use are: pH, intracellular pH; NHE, Na\(^+\)/H\(^+\) exchanger; PI3K, phosphoinositide 3-kinase; βi, intrinsic buffering capacity; βc, intracellular buffering capacity; PBS, phosphate-buffered saline; DMA, 5-(N,N-dimethyl)-amiloride.
response, and what role the NHE plays in the aberrant regulation of both intracellular and extracellular tumor pH have not yet been fully elucidated.

This study was undertaken to characterize the unique effects of serum deprivation, which mimics in vivo tumor conditions, on the basic properties of the Na\(^+\)/H\(^+\) exchanger of tumor cells and on their invasive potential: (a) is the response qualitatively different between tumor and normal cells, (b) does the response differ quantitatively between tumor cells of differing neoplastic status, (c) which modifications of the Na\(^+\)/H\(^+\) exchanger are responsible for its altered activity, (d) which signal transduction pathways underlie this altered regulation of the exchanger, and (e) is the altered Na\(^+\)/H\(^+\) exchange activity involved in increased tumor cell invasive capacity. To this scope the studies were simultaneously conducted on three well studied human mammary epithelial cell lines that represent distinct phases of neoplastic progression. These are a highly differentiated, spontaneously immortalized, non-tumor epithelial cell line, MCF-10A (29); a less differentiated, very aggressive, very invasive metastatic cell line, MCF-7 (30); and a undifferentiated, very aggressive, very invasive metastatic cell line isolated from a pleural infusion of a metastatic tumor, MDA-MB-435 (31).

We demonstrate that the tumor cell response is the opposite of normal cells to serum deprivation; in the normal cells serum deprivation results in a decrease in NHE activity, whereas in the tumor cells NHE activity was up-regulated by serum deprivation and this stimulation of NHE activity increases with an increasing neoplastic state. In the tumor cells the kinetics of the stimulation of exchanger activity with serum deprivation increases with an increasing neoplastic state. These are a highly differentiated, spontaneously immortalized, non-tumor epithelial cell line, MCF-10A (29); a less differentiated, very aggressive, very invasive metastatic cell line isolated from a primary tumor, MCF-7 (30); and a undifferentiated, very aggressive, very invasive metastatic cell line isolated from a pleural infusion of a metastatic tumor, MDA-MB-435 (31).

We demonstrate that the tumor cell response is the opposite of normal cells to serum deprivation; in the normal cells serum deprivation results in a decrease in NHE activity, whereas in the tumor cells NHE activity was up-regulated by serum deprivation and this stimulation of NHE activity increases with an increasing neoplastic state. In the tumor cells the kinetics of the stimulation of exchanger activity with serum deprivation was consistent with an increase in the affinity of the internal proton regulatory site without a change in either sodium K\(_o\) or exchanger protein expression. We observe that during serum deprivation there is a reversal of the usual, positive phosphoinoside 3-kinase (P13K) regulatory pathway for NHE1 found in the presence of serum such that the serum deprivation-dependent activation of tumor cell NHE activity was potentiated by inhibition of P13K. Our data demonstrate that with serum deprivation, tumor cells developed an increased capacity for acid extrusion into their environment and, subsequently, to invade. We conclude that an increased NHE activity is the probable tumor extracellular acidification mechanism during serum deprivation and this increase is directly responsible for the increased invasive potential observed in these conditions.

**EXPERIMENTAL PROCEDURES**

**Cells**—MCF-10A is a spontaneously immortalized, untransformed human breast cell line derived from a patient with mild fibrocystosis (29); MCF-7, is a human breast tumor cell line derived from a primary, non-malignant tumor (30) and MDA-MB-435, is a human breast tumor cell line derived from a pleural effusion of a malignant tumor (31). MCF-10A cells were routinely grown in Dulbecco's modified Eagle's medium high glucose (4500 mg/l) supplemented with NaHCO\(_3\) (3700 mg/l), 5% (v/v) heat-inactivated fetal bovine serum, 40 units/ml insulin, 0.5 µg/ml hydrocortisone, 10 ng/ml epidermal growth factor, l-glutamine (2 mM), sodium-pyruvate (1 mg/ml), and penicillin (100 units/streptomycin (100 µg/ml). MCF-7 and MDA cells were grown in Dulbecco's modified Eagle's medium high glucose (4500 mg/l) supplemented with NaHCO\(_3\) (3700 mg/l), 10% (v/v) heat-inactivated fetal bovine serum, l-glutamine (2 mM), sodium-pyruvate (1 mg/ml) and penicillin (100 units/streptomycin (100 µg/ml). All three lines were grown in a 5% CO\(_2\)/95% air humidified incubator at 37 °C.

For some experiments, 200 µl of complete medium containing 200,000 cells were seeded onto a glass coverslip. After seeding, cells were returned to the incubator for at least 2 h to allow attachment, after which the unattached cells were removed, and the attached cells were immersed in fresh complete medium and returned to culture. With this routine seeding density, cells formed a confluent monolayer in 2 days and were immediately used for experiments. To be sure that pH\(_i\) was measured in healthy cells, trypan blue exclusion measurements were performed on a representative coverslip before continuing the experiment. If more than 5% of the cells could not exclude the dye, experiments were not carried out.

**Expression Vector**—A pGEX-2T plasmid containing the dominant-negative p85α subunit of PI3 kinase (pBSSINT Tag) was kindly provided by M. Falasca (Consorzio Mario Negri Sud, San Donato Milanese, Milano, Italy). Transient transfections on monolayers on glass coverslips were performed with the Lipotax reagent (Stratagene) according to the manufacturer's instructions. 5 µg of plasmid cDNA or empty vector was incubated with 100 µl of Lipotax reagent in 2 ml of simple Dulbecco's modified Eagle's growth medium for 30 min at room temperature. 200 µl of culture were pipetted onto confluent monolayers on glass coverslips and placed in an incubator at 5% CO\(_2\) and 37 °C for 6 h. The medium was replaced with fresh complete medium (10% serum) for 24 h in normal growth conditions. Cells were then treated, and NHE activity was measured.

**Perfusion Solution**—During all experiments presented, cells were initially perfused with medium resembling culture medium in its ionic content but without added CO\(_2\), NaCl, or serum. After perfusion, coverslips with confluent monolayers were inserted into an angle of 60° in a fluorometer cuvette designed to permit easy solution change with multiple perfusion solutions as described previously (32). Fluorescence was monitored in a Shimadzu RF 5000 spectrophotometer using alternately 440 nm (pH-insensitive) and 490 nm (pH-sensitive) as excitation wavelengths utilizing a xenon light source (5-nm bandwidth). Emission was measured at 530 nm (15-nm bandwidth). pH\(_i\) was calculated from the fluorescence emission ratio of the two excitation wavelengths using a standard calibration procedure based on the use of nigericin (0.5 µM) in high potassium K-clamp ringer (20 mM NaCl, 110 mM KCl, 1 mM CaCl\(_2\), 1 mM MgSO\(_4\), 18 mM glucose, 20 mM Heps, pH 7.4). A second medium was also used in which n-methylglucamine completely replaced Na\(^+\) ("n-methylglucamine ringer"). The osmolarity in these mediums, as determined by an osmometer, was identical to that of the culture medium. In some experiments, Na\(^+\) and n-methylglucamine monolayers were blended together to give intermediate Na\(^+\) concentrations. To manipulate intracellular pH, cells were exposed to 135 mM Na\(^+\) medium to which 20 mM NH\(_4\)Cl was added from a stock solution of 4M.

**pH Determination**—Cytosolic pH was measured spectrofluorimetrically at 37 °C with the fluorescent pH-sensitive probe, BCECF, trapped intracellularly in cell monolayers grown on glass. Cells were loaded for 1 h at room temperature with the acetoxyethyl ester of BCECF (10 µM) in Na\(^+\) ringer. After loading, coverslips with confluent monolayers were inserted at an angle of 60° in a fluorometer cuvette designed to permit easy solution change with multiple perfusion solutions as described previously (32). Fluorescence was monitored in a Shimadzu RF 5000 spectrophotometer using alternately 440 nm (pH-insensitive) and 490 nm (pH-sensitive) as excitation wavelengths utilizing a xenon light source (5-nm bandwidth). Emission was measured at 530 nm (15-nm bandwidth). pH\(_i\) was calculated from the fluorescence emission ratio of the two excitation wavelengths using a standard calibration procedure based on the use of nigericin (0.5 µM) in high potassium K-clamp ringer (20 mM NaCl, 110 mM KCl, 1 mM CaCl\(_2\), 1 mM MgSO\(_4\), 18 mM glucose, 20 mM Heps, 1 mM KH\(_2\)PO\(_4\)) buffered at pH values from 6.5 to 8.5 in steps of 0.5 pH units.

**Determination of Intrinsic Buffering Capacity (β)**—The activity of the Na\(^+\)/H\(^+\) exchanger was measured by monitoring pH\(_i\) recovery after an intracellular acid load produced with the NH\(_4\)Cl prepulse technique (33). The initial rate of Na\(^+\)-dependent alkalization was determined by linear regression analysis of the first 15 points taken at 4-s intervals after the readdition of sodium. The use of CO\(_2\)/HCO\(_3\)-free solutions minimizes the likelihood that Na\(^+\)-dependent HCO\(_3\) transport was responsible for the observed pH\(_i\) changes.

**Expression of Intracellular Buffer Capacity (β)**—The pH\(_i\) dependence of intracellular buffer capacity (β), i.e., the buffering power of all non-HCO\(_3\)-CO\(_2\)-buffers, was computed by the NH\(_4\) pulse method (33). β refers to the ability of intrinsic cellular components, excluding HCO\(_3\)-CO\(_2\), to buffer changes in pH, and is defined as the moles of H\(^+\) required to produce a one-unit shift in pH. Buffering capacity is calculated as the product of the measured buffer capacity (β) and the concentration of the buffer which makes up the difference between the pH of the pH pulse and the pH of the cell monolayers. Buffer capacity is calculated as follows.

\[
\beta = [\text{NH}_4]\text{pH}^+ \quad \text{(Eq. 1)}
\]

The actual activity of the exchanger in terms of proton flux rate (mM H\(^+\)/liter of intracellular fluid. Estimations of β were carried out in HEPES-buffered solutions, and extracellular sodium was removed to block all Na\(^+\)-dependent pH regulatory mechanisms. β was calculated from the Henderson-Hasselbach equation from the measured pH\(_i\) changes and the calculated changes of [NH\(_4\)\_] during stepwise reductions of [NH\(_3\)\_] in HCO\(_3\)-CO\(_2\)-free solution. One assumes that [NH\(_4\)\_] is equal to [NH\(_3\)\_], a low NH\(_4\) conductance, and a pK\(_b\) of 9.25. Lowering [NH\(_3\)\_] thus decreases a decrease in both [NH\(_3\)\_] and [NH\(_4\)\_], and the buffering capacity is calculated as follows.

\[
V_{H_\text{max}} \times (\text{mM/min}) = \beta (\text{mM/pH}) \times \text{pH/t (pH/min)} \quad \text{(Eq. 2)}
\]

**Invasion and Motility**—A quantitative measure of the degree of invasion or motility of the cells was obtained in Transwell cham-
Regulation of NHE1 in Human Breast Cancer

RESULTS

Characterization of the Neoplastic State Represented by These Cells—The effect of increasing serum concentration on invasive capacity as measured by the cells ability to cross a layer of matrigel in a Boyden Chamber was determined (Fig. 1). MDA-MB-435 cells (squares) had a 2-fold greater basal invasive capacity in the absence of serum than did MCF-7 cells (circles). In both cell lines invasion was concentration-dependent and already stimulated at the lowest serum concentration used (0.0025%). However, the slope of the serum invasion-response curve was higher in the metastatic MDA-MB-435 cells. Further, whereas the invasive response of MCF-7 cells to serum concentration slowed down after about 0.25% serum, the MDA-MB-435 cells continued an linear response of invasive capacity to increasing neoplastic state: 6.98 ± 0.07 (n = 43) to 7.26 ± 0.06 (n = 37) to 7.53 ± 0.05 (n = 52) for MCF-10A, MCF-7, and MDA-MB-435 cells, respectively. Steady state lactate production also increased with increasing neoplastic state: 271.8 ± 19.4 (n = 6) to 637.8 ± 59.9 (n = 7) to 840.3 ± 118.6 (n = 5) nmol/h/10^6 cells for MCF-10A, MCF-7, and MDA-MB-435 cells, respectively. The effect of serum deprivation on lactate production was then measured to determine if there are differences between normal and tumor cells and with neoplastic state. Serum deprivation resulted in a decrease in steady-state lactate production in all three cell lines: 179.6 ± 15.3 (n = 10, p < 0.001) to 526.5 ± 43.8 (n = 6, p < 0.001) to 771.7 ± 88.4 (n = 7, p < 0.01) nmol/h/10^6 cells for MCF-10A, MCF-7, and MDA-MB-435 cells, respectively. As can be seen, although lactate production decreased with serum deprivation, the effect was lessened with increasing neoplastic state. All together these data show that resting pH_i, lactate production, serum independence of growth, and invasiveness increased with increased neoplastic status and support the use of these cell lines to study the change in regulation of pH homeostasis with neoplastic progression.

Effect of Serum Deprivation on Intrinsinc Buffering Capacity (β)—Cytoplasmic buffer capacity (β) is an important mechanism to maintain pH_i at a constant level during acute pH_i displacements. The response of the relation of β to pH_i (33) in serum deprived and nondeprived cells for each cell line was measured next. As can be seen in Fig. 2, all three cell lines had a linear relationship of β to intracellular pH. Serum deprivation had a strong negative impact on the β of the normal cells, reducing the slope of the curve by about 4-fold, whereas in both tumor cell lines the relationship of β versus pH_i was unaffected.

Na^+ /H^+ Exchanger Activity Is Inhibited in the Normal Cells and Stimulated in the Cancer Cells by Serum Deprivation—All studies were conducted in nominally HCO_3^- -free HEPES-buffered solutions to measure only the Na^+ /H^+ exchange. BCECF-loaded cells initially perfused with HEPES-buffered bicarbonate-free Na^- medium were exposed for 5 min to 20 mM NH_4Cl. The medium was replaced by a Na^- -free, n-methylglucamine chloride solution resulting in an acidification of the cytoplasm. When the cells reached a stable acid pH_i, the addition of Na^- medium produced a rapid rise in pH_i, and the initial rate of cellular pH_i-recovery (dPH_i/dt) was determined. In all three cell lines pH_i recovery was completely inhibited by 2 μM 5-[(N,N-
dimethyl)-amiloride (DMA), a specific inhibitor of NHE (data not shown). Fig. 3 illustrates representative experiments comparing pH recovery in serum deprived (D) and nondeprived (ND) monolayers in all three cell lines at 135 mM external sodium. As can be seen in MCF-10A cells, 24 h of deprivation resulted in a reduction in dpH/dt, whereas 24 h of deprivation in both the tumor cells (MCF-7 and MDA-MB-435) stimulated dpH/dt.

The Na+/H+ exchanger activity from the initial pH recovery was then calculated as H+ extrusion rate from the $\beta_1$, as described under “Experimental Procedures.” The average alteration in Na+/H+ exchanger activity in all three cell lines with different times of serum deprivation is shown in Fig. 4. As can be seen, the inhibition of transport activity in the normal cells (MCF-10A, inset) was already maximum by 8 h of serum removal. In the two tumor cell lines serum deprivation-dependent up-regulation of NHE activity differed in both the time course of stimulation and in amplitude. The cells derived from a primary tumor (MCF-7, lightly dotted bar) had their maximum response at two days of deprivation with approximately a 1.65-fold increase over nondeprived cells. The malignant cells (MDA-MB-435, stippled bar) had a later maximum response (three days) and a greater stimulation of activity at all time points with a maximum stimulation of almost 3-fold over non-deprived cells. The data demonstrate that tumor cells have an opposite response than normal cells to serum deprivation and that the extent of the tumor-specific response increases with increasing neoplastic state.

 Serum Deprivation Has No Effect on NHE1 Expression—To determine if the observed changes in $V_{max}$ are dependent on a change in transporter expression, the effect of serum deprivation on the expression and glycosylation state of the NHE1 isoform in the three cell lines was analyzed by Western blot of this isoform in a crude membrane fraction using antibodies generated against a fusion protein of NHE1 (38). Membrane protein was loaded in equal amounts (60 $\mu$g) from all three cell lines and treatments (Fig. 5A). The antibody recognized a protein in all three cell lines. Although the amount of protein decreased with increasing neoplastic state (Fig. 5B), serum deprivation did not result in any change in NHE1 protein expression. Although glycosylation did not change with serum deprivation, the relative level of glycosylation of NHE1 was reduced with the increased neoplastic state; in the normal cell line MCF-10A NHE1 was highly glycosylated with a major band being at about 118 kDa, whereas in MDA-MB-435 cells NHE1 does not seem to be glycosylated as there was no recognition by anti-NHE1 of a form of greater $M_\text{r}$ than 97 kDa. Reduced or altered glycosylation of membrane proteins is common in cancer cells (39) but how this is related to the transformed phenotypes is still unclear. Little is known about the role of glycosylation in normal Na+/H+ exchange function. Human NHE1 has both N- and O-linked sugars that do not seem to be essential for its kinetics or function (40).

 Effect of Serum Deprivation on NHE Sodium Kinetics—We next looked at the effects of varying extracellular Na+ on the activity of the exchanger to determine if alterations in the sodium kinetics of the exchanger could account for the observed deprivation-dependent changes in activity. The effect of increasing external Na+ concentration on the initial rate of proton extrusion by the Na+/H+ exchanger before and after maximum serum deprivation in all three cell lines is illustrated in Fig. 6. Initial Na+ dependent recovery from an acid load was measured in the presence of 0, 6.75, 13.5, 27, 67, and 135 mM sodium at ringer pH 7.4. The rate of Na+-dependent H+ extrusion followed simple Michaelis-Menten saturation kinetics in all three cell lines. Analysis of the data by nonlinear regression yielded similar sodium affinity constants ($K_m$) for all three cell lines in the non-serum-deprived state (H ~30 mM). In the non-tumor cells, serum deprivation resulted in an increase in sodium affinity ($K_m = 23.9 \pm 2.7$ mM) and a 47% decrease in $V_{max}$ (from 42 ± 1.8 to 22 ± 0.9 mM H+ /min). In the tumor cells the $V_{max}$ was increased 173% in MCF-7 cells (from 9.3 ± 0.4 to 16.1 ± 0.4 mM H+ /min, $p < 0.001$) and 270% in MDA-MB-435 (7.7 ± 0.8 to 20.8 ± 0.6 mM H+ /min, $p < 0.001$), whereas the $K_m$ for sodium was unchanged. These kinetic values were utilized to plot the theoretical curves for this relationship shown in Fig. 6.

Serum Deprivation Has Opposite Effects on the Kinetics of the Allosteric Proton Regulatory Site in Nontumor and Tumor Cells—As NHE activity is finely regulated by intracellular proton concentration via an allosteric proton regulatory site, the effect of serum deprivation on the dependence of NHE activity to pH$_{i}$ was analyzed essentially as described (41). Fig. 7 shows the relationship of NHE activity in serum-derived (circles) and non-serum-deprived (squares) cells. In the normal cells (MCF-10A) serum deprivation shifted the pK value for intercellular H+ to acidic values (acidic shift) and reduced the...
slope of the relationship. Kinetic analysis (Table I) revealed that serum deprivation reduced the $V_{\text{max}}$ by approximately 50% and the Hill coefficient ($n_{\text{app}}$) from approximately 2 to 1. In both the carcinoma cell lines the pH$_i$ versus activity curve for serum-deprived cells was shifted to the right of the curve for nondeprived cells (alkaline shift) without a shift in the slope. This alkaline shift was greater in the highly invasive, malignant cell line (MDA-MB-435) than in the in situ carcinoma cell line (MCF-7). Kinetic analysis demonstrated that the $V_{\text{max}}$ and affinity increased in both of the cancer cells upon serum deprivation without a change in the Hill coefficient (Table I). These data suggest that serum deprivation in the cancer cells increases NHE activity by increasing the affinity of the internal proton regulatory site of the NHE for protons. The observed alkaline shift in serum-deprived conditions in the tumor cells is consistent with an increased capacity for net acid extrusion (42).

PI3K Regulation of NHE Activity in Tumor Cells Is Reversed

by Serum Deprivation—Recently, PI3K has been demonstrated to be involved in integrin-stimulated invasion in the breast carcinoma cell line, MDA-MB-231 (43). To determine which role PI3K may play in the stimulation of tumor cell NHE activity by serum deprivation, the effect of the inhibition of PI3K by the specific inhibitor, wortmannin (10 nM), or by transfection of a dominant-negative mutant of the regulatory subunit p85α of PI3K, was analyzed. The MDA-MB-435 cell line and 24-h treatment time were chosen for these experiments to maximize the serum deprivation stimulatory response and minimize the time under pharmacological influence. The effect of the inhibition of PI3K by 10 nM wortmannin or by transfection of the dominant-negative mutant on NHE activity in non-serum-deprived (ND) or 24 h-deprived (D) MDA-MB-435

Fig. 3. Effect of serum deprivation on Na$^+$-dependent pH$_i$ recovery after an NH$_4$Cl-induced acid load: typical experiment. Cells were acidified by a 20-min pulse of 30 mM NH$_4$Cl during the dye-loading procedure. The trace begins at the start of perfusion of the monolayer with HEPES-NMEG solution (pH 7.4). There was no recovery of pH$_i$ under this condition. When the monolayer was perfused with 135 mM Na$^+$ nominally bicarbonate-free HEPES solution (pH 7.4) a rapid recovery of pH$_i$ commenced. The recovery in a non-serum-deprived monolayer (ND) is compared with that in a serum-deprived monolayer (D) measured at 135 mM sodium in representative experiments.

Fig. 4. Time course of alteration of Na$^+$/H$^+$ exchange after serum deprivation. pH$_i$ recoveries were measured as in Fig. 3 and the initial rate of the NHE activity was calculated as Na$^+$-dependent H$^+$ efflux as described under “Experimental Procedures.” The inset is the data for MCF-10A cells after 8 and 24 h of serum deprivation. Data for MCF-7 cells are shown in the dotted bars, whereas the stippled bars represent data for MDA-MB-435 cells. Data are the mean ± S.E. of between 5 and 20 observations for each condition.

Fig. 5. Western blot analysis of NHE1 expression. A, 60 μg of protein of a crude membrane fraction was separated in 7.5% acrylamide SDS-polyacrylamide gel electrophoresis and blotted as described under “Experimental Procedures.” ND, nondeprived of serum; D, deprived of serum for 1, 2, and 3 days for MCF-10A, MCF-7, and MDA-MB-435 cells, respectively. B, the relative optical density of each band was measured as under “Experimental Procedures” ($n = 3$ independent experiments).
monolayers can be seen in Fig. 8. The inhibition of PI3K by either wortmannin or the dominant-negative mutant resulted in the same pattern; they inhibited the NHE activity in the nondeprived cells, whereas strongly potentiating the stimulation of NHE activity by serum deprivation. Importantly, whereas serum deprivation for 8 h was an insufficient time to develop a measurable up-regulation of NHE activity, incubation with wortmannin for 8 h already reversed the regulatory pattern from inhibitory in non-serum-deprived cells to stimulatory in the deprived cells (data not shown). This suggests that the serum deprivation-dependent reversal in signal transduction takes place early in the process, long before alterations in NHE activity manifest.

Serum Deprivation Increases the Motility and Invasive Capacity of MDA-MB-435 Cells: Effects of DMA and Wortmannin—The effect of serum deprivation and the role of the NHE on the ability of MDA-MB-435 cells to cross a collagen layer in a Boyden Chamber (motility) and to infiltrate a confluent and tight (\( \approx 10000 \, \Omega \times \text{cm}^2 \)) monolayer of non-tumor MCF-10A breast epithelial cells (invasivity) were analyzed next. MDA-MB-435 cells were chosen for this assay as the serum deprivation-dependent increase in NHE activity was much greater in these cells. As seen in Fig. 9, 24 h serum deprivation significantly increased both MDA-MB-435 motility (Fig. 9A) and the invasivity (Fig. 9B). Furthermore, both of these processes were strongly inhibited by the specific NHE inhibitor, DMA, leading to the hypothesis that the NHE plays a fundamental role in these processes.

To further determine if the potentiation of NHE up-regulation in MDA-MB-435 correlates with increased invasive potential, the effect of a 24-h incubation with 10 nM wortmannin on invasive capability for both serum deprived (D) and nondeprived (ND) MDA-MB-435 cells was analyzed (Fig. 9C). As can be seen, invasive capacity closely followed NHE activity (see Fig. 8) in that wortmannin treatment slightly inhibited invasion in the non-serum-deprived cells and potentiated invasion in the serum-deprived cells.

Changes in Intracellular ATP Do Not Correlate with Changes in Tumor Cell NHE Activity—As the alterations in sodium and proton kinetics of the NHE in the normal MCF-10A cells are similar to those reported during ATP depletion in normal cells (44), the level of cellular ATP was measured before and after serum deprivation to determine if the alterations in ATP levels in response to serum deprivation are the same in normal and tumor cells. Serum deprivation resulted in a decrease in cellu-
MCF-10A cells were serum-deprived for 24 h, MCF-7 cells for 48 h, and MDA-MB-435 cells for 72 h. Values are mean ± SE for \( V_{\text{max}} \) (mM H\(^+\)/min), and H\(^+\) apparent \( K^0 \) (\( \mu \text{m} \)) measured at 135 mM external sodium. The Hill coefficient (\( n_{\text{app}} \)) was derived at 135 mM external sodium. Confluent cell monolayers were either kept in serum repleat medium (ND) or placed in serum-free growth medium (D) for the indicated time prior to a measurement of H\(^+\). Significance is between the difference of the values in deprived and nondeprived cells.

| Kinetic parameter | MCF-10A | MCF-7 | MDA-MB-435 |
|-------------------|---------|-------|------------|
| \( V_{\text{max}} \) | 38 ± 1.8 | 10 ± 0.4 | 12 ± 0.0 |
| \( K^0 \) for H\(^+\) | 0.13 ± 0.05 | 0.18 ± 0.09 | 0.20 ± 0.07 |
| \( n_{\text{app}} \) | 2.1 | 1.8 | 2.2 |

\( a^* \) \( p < 0.001 \).
\( b \) \( p < 0.001 \).

**FIG. 8. Role of PI3K in NHE up-regulation by serum deprivation.** To examine the role of PI3K in the serum deprivation activation of NHE activity, MDA-MB-435 monolayers were serum-deprived (D) or left in fresh complete growth medium (ND) for 24 h in the absence (cont) or presence of 10 \( \mu \text{M} \) wortmannin (Wort) for 24 h or after the cells had been transfected with 5 \( \mu \text{g} \) of pGEX-p85INT Tag encoding a dominant-negative mutant of the p85 regulatory subunit of PI3K (p85dn). Exchanger activity was assayed as under “Experimental Procedures.” Data are the mean ± S.E. of between 8 and 15 observations for each condition.
primary due to an increased affinity for protons that increased the maximum transport rate (Table I). This alkaline shift of the pH, dependence of NHE activity suggests that the tumor cells developed an increased capacity for acid extrusion into its environment. The metastatic MDA-MB-435 cells had a much larger alkaline shift of the kinetics than did the primary tumor MCF-7 cells, indicating a greater ability to extrude protons in the MDA-MB-435 cell line. This is in line with a study demonstrating a much greater ability to acidify the extracellular medium in MDA-MB-231 cells than in MCF-7 cells (46).

Although ATP levels also decreased in the cancer cell lines with serum deprivation, the NHE was up-regulated in those lines with alterations in both the sodium kinetics (Fig. 6) and proton kinetics (Fig. 7 and Table I) that were different than those in the normal, MCF-10A cells. This suggests that tumor cells have an ATP-independent mechanism that increases NHE activity even during the reduction of the cellular ATP levels. In contrast, a mechanism for the reduction in the transport activity of the NHE in the normal (MCF-10A) cells is suggested by data from ATP depletion studies. A remarkable similarity exists between the effects of serum deprivation in MCF-10A cells in the present study and those reported by either ATP depletion (44) or by serum deprivation (47) in normal cells. Inhibition of NHE activity was observed in both those cases and was due predominantly to an acidic shift of the pH, dependence of the rate of transport and a reduction in the stoichiometry of the proton allosteric site from 2 to 1 as was observed in the present study (Fig. 7 and Table I). Further, the inhibition of NHE activity was accompanied by an decrease in the sodium half-saturation constant ($K_m$) in both MCF-10A cells (Fig. 6) and in the other study on serum deprivation (47).

The serum deprivation-dependent up-regulation of the NHE in both early and late neoplastic stages underlines its importance in the process of carcinogenesis and suggests that a fundamental, keystone alteration underlies its development, most probably somewhere in the intracellular signal transduction network. The present study begins to define the alterations of signal transduction pathways unique to tumor cells when confronted with serum deprivation, an environmental condition normal to tumors. We observe that during serum deprivation there is a reversal of the usual, positive PI3K regulatory pathway for NHE1 found in the presence of serum (Fig. 8). We postulate that it is this inversion of PI3K regulatory action that contributes, in part, to the up-regulation of the NHE by serum deprivation in tumor cells and hence to malignant progression. It would seem that a delicate balance between positive and negative actions of a kinase can determine the specificity and/or magnitude of a physiological or pathophysiological response. A similar scenario has been observed in T-cell homeostasis where expression of an activated form of PI3K inhibited T-cell antigen receptor-mediated responses, whereas expression of a dominant-negative mutant potentially enhanced T-cell antigen receptor controlled NF-AT induction (48).

The major direct targets of PI3K are the protein kinase Akt, p70S6 kinase and noncanonical forms of protein kinase C, all of which are thought to be involved in one way or another with control of neoplastic phenotypes (49). We observed that inhibiting p70S6 kinase with rapamycin (data not shown) had no effect on either steady state NHE activity or on its activation by serum deprivation. PI3K can interact with the small GTP-binding protein Rac1 to produce actin rearrangement and membrane ruffling (50), regulate Tiam1-dependent motility (51), and transduce integrin-stimulated invasion in MDA-MB-435 (43, 52). However, the results of the present study also potentially unify recent observations concerning alternative mechanisms underlying tumor invasive potential: the RhoA-ROCK activation of NHE (53) and the RhoA-ROCK mediation of serum-stimulated invasion (54). The involvement of the Rho family GTPase in serum deprivation-dependent regulation of NHE and invasion are currently under investigation in our laboratory.

Tumor invasion and metastasis associated with neoplastic progression are the major causes of cancer deaths. The invasive process occurs through a complex series of interactions with the host tissue in which the infiltration and penetration of the normal tissue by the cancer cell takes place by three biochemical and physiological steps: tumor cell attachment to basement membranes or extracellular matrices, local degradation of these structures directly by acid extrusion and secretion of acid-dependent proteases, and increased tumor cell locomotion into the modified region. Both the second and third processes...
are regulated by extra- and intracellular pH, respectively. The tumor microenvironment has been shown to be critical in controlling invasive capacity and subsequent malignant progression (10, 11, 21) by increasing the activity one or more of the above steps. We have identified an activated NHE1 as the mechanism that continues to acidify the extracellular environment in nutrient-depleted conditions. Further, we demonstrate that this activated NHE drives an increased motility and invasive capability. NHE has been shown to play a fundamental role in motility by selectively regulating cytoskeletal events such as focal adhesion assembly and turnover (55, 56). These results are consistent with our findings and give further support to the idea that by activating NHE, serum deprivation increases tumor invasive potential via two complementary mechanisms: an increased acid extrusion by the cancer cell and an increased capability for motility.

In conclusion, our results demonstrate that serum deprivation results in an up-regulation of the NHE present only in cancer cells with the subsequent increase in their invasive potential. These data suggest that tumor cells have not only adapted to the specialized conditions of their microenvironment but have developed completely different regulatory patterns that allow them to take advantage of these conditions to maximize their neoplastic potential. As the tumor microenvironment is heterogeneous and unstable within the tumor, this adaptive ability may have evolved to permit local motility toward zones with a more hospitable microenvironment.

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Phosphoinositide 3-Kinase Is Involved in the Tumor-specific Activation of Human Breast Cancer Cell Na\textsuperscript{+}/H\textsuperscript{+} Exchange, Motility, and Invasion Induced by Serum Deprivation

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