Control of intracellular ion composition is a fundamental requirement of all living organisms. As a result of specialization, epithelial cells use their ion transporting machinery not only for "housekeeping" purposes, but also for regulation of transport of ions and water across the epithelium. One example of such specialization is the family of Na\(^+/\)H\(^+\) exchangers (NHEs). Six isoforms of NHE have been cloned to date (33, 47). All of the isoforms are membrane proteins that perform Na\(^+/\)H\(^+\) exchange with stoichiometry 1:1. The two isoforms best characterized to date are NHE1 and NHE3. NHE1, the ubiquitously present exchanger, is believed to perform housekeeping functions, which include regulation of intracellular pH (pHi), cell volume, and cell proliferation. In most polarized epithelial cells, NHE1 is located at the basolateral (BL) membrane domain (9, 31). The other well-characterized isoform is NHE3. It has been found predominantly in the brush border (BB) of renal, intestinal, and salivary gland epithelium (4, 14, 36). In the kidney, NHE3 is involved in Na\(^+/\)HCO\(_3\)\(^-\) and probably NH\(_4\)\(^+\) absorption (35). In the intestine, NHE3 is believed to play a major role in 1) neutral NaCl absorption (23), 2) the increased ileal Na\(^+\) absorption, which is stimulated via neurohormonal signals after meals (24, 50), and 3) the decreased Na\(^+\) absorption, which contributes to secretory diarrhea (10). NHE3 is present in jejunal and ileal villus cells, colonic surface cells, and in the upper crypt cells of small intestine and colon in humans, rabbit, and rat (9).

Most of our knowledge concerning the regulation of NHE3 activity comes from studies of the exchanger molecules exogenously expressed in nonepithelial and some epithelial cell lines. The results of these studies indicate that most of the regulation of NHE3 activity occurs via changes in maximal velocity (V\(_{\text{max}}\)) of the exchanger, in contrast to the regulation of NHE1, which occurs mostly by changes in K\(_{\text{m}}\) (H\(^+\)). Growth factors, serum, and okadaic acid stimulate NHE3 activity, whereas protein kinase C (PKC), carbachol, and hyperosmolality inhibit the exchanger (9). Recent studies of regulation of NHE3 in native ileum indicate that activation of some signal transduction pathways is highly polarized in expression (16, 17). Moreover, some evidence suggests that the regulation of NHE3 in polarized epithelial cells involves cytoskeletal proteins (e.g., villin), which are unique to some specialized epithelial cell types (18). These facts may create problems when extrapolating the results of in vitro studies on NHE3 regulation to native tissue. Published studies of regulation of endogenous NHE3 in epithelial cells are limited to kidney-derived OK cells, and most in vitro studies have been performed using cells transfected with NHE3 cDNA. The latter approach may be

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
complicated by a separate set of problems, including those related to possible overexpression and mistargeting of membrane proteins. In this communication we describe a gradual development of activity, membrane targeting, and regulation of endogenous NHE3 in three clones of the human colonic adenocarcinoma cell line Caco-2. This gradual development was clone specific and appeared to result from an increasing number of cells expressing NHE3 at the BB and not from parallel, progressive differentiation of targeting and regulatory mechanisms within individual cells. In mature monolayers of two of three tested Caco-2 clones, both the polarity of expression as well as the regulation of NHE3 by PKC and epidermal growth factor (EGF) closely resembled those described for native mammalian intestinal epithelial cells.

MATERIALS AND METHODS

Cell culture. Three clones of Caco-2 cell line originally derived from human colonic adenocarcinoma were studied. Clone ATCC was purchased from American Type Culture Collection (Rockville, MD) and was used between passages 27 and 47. Two other clonal lines derived from the original Caco-2 cell population, PF-11 and TC-7, were characterized previously (7). Clonal line PF-11 was originally an "early" passage (passage 29) and was studied between 35 and 45 subsequent passages. Clonal line TC-7 was originally a "late" passage (passage 198) and was studied between 42 and 49 subsequent passages. These two clonal lines differ significantly in monolayer morphology, glucose consumption rate, glycogen accumulation, and sucrase-isomaltase expression at the BB (7). All cells were plated at a density 5–8 x 10^4 cells/cm² into tissue culture inserts equipped with 0.45-µm pore size, high-pore density Falcon PET membranes (Becton Dickinson Labware, Franklin Lakes, NJ) and grown in DMEM supplemented with 0.1 mM nonessential amino acids, 1 mM pyruvate, penicillin (50 U/mL), streptomycin (50 µg/mL) and 10% fetal bovine serum, in a 10% CO₂-humidified incubator at 37°C. The same batch of serum was used in all experiments. In some experiments, cells were plated on the Falcon polyethylene terephtalate (PET) membranes covered with human laminin (1 µg/cm²; Sigma Chemical, St. Louis, MO).

For fluorometry, cells were grown as polarized monolayers on small pieces of Falcon PET membranes glued over an aperture in rectangular plastic coverslips ("filterslips") as described previously (27). Filterslips with attached cells were kept in standard 12-well culture plates, and culture medium was replaced every other day.

Measurement of Na⁺/H⁺ exchange rate. A fluorometric method based on pH-sensitive fluorophore 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein-acetoxyethyl ester (BCECF-AM; Molecular Probes, Eugene, OR) was used for functional characterization of Na⁺/H⁺ exchange separately at the apical (AP) and BL surfaces of the monolayers, as described in detail previously (27, 48). Briefly, monolayers grown on filterslips and serum deprived for 8–12 h were exposed to BCECF-AM (5 µM) and NH₄Cl (40 mM, to promote subsequent intracellular acidification) in Na⁺ medium [in mM, 131 NaCl, 5 KCl, 2 CaCl₂, 1 MgSO₄, 0.8 Na₂HPO₄, 0.2 NaH₂PO₄, 25 glucose, 20 HEPES, pH 7.4] for 50 min at room temperature. Filterslips were then mounted in a cuvette, placed in the fluorometer (SPF 500C; SLM, Urbana, IL), and perfused at both monolayer surfaces with tetrarmethylammonium (TMA) medium (identical to Na⁺ medium except that Na⁺ salts were replaced by TMA salts) to allow for rapid intracellular acidification. The TMA medium was subsequently replaced with Na⁺ medium at one monolayer surface. Changes in pH, were monitored by alternating the excitation wavelengths between 440 and 500 nm and collecting the emission signal at 530 nm. Rates of Na⁺-dependent intracellular alkalinization (efflux of H⁺, in µM/s) were calculated for a given pH, within the linear phase of the initial rate of intracellular alkalinization, as the product of ∆pH/∆f and buffering capacity) using Enzfit software ( Biosoft) (48). The buffering capacity of Caco-2 cells was determined separately, as described elsewhere (6, 48). The monolayers’ integrity was systematically tested by adding 1 mM amiloride (which, at this concentration, inhibits both NHE1 and NHE3) to the AP superfusate (TMA medium) in the presence of 131 mM Na⁺ in the BL superfusate, and comparing the rate of intracellular alkalinization with that observed in the absence of amiloride. In these experiments, <3% of tested monolayers exhibited inhibition of BL Na⁺/H⁺ exchange, which suggested a leak of amiloride from AP to BL side and, therefore, a break in the integrity of the monolayer.

To differentiate among NHE1, NHE2, and NHE3 activity at a given monolayer surface, we used two known Na⁺/H⁺ exchange inhibitors: HOE-694 and amiloride. HOE-694 is a benzoylguanidine derivative initially characterized using PS120 fibroblasts transfected with various NHE isoforms (8). In these cells, the compound’s inhibitor constant for NHE3 was 650 µM, whereas its inhibitor constant for NHE1 was only 0.15 µM. In our hands (see results) complete inhibition of endogenous NHE1 in Caco-2 cells without any effect on NHE3 activity was observed with a HOE-694 concentration of 20 µM. In the presence of 131 mM Na⁺, the IC50 for NHE2 transfected into PS120 fibroblasts was 20.5 µM (see below). We used 1 mM amiloride (instead of HOE-694) for inhibition of NHE3, inasmuch as we were not able to solubilize HOE-694 in Na⁺ buffer at a concentration higher than 1 mM (which was required for complete inhibition of NHE3). The NHE1 activity was calculated by subtraction of the exchange rate observed at a given pH, in presence of 20 µM HOE-694 from the total exchange rate observed at a chosen monolayer surface in the presence of 131 mM Na⁺. The Na⁺/H⁺ activity observed in the presence of 131 mM Na⁺/H⁺ exchange inhibitors was due to NHE3 only, and it was predictably inhibited by 1 mM amiloride. NHE2 was not expressed in any of the Caco-2 clones examined (see below).

In experiments with EGF (200 ng/mL) and phorbol 12-myristate 13-acetate (PMA, 1 µM) (Sigma Chemical), the tested substances were present in Na⁺ medium during the final 20 min of incubation with BCECF, and then in both AP and BL media during the entire perfusion. In some experiments, separate groups of monolayers were exposed to PMA in the presence of the PKC inhibitor 1-(5-isoquinolinylsulfonyl)-2-methylpiperezine (H-7, 65 µM; Seikagaku Kogyo, Tokyo, Japan), which was preceded by 20 min of preincubation with H-7 alone.

Inhibition of AP and BL Na⁺/H⁺ exchange activity by amiloride and HOE-694. Examination of the response kinetics of Na⁺/H⁺ exchange to amiloride and HOE-694 was performed separately at the AP and BL surfaces of Caco-2 monolayers by using the BCECF fluorometric method as previously described (48). Caco-2 monolayers at 17 postconfluent days (PCD) were superfused at the chosen monolayer surface with Na⁺ medium without or with various concentrations of the inhibitors. The opposite surface of the monolayer was perfused with TMA medium. Na⁺/H⁺ exchange kinetics were then calculated using Enzfit software, and the rates were compared at the same pH, within the linear portion of
The kinetic curves. The dose-response curves were obtained using a computer-enhanced curve-fitting algorithm (Micral Origin, Micral Software).

Inhibitory effects of HOE-694 on NHE1 and NHE2 in PS120 fibroblasts were evaluated using PS120 cells transfected with cDNA encoding for NHE1 and NHE2, respectively (41, 42). The rate of intracellular alkalinization in the presence of 131 mM NaCl and different concentrations of HOE-694 was examined as previously described for Caco-2 cells, with the exception that PS120 cells were cultured on glass coverslips and were therefore superfused only at the cell surface.

Localization of intracellular NHE1 and NHE3 by indirect immunofluorescence. Labeling of intracellular NHE1 and NHE3 was performed as described in detail previously (15).

Briefly, Caco-2 monolayers grown on PET membranes were rinsed with PBS and fixed with 3% paraformaldehyde in PBS, pH 7.4, for 45 min. The permeabilization and blocking of nonspecific binding sites were performed in one step by incubating the monolayers with PBS containing 0.075% saponin, 1% BSA (Goldmark Biologicals, Phillippsburg, NJ), and 15% normal goat serum (buffer PBG; Jackson Immunoresearch, West Grove, PA) for 45 min. Monolayers were then incubated with anti-NHE1 polyclonal antibody (Ab) 1380 (1:50 dilution) or with anti-NHE1 Ab 1950 (1:50 dilution) in buffer PBG for 1 h, followed by washing with PBS containing 0.05% saponin and 1% BSA. Both antibodies were raised in rabbits and have been shown previously (by immunohistochemistry and Western analysis) to specifically recognize respective exchanger molecules expressed in PS120 fibroblasts as well as endogenous exchangers in rabbit and human intestinal epithelia and not to cross-react with each other or with NHE2 (14, 41). For control of nonspecific binding, the antisera were substituted with preimmune rabbit serum at 1:1,000 dilution. This antibody was shown previously not to cross-react with rabbit NHE3 and NHE1 (41) and to specifically recognize human NHE2 (14).

Statistical analysis. Numerical data are expressed as means ± SD, and the significance of difference between experimental groups was analyzed by two-tailed Student’s t-test.

RESULTS

General characteristics of the studied Caco-2 clones. Once confluence was reached (4–5 days), all three clones studied exhibited morphological heterogeneity, as described previously (44). At the level of phase contrast microscopy, islands of well-demarcated polygonal cells were interspersed with areas covered with more irregular, larger cells. Formation of domes was observed within 2–3 PCD in monolayers grown on solid substrate. The ATCC and PF-11 clones were more heterogeneous and produced significantly more domes than the TC-7 clone (not shown). Moreover, TC-7 cells had a higher mitotic index than the other two clones (doubling time of ~24 and 36 h, respectively), consistent with previous reports (7). In the PF-11 clone, the average cell density at confluence was ~3.1 × 10³/cm², and gradually increased to ~7.2 × 10³/cm² at 3–4 PCD, after which time the cell density remained relatively stable for up to 30 PCD. Similar results were observed in TC-7 and ATCC clones.

Gradual development of polarized Na⁺/H⁺ exchange activity in Caco-2 monolayers. Polarized expression of NHE1 and NHE3 activities was investigated starting as soon as the formation of the occluding junctions was completed, e.g., between 48 and 72 h postconfluency (38). A representative pattern of Na⁺-dependent intracellular alkalinization at 3 PCD is shown in Fig. 1A. Exposure of BL monolayer surface to Na+ medium resulted in a rapid intracellular alkalinization, with
the average H$^+$ efflux rate being $733 \pm 83$ µM/s ($n = 8$) within the linear portion of the alkalization curve. The pH$_i$ reached plateau at $7.12 \pm 0.08$ within 12–14 min (not shown). This BL Na$^+$/H$^+$ exchange was completely suppressed by 20 µM HOE-694, indicating that it was entirely due to the activity of NHE1. Significant Na$^+$/H$^+$ exchange activity was also noted when the monolayers were perfused with Na$^+$ medium at the AP surface. However, only ~67% of this activity was suppressed by 20 µM HOE-694, the residual activity requiring 1 mM amiloride for complete inhibition. These data suggest the presence of activities of both NHE1 and NHE3 (~67 and 33% of NHE1 and NHE3, respectively) at the AP surface of the early postconfluent monolayers (Fig. 1A and Table 1). Lack of detectable NHE2 in these cells (Fig. 2) speaks against the possibility of significant contribution of this isoform to the observed “amiloride sensitive”AP and/or BL Na$^+$/H$^+$ exchange at this early postconfluent period. With increasing time postconfluency, a progressively higher activity of NHE3 was observed at the AP surface of the Caco-2 cell monolayers, and this was paralleled by a significant decrease of NHE1 activity at this membrane.

### Table 1. Comparison of total apical Na$^+$/H$^+$ exchange and relative contribution of NHE3 to this exchange in 3 Caco-2 cell clones

<table>
<thead>
<tr>
<th>Caco-2 Clone</th>
<th>Tot$_{AP}$ exchange, µM H$^+$/s</th>
<th>NHE3$_{AP}$, %</th>
<th>Tot$_{AP}$</th>
<th>NHE3$_{AP}$, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>PF-11</td>
<td>284 ± 30</td>
<td>33 ± 6</td>
<td>468 ± 32t</td>
<td>96 ± 9t</td>
</tr>
<tr>
<td>ATCC</td>
<td>230 ± 38</td>
<td>29 ± 4</td>
<td>410 ± 52t</td>
<td>93 ± 10t</td>
</tr>
<tr>
<td>TC-7</td>
<td>51 ± 12*</td>
<td>5 ± 8*</td>
<td>79 ± 15*</td>
<td>8 ± 5*</td>
</tr>
</tbody>
</table>

Values are means ± SD from 8 monolayers from 2 separate experiments. Total apical exchange activity (Tot$_{AP}$) is expressed as H$^+$ efflux rate at intracellular pH (pH$_i$) 6.40 with 131 mM Na$^+$ present at apical monolayer surface, as evaluated 3 (3 PCD) and 22 (22 PCD) days postconfluency. Percent contribution of NHE3 activity to total apical exchange (NHE3$_{AP}$) was evaluated using 20 µM HOE-694, as described in MATERIALS AND METHODS, and is presented as percent of total exchange rate at pH$_i$ 6.40. *Significantly different ($P < 0.01$) from any value in same column. †Significantly different ($P < 0.01$) from respective value at 3 PCD.
Sensitivity of AP and BL Na⁺/H⁺ exchange activity to HOE-694 and amiloride. Evaluation of the dose-response kinetics of AP and BL Na⁺/H⁺ exchange for HOE-694 and amiloride was performed using clone PF-11 at 17 PCD, i.e., at the time of stable polarized expression of NHE1 and NHE3. The dose-response curve obtained for HOE-694 applied basolaterally suggested the presence of a single isoform of NHE, with IC₅₀ equaling 0.13 μM (Fig. 4, top). In contrast, the curve obtained when the inhibitor was applied at the AP surface of Caco-2 monolayers suggested the presence of two NHE isoforms, with IC₅₀ of the “HOE-694-sensitive” component being ~0.11 μM and the IC₅₀ for the “HOE-694-resistant” component being 655 μM. Moreover, the HOE-694-sensitive component was apparently responsible for only ~9% of the overall AP Na⁺/H⁺ exchange. These values are consistent with the predominant contribution of NHE3, and only a small contribution of NHE1 to the total AP Na⁺/H⁺ exchange in this Caco-2 clone at 17 PCD. The purely sigmoidal shapes of the dose-response curves obtained for the HOE-694-sensitive Na⁺/H⁺ exchange in the AP and the BL monolayer surfaces speaks against the presence of NHE2 activity in these monolayers. Computer-enhanced analysis of both curves (Fig. 4, top) suggested the presence of only one exchanger with IC₅₀ of ~12 μM. This was similar to the IC₅₀ obtained from PS120 cells transfected with NHE1 cDNA (0.25 μM, Fig. 5). Because the IC₅₀ for NHE2 was 20.5 μM (Fig. 5, PS120 cells), contribution of NHE2 to the AP and/or BL Na⁺/H⁺ exchange in Caco-2 cells would result in a bimodal shape of the respective segments of the dose-response curves.

The dose-response patterns obtained for amiloride are shown in Fig. 4, bottom. Similar to the patterns shown in Fig. 4, top for HOE-694, they suggest that the BL Na⁺/H⁺ exchange was entirely due to NHE1 activity (IC₅₀ 2.1 μM), and that both NHE3 (~92% of overall AP activity, IC₅₀ 115 μM) and NHE1 (~8% of overall AP...
activity, IC$_{50}$ 2.1 µM) were present at the AP surface of these monolayers.

Subcellular localization of NHE1 and NHE3 by immunofluorescence. NHE3 was found at the AP surface of a small number of Caco-2 cells (clone PF-11) shortly after postconfluence, and the number of positively labeled cells increased progressively with time, from ~15% positive cells seen at 3 PCD to ~52% at 11 PCD and 85% at 22 PCD (Table 2). Similar results were obtained with ATCC monolayers, whereas only very small numbers of NHE3-positive cells were found in TC-7 cells, regardless of the postconfluent culture period (Table 2).

Because the extracellular matrix and in particular laminin appear to affect the functional maturation of Caco-2 cells (45), we tested the effect of coating the permeable culture substrate with exogenous laminin on the expression of NHE3 in the three clones of Caco-2 cells. In PF-11 and ATCC clones the presence of laminin resulted in a significant increase in the number of NHE3 expressing cells at 3 and 11 PCD, with no significant effect of laminin seen at 22 PCD (Table 2). The effect of laminin on the number of NHE3-positive cells was paralleled by the NHE3 activity of the monolayers. In contrast, no significant effect of laminin on NHE3 expression was observed in TC-7 clone at any postconfluent period examined (Table 2).

The representative pattern of expression of surface NHE3 labeled by indirect immunofluorescence in Caco-2 (PF-11) monolayer at 17 PCD is shown in Fig. 6. The labeled cells were aggregated in irregular islands, interspersed with areas of cells that exhibited a much weaker fluorescent signal. With use of confocal microscopy, two distinctive morphological patterns of labeled BB could be recognized: 1) a relatively smooth and uniform “standard” pattern and 2) a “flower” pattern, with labeling aggregated into small clusters on the surface of a single cell. These patterns were consistent with the description of immunolabeling of some BB hydrolases (sucrase-isomaltase, alkaline phosphatase, dipeptidylpeptidase IV, lactase) in Caco-2 cells in earlier reports (44, 46). Reconstruction of the images in the vertical (XZ) plane revealed that the immunolabeling was exclusively present in the AP cell area corresponding to the BB and a narrow zone of subapical cytoplasm (Fig. 6B). No significant labeling was observed in the areas corresponding to the BL surface.

Because Ab 1380 was used at a relatively low dilution, the possibility of nonspecific binding had to be considered. Three pieces of evidence speak against this possibility. First, no significant binding was observed when preimmune serum was used instead of Ab 1380 (Fig. 6C). Second, in late postconfluent monolayers of...
immunostained with anti-NHE3 antibody and analyzed by confocal microscopy as described in MATERIALS AND METHODS. Positive labeling PC on plain PET membranes (Plain) or membranes covered with laminin (Laminin). Monolayers were fixed at indicated intervals,

protein of molecular mass from the lysates of PS120 fibroblasts transfected with 85-kDa band observed in the immunoblots obtained Western analysis.

quantitation of NHE3 by surface biotinylation and fluent period.

Caco-2 monolayers and with a presence of this exchanger also at the BB, especially in the early postconfluent period.

BL localization of NHE1 activity in fully matured Caco-2 monolayers (3 PCD), the NHE1 labeling was present predominantly in the BL areas, but some weak labeling was also occasionally observed at the BB (not shown). These findings were consistent with the predominantly BL localization of NHE1 activity in fully matured Caco-2 monolayers and with a presence of this exchanger also at the BB, especially in the early postconfluent period.

Subcellular localization of NHE1 and NHE3 and quantitation of NHE3 by surface biotinylation and Western analysis. Surface biotinylation experiments were performed to confirm the pattern of NHE3 and NHE1 intracellular localization suggested by microscopic studies. In Caco-2 monolayers at 17 PCD (clone PF-11), a prominent protein band of molecular mass, ~85 kDa, was observed in the immunoblots of cell lysates obtained from monolayers biotinylated at the AP surface (Fig. 8, top). This band corresponded to the 85-kDa band observed in the immunoblots obtained from the lysates of PS120 fibroblasts transfected with rabbit NHE3. In contrast, no detectable biotinylated protein of molecular mass ~85 kDa was detected in the Caco-2 lysates obtained from monolayers biotinylated at the BL surface (Fig. 8, top). These data confirmed the exclusive AP targeting of endogenous NHE3 in Caco-2 cells. On the other hand, performance of a similar procedure but with use of anti-NHE1 Ab 1950 resulted in detection at the BL monolayer surface of significant amounts of a protein of molecular mass ~110 kDa, which corresponded to similar band obtained from

TC-7 cells in which BB was fully and uniformly developed (as judged by labeling with PHA-E, data not shown) virtually no immunolabeling of NHE3 was observed, and this corresponded to the very low AP NHE3 activity observed in these cells (Table 1). Finally, the same Ab 1380 was shown to specifically detect NHE3 in PS120 cells as well as at the BB of native human and rabbit intestinal epithelia (14).

In contrast to NHE3, immunolabeling of NHE1 in Caco-2 monolayers with anti-NHE1 Ab 1950 revealed a "chicken wire" pattern suggesting BL localization of the exchanger. This was confirmed by images reconstructed in the XZ plane (Fig. 7). In early postconfluent monolayers (3 PCD), the NHE1 labeling was present predominantly in the BL areas, but some weak labeling was also occasionally observed at the BB (not shown). These findings were consistent with the predominantly BL localization of NHE1 activity in fully matured Caco-2 monolayers and with a presence of this exchanger also at the BB, especially in the early postconfluent period.

Table 2. Effect of laminin on relative number of cells expressing brush-border NHE3 and on apical NHE3 activity in 3 Caco-2 cell clones analyzed at 3, 11, and 22 PCD

<table>
<thead>
<tr>
<th>Clone</th>
<th>3 PCD</th>
<th>11 PCD</th>
<th>22 PCD</th>
</tr>
</thead>
<tbody>
<tr>
<td>PF-11</td>
<td>15 ± 4</td>
<td>38 ± 6*</td>
<td>52 ± 10</td>
</tr>
<tr>
<td>ATCC</td>
<td>14 ± 6</td>
<td>30 ± 5*</td>
<td>46 ± 11</td>
</tr>
<tr>
<td>TC-7</td>
<td>3 ± 2</td>
<td>5 ± 4</td>
<td>4 ± 2</td>
</tr>
</tbody>
</table>

Values are means ± SD from 500 cells counted in each group in 2 separate experiments. Three Caco-2 cell clones were cultured for up to 22 PCD on plain PET membranes (Plain) or membranes covered with laminin (Laminin). Monolayers were fixed at indicated intervals, immunostained with anti-NHE3 antibody 1380, and analyzed by confocal microscopy as described in MATERIALS AND METHODS. Positive labeling was defined as apical surface emission signal of intensity at least fourfold higher than cytoplasmic background. Parallel monolayers were examined for NHE3 activity by 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein fluorometric method. Relative increases of NHE3 activity over respect control values (percent of control) are shown in parentheses in laminin columns. These values represent means ± SD from 5 monolayers/group in 2 separate experiments. *Significantly different (P < 0.01) from respective plain value.

Fig. 6. Representative images of Caco-2 monolayer (clone PF-11, 17 PCD) immunolabeled with anti-NHE3 Ab 1380. A: combines multiple images obtained in XY plane by confocal microscopy to present a 15-µm thick optical section at brush-border (BB) level. Note uneven expression of immunoreactive NHE3 at surface of adjacent cells and the presence of 2 distinctive patterns of the BB labeling, namely smooth "standard" pattern (S) and "flower" pattern (F). B: reconstruction of monolayer obtained in vertical (XZ) plane. Note predominant immunolocalization of NHE3 at the BB and adjacent, narrow zone of subapical cytoplasm. C: reconstruction in XZ plane of image of control monolayer incubated with preimmune serum instead of Ab 1380. Dashed lines, surface of permeable culture support. Bars correspond to 20 µm.
were conducted using clone PF-11. A representative EGF (200 ng/ml) on endogenous NHE3 in Caco-2 cells and EGF. Because only lysates obtained from TC-7 cells (data not shown). These findings confirmed a predominantly BL targeting of NHE3 in the examined Caco-2 clones and also excluded the possibility that NHE3 was not detected at the BL membrane domain due to poor accessibility of this domain to biotin.

The relative abundance of BB NHE3, as measured by cell surface biotinylation (and normalized by the number of cells) steadily increased during postconfluent maturation (Fig. 9). At 3 PCD, surface NHE3 constituted 19% and at 11 PCD it was 56% of the total amount of NHE3 (100%) found at 22 PCD (Fig. 9, A and C). Interestingly, the pattern of increase of the total cellular NHE3 amount closely followed the pattern observed for the amount of NHE3 at the BB (Fig. 9, B and C). Similar results were obtained from the ATCC clone, whereas virtually no NHE3 protein was detected in either biotinylated preparations or in the whole cell lysates obtained from TC-7 cells (data not shown). Because only ~18% of the total cellular NHE3 is present in the cytoplasm of mature Caco-2 cells (clone PF-11) (15), these data suggest that most of the total cellular NHE3 was targeted to BB of Caco-2 cells (PF-11 and ATCC) regardless of the length of the postconfluent culture period.

Regulation of activity of endogenous NHE3 by PMA and EGF. Investigations on the effects of PMA (1 µM) or EGF (200 ng/ml) on endogenous NHE3 in Caco-2 cells were conducted using clone PF-11. A representative pattern of the effect of PMA on the initial rate of Na+/H+ exchange in monolayers at 17 PCD is shown in Fig. 10A and is consistent with our previous report (15). In the experiment shown, PMA inhibited the control H+ efflux rate by 27% (from 380 to 273 µM H+/s at pH 6.40). In three separate experiments, the average inhibition of NHE3 by PMA was 28 ± 5% of control value (Fig. 11). This effect was completely abolished by the PKC inhibitor H-7 (Fig. 11), suggesting the involvement of the PKC signaling transduction pathway in the observed PMA-mediated inhibition of NHE3 activity. H-7 alone did not have any significant effect on the control Na+/H+ exchange rate. Similar magnitude of PMA-induced inhibition of NHE3 was observed in early postconfluent PF-11 monolayers, although the overall activity of NHE3 was much lower at that time. At 7 PCD PMA (1 µM) inhibited NHE3 activity by 25 ± 3% (means ± SD, n = 8 monolayers),
whereas exposure to PMA in the presence of H-7 resulted in a nonsignificant change of activity compared with control. Twenty minutes of exposure of PF-11 cells (17 PCD) to 1 µM PMA did not result in a significant change in the BB length [3.8 ± 0.5 µm in control cells vs. 3.5 ± 0.4 (SD) µm in PMA-treated cells, n = 30 cells from 5 monolayers for each condition].

On the other hand, the presence of EGF in the AP and BL superfusates resulted in a small but significant increase of the initial rate of intracellular alkalinization. As shown in Fig. 10B, EGF stimulated the exchange rate by ~19% over control (from 326 to 385 µM H⁺/s at pH 6.40). The average stimulation observed in three separate experiments was 18 ± 6% over control (Fig. 11). Similarly small but significant stimulation of NHE3 activity by EGF was observed at 7 PCD (21 ± 5% over control, means ± SD, n = 8 monolayers). Exposure to EGF resulted in a small but significant increase in the length of the BB [4.2 ± 0.6 µm in control cells vs. 5.5 ± 0.6 (SD) µm in EGF-treated cells, 17 PCD, P < 0.01, n = 30 cells from 5 monolayers for each condition].

**DISCUSSION**

In this report we present data indicating that NHE3 is endogenously expressed exclusively at the BB of the human colonic adenocarcinoma cells Caco-2. The magnitude of the AP Na⁺/H⁺ exchange due to NHE3

![Fig. 9. Relative abundance of NHE3 protein at BB and in whole cell lysates obtained from Caco-2 monolayers at 3, 11, and 22 PCD. Biotinylated AP surface protein preparations or whole cell lysates obtained from PF-11 clone cultured for indicated periods were subjected to Western analysis, and bands corresponding to NHE3 were quantitated by densitometry as described in MATERIALS AND METHODS. A: representative pattern of NHE3 abundance in biotinylated preparations. B: representative pattern obtained from whole cell lysates. Material separated in each lane of A and B was obtained from similar number of cells (differences in cell number among lanes were ≤5% of mean cell number). C: comparison of patterns of increase of the surface (open bars) and the whole cell (shaded bars) amounts of NHE3, expressed as percent of respective amounts found at 22 PCD (shown as 100% for each group). Data in C are means ± SD from 3 independent experiments. Data shown by line tracing (●) represent average NHE3 activity measured in parallel monolayers at respective postconfluent periods and are presented as percent of activity observed at 22 PCD.

![Fig. 10. Inhibition by phorbol 12-myristate 13-acetate (PMA, A) and stimulation by epidermal growth factor (EGF, B) of activity of endogenous NHE3 in Caco-2 cells (clone PF-11, 17 PCD). Monolayers on filterslips were preincubated with PMA (1 µM) or EGF (200 ng/ml) for 20 min, and the Na⁺-dependent rate of intracellular alkalinization was evaluated using fluorometric method, as described in MATERIALS AND METHODS. Data show means ± SD of rates observed in 9 monolayers in 3 separate experiments for each treatment. Dotted lines, least-square linear fit curves for control condition (CTR) and for monolayers exposed to PMA or monolayers exposed to EGF. Arrows, onset of exposure of the AP surface of monolayers to 131 mM Na⁺.](G300/DEVELOPMENT-OF-ENDOGENOUS-NHE3-IN-CACO-2-CELLS)
activity increased gradually between 3 and 22 PCD in clones PF-11 and ATCC of Caco-2 cells, the increase being a result of progressively increasing number of NHE3-expressing cells. This was in contrast with clone TC-7, in which NHE3 contributed only marginally to the total AP Na⁺/H⁺ exchange up to 22 PCD examined. In all three clones, the housekeeping isoform NHE1 was present predominantly at the BL membrane domain and, to much lesser degree, also at the AP domain. The AP expression of NHE1 decreased dramatically during the postconfluent differentiation of clones PF-11 and ATCC, and it contributed only 4–7% to the total BB exchange activity in the mature monolayers. In TC-7 clone, however, NHE1 was responsible for the majority of the AP Na⁺/H⁺ exchange, even in mature monolayers. Neither clone expressed, in our hands, detectable amounts of NHE2. Stimulation of the PKC signal transduction pathway by PMA resulted in an inhibition of NHE3 activity in clones expressing significant amounts of NHE3 at the BB (ATCC and PF-11), whereas exposure to EGF resulted in small but significant stimulation of the exchanger’s activity.

Our studies demonstrate a gradual development of the functional expression of endogenous NHE3 at the BB of Caco-2 cells, which increased almost fivefold during 22 days postconfluency. This pattern closely resembled the previously reported gradual development of expression of sucrase-isomaltase, lactase, amylase, cathepsin D, and alkaline phosphatase at the BB of Caco-2 cells (46). Two major mechanistic scenarios could explain the phenomenon of time-dependent increase in morphological and functional expression of NHE3 in the examined Caco-2 clones. In the first scenario, all the cells gradually, and in parallel, differentiate into a mature, polarized phenotype. In such a situation, the majority of the cells in the immediate postconfluent period would exhibit a poorly differentiated phenotype with little “per cell” expression of NHE3. In the second scenario, the gradual increase in the AP NHE3 activity of the cultured population as a whole would occur by a progressively increasing number of cells expressing the exchanger at the BB. Data presented in this communication support the second possibility. Indeed, with increasing time postconfluence, we observed an increasing amount of BB NHE3 (biotinylation experiments, which paralleled an increasing amount of total cellular NHE3 and an increase in NHE3 activity at the BB, but which was not accompanied by any significant increase in the cell number (Fig. 9). Thus it seems that once Caco-2 cells were capable of expressing NHE3, both the targeting and regulatory mechanisms were fully operational, and it was the increasing number of the phenotypically differentiated cells which was solely responsible for the time-related increase in the monolayers’ NHE3 activity and NHE3 content. Similar conclusions were drawn by Vachon et al. (46) to explain the time-dependent increase in expression of BB hydrolases in Caco-2 cells. The authors suggested that the gradual increase of activity of hydrolases reflected the changing “transient mosaic pattern” of expression, resulting from a steadily increasing number of functionally differentiated cells in the cultured population. Some evidence suggests that this transient mosaic pattern results from an uncoordinated pattern of expression of genes involved in some aspects of differentiation of Caco-2 cells, one result of which might be a mosaic deposition of heterotrimeric laminin (a basement membrane component essential for terminal differentiation of enterocytes) (45). The latter hypothesis is supported by our observation that the presence of exogenous laminin significantly accelerated the development of NHE3 expression in PF-11 and ATCC clones (Table 2).

The comparison of NHE3 expression with that of BB hydrolases, and especially sucrase-isomaltase, should be done cautiously, however. Although expression of sucrase-isomaltase was reported to be severalfold higher in late TC-7 clone than in early PF-11 clone (7), the opposite was observed by us in respect to NHE3 expression. Clearly, many factors, including amount and composition of the deposited extracellular matrix, differential gene expression in various clones, and even the passage number of the same clone, are involved in the in vitro differentiation of Caco-2 cells. Therefore, in this study the term “differentiation” has been replaced by the term “maturation” whenever we discussed the phenomenon of time-dependent increase in the overall expression of NHE3 by Caco-2 monolayers. However, this in vitro phenomenon may, indeed, reflect the changes actually occurring during enterocyte differen-
G302 DEVELOPMENT OF ENDOGENOUS NHE3 IN CACO-2 CELLS

tiation along the crypt-villus axis. A mosaic pattern of expression of BB hydrolases and some other BB proteins has been described during development of the gut in rats and humans (3, 39), as well as in certain pathological conditions in humans (25, 29). Moreover, NHE3 was found predominantly in the BB of villus and upper crypt epithelial cells and only in small amounts in the lower crypt cells of jejunum and ileum in humans, thus supporting the notion that the gradually increasing expression of some BB enzymes and transport proteins parallels some aspects of terminal enteroocyte differentiation.

The pattern of development of functional expression of NHE1 at the BL membrane domain reported here did not parallel that of NHE3. The expression of NHE1 was maximal at 5-7 PCD, shortly after the monolayers reached a maximal cell density. Therefore, the expression of NHE1 during Caco-2 in vitro maturation seems to be regulated differently from that of NHE3, and NHE1 is rather ubiquitous expressed in these cells already at the early stages of postconfluent growth. This conclusion is supported by the observation that immunoreactive NHE1 was detected at the BL membrane domain of all cells as early as 3 PCD (data not shown). NHE1 was also targeted to AP membrane domain, although both the absolute and relative amount of this isoform at the BB dramatically decreased during postconfluent maturation. It is unlikely that the BB amiloride-sensitive Na+/H+ exchange activity was contaminated with, or represented by, an endogenous NHE2, because no NHE2 protein was detected in these monolayers by Western analysis (Fig. 2). Although NHE1 has not been detected in the BB of native intestinal epithelia examined so far, such “mistargeting” of other basolaterally targeted proteins in in vitro epithelial cell models as well as in human kidney epithelium (polycystic kidney disease) has been described (11, 20, 32, 49). Nevertheless, in mature Caco-2 cells only 4% of total NHE1 activity was localized at the BB. This is in contrast with a much higher proportion of apically targeted NHE3 found in Caco-2 cells as well as in Madin-Darby canine kidney (MDCK) and HT-29 by Noel et al. (32). The reason for this discrepancy is not clear.

In contrast to NHE1, both functional and morphological expression of NHE3 was exclusively AP in all three Caco-2 clones examined. This exclusively AP functional expression of NHE3 paralleled the subcellular distribution of the exchanger molecules, as indicated by the results of immunofluorescent studies as well as cell surface biotinylation. Although we never found NHE3 at the BL membrane domain of the three Caco-2 clones studied, the magnitude of the AP expression of this isoform was significantly smaller in TC-7 cells than in the other two clones. This observation suggests differential regulation of expression of BB proteins among various Caco-2 clones. Our data emphasize the need for a careful characterization of a studied Caco-2 clone with respect to the expression of the protein(s) of interest. Full expression of these proteins may require relatively long postconfluent growing time and, additionally, the magnitude of expression may differ significantly among clones. It is important to notice that the significant differences in expression of NHE3 between clone TC-7 and the other two clones examined occurred in otherwise morphologically well-differentiated cells (7). Moreover, although TC-7 cells expressed significantly less NHE3 than the PF-11 clone, they were previously reported to express more sucrase-isomaltase and dipeptidylpeptidase IV (7). Despite these differences in the magnitude of NHE3 expression among the clones, NHE3 has not been targeted to the BL membrane domain in any clone and at any time postconfluence, a finding consistent with results of in vivo studies, in which NHE3 was exclusively found at the BB of ileal and colonic epithelium in human, rat, and rabbit (5, 14). The presence of NHE3 at the BB of Caco-2 cells has been reported by us previously using morphological and biochemical approaches (15). Also, the activity of an endogenous Na+/H+ exchanger with NHE3 characteristics at the AP surface of Caco-2 cells has been suggested by results reported earlier by Osypiw et al. (34). Using a fluorometric method, the authors observed the activity of a relatively amiloride-resistant (IC50 287 µM) Na+/H+ exchanger at the BB of Caco-2 cells, which most probably represented NHE3. The authors also observed a Na+/H+ exchanger at the BL membrane domain of Caco-2 cells, which probably represented NHE1, although no details concerning activity of this exchanger at low amiloride concentrations was reported. In contrast, no evidence of Na+/H+ exchange at the BB of Caco-2 monolayers (ATCC clone) was reported by Watson and colleagues (48) from our laboratory. These authors described the presence of an amiloride-sensitive Na+/H+ exchanger (NHE1) at the BL membrane domain of Caco-2 monolayers, but they did not observe any significant Na+/H+ exchange at the AP domain. One possible explanation for this discrepancy is that these studies were conducted at an early postconfluent period, when relatively little endogenous NHE3 activity is present at the BB. More recently, McSwine and colleagues (26) characterized a clonal line of Caco-2 cells (C2/bbe), which also lacked endogenous NHE3 expression. These data suggest that a subpopulation of cells with no or with a very small capacity for NHE3 expression did exist in the originally derived Caco-2 adenocarcinoma cell line and that therefore some clones obtained by dilutional (“single cell”) cloning may remain incapable of expressing a significant quantity of the exchanger. This hypothesis is supported by our observation that ~10% of all cells in the PF-11 clone and ~17% of all cells in the ATCC clone did not express NHE3 for up to 30 PCD, and that very low levels of NHE3 protein and activity were found in some single cell clones obtained in our laboratory from the PF-11 cell clone (data not shown). Finally, although the presence of exogenous laminin significantly accelerated development of NHE3 expression in PF-11 and ATCC clones, laminin was not capable of inducing NHE3 expression in all the cells in the cultured populations. Moreover, laminin had no effect on NHE3 expression in TC-7 cells. These findings would suggest an early block
The activity of endogenous NHE3 in Caco-2 cells was inhibited by PMA, and it was stimulated by EGF. We believe that the observed inhibitory effect of PMA on NHE3 was due to stimulation of PKC activity, because it was abolished by H-7, a PKC inhibitor. We recently reported that ~50% of the PKC-induced inhibition of NHE3 in Caco-2 cells is due to redistribution of the exchanger molecules from the BB into the subapical cytoplasmic compartment (15). Magnitude of the NHE3 inhibition (~28%) resembles that reported for other cell types, including OK cells, PS120 fibroblasts, gallbladder epithelial cells, and Caco-2 cells transfected with NHE3 cDNA (2, 13, 21, 26, 40). On the other hand, the stimulatory effect of EGF, although statistically significant, was of lower magnitude than that reported for fibroblast growth factor in PS120 fibroblasts (22, 51). One possible reason for the relatively small stimulatory effect of EGF could be the autocrine downregulation of the EGF receptors by transforming growth factor-α, a growth factor reported to be secreted by Caco-2 cells (1). Such a downregulation of EGF receptors was shown previously in type 2 pneumocytes (30), and one preliminary report suggests a similar phenomenon occurring during postconfluent differentiation of Caco-2 cells (19).

We attempted to answer this question by pretreatment of PF-11 Caco-2 monolayers with suramin (1 mM), the polyanionic compound that complexes many growth factors and thus may prevent downregulation of the growth factor receptors (30). However, this approach did not result in a significant increase of the stimulatory effect of EGF on NHE3 activity (data not shown).

Importantly, the observed inhibitory effect of PMA and stimulatory effect of EGF on NHE3 activity in Caco-2 cells resembled those described for native ileal Na⁺-absorbing epithelial cells. In the rabbit ileum, carbachol was shown to inhibit neutral NaCl absorption, which was associated with an asymmetric increase of PKC activity at the BB of ileal epithelium, translocation of phospholipase C (PLC)-γ to the BB, and activation of AP, but not BL, phosphatidylinositol 4,5-bisphosphate-PLC activity (16). This translocation of PKC to the BB and an increase in the kinase activity strongly support the hypothesis that, at least in the intestine, the PKC signal transduction pathway is involved in the inhibitory regulation of the NHE3. On the other hand, EGF has been shown to stimulate NaCl absorption and NHE3 activity in native ileal epithelium, a process requiring an increased activity of phosphatidylinositol 3-kinase (PI3K) (17). A similar dependence of EGF stimulation of NHE3 on PI3K activity has also been reported for Caco-2 cells transfected with rabbit NHE3 (17). However, the molecular mechanism of the acute stimulation of NHE3 by EGF in intestinal epithelial cells remains unclear. Because the stimulation is caused by changes in \( V_{max} \) (21), increased number of NHE3 molecules within the BB membrane is a highly probable mechanism. This in turn could result from increase rate of exocytic insertion of NHE3 molecules into the BB, from decreased rate of endocytic retrieval of the exchanger from the BB, or both. The mechanism involving a rapid subcellular redistribution of NHE3 is suggested by the results published by Hardin and colleagues (12). The authors observed a significant increase in BB height and surface area after acute exposure of rabbit jejunal epithelium to EGF, which suggested a rapid, EGF-induced, cytoskeletal rearrangement and/or exocytic insertion of preformed membranes into the BB. In our hands, exposure to EGF resulted in a small but significant increase in the BB height in Caco-2 cells, an observation that would support the redistribution hypothesis. It is not clear, however, why we did not observe a decrease in the BB height after exposure of the cells to PMA, in which case endocytic retrieval is known to take place (15). One possibility is that the changes in surface area of the BB after exposure to PMA might be too small to be detected just by measuring the monolayer height using optical microscopy. Alternatively, surface area might not change following exposure to PMA even with removal of BB membrane if compensatory changes in the BB cytoskeleton occur, as has been reported previously (28, 37, 43).

In conclusion, our data demonstrate that the PF-11 and ATCC clones of Caco-2 cells represent a promising “physiological” model for studying the development and regulation of endogenous NHE3 in the intestinal epithelium. In mature monolayers, both the polarized AP expression of NHE3 as well as regulation of the exchanger by PKC and receptor tyrosine kinase signal transduction pathways closely resemble the behavior of endogenous NHE3 in the ileal and colonic epithelium. The fact that Caco-2 cells are derived from human colonic epithelium adds value to this in vitro model, because studies on native human intestinal epithelium are limited for many reasons.

We thank Dr. Ann Hubbard for advice and critique during the development of the project. We also thank Dr. Shaoyou Chu and Greg Martin for expert advice and help with the confocal microscopy and immunostaining, and Dr. Hans-Jochen Lang from Hoechst for providing us with HOE-694 compound.

This work was supported by the National Institute of Diabetes and Digestive and Kidney Diseases Grants RO1-DK-26523, PO1-DK-44484, R29-DK-43778, T32-DK-07632, and K08-DK-02557; the Meyerhoff Digestive Diseases Center; and the Hopkins Center for Epithelial Disorders.

Part of this work was presented at Digestive Disease Week (Washington, DC, May 10–16, 1997), and was published in abstract form (Gastroenterology 112: A372, 1997).

Present addresses: M. H. Montrose, Dept. of Physiology, Univ. of Indiana School of Medicine, Indianapolis, IN 46202-5120; F. Sanchez de Medina, Dept. of Pharmacology, Univ. of Granada, 18071 Granada, Spain.

Address for reprint requests and other correspondence: M. Donowitz, Johns Hopkins Univ., School of Medicine, Division of Gastroenterology, 918 Ross Research Bldg., 720 Rutland Ave., Baltimore, MD 21205 (E-mail: mdonowitz@welchlink.welch.jhu.edu).

Received 13 November 1998; accepted in final form 15 April 1999.

REFERENCES


