DIFFERENTIAL LOCALIZATION OF Na+/H+ EXCHANGER ISOFORMS (NHE1 AND NHE3) IN POLARIZED EPITHELIAL CELL LINES

Josette Noel*, Danièle Roux* and Jacques Pouysségur†
Centre de Biochimie-CNRS, Université de Nice, Parc Valrose, 06108, Nice, France

*These authors have contributed equally to this work
†Author for correspondence (e-mail: pouyssegur@unice.fr)

SUMMARY

Na+/H+ exchangers (NHEs) are transporters that exchange sodium and proton ions across the plasma membrane at the expense of their chemical gradient. In higher eukaryotes these transporters exist as multiple specialized isoforms. For example, NHE1, the ubiquitously expressed form is a major pH-regulating system whereas the epithelial NHE3 isoform is specialized in transepithelial Na+ transport. NHE1 and NHE3 can be very well distinguished pharmacologically with the HOE694 specific inhibitor and immunologically with specific polyclonal and monoclonal antibodies. With these molecular tools we investigated the specific steady state expression of the two NHE isoforms in polarized epithelial cells in culture.

Endogenous NHE3 in OK cells or NHE3-VSVG transfected in either OK or MDCK cells showed an exclusive expression of the transporter at the apical membrane. Overexpression of NHE3 did not result in any spill over on the basal lateral side. These results obtained by functional measurement of NHE3 activity were fully consistent with its detection only at the apical side by immunofluorescence and confocal microscopy. By contrast, using the same cells, the same culture conditions and the same detection methods, we clearly detected NHE1 at both specialized membranes of four different polarized epithelial cell lines. Furthermore, biotinylation of cell surface proteins of MDCK, OK and HT-29 cells followed by immunoprecipitation of NHE1 revealed expression of the transporter at both sides of the polarized epithelial cells. Interestingly, the cell surface expression correlated well with the corresponding NHE1 activities. In addition, immunodetection by fluorescence microscopy was found to be qualitatively consistent with the above-reported results.

We therefore conclude that the epithelial and more specialized NHE3 isoform is exclusively restricted to the apical side of epithelial cells. In marked contrast, both endogenous or ectopically expressed NHE1 isoform, have the capacity to be expressed in both the apical and basal lateral membranes of polarized cells in cultures.

Key words: Na+,H+ exchanger, NHE isoform

INTRODUCTION

The Na+/H+ exchanger (NHE) is a plasma membrane protein that functions in higher eukaryotic cells primarily for the maintenance of intracellular pH but also contributes to the transepithelial transport of Na+ (for reviews see Grinstein et al., 1989; Tse et al., 1993a; Noël and Pouysségur, 1995). H+ extrusion is ensured by the extreme sensitivity of NHE to intracellular H+ and by the inwardly-directed Na+ gradient established by the Na+, K+ ATPase. NHE is therefore powerful for restoring intracellular pH towards neutrality in response to acid-load insults (Pouysségur et al., 1984). In intestine and renal epithelial cells, NHE, expressed at the luminal side contributes primarily in transepithelial transport of Na+, the plurality of functions being dictated by the location and the kinetic parameters of the NHE molecules.

The first NHE to be cloned and referred to as NHE1 is a glycoprotein of 110 kDa (Sardet et al., 1989, 1990) that exists in the membrane as a homodimer (Fafournoux et al., 1994). The N-terminal half of the molecule possesses multiple transmembrane segments (10 to 12) and constitutes the transporter unit. The C-terminal cytoplasmic domain of about 300 amino acids and confocal microscopy. By contrast, using the same cells, the same culture conditions and the same detection methods, we clearly detected NHE1 at both specialized membranes of four different polarized epithelial cell lines. Furthermore, biotinylation of cell surface proteins of MDCK, OK and HT-29 cells followed by immunoprecipitation of NHE1 revealed expression of the transporter at both sides of the polarized epithelial cells. Interestingly, the cell surface expression correlated well with the corresponding NHE1 activities. In addition, immunodetection by fluorescence microscopy was found to be qualitatively consistent with the above-reported results.

We therefore conclude that the epithelial and more specialized NHE3 isoform is exclusively restricted to the apical side of epithelial cells. In marked contrast, both endogenous or ectopically expressed NHE1 isoform, have the capacity to be expressed in both the apical and basal lateral membranes of polarized cells in cultures.

Key words: Na+,H+ exchanger, NHE isoform

Differential localization of Na+/H+ exchanger isoforms (NHE1 and NHE3) in polarized epithelial cell lines

Josette Noel*, Danièle Roux* and Jacques Pouysségur†
Centre de Biochimie-CNRS, Université de Nice, Parc Valrose, 06108, Nice, France

*These authors have contributed equally to this work
†Author for correspondence (e-mail: pouyssegur@unice.fr)
NHE2 and NHE3 at the apical surface of epithelial cells confers a specialized function to these isoforms, pointing out the importance of the mechanisms involved in membrane protein sorting. A large effort has been devoted to this question over the last decade and although considerable progress and understanding has been made, little information is available on the signals that direct polytopic transmembrane proteins to their final destination (Matter and Mellman, 1994).

Our major goal was to establish an epithelial cellular model amenable to molecular genetics and in which we could: (i) demonstrate differential membrane targeting of transfected NHE isoforms; and (ii) define the NHE molecular determinants that specify their restricted membrane localization. In this report we demonstrate that endogenous or ectopically expressed NHE1 in Madin-Darby canine kidney (MDCK) or opossum kidney (OK) cells, is functionally expressed at both apical and basal lateral sides of these polarized epithelial cells. In contrast in the same cells (MDCK and OK), endogenous or ectopically expressed NHE3 is totally restricted to their apical membrane.

MATERIALS AND METHODS

Materials

5-(N-methyl-N-propyl)amiloride (MPA) was a gift from E. Cragoe Jr (Merck Sharp & Dohme), HOE694 (3-methyl sulfonyl-4-piperidinobenzoxyl) guanidine methanesulfonate was a gift from D. W. Scholz (Hoechst, Cardiovascular department, Frankfurt). P5D4 monoclonal antibody directed against the VSVG epitope was a gift from Dr T. Kreis (EMBL, Heidelberg). RPE28 polyclonal antibodies directed against the carboxyl tail of human NHE1 was prepared as previously described (Sardet et al., 1990). FITC-conjugated anti-mouse, anti-rabbit IgGs and 22NaCl (carrier-free) were from the Radiocalbio Chemical Centre (Amersham). All other chemicals were of the highest purity available. Rat NHE3 cDNA was kindly provided by Dr J. Orlowski, McGill University, Montréal (Orlowski et al., 1992). Opossum kidney (OK) epithelial cell line (Montrose and Mürer, 1990) and MDCK high resistance colon cell lines were kindly provided by Dr H. Mürer (University of Zurich, Zurich). MDCK low resistance clone, used here for most of the studies presented, Caco-2 and HT-29 colonic cell lines were kindly provided by Prof. D. Louvard (Curie Institute, Paris).

Plasmid constructions

Wild-type human NHE1 cDNA previously isolated (Sardet et al., 1989) was subcloned into the eukaryotic expression vector pECE under the control of the SV40 promoter (Ellis et al., 1986). NHE1 cDNA (~53 to 3,552 bp) was inserted into HindIII/EcoRI digested pECE as previously reported (Wakabayashi et al., 1992). A point mutation in human NHE1, a Leu163Phe substitution, was found to confer resistance to methylpropyl amiloride (MPA) (Counillon et al., 1993b). NHE1-L163F was subcloned in the pECE vector as for wild-type NHE1.

A vesicular stomatitis virus glycoprotein (VSVG) epitope (Kreis, 1986) was inserted at the end of the carboxyl tail of NHE3 as previously reported (Fafournoux et al., 1994). NHE3 was truncated at the Nhe1 site (2,436 bp) substituting the last 47 amino acids of the carboxyl tail with a stretch of 22 amino acids containing the VSVG epitope at the new carboxyl end. The residues of the following stretch (Pro Pro Arg Gly Arg Gly)-(Gly Pro Pro Gly Pro)-(Tyr Thr Asp Ileu Glu Met Asn Arg Leu Gly Lys-COOH) represent, respectively, the Bluescript plasmid linker (residues 1-6), the spacer (residues 7 to 11), and the VSVG epitope (residues 12 to 22). This NHE3-VSVG cDNA was subcloned into the KpnI site of the pECE vector.

Cell culture and transfection

PS120 is a Chinese hamster lung fibroblast clone, lacking Na+/H+ exchange activity (Pouysségur et al., 1984) that was derived from CCL39 (ATCC). All cell lines were maintained in culture medium (DMEM) supplemented with 25 mM NaHCO3, 10% fetal calf serum, penicillin (50 units/ml), and streptomycin (50 μg/ml) and incubated at 37°C in the presence of 5% CO2. Culture medium for Caco-2 and MDCK was DMEM (H21, Gibco) supplemented with 1% non essential amino acids. Culture medium for OK cells was DMEM-H21/Ham-F12 (1:1). For polarity experiments only, cells were grown at confluency on transparent cell culture membrane inserts with a 0.45 µm pore size and 10 mm diameter (Falcon, catalogue no. 3180), otherwise for passages, transfections and subcloning, cells were grown on tissue culture Petri dishes. Unless otherwise indicated all transfections of either NHE1 or NHE3 molecules were performed as follows: cells were plated at 5x105 cells in a 10 cm tissue culture dish one day before addition of 25 μg of NHE expression vector co-precipitated with CaPO4 as reported previously (Wakabayashi et al., 1992). Selections were based on resistance to acid load conferred by the expression of the NHE cDNA transgene. Under appropriate pharmacological conditions the transfected NHE molecule can be easily selected (see below).

Selection of stable OK transfectants expressing either NHE1 or NHE3-VSVG

Prior to transfection, OK cells were submitted to a H+-suicide selection that enriched the population in OK cells expressing a low level of endogenous Na+/H+ exchange. This selection, applied twice, consists of loading the cells with 130 mM LiCl for 2 hours, before exposing them to a Na+- and Li+-free isotonic solution at low external pH (5.5). Cells expressing high levels of endogenous Na+/H+ exchange activity are killed by H+ uptake driven by the outward-directed Li+ gradient (see Pouysségur et al., 1984 for details). Resistant OK cells, usually one third of the population, served as recipients for transfection of either NHE1 or NHE3-VSVG.

OK cells transfected with pECE-NHE1 were selected by application of the following acid-loading test. One day after trypsinization (to reduce cell-cell contact), cells are loaded with NH4+ for 1 hour at 37°C in Hepes-buffered saline (HBS) containing 50 mM NH4Cl and 70 mM choline chloride (ChoCl). Cells are rapidly washed twice with 120 mM ChoCl, pH 7.0 and further incubated for 1 hour in HBS containing 30 mM NaCl and 90 mM ChoCl, pH 7.5 (recovery medium). Then the cells are returned to the regular bicarbonate-containing medium. This acid-load selection was applied 4 times over a period of 3 weeks and the resultant stable transfectants were used for biochemical studies (RNA and protein expression), as well as for transport, pharmacology and immunolocalization studies performed on polarized epithelia.

Selection of stable MDCK transfectants expressing either NHE1-L163F or NHE3-VSVG

Conditions of transfection and selection were identical to those described above for OK cells with a slight modification in the recovery medium following acid loading. In this case, cells were incubated for 1 hour in HBS containing 120 mM NaCl supplemented with either 10 μM of MPA or 100 μM HOE694, pH 7.5. These inhibitor concentrations block the low endogenous NHE3 activity, allowing selection of the transfected NHE3 at a higher threshold value.

Measurement of Na+/H+ exchange activity (22Na+ uptake)

MPA- or HOE694-sensitive 22Na+ uptake was measured following an acid load generated by the NH4+-prepulse technique as previously described (Pouysségur et al., 1984). Unless otherwise specified, OK, MDCK and transfectant-derivatives were seeded on 10 mm membrane.
inserts (0.45 μm) at 0.6×10^6 cells/cm² and grown up to 11 days at confluence with a medium change every two days. 22Na⁺ uptake was performed either at 4, 8 or 11 days of cultures as follows: cells are loaded (upper and lower compartments) with NH4Cl for 1 hour at 37°C in Hepes (15 mM)-buffered saline (HBS) containing 50 mM NH4Cl and 70 mM choline chloride. 1 mM MgCl₂, 1.8 mM CaCl₂, 5 mM KCl, 5 mM glucose, pH 7.5. Medium was removed and cells rapidly washed twice with the uptake Na⁺-free medium: 120 mM choline chloride, 1 mM MgCl₂, 1.8 mM CaCl₂, 15 mM Hepes, pH 7.4. Cells were then incubated (upper and lower compartments) with the same medium supplemented with 1 mM ouabain at 37°C and uptake was initiated by the addition of the uptake medium containing 1 μCi/ml 22Na⁺ either on the upper or on the lower compartment. Uptakes were stopped after 3 minutes by three rapid washes with ice-cold phosphate buffered saline. We checked that 22Na⁺ uptakes were linear with time for at least 10 minutes, therefore a 3 minute uptake is a good reflection of the initial transport rate. Three separate determinations of 22Na⁺ uptake were performed in duplicate: (1) in the absence of inhibitor (A value); (2) in the presence of 10 μM or 100 μM HOE694 (B value); and (3) in the presence of 10 mM HOE694 (C value). A-C represents the total NHE activity; A-B represents NHE1 activity of the wild-type or mutated NHE1 (NHE1-L163F); B-C represents the NHE3 activity. We derived this protocol from the respective NHE pharmacology profiles (see Results). Cells were then solubilized in 0.1 M NaOH, radioactivity counted in a γ-spectrometer and protein concentration measured on the corresponding samples.

## Northern and western blot analyses
Total RNA was extracted from untransfected and stably transfected cells by using the LiCl precipitation method (Aufray and Rougeon, 1980) and poly(A) RNA was selected on oligo(dT)-cellulose columns (Pharmacia, type 7), separated on 1% agarose gels, blotted on Hybond nylon membranes and hybridized at 65°C with the following probes: rat NHE3 cDNA (Kpn1-Apal fragment of 2,391 bp) and human NHE1 cDNA (BamHI fragment of 1,915 bp). Washing of nylon membranes was performed in stringent conditions (65°C). For protein detection, crude membrane fractions prepared as previously reported (Fafournoux et al., 1994) were solubilized and protein separated by SDS-PAGE on a 10% (w/v) polyacrylamide gel. After electrophoretic transfer to Hybond C membranes, proteins were revealed by overnight incubation with Rp28 (1:1,000) or P5D4 (1:500) antibodies at 4°C. Membranes were washed in saline solution containing 0.05% Triton X-100 and incubated with horseradish-peroxidase-conjugated goat anti-rabbit or anti-mouse IgG (1:2,000). The western blots were revealed using the enhanced chemiluminescence (ECL) detection system (Amersham).

## Immunofluorescence and confocal microscopy
Cells were grown on coverslips at confluency for 6-8 days. After washing with PBS, cells were fixed at ~20°C for 10 minutes with methanol/aceton (7:3, v/v). Fixative solution was removed, cells were left to dry for 10 minutes before rehydration for 10 minutes at room temperature (5 minutes in PBS followed by 5 minutes in PBS containing 2% BSA and 0.2% gelatin). The same result was obtained with 3% paraformaldehyde for 30 minutes and permeabilization with 2% Triton X-100 for 4 minutes. Primary antibodies were incubated for 2 hours at room temperature in PBS/BSA/gelatin at the following dilutions: 1:500 for P5D4 (anti-VSVG epitope) and 1:500 for Rp28 (anti-NHE1). Cells were then washed 4 times with PBS, once with PBS/BSA/gelatin and incubated for 60 minutes at room temperature with fluorescein-conjugated secondary antibody to a dilution of 1:50 for the anti-mouse and 1:200 for the anti-rabbit. Finally, cells were washed 4 times with PBS, mounted with Citifluor and examined under epifluorescent illumination with excitation-emission filters for fluorescein. Images were visualized using the Nikon Diaphot microscope and photographed with Fujichrome 1600 ASA film. For confocal microscopy, nuclei were stained with propidium iodide, washed in PBS and mounted with Citifluor (Sigma).

## Cell surface biotinylation

Cell surface biotinylation was performed as described (Sargiacomo et al., 1989; Le Bivic et al., 1989). Immunoprecipitation of NHE1, biotinylated from either the apical or the basolateral sides was performed as previously described (Fafournoux et al., 1994). Biotinylated NHE1 proteins were revealed by using the streptavidin-horseradish peroxidase reagent combined with the chemiluminescence kit (ECL). Total NHE1 proteins were revealed with the Rp28 anti-NHE1 antibody following reagent stripping by incubation in the stripping buffer (63 mM Tris-HCl, 2% SDS, 100 mM β-mercaptoethanol, pH 6.7) at 50°C for 30 minutes.

## RESULTS

### Strategy for functional expression and identification of NHE molecules in polarized epithelial cells

After a survey of different epithelial cell lines often used for studies on membrane sorting (Caco-2, HT-29, MDCK, OK), we selected MDCK and OK strains, first for their good transfectability and second for their well established polarized features. The two NHE isoforms that we decided to examine and compare are NHE1, the most sensitive isoform to amiloride and derivatives and NHE3, the amiloride resistant epithelial isoform (Tse et al., 1993a; Noël and Pouysségur, 1995). In fact, for the studies presented here, we have used another NHE specific inhibitor, HOE694, which is capable of fully distinguishing NHE1 from NHE3 in Na⁺ flux studies when expressed in PS120 fibroblasts, since their respective Kᵢ values differ by a factor of 4,000 (Counillon et al., 1993a; see Fig. 1). Two strategies have been adopted to distinguish functional transfected molecules. In the case of MDCK cells, as will be apparent later, no endogenous NHE3 activity was detectable; therefore functional expression of NHE3 was monitored by the occurrence of a Na⁺/H⁺ exchange activity sensitive to HOE694 in the mM range. NHE1, however, is expressed in MDCK cells and its activity is sensitive to HOE694 in the μM range. To facilitate the selection and identification of exogenous NHE1, we used a point mutant NHE1-L163F, whose mutation increases the HOE694 Kᵢ value by 25-fold (Counillon et al., 1993a,b).

The situation is different for OK cells. These cells do not possess detectable NHE1 activity (Helmle-Kolb et al., 1990, 1993), however, they have an endogenous NHE activity that is pharmacologically apparented to NHE3 (see later). To facilitate the selection and characterization of rat NHE3, we first isolated a subpopulation of OK cells that displays very low NHE activity. With this low NHE activity background we have been able to use our acid loading strategy to select stable NHE3 transfectants. In addition, to monitor the transfected NHE molecules by their differential pharmacology on H⁺-activated Na⁺ influx, immunological probes were developed to specifically visualize NHE subcellular localization. As shown in Fig. 1, NHE1 molecules are detected with the polyclonal antibody RP28 directed against C-terminal determinants (Sardet et al., 1990), and NHE3 by the introduction of the vesicular stomatitis virus glycoprotein epitope (VSVG) in its carboxyl tail (see Materials and Methods for details).

### Expression of NHE1 and NHE3 in MDCK and OK epithelial cells

Cell populations that have been stably transfected either with NHE1, NHE1-L163F or NHE3 were analyzed for mRNA and
protein expression. Fig. 2 depicts mRNA expression in untransfected and stably transfected MDCK and OK cell populations. In Fig. 2A, NHE3 cDNA probe detects a positive signal only in NHE3-transfected cells (lane 3 for OK cells and lane 6 for MDCK cells). NHE1 cDNA probe detects a positive signal only in NHE1-transfected cells (Fig. 2B, lane 2 and lane 5). Only after longer exposure of the northern blots is NHE1 specific endogenous mRNA detected in MDCK but not in OK cells. No endogenous NHE3 mRNA could be detected, however, in either cell line, even in the poly(A) enriched RNA population (Fig. 2A,B, right).

Fig. 1. Molecular features of NHE isoforms used for ectopic expression in epithelial cells. The two human NHE1 and the rat NHE3 used in this study are distinguishable pharmacologically. NHE1-L163F possesses a point mutation in the 4th transmembrane segment that augments by 40-fold the $K_i$ for the specific inhibitor HOE694, whereas rat NHE3 is 4,300-fold more resistant than NHE1 to the same inhibitor. In addition, both NHE1 and NHE3 can be immunologically recognized by their cytoplasmic tail (RPc28 epitope for NHE1 and VSVG epitope for NHE3-VSVG) grafted to NHE3 (see Materials and Methods). All cDNAs were inserted into the pECE vector for stable transfection.

Fig. 2. mRNA expression of NHE transfected cDNAs into MDCK and OK epithelial cell lines. Total RNA or poly(A) selected RNA were prepared from untransfected and stably transfected cells with either NHE1, NHE1-L163F or NHE3-VSVG cDNAs as indicated in Material and Methods, separated on 1% agarose gel and blotted on nylon for hybridization. In A blotted material was hybridized with NHE3 cDNA probe and in B with NHE1 cDNA probe. Each lane corresponds to either 10 µg of total RNA or poly(A) RNA as indicated, derived from: lane 1, non-transfected OK cells; lane 2, NHE1-transfected OK cells; lane 3, NHE3-VSVG-transfected OK cells; lane 4, non-transfected MDCK cells; lane 5, NHE1-L163F-transfected MDCK cells; lane 6, NHE3-VSVG-transfected MDCK cells. The different exposure times are indicated for each experiment.

Fig. 3 shows the Na⁺/H⁺ exchange proteins expressed following NHE cDNAs transfection in MDCK and OK epithelial cell lines. Crude membrane proteins were prepared from untransfected and stably transfected cell populations and separated by SDS-PAGE on a 10% polyacrylamide gel. After electrotransfer to nitrocellulose, proteins were blotted and hybridized (top) with a polyclonal antibody specific for NHE1 (RPc28) or (bottom) with a monoclonal antibody (P5D4) specific for the VSVG epitope grafted on transfected NHE3 cDNA. Each lane corresponds to 50 µg of membrane protein derived from: lane 1, untransfected OK cells; lane 2, NHE1-transfected OK cells; lane 3, NHE3-VSVG-transfected OK cells; lane 4, untransfected MDCK cells; lane 5, NHE1-L163F-transfected MDCK cells; lane 6, NHE3-VSVG-transfected MDCK cells. The double arrow (top left) points to the mature and immature forms of NHE1 in lane 4 (untransfected) and lanes 2, 5 (transfected). The single arrow indicates the position of NHE3. Note the absence of the immature form and its lower molecular mass (≈80 kDa) consistent with its lack of N- and O-glycosylation sites. Standards are, from top to bottom, 106, 80 and 50 kDa.
L163F and NHE3 can be used as direct selective markers with the appropriate H⁺-suicide selective protocols, here described.

**Level of expression and pharmacological profiles of transfected NHE molecules**

NHE1 and NHE3 molecules have been introduced in epithelial cells and selected on the basis of their capacity to survive a lethal acid load. The molecules are therefore functionally expressed in the plasma membrane. We then analyzed the $V_{\text{max}}$ values and pharmacological profile of endogenous and transfected NHE molecules to evaluate the contribution of each population of transporters (endogenous and transfected) in the measured Na⁺/H⁺ exchange activity.

Fig. 4A shows the HOE694-sensitivity of both NHE1 and NHE3 transfected in OK cells. The endogenous NHE activity of OK cells, like NHE3, is extremely resistant to HOE694 (they have a $K_i$ of about 500-700 μM), whereas NHE1 expressed in these cells has a $K_i$ of 0.1 μM, a value similar to that of NHE1 expressed in PS120 fibroblasts (Counillon et al., 1993a). Regarding the $V_{\text{max}}$ values of $^{22}$Na⁺ uptake, it was stimulated 16- and 10-fold for NHE1 and NHE3, respectively (values varied from 1,295 to 21,448 and 13,588 cpm/minutes per mg protein). These very high values are nevertheless relative to the very low NHE endogenous activity of OK cells that we precisely selected for this study (see Materials and Methods). Fig. 4A shows clearly that $^{22}$Na⁺ uptake inhibited by 10 μM of HOE694 is attributable to NHE1, whereas the residual activity inhibited by 10 mM HOE694 reflects the activity of NHE3. This pharmacological dissection was exploited in the next section to evaluate the contribution of each NHE isoform in either side of the polarized epithelial membrane to the total HOE694-sensitive $^{22}$Na⁺ uptake.

---

**Fig. 4.** Pharmacological profile of Na⁺/H⁺ exchange activity towards HOE694 of NHE1 and NHE3 expressed in OK and MDCK cells. (A) Untransfected (□) OK cells as well as OK stable transfectants expressing either NHE1 (○) or NHE3-VSVG (■) were grown to confluency on plastic dishes and submitted to an acid load. Initial rates of $^{22}$Na⁺ uptake were analyzed at different concentrations of the specific inhibitor HOE694, and expressed as the percentage of respective maximal uptake values. Note that the NHE endogenous activity of OK cells has the same $K_i$ value as the rat NHE3. (B) Untransfected (○) MDCK cells as well as stable MDCK-transfectants expressing either NHE1-L163F (Δ) or NHE3-VSVG (■) were grown to confluency and initial rates of $^{22}$Na⁺ uptake are expressed as in A.
With MDCK cells the situation is somewhat opposite to that found with OK cells (Fig. 4B). In untransfected cells the pharmacology profile, towards HOE694, suggests a single population of NHE molecules that is apparently NHE1. Although the $K_i$ for HOE694 is 10-fold higher than for the human NHE1, we believe that this NHE activity represents dog NHE1 as attested by northern analysis and recognition of the molecule with anti-NHE1 antibodies (Figs 2 and 3).

Transfection and selection of human NHE1-L163F into these cells also gives a pharmacological profile characteristic of an homogenous population with a $K_i$ for HOE694 of 4 $\mu$M. This high $K_i$ value is due to the point mutation L163F introduced within the 4th transmembrane segment of NHE1 (Counillon et al., 1993b). Because the $K_i$ difference between dog NHE1 and transfected NHE1-L163F is small, the two populations of exchangers cannot be separated on the curve. Transfection of NHE1-L163F, however, has increased the Vmax by 3.6-fold (from 4,451 to 16,359 cpm/minutes per mg protein, $n=2$). In contrast when rat NHE3 is expressed in MDCK cells, a biphasic profile of HOE694 inhibition is obtained. The first wave of inhibition, which corresponds to about 10% of total NHE activity, represents endogenous NHE1; the second and prominent wave of inhibition is characteristic of the newly expressed and HOE694-resistant NHE3 isoform. The Vmax is increased by 6-fold (from 4,451 to 27,480 cpm/minutes per mg protein, $n=2$). Here, inhibition of NHE activity with 100 $\mu$M and 10 mM of HOE694 will discriminate, respectively, the values of NHE1 (endogenous and transfected) from NHE3 (see next section).

Functional expression of NHE1 and NHE3 in polarized epithelial cell lines

OK cells were maintained on semi permeable membrane inserts confluent for several days to obtain a polarized epithelial cell monolayer. Following intracellular acidification, Na⁺/H⁺ exchange activity measured by initial $^{22}$Na⁺ uptake rates was expressed either as total NHE, NHE1 or NHE3 activity (Fig. 5). These activities were discriminated on the basis of their differential sensitivity to HOE694 (see Materials and Methods for calculation). The central panel of Fig. 5A shows that NHE1 transfected in OK cells is delivered both to the basal lateral and apical membranes with a marked preference for the apical side (79% of total NHE1 activity). In sharp contrast, expression of NHE3, in these cells, shows a full recovery at the apical membrane (100%, Fig. 5A, right panel), as observed for the endogenous isoform of untransfected OK cells.

Similar experiments with MDCK cells are shown in Fig. 5B: transfection of either NHE1 or NHE3 encompassed by 2- to 3-fold the endogenous NHE activity of MDCK (Fig. 5B, left panel). The central panel shows that NHE1 introduced in these cells, is delivered at both sides of the polarized epithelial cells, here again with a marked preference for the apical side (72% of total NHE1). It is interesting to note that the endogenous NHE1 activity is also distributed between both membranes with an enrichment at the apical face. Functional expression of NHE3, however, is fully restricted to the apical membrane (100%; Fig. 5B, right panel). Fig. 5A and B are representative of two independent transfection experiments. Selected transfected clones behave similarly to the transfected cell population (data not shown).

Given the results shown above for the dual but quite different NHE1 membrane expression in the two renal epithelial cell lines MDCK and OK cells (Fig. 5A and B), we...
extended our study to three other renal and intestinal cell lines, respectively, the highly resistant strain I of MDCK, Caco-2 and HT-29 cells. The results of all these studies are summarized in Table 1. It is clear from these studies that NHE1 is distributed to both apical and basolateral membranes but in different proportions according to the cell line where it is expressed.

**Fig. 7.** Cellular distribution of stably expressed NHE1 and NHE3-VSVG in polarized MDCK and OK cells as revealed by indirect immunofluorescence and analyzed by confocal laser microscopy. (A) NHE1 transfected MDCK cells. Both the endogenous and the exogenous NHE1-L163F are revealed with the antibody. (B) NHE1 transfected OK cells. The antibody recognizes only the exogenous NHE1. Three different ‘en face’ plans are shown from the basal side (left) to the apical side (right) of the cells. (C) NHE3-VSVG-transfected MDCK cells. An XZ plan reveals the exclusive apical expression of NHE3-VSVG proteins. Cells were grown, fixed, and revealed as indicated in legend of Fig. 6. Nuclei and nucleolei were labelled with propidium iodide and appear in red color. NHE1 or NHE3-VSVG appear in green (FITC-labelled secondary antibodies). Bar, 10 μm.
Cellular distribution of NHE1 and NHE3 expressed in polarized epithelial cells revealed by immunolocalization

As a complementary approach to transport studies and pharmacology dissection, we then determined the subcellular localization of NHE1 and NHE3 in MDCK and OK-transfected cells by immunolabelling.

In untransfected MDCK cells, the polyclonal antibodies directed against human NHE1 (RPc28) detected a typical basal lateral signal that we interpreted as endogenous NHE1 (Fig. 6A). The same pattern, characteristic of basal lateral proteins, is enhanced in cells transfected with the human NHE1 cDNA. In particular, the lateral signal is stronger (Fig. 6B). A picture very similar to MDCK emerged from OK cells transfected with NHE1 (basal lateral labeling, Fig. 6E). In sharp contrast, however, is the expression of the epitope-tagged NHE3-VSVG expressed either in MDCK or OK cells (Fig. 6D and F). A very strong apical immunolocalization is seen in the transfected cells with a total exclusion from the basal and lateral sides. These observations are reinforced by the confocal laser microscopy pictures shown in Fig. 7 where a cobblestone pattern and punctate labeling are seen for NHE1 (Fig. 7A and B), characteristic of, respectively, a lateral and apical expression of the protein. The confocal laser microscopy picture, specially for OK cells, was thus clear enough to confirm expression of NHE1 at both apical and basolateral membranes of these cells (Fig. 7B). In contrast, a discrete NHE3 specific labelling is seen only on the apical surface of MDCK cells (Fig. 7C).

We therefore conclude that in these two different types of cultured epithelial cells, endogenous and ectopic expression of NHE3 is strictly restricted to the luminal side, whereas endogenous or exogenously expressed NHE1 shows dual membrane targeting.

Membrane surface expression of NHE1 in three epithelial cell types

Functional Na⁺ studies revealed a much more pronounced expression of NHE1 at the apical side (70 to 80%) than at the basal lateral side (Fig. 5) whereas results of immunolocalization, at least judged by fluorescence microscopy, suggest a major NHE1 expression at the basal lateral side. To resolve this apparent discrepancy, we carried out protein surface biotinylation followed by immunoprecipitation of NHE1 molecules. These analyses were conducted under the same culture conditions as those used for Na⁺ flux studies and with the same transfected cell populations of MDCK and OK cells. In addition we included in the study another cell line, clone 19A of HT-29, which expresses 75% of NHE1 activity at the basal lateral side. Fig. 8A shows that NHE1 is preferentially expressed in the apical membrane of both MDCK and OK cells with a higher Ap/Bl ratio in OK cells, a result that strikingly parallels quantitation of functional NHE1 protein (Fig. 8C). Interestingly, this correlation extends also to HT-29 cells (Fig. 8A and C). These results therefore validate our conclusion based on NHE1 functional studies, establishing a dual plasma membrane expression of NHE1 in polarized cells in culture.

DISCUSSION

We reported in this paper the steady state expression of two Na⁺/H⁺ exchanger isoforms, NHE1 and NHE3, in cultured epithelial cells. The two kidney cell lines chosen, MDCK and OK, have been widely used for membrane protein targeting and physiological studies on cell polarity. NHE1 and NHE3 isoforms were selected because they represent two extremes within the NHE family as far as the amiloride-sensitivity and tissue distribution are concerned (Orialkowski et al., 1992; Tse et al., 1992; Counillon et al., 1993a). We found that NHE3 is strictly expressed at the apical membrane of polarized epithelial cells, whereas NHE1 is distributed in both apical and basal lateral membranes of the cell. This finding has been established in both cell lines and was derived from the use of three approaches: immunofluorescence labelling, quantitation of H⁺-activated Na⁺ influx differentially sensitive to HOE694 and specific cell surface biotinylation of NHE1 molecules.

NHE3 expression

Although the immunological approach is qualitative, it is clearly established that NHE3 is ‘purely’ apically expressed, since high levels of expression obtained by H⁺-suicide selection did not induce NHE3 spill-over on the basal lateral side of polarized cells. Indeed OK- and MDCK-transfected clones, expressing NHE3 activities exceeding up to ten times that of the original untransfected cells, showed a strict NHE3 immunolabelling at the apical surface. This result is fully consistent with NHE3-dependent Na⁺ influx being found exclusively at the apical pole. We therefore conclude that both MDCK and OK cells have the full capacity to specifically target NHE3 molecules to the apical membrane even when the transporter is overexpressed. This finding is in good agreement with previous work from Mürer’s group on the endogenous NHE activity of OK cells (Montrose and Mürer, 1990; Helmle-Kolb et al., 1990) and with recent in situ immunodetection of NHE3 in the brush border of rat intestine (Bookstein et al., 1994) and in the apical membrane of rat proximal and medullary tubules (Biemesderfer et al., 1993).

NHE1 expression

The situation with NHE1 is more complex and somehow unexpected. Previous results have documented the expression of NHE1 at the basal lateral membrane of Caco-2 (Watson et al., 1991) and HT-29 cell lines (Table 1) and when analyzed in situ by immunohistochemistry, NHE1 was detected only at the basal lateral membrane of both renal (Biemesderfer et al., 1992) and intestinal cells (Bookstein et al., 1994). Therefore the unexpected finding was that not only was NHE1 expressed at both sides of OK and MDCK cell membranes but with a marked preference for the apical side (70% in MDCK and 80% in OK cells as determined by Na⁺ uptake studies). Although immunofluorescence

Table 1. Membrane distribution of endogenous NHE isoforms in untransfected intestinal and renal cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Tissue origin</th>
<th>Isoform</th>
<th>Apical</th>
<th>Basolateral</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caco-2</td>
<td>Human colon</td>
<td>NHE1</td>
<td>55%</td>
<td>45%</td>
</tr>
<tr>
<td>HT-29</td>
<td>Human colon</td>
<td>NHE1</td>
<td>25%</td>
<td>75%</td>
</tr>
<tr>
<td>MDCK, strain I</td>
<td>Dog kidney</td>
<td>NHE1</td>
<td>21%</td>
<td>79%</td>
</tr>
<tr>
<td>MDCK, strain II</td>
<td>Dog kidney</td>
<td>NHE1</td>
<td>40%</td>
<td>60%</td>
</tr>
<tr>
<td>OK</td>
<td>Opossum kidney</td>
<td>NHE3</td>
<td>100%</td>
<td>–</td>
</tr>
</tbody>
</table>

°Na⁺ uptake sensitive to specific concentration of HOE694 was measured in both sides and expressed as the percent of total uptake. Cells were grown on Falcon inserts for 6 to 10 days according to requirements of each cell line.
The sorting machinery could have been saturated by high targeting of NHE3 at the apical membrane (data not shown). (iii) of NHE3 into NHE1-transfected cells did not prevent exclusive MDCK or OK cells (data not shown). Furthermore introduction found not to be modified upon NHE1 transfection in either polarity (Stevenson et al., 1986; Stevenson and Begg 1994) was polarity. This is not the case because the ZO-1 (Zona occludens and 8C). (ii) NHE1 transfection could have altered cellular side of the filter. Moreover, quantification of membrane surface artefact in quantifying specific 22Na + uptake from the basolateral done with MDCK and OK cells are not due to a methodology problem of NHE inhibitor accessibility at the basolateral side of the insert. The NHE1 distribution obtained in clone 19A of MDCK and OK cells and in untransfected HT-29 cells.

(A) Quantification of NHE1 biotinylated from the apical (Ap) or the basal lateral (Bl) side. After Triton lysis, NHE1 proteins were immunoprecipitated as previously described (Fafournoux et al., 1994), run on a 7.5% SDS-PAGE gel and electrotransferred to PVDF membrane (Millipore). Biotinylated NHE1 proteins were revealed with streptavidin-HRP. (B) Total cellular NHE1 expression. Following stripping of streptavidin-HRP, the anti-NHE1 antibody (RPc28) was used to reveal all NHE1 proteins (apically, basolaterally and intracellularly expressed proteins). (C) The relative specific NHE1 activity reported here (see Table 1) indicates that a good correlation exists between the surface expression of NHE1 and its functional transport activity. Cells were grown on filters for seven (MDCK and OK cells) and ten days for HT-29 cells. This is a representative experiment from three similar ones.

Future studies should now concentrate on the determinants expression levels of the transporter. This is very unlikely because the Ap/Bl ratio of NHE1 activity is identical whether we consider MDCK low expressors (basal activity of untransfected cells) or high expressors (5-fold the basal level). (iv) Formation of heterodimers between endogenous NHE3 and transfected NHE1 could have driven some NHE1 molecules to the apical membrane of OK cells. This possibility can be easily excluded. In experiments conducted with fibroblasts we previously showed that co-expression of NHE1 and NHE3 in the same cells led only to the formation of homodimers NHE1/NHE1, NHE3/NHE3 (Fafournoux et al., 1994). (v) Finally we could argue that the cellular models used are not adequate for basal lateral targeting. This point also appears unlikely because MDCK cells have been widely used for protein targeting showing basolateral sorting for a variety of transmembrane proteins, for example Na+,K+-ATPase (Gottardi and Caplan, 1993), low density lipoprotein (LDL) receptor (Matter et al., 1992), NGF receptor (Le Bivic et al., 1991), and α2-adrenergic receptor (Keef er and Limbird, 1993). In addition, the endogenous NHE1 expressed in HT-29, Caco-2 and high electrical resistance MDCK strains is distributed both at the apical and basolateral membranes, although the Ap/Bl ratio varied from one cell line to the other (Table 1). Therefore we are left with the second explanation suggesting that bilateral targeting of NHE1 is an intrinsic feature of the protein.

This apparent ‘permissivity’ in membrane targeting is not unique and might even be a general rule shared by basal lateral proteins that ensure, like NHE1, cellular constitutive functions in virtually all cell types. The Na+,K+-ATPase is a typical example of a polytopic membrane protein for which the same protein subunit has been found expressed at the apical membrane (Marrs et al., 1995; Ghosh et al., 1990; Gunderson et al., 1991) or to both the apical and lateral membranes (Wilson et al., 1991; Hammerton et al., 1991). NHE1 is primarily detected in the basal lateral membranes of intestinal and kidney cells (Biemesderfer et al., 1992; Bookstein et al., 1994), found in both membranes of cultured MDCK or OK cells (this study) and in the brush border membrane of placental epithelial cells (Kulanthaivel et al., 1992). LDL receptor endowed with basal lateral targeting signals (Matter et al., 1992; Matter and Mellman 1994) provides another example of bilateral expression. In transgenic mice, LDL receptor is found in the basal lateral membrane of intestine and liver epithelial cells, whereas it is expressed at the apical membrane of kidney proximal tubules (Pathak et al., 1990). Because some of the basal lateral signals that have been identified in several single transmembrane proteins overlap with the tyrosine-dependent endocytotic signals (Matter and Mellman, 1994), it has been proposed that accumulation of these proteins could specify delivery to functionally (most endocytically active site) rather than topologically defined plasma membrane domains (Gottardi and Caplan, 1993). In the case of the Na+,K+-ATPase that is delivered to both membranes of MDCK cells, retention of the active enzyme to the basal lateral membrane, probably by interaction with the membrane cytoskeleton (ankyrin, fodrin) (Hammerton et al., 1991) and specific cadherins (Marrs et al., 1995), and inactivation at the apical side, has been proposed as a mechanism for establishing the polarity of this transporter. In any event NHE1, with its bilateral delivery seems to belong to this category of housekeeping proteins that might have evolved multiple signals, allowing more plasticity for protein targeting in a wide variety of cells.

analysis by confocal optical sectioning confirmed that NHE1 is expressed at the apical surface of OK and MDCK cells (punctate labelling above nuclei in Fig. 7A and B), NHE1 immunolabelling of the lateral side of both cell types appeared as a very prominent signal. Two explanations could account for the bilateral NHE1 distribution: (1) it results from a methodology artefact; (2) it is a characteristic of the NHE1 protein. Several points must be considered in regard to the first explanation. For instance: (i) a problem of NHE inhibitor accessibility at the basolateral side of the insert. The NHE1 distribution obtained in clone 19A of intestinal HT-29 cells (see Table 1) grown on the same filters and in the same conditions demonstrates clearly that the observations done with MDCK and OK cells are not due to a methodology artefact in quantifying specific 22Na + uptake from the basolateral side of the filter. Moreover, quantification of membrane surface expression of NHE1 in these three cell types indicates that a fairly good correlation exists between the quantity of proteins expressed at the membrane and their specific activity (Fig. 8A and 8C). (ii) NHE1 transfection could have altered cellular polarity. This is not the case because the ZO-1 (Zona occludens protein 1) pattern characteristic of epithelial cell integrity and polarity (Stevenson et al., 1986; Stevenson and Begg 1994) was found not to be modified upon NHE1 transfection in either MDCK or OK cells (data not shown). Furthermore introduction of NHE3 into NHE1-transfected cells did not prevent exclusive targeting of NHE3 at the apical membrane (data not shown). (iii) The sorting machinery could have been saturated by high
and mechanisms that provide bilateral expression of NHE1 and restriction of NHE3 to the apical side of the plasma membrane. Chimeric molecules between NHE1 and NHE3 have clearly shown that the large cytoplasmic domain of these polytopic membrane proteins is essential for correct delivery to the plasma membrane and for specification of their final membrane insertion (Noël, Roux, Wakabayashi and Pouysségur, in preparation).

We thank R. Elliot, S. Robin and D. Louvard from the Institut Pasteur, Paris, for their estimable contribution in confocal microscopy as well as A. LeBivic from CNRS, Marseille for judicious advice. C. Cibre is acknowledged for the photographic work and M. Valetti for secretarial assistance. Research was funded by CNRS, Association pour la Recherche sur le Cancer (ARC), the Commissariat à l’Energie Atomique (CEA) and the Institut National de la Santé et de la Recherche Médicale (INSERM). J. Noël received a fellowship from the Medical Research Council of Canada.

REFERENCES


(Received 25 September 1995 - Accepted 1 January 1996)