

# Properties of the Na<sup>+</sup>/H<sup>+</sup> exchanger protein

## Detergent-resistant aggregation and membrane microdistribution

Bonnie L. Bullis<sup>1</sup>, Xiuju Li, Carmen V. Rieder<sup>1</sup>, Dyal N. Singh<sup>2</sup>, Luc G. Berthiaume<sup>1,3</sup> and Larry Fliegel<sup>1</sup>

Departments of <sup>1</sup>Biochemistry, CIHR Membrane Protein Group, <sup>2</sup>Anatomy and <sup>3</sup>Cell Biology, Faculty of Medicine and Dentistry, University of Alberta, Edmonton, Alberta, Canada

The Na<sup>+</sup>/H<sup>+</sup> exchanger is a ubiquitous membrane protein of bacteria, plants and mammals. The first isoform discovered (NHE1) is present on the mammalian plasma membrane and transports one H<sup>+</sup> out of cells in exchange for one extracellular Na<sup>+</sup>. With solubilization in standard SDS/PAGE buffer, this protein had a high tendency to aggregate when subjected to elevated temperature. The aggregates were stable and did not dissociate in high concentrations of SDS or 2-mercaptoethanol. We examined the distribution of the Na<sup>+</sup>/H<sup>+</sup> exchanger within membrane subfractions. The Na<sup>+</sup>/H<sup>+</sup> exchanger was found both in caveolin-containing fractions and, in lesser amounts, in higher density membrane fractions where the bulk of proteins were contained. Treatment with cytochalasin D caused only a minor reduction of the amount of Na<sup>+</sup>/H<sup>+</sup> exchanger present in caveolin-

enriched fractions suggesting an intact cytoskeleton was not important for NHE1 localization to these microdomains. Treatment of cells with methyl β-cyclodextrin had a small stimulatory effect on Na<sup>+</sup>/H<sup>+</sup> exchanger activity and reduced the amount of Na<sup>+</sup>/H<sup>+</sup> exchanger in low density membrane fractions. Our study demonstrates that SDS cannot maintain the protein in a monomeric state suggesting that strong hydrophobic interactions are responsible for this temperature dependent aggregation behavior. In addition a large proportion of the Na<sup>+</sup>/H<sup>+</sup> exchanger protein is found to be enriched in low density caveolin-containing fractions.

**Keywords:** caveolin; intermolecular hydrophobic interactions; lipid rafts; Na<sup>+</sup>/H<sup>+</sup> exchanger; SDS-resistant aggregation.

The Na<sup>+</sup>/H<sup>+</sup> exchanger is an integral membrane protein that is present in yeast and *Escherichia coli* and in mammalian cell types. In higher eukaryotes, it removes an intracellular H<sup>+</sup> and exchanges it for an extracellular Na<sup>+</sup>, thereby protecting cells from intracellular acidification. The first known isoform of the protein was cloned in 1989 [1] followed by identification of six other isoforms to date, NHE2–NHE7 [2]. The human NHE1 protein is an 815 amino acid plasma membrane glycoprotein that is widely distributed in mammalian tissues [2]. Growth factor-induced phosphorylation acts to increase its activity resulting in a rise in intracellular pH [3]. Studies have suggested that the NHE1 protein has an N-terminal, hydrophobic, membrane-associated domain of about 500 amino acids, and a C-terminal, hydrophilic domain that contains about 315 amino acids. The membrane-associated domain has 12 transmembrane segments and one membrane-associated segment [1,4]. The protein is known to exist as a dimer and the NHE1 isoform specifically dimerizes to itself and not to other isoforms of the Na<sup>+</sup>/H<sup>+</sup> exchanger [5].

Although the NHE1 isoform of the Na<sup>+</sup>/H<sup>+</sup> exchanger is of great physiological importance, playing a role in intracellular pH regulation and cell proliferation and differentiation, there have been few studies with the intact

protein. The relatively low level of expression and the difficulty in overexpressing membrane proteins has made characterization of the intact protein difficult. In this study, we have examined the characteristics of the protein in detergent (SDS). We developed procedures for maximizing the detection of the protein and characterize SDS-resistant aggregation of the protein. We [6] and others [7] have shown that the Na<sup>+</sup>/H<sup>+</sup> exchanger is localized to specific foci in some cell types. We therefore also examined the membrane distribution of the protein using our improved Western blotting procedure. Our results are the first examination of the aggregation behavior and membrane microdistribution of the NHE1 isoform of the Na<sup>+</sup>/H<sup>+</sup> exchanger.

## MATERIALS AND METHODS

### Materials

Restriction endonucleases and DNA modifying enzymes were obtained from Boehringer Mannheim (Laval, Quebec, Canada) and Bethesda Research Laboratories (Gaithersburg, MD, USA). Anti-hemagglutinin (HA) Ig for Western blotting was purchased from Boehringer Mannheim (Laval, Quebec, Canada) or Berkeley Antibody Co. (Richmond, CA, USA). For immunoprecipitation anti-HA Ig was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-NHE1 monoclonal antibody was from Chemicon Int. Inc. (Temecula, CA, USA) and calpain I inhibitor was from Calbiochem (San Diego, CA, USA). Anti Na<sup>+</sup>/K<sup>+</sup> ATPase Ig (6F) was from the Developmental Studies Hybridoma Bank (Iowa City, Iowa, USA). Rabbit polyclonal anti-caveolin Ig was from Transduction Laboratories (Lexington, KY, USA). Cholera toxin

Correspondence to L. Fliegel, 347 Medical Science Bldg., Department of Biochemistry, Faculty of Medicine, University of Alberta, Edmonton, Alberta T6G 2H7, Canada T6G 2H7.

Fax: +780 4920886, Tel: +780 4921848,

E-mail: lfliegel@ualberta.ca

Abbreviations: HA, hemagglutinin.

(Received 19 July 2002, accepted 22 August 2002)

coupled to Alexa Fluor 488 was from Molecular Probes, Inc. (Eugene, OR, USA). All other chemicals not listed were of analytical or Molecular Biology grade and were purchased from Fisher Scientific (Ottawa, Ontario, Canada), Sigma (St. Louis, MO, USA) or BDH (Toronto, Ontario, Canada).

### Preparation of proteins from tissues and cells in culture

Organs were harvested from adult mice and immediately frozen in liquid nitrogen. Tissues were then placed in a buffer containing 1 M NaCl, 100 mM Tris pH 7.4, 0.1 mM phenyl methanesulfonyl fluoride, 0.1 mM benzamide, 37.5  $\mu$ M ALLN (calpain I inhibitor) and a proteinase inhibitor cocktail [6] for homogenization. Samples were homogenized at 4 °C for 30 s, incubated on ice for 30 s, and then homogenized again for 30 s using an Omni International 2000 electric homogenizer. To obtain crude membrane fractions (which contained the NHE1 protein within cells), homogenates were subjected to a series of centrifugation steps [8]. Initial centrifugation was for 10 min at 1100 *g*. The pellet was discarded and the supernatant centrifuged at 9000 *g* for 15 min. The resulting pellet was again discarded and the supernatant was centrifuged at 100 000 *g* for 1 h to obtain a fraction enriched in crude microsomes. The pellet containing the membrane fraction was resuspended in the same buffer as described above with the addition of 1% SDS to aid in solubilization. Total protein was quantified using the Bio-Rad D<sub>C</sub> Protein Assay kit.

AP-1 cells stably transfected with the HA-tagged Na<sup>+</sup>/H<sup>+</sup> exchanger were maintained in alpha-MEM medium with G418 as described earlier [9]. For analysis of NHE1, total cell lysates were used and were prepared and subjected to SDS/PAGE essentially as previously reported [10]. The standard buffer used for solubilization of Na<sup>+</sup>/H<sup>+</sup> exchanger containing samples contained 2% SDS, 10% glycerol, 43.25 mM Tris/HCl, pH 6.8, 0.05 mM bromophenol blue. The temperature of incubation varied as described in the text and the entire sample treated was used for electrophoresis. After treatment at various temperatures samples were maintained at room temperature prior to SDS/PAGE.

The Na<sup>+</sup>/H<sup>+</sup> exchanger from rat myocyte proteins was immunoprecipitated with a rabbit polyclonal antibody against the cytoplasmic domain of the protein as described earlier [10]. Immunoprecipitation of the Na<sup>+</sup>/H<sup>+</sup> exchanger from transfected AP-1 cells was with a rabbit polyclonal against the HA tag (Santa Cruz Biotechnology, Inc.).

Preparation of caveolin-enriched membrane fractions using sucrose density centrifugation AP-1 cells stably transfected with the HA-tagged Na<sup>+</sup>/H<sup>+</sup> exchanger [9] were grown to near confluence in 100-mm dishes and were used to prepare caveolin-enriched membrane fractions by a detergent-free (sodium carbonate) method [11]. After two washes with ice-cold NaCl/P<sub>i</sub>, two confluent dishes were scraped into 2 mL of 500 mM sodium carbonate, pH 11.0. Homogenization was carried out using 10 strokes of a tight-fitting Dounce homogenizer followed by three 10-s bursts of a Polytron tissue grinder (Brinkmann Instruments, Westbury, NY, USA) and then three 20-s bursts of sonication (XL Sonicator, Hert Systems, Farmingdale, NY, USA) to more thoroughly disrupt the cellular membranes [11]. The

homogenates were adjusted to 40% sucrose by addition of 2 mL of 80% sucrose and placed at the bottom of an ultracentrifuge tube. A 5–40% discontinuous sucrose gradient was formed above by placing 4 mL of 30% sucrose solution above the 4 mL sample (40% sucrose) and then 4 mL of 5% sucrose solution above the 30% sucrose solution. The tubes were centrifuged at 39 000 r.p.m. for 16–20 h in an SW40Ti rotor (Beckman Instruments, Palo Alto, CA, USA). A light scattering band was observed at the 5–30% sucrose interface that contained caveolin-rich membranes but excluded most other cellular proteins as judged by Coomassie blue staining of proteins. Twelve 1-mL fractions were removed from the top of the tubes and equal portions of each were analyzed by SDS/PAGE followed by Western blot analysis. In some experiments, AP-1/Na<sup>+</sup>/H<sup>+</sup> exchanger containing cells were treated with cytochalasin D (0.5  $\mu$ g/mL, 2 h 37 °C) as described earlier [12]. To disrupt lipid rafts and examine effects on Na<sup>+</sup>/H<sup>+</sup> exchanger activity we treated cells with 10 mM methyl  $\beta$ -cyclodextrin for 30 min at 37 °C in serum-free medium, as described earlier [13,14].

In some experiments, membrane fractions were isolated using a procedure that contained Triton X-100 [15,16]. After two washes with cold NaCl/P<sub>i</sub>, two confluent dishes were scraped into 1 mL of MBS (25 mM Mes, pH 6.5, 0.15 M NaCl and 1% Triton X-100). Cells were solubilized at 4 °C for 30 min and scraped from the dishes and collected. Cells were homogenized with a Dounce homogenizer as described above. The homogenates were made to 40% sucrose by adding 2 mL of 80% sucrose 2  $\times$  MBS in a glass tube, mix by vortexing and placed at the bottom of an ultracentrifuge tube. A 5–40% discontinuous sucrose gradient in MBS was made as described above and samples were centrifuged and collected as described above.

For some experiments we used Triton X-100 to re-extract membranes that were isolated using the sodium carbonate containing procedure [11] essentially as described earlier by others [17]. Samples from fraction 5, that contained the HA-tagged Na<sup>+</sup>/H<sup>+</sup> exchanger were made to a final concentration 50 mM Tris, pH 7.4, 150 mM NaCl and 5 mM EDTA  $\pm$  1% Triton X-100. Samples were incubated for 20 min at 4 °C then pelleted in a TLA100.3 rotor at 80 000 r.p.m. for 20 min. The supernatants and pellets were collected and analyzed for the presence of Na<sup>+</sup>/H<sup>+</sup> exchanger by Western blotting.

Na<sup>+</sup>/H<sup>+</sup> exchanger activity was measured in treated and paired mock-treated cells. Acid load was induced with ammonium chloride as described earlier [18]. There was no difference in the degree of acidification in experimental and mock-treated cells. Results were compared with a paired Student's *t*-test and considered significant when *P* < 0.05 (*n* = 8).

### NHE1 Western blot analysis

Crude membrane fractions containing 60–100  $\mu$ g total protein were run on 10% polyacrylamide gels followed by transfer to nitrocellulose membranes, essentially as described earlier [9]. For immunoblotting with anti-NHE1 Ig (Chemicon) nitrocellulose membranes were incubated overnight at 4 °C in 10% milk/Tris/NaCl (Tris/NaCl = 20 mM Tris, 137 mM NaCl, pH 7.6), and then washed four times for 15 min each in Tris/NaCl at room temperature.

Membranes were probed at 4 °C overnight in the absence of milk powder with anti-NHE1 monoclonal antibody (Chemicon) at a concentration of 1 : 2000 in Tris/NaCl. Following four washes of 15 min each with Tris/NaCl, membranes were incubated with 1 : 5000 goat anti-mouse Ig in Tris/NaCl at room temperature for 1 h. After three 5-min washes in Tris/NaCl, the Amersham Enhanced Chemiluminescence reaction was used to visualize immunoreactivity as described earlier [10]. For samples containing HA-tagged Na<sup>+</sup>/H<sup>+</sup> exchanger the immunoblotting procedure was essentially as described earlier [9]. Analysis of the relative amounts of protein present in membrane fractions was as described earlier [19].

### Immunocytochemistry

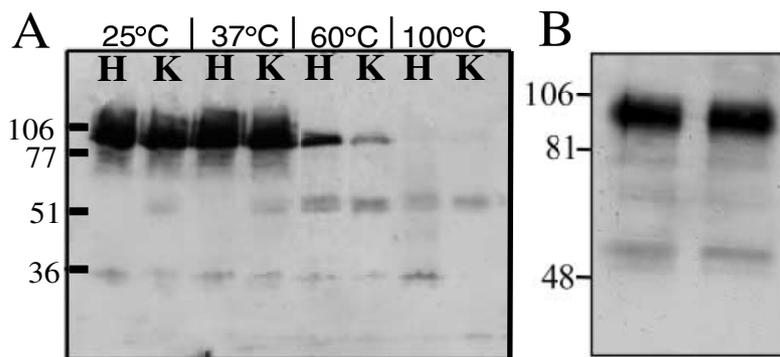
To determine the intracellular localization of NHE1 protein *in vivo* in relation to lipid rafts AP-1 cells stably transfected with pYN4 + plasmid containing the Na<sup>+</sup>/H<sup>+</sup> exchanger [9] were grown on coverslips. At 60% confluence cells on coverslips were washed three times with 1 × NaCl/P<sub>i</sub>, pH 7.4. Following washes, the cells were fixed in 4% paraformaldehyde for 10 min. Fixation was terminated with 100 mM glycine in NaCl/P<sub>i</sub> pH 7.4 for 15 min. Cells were then washed twice with 0.1% Triton-X 100 and 0.1% BSA in NaCl/P<sub>i</sub> (TA-NaCl/P<sub>i</sub>) and then permeabilized by incubation in the same solution for 30 min. The cells were then washed with three changes of TA-NaCl/P<sub>i</sub> followed by blocking in 5% fetal bovine serum or goat serum for 20 min. After washing with 3 quick changes of TA-NaCl/P<sub>i</sub>, coverslips were incubated overnight at 4 °C with the anti-HA Ig (dilution 1 : 200). Control coverslips were incubated in TA-NaCl/P<sub>i</sub> only. Following reaction with the anti-HA Ig, coverslips were washed three more times in TA-NaCl/P<sub>i</sub> and reacted with the fluorescently labeled (Rhodamine) goat anti-mouse Ig in TA-NaCl/P<sub>i</sub> (1 : 500) for 1 h at room temperature. The fluorescently labeled cells were washed three times with 1 × NaCl/P<sub>i</sub> pH 7.4 and mounted in 50% glycerol containing 1% propyl gallate. To compare the localization of NHE1 with lipid raft distribution we used cholera toxin to examine staining of GM1 ganglioside essentially as described by others [13]. Cholera toxin (coupled to Alexa Fluor 488, Molecular probes, dilution

1 : 200) was reacted immediately after the reaction with the primary antibody against the HA tag as described above. Cells were visualized with a Zeiss fluorescent microscope equipped with appropriate filters.

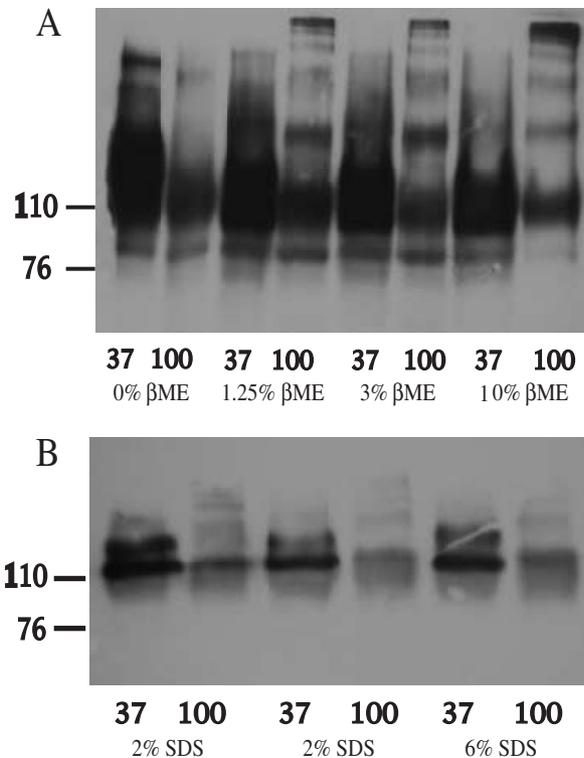
### RESULTS

To examine the Na<sup>+</sup>/H<sup>+</sup> exchanger protein in intact murine tissues we used Western blotting with a monoclonal antibody against the cytoplasmic domain of the Na<sup>+</sup>/H<sup>+</sup> exchanger. Samples of heart and kidney crude membranes were subjected to a 5-min incubation at either 25, 37, 60 or 100 °C prior to loading the samples into SDS/PAGE. Western blotting (Fig. 1A) showed that with incubation at either 60 or 100 °C, the amount of NHE1 immunoreactive protein dramatically declined. This occurred in both the heart and kidney membranes. To ensure that the antibody against the Na<sup>+</sup>/H<sup>+</sup> exchanger was reacting with the appropriate protein we immunoprecipitated NHE1 from a rat heart extract using a rabbit polyclonal antibody raised against the C-terminal 178 amino acids of the protein. The immunoprecipitate was run on SDS/PAGE and probed with anti-NHE1 monoclonal and a 100–105 kDa band was evident (Fig. 1B). The results confirmed that the anti-NHE1 monoclonal was reacting with the Na<sup>+</sup>/H<sup>+</sup> exchanger protein.

To determine if the same phenomenon occurred in an entirely different system we examined the Na<sup>+</sup>/H<sup>+</sup> exchanger protein (NHE1 isoform) that was transfected into AP-1 cells. The HA-tagged protein was immunoprecipitated, solubilized with SDS and subjected to incubation at either 37 or 100 °C for 5 min. We varied the concentration of 2-mercaptoethanol to determine if this influenced the effect. The results are shown in Fig. 2A. Boiling the samples caused aggregation of the immunoprecipitated NHE1 protein that was reduced in amount at the 105–110 kDa size. The amount of 105–110 kDa protein was reduced and in many instances evidence of aggregation was evident at the top of the gels. The amount of aggregate present at the top of the Western blot of the gels was not equal in amount to that lost at the lower molecular mass. This was probably due to reduced efficiency of electrophoretic transfer of the larger size aggregate. There was no effect of varying the



**Fig. 1.** Western blot analysis of the endogenous Na<sup>+</sup>/H<sup>+</sup> exchanger protein from mammalian heart and kidney. (A) Western blot of adult heart (H) and kidney (K) crude membrane preparations. Samples were heated for 5 min at 25, 37, 60 or 100 °C prior to loading the SDS/PAGE gel. After transfer samples were immunoblotted with anti-NHE1 monoclonal antibody. The size and relative position of molecular mass markers are indicated. (B) Western blot of NHE1 protein immunoprecipitated from rat heart myocytes with an antibody against the C-terminal 178 amino acids of the protein. The immunoprecipitate was then probed with the anti-NHE1 monoclonal antibody.



**Fig. 2.** Effect of 2-mercaptoethanol and SDS on temperature-dependent aggregation of the  $\text{Na}^+/\text{H}^+$  exchanger. The hemagglutinin-tagged  $\text{Na}^+/\text{H}^+$  exchanger protein was expressed and immunoprecipitated from AP-1 cells as described in the Materials and methods. The immunoprecipitated protein was used for Western blotting under varying conditions. (A) Effect of varying concentrations of 2-mercaptoethanol on aggregation of the  $\text{Na}^+/\text{H}^+$  exchanger. Samples were incubated with the indicated concentration of 2-mercaptoethanol at either 37 or 100 °C. After SDS/PAGE and transfer, immunoblotting was with an antibody against the hemagglutinin tag. (B) Samples were incubated in the presence of 2, 4 or 6% SDS for 5 min at either 37 or 100 °C. After SDS/PAGE and transfer, immunoblotting was carried out as described in (A).

2-mercaptoethanol concentration on the amount of protein aggregation. Increasing the SDS concentration from the standard 2% to either 4 or 6% also did not prevent protein aggregation. We sometimes observed that the NHE1 protein ran as multiple species of approximately 110 kDa in size. The reason for this is not yet known but could be due to the presence of other tightly associated proteins such as calmodulin [20].

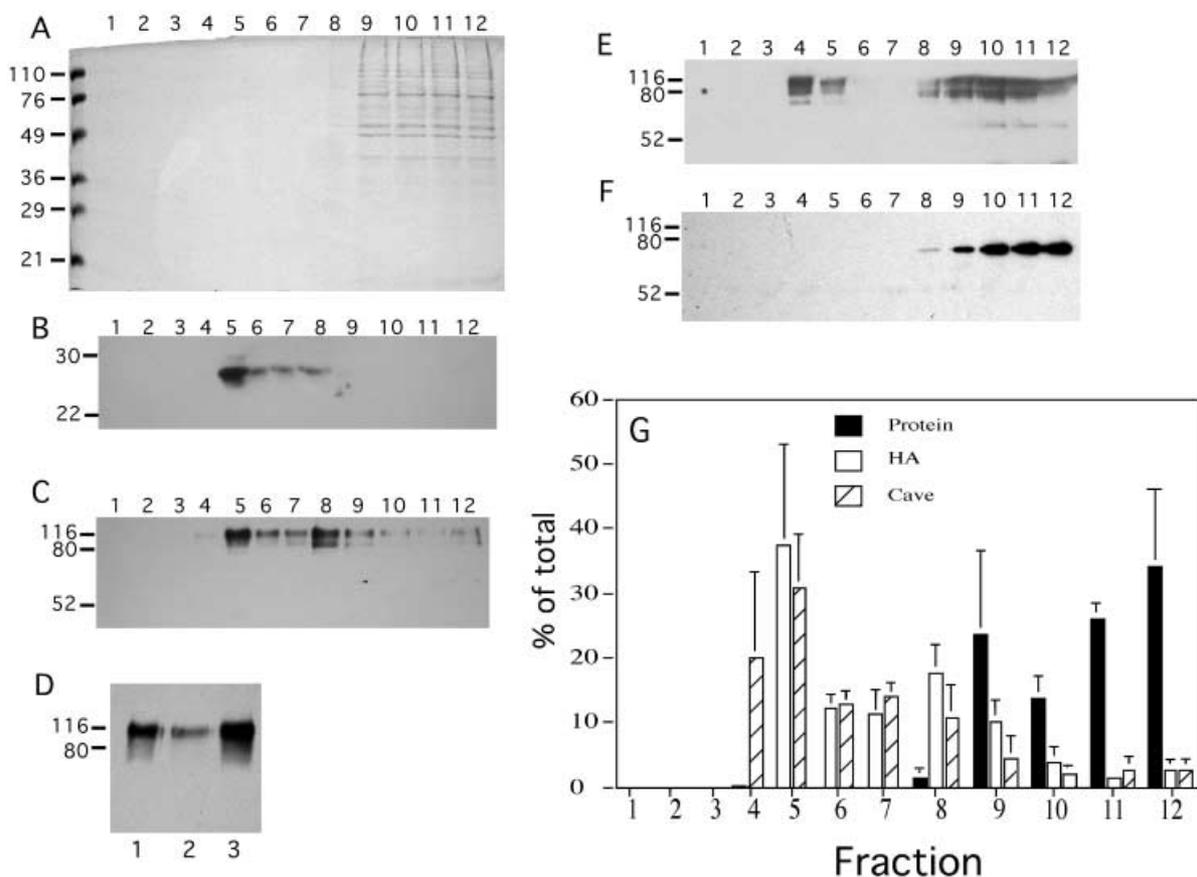
Recently, various membrane proteins have been demonstrated to target to microdomains of the membrane known as lipid rafts. Lipid rafts are known to be detergent resistant and enriched in caveolin [21–23]. We examined if the  $\text{Na}^+/\text{H}^+$  exchanger might also be targeted to these lipid glycosphingolipid- and caveolin-enriched fractions. To test this hypothesis, we isolated membrane fractions from mammalian cells and examined the distribution of the  $\text{Na}^+/\text{H}^+$  exchanger and of caveolin, a marker of lipid rafts. To avoid possible detergent-induced artifacts that could either alter the distribution of the  $\text{Na}^+/\text{H}^+$  exchanger or the constituency of the lipid rafts, we used an established detergent-free lipid raft isolation procedure for some of our

studies [11]. Figure 3A shows the protein distribution of the lipid raft fractions. Little protein was found in the earlier fractions (4–5) that contain the lipid rafts (summarized in Fig. 3G) [21–23]. As seen in Fig. 3B,G, caveolin was mainly found in the low density fractions of lanes 4 and 5, at the 5–30% interface. A small amount of caveolin was also found in fractions 6–8. In contrast, Western blotting of the  $\text{Na}^+/\text{H}^+$  exchanger protein (Fig. 3A,G) revealed that it was present in fractions 5–12. Fraction 5 contained a very reproducible large amount of  $\text{Na}^+/\text{H}^+$  exchanger protein. Smaller amounts were found in fractions 6–12. We and others have shown that fractions 8–12 contain noncaveolar membranes including plasma membranes, endoplasmic reticulum, Golgi apparatus and lysosomes [11]. These results showed that the NHE1 protein was found in significant amounts in both the raft and nonraft fractions.

To confirm that the  $\text{Na}^+/\text{H}^+$  exchanger is present in low density membrane fractions we used two procedures. First, we isolated membrane fractions by standard procedures in the presence of Triton X-100. The results are shown in Fig. 3E,F. The  $\text{Na}^+/\text{H}^+$  exchanger was present in the low density fractions (4 and 5) and was again present in the higher density fractions 8–12. The higher density fractions again contained the majority of the total protein present in the membranes (not shown). To confirm that the higher density fractions contained 'nonraft' proteins, we used an antibody against  $\text{Na}^+/\text{K}^+$  ATPase. The results (Fig. 3F) show that  $\text{Na}^+/\text{K}^+$  ATPase is present in the highest density fractions (9–12).

Another experiment was performed to confirm that the  $\text{Na}^+/\text{H}^+$  exchanger was present in detergent resistant low density fractions, typical of 'raft-like' membranes. Low density fractions (fraction 5) isolated using the bicarbonate-based procedure [11] were treated with Triton X-100 to solubilize any non raft-containing membranes. The results are shown in Fig. 3D. The supernatant solubilized with Triton X-100 contained relatively small amounts of  $\text{Na}^+/\text{H}^+$  exchanger. In contrast, the pellet of the Triton X-100 extracted sample did not decrease in amount of  $\text{Na}^+/\text{H}^+$  exchanger protein, and in fact appeared to increase in the relative concentration of  $\text{Na}^+/\text{H}^+$  exchanger present per protein applied. When calculating the total volumes and concentrations of the fractions, together with the relative amount of  $\text{Na}^+/\text{H}^+$  exchanger present in the supernatant and the pellet [19], it was found that over 80% of the  $\text{Na}^+/\text{H}^+$  exchanger remained in the Triton X-100 insoluble fraction.

The  $\text{Na}^+/\text{H}^+$  exchanger has been reported to be linked to the cytoskeleton possibly providing a link between actin binding proteins and the plasma membrane [24]. We used cytochalasin D to disrupt the cytoskeleton to determine if this would affect the distribution of the protein within lipid rafts. Cytochalasin D caused caveolin to be more widely distributed throughout the membrane fractions (Fig. 4A). Cytochalasin D caused a slight reduction in the relative amount of the  $\text{Na}^+/\text{H}^+$  exchanger in fraction 5 and a slight enrichment in the relative amount present in fractions 8–12 though the majority of the NHE1 protein remained unchanged in its distribution (Fig. 4A,B). In a separate experiment, we examined the effect of methyl 2-cyclodextrin on the distribution of the  $\text{Na}^+/\text{H}^+$  exchanger within the membrane fractions. Methyl  $\beta$ -cyclodextrin treatment depletes plasma membrane cholesterol and disrupts low



**Fig. 3. Distribution of caveolin and the Na<sup>+</sup>/H<sup>+</sup> exchanger in membrane subfractions from AP-1 cells transfected with the NHE1 isoform of the Na<sup>+</sup>/H<sup>+</sup> exchanger.** Membranes were fractionated as described in the Materials and methods and separated by SDS/PAGE. Membrane fractions for A–D and G were prepared using a detergent free, sodium carbonate-based procedure [11]. Membranes for E and F were prepared using a detergent-based procedure that contained Triton X-100 [15]. (A) Coomassie blue stain of 30- $\mu$ L samples of fractions. (B) Immunoblotting with anti-caveolin Ig. (C) Immunoblotting with anti-NHE1 (HA-tag) monoclonal antibody. (D) Results of re-extraction of membrane fractions containing the Na<sup>+</sup>/H<sup>+</sup> exchanger (fraction 5) prepared as described for A–C. Membrane fractions were re-extracted with 1% Triton X-100 pelleted and the supernatant and pellets were subjected to SDS/PAGE and immunoblotting as described in the Materials and methods. Lane 1, untreated fraction 5, 5  $\mu$ g; lane 2, 7  $\mu$ g of supernatant of the Triton X-100-treated fraction 5; lane 3, pellet of Triton X-100-treated fraction 5, 4.5  $\mu$ g. (E) Immunoblotting with anti-NHE1 (HA-tag) monoclonal antibody of membranes prepared in the presence of Triton X-100. F, Immunoblotting with anti-(Na<sup>+</sup>/K<sup>+</sup> ATPase) Ig of membranes prepared in the presence of Triton X-100. (G) Summary of Na<sup>+</sup>/H<sup>+</sup> exchanger and caveolin distribution in membrane fractions from AP-1 cells transfected with the NHE1 isoform of the Na<sup>+</sup>/H<sup>+</sup> exchanger. Membrane fractions were prepared and visualized as described in Fig. 3A–C. The relative distribution of proteins in the gels and Western blots was analyzed as described earlier [19]. Results are typical of at least three independent determinations.

density lipid rafts [13,14]. This treatment changed the distribution of the Na<sup>+</sup>/H<sup>+</sup> exchanger and caveolin. The Na<sup>+</sup>/H<sup>+</sup> exchanger (Fig. 4C) was almost absent from fraction 5 and was present in much greater amounts in fractions 6 and 7 and in fraction 12. The distribution of caveolin was greatly changed. It was almost absent from fractions 4 and 5 and was spread throughout the other fractions, with the greatest amount being in fraction 12.

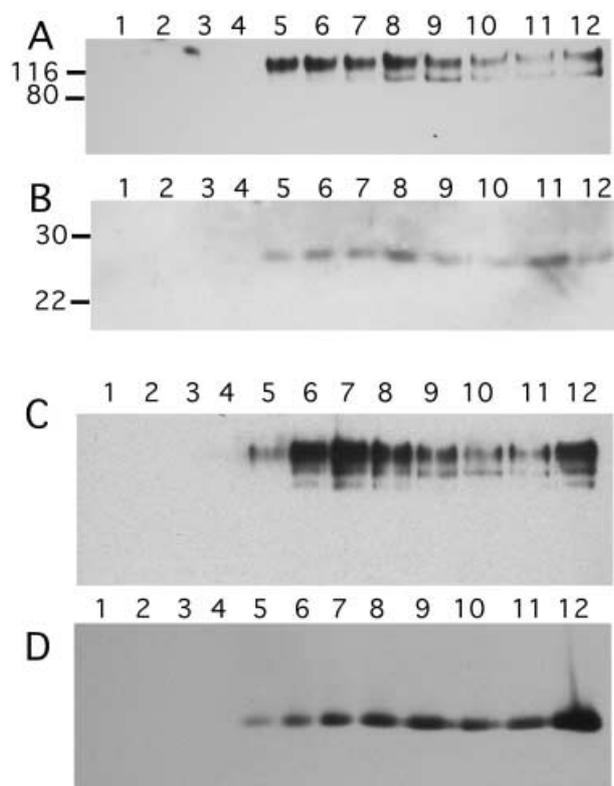
As an alternative method of examining NHE1 colocalization with lipid rafts we used immunocytochemical staining of NHE1 in combination with cholera toxin staining of GM1 gangliosides. Figure 5A illustrates immunocytochemical staining of the Na<sup>+</sup>/H<sup>+</sup> exchanger and Fig. 5B illustrates cholera toxin staining of the GM1 gangliosides. The combined image (Fig. 5C) illustrates in yellow where cholera toxin and the Na<sup>+</sup>/H<sup>+</sup> exchanger distribution overlap. It demonstrates that there are clearly significant

areas of overlap though there are definitely areas that do not overlap. These results demonstrate that there are some areas of colocalization plus other areas that clearly show no colocalization.

To determine whether rafts were essential to NHE1 activity we used cyclodextrin to deplete rafts of cholesterol and disrupt them as described earlier [13,14]. Figure 6 shows that treatment with methyl  $\beta$ -cyclodextrin did not impair NHE1 activity, and a slight, significant stimulation of Na<sup>+</sup>/H<sup>+</sup> exchanger activity was noted.

## DISCUSSION

The NHE1 isoform of the Na<sup>+</sup>/H<sup>+</sup> exchanger is a low abundance plasma membrane protein responsible for pH regulation in a variety of mammalian cell types [2,25]. While numerous studies have characterized activity and regulation



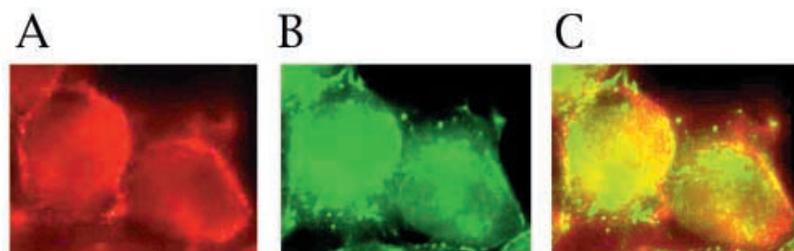
**Fig. 4.** Distribution of caveolin and the  $\text{Na}^+/\text{H}^+$  exchanger in membrane subfractions from AP-1 cells after various treatments. Membranes were fractionated as described for Fig. 3A–C. Membrane fractions used in A and B were from cells treated with cytochalasin D as described in the Materials and methods. (A) Immunoblotting with anti-NHE1 (HA-tag) monoclonal antibody of fractions from cytochalasin D-treated cells. (B) Immunoblotting with anti-caveolin Ig of membrane fractions from cytochalasin D-treated cells. Membrane fractions used in C and D were from cells treated with methyl  $\beta$ -cyclodextrin as described in the Materials and methods. (C) Immunoblotting with anti-NHE1 (HA-tag) monoclonal antibody of fractions from methyl  $\beta$ -cyclodextrin-treated cells. (D) Immunoblotting with anti-caveolin Ig of membrane fractions from methyl  $\beta$ -cyclodextrin-treated cells.

of the  $\text{Na}^+/\text{H}^+$  exchanger, biochemical characterization of the properties of the full length protein has proven difficult because of its low abundance and because of the difficulty in overexpressing integral membrane proteins. In this study, we examined the behavior of the protein expressed in mammalian cells to gain insights into its character and to

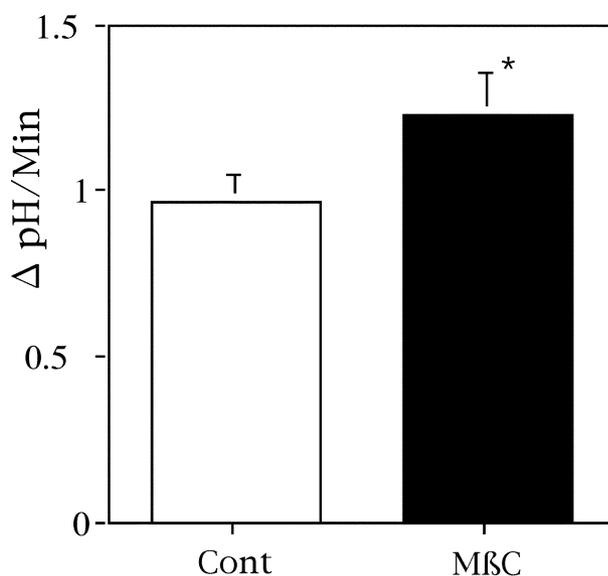
optimize future analysis of the protein. Our initial experiments demonstrated that the endogenous NHE1 protein of both heart and kidney aggregated in highly denaturing SDS containing buffer. Incubation at elevated temperatures dramatically decreased the amount of protein that was detectable by Western blot analysis. The same phenomenon occurred with immunoprecipitated protein expressed in the Chinese hamster ovary (CHO) cell line, AP-1 cells (Figs 1 and 2). The effect was not altered by changes in 2-mercaptoethanol concentration or by elevation of detergent concentrations. It was clear that this temperature-dependent effect was inducing aggregation of the protein because aggregates could often be seen on the top of the SDS/PAGE gels depending on the conditions of electrophoresis (Fig. 2). The effect was not due to overexpression of the  $\text{Na}^+/\text{H}^+$  exchanger protein because we also found that it occurred with the endogenous protein (Fig. 1) that is present in low levels in the heart and kidney.

SDS-resistant protein aggregation has been demonstrated in a variety of proteins including for the vesicular monoamine transporter [26], for the *E. coli* inner membrane glycerol facilitator [27] and for human testis-enhanced gene transcript [28]. It has been suggested that proteins susceptible to SDS-resistant aggregation retain a significant level of structure in the presence of SDS [26]. Several membrane proteins such as bacteriorhodopsin [29] can maintain a substantial amount of their structure even in the presence of SDS. Our results suggest that the  $\text{Na}^+/\text{H}^+$  exchanger may belong to this category of membrane proteins. It has been shown that heat-induced aggregation of membrane proteins may occur more in some cases when there is a relatively higher concentration of protein [28]. However, we found that the aggregation behavior of the  $\text{Na}^+/\text{H}^+$  exchanger occurred for both the more concentrated, immunoprecipitated protein and for the unpurified endogenous  $\text{Na}^+/\text{H}^+$  exchanger protein in crude membrane preparations. Because of the key role the protein plays in calcium overload, several studies have recently examined expression of the  $\text{Na}^+/\text{H}^+$  exchanger in development and in various pathophysiological states [30–32]. Our results suggest that treatment of samples proteins at inappropriate conditions could easily distort detection of the protein or prevent its detection altogether in some cases.

Temperature-induced aggregation of proteins in SDS occurs in membrane proteins. It is suggested that this aggregation is usually the result of their hydrophobicity [28,33]. The  $\text{Na}^+/\text{H}^+$  exchanger is predicted to have 12 transmembrane segments and one membrane-associated



**Fig. 5.** Colocalization of the  $\text{Na}^+/\text{H}^+$  exchanger with GM1 ganglioside in AP-1 cells. AP-1 cells were immunostained with anti-HA (tag) Ig for the  $\text{Na}^+/\text{H}^+$  exchanger and with cholera toxin for staining of GM1 ganglioside. (A) Immunostaining of  $\text{Na}^+/\text{H}^+$  exchanger. (B) Cholera toxin staining of GM1 ganglioside. (C) Colocalization of the  $\text{Na}^+/\text{H}^+$  exchanger and GM1 ganglioside.



**Fig. 6.** Effect of treatment with methyl  $\beta$ -cyclodextrin on the rate of Na<sup>+</sup>/H<sup>+</sup> exchanger recovery from an acute acid load. Cells were treated with 10 mM methyl  $\beta$ -cyclodextrin or 'mock' treated and Na<sup>+</sup>/H<sup>+</sup> exchanger activity was measured after transient induction with an acid load as described in the Materials and methods. \*Significantly different from control at  $P < 0.05$ .

segment [1,4]. This large amount of hydrophobic amino acids is probably responsible for the aggregation behavior. It is not clear at the present time whether the aggregation is homotypic or heterotypic. It is of note that we often found several forms of the Na<sup>+</sup>/H<sup>+</sup> exchanger of slightly different apparent mobility. This could suggest association with other different proteins including accessory proteins.

We [6] and others [7] have shown that the Na<sup>+</sup>/H<sup>+</sup> exchanger is localized to specific foci in some cell types. Because of this property and because of the large amount of functional hydrophobic residues present in the protein, we examined whether the protein was present in caveolin-enriched, low density membrane fractions. Various membrane proteins are specifically targeted to these fractions, and often this can involve an increase in their hydrophobicity by addition of lipid anchors such as myristate or palmitate. These membrane subdomains are reported to be enriched in glycosphingolipids, free cholesterol, saturated phospholipids and some specific proteins [34,35]. The function of such subdomains of the membrane proteins may be to aid in targeting, and possibly to aid in signaling by localizing specific receptors [23]. Several lines of evidence showed that the Na<sup>+</sup>/H<sup>+</sup> exchanger was present in these low density membrane fractions. First, we showed that it colocalized with caveolin in the cholesterol enriched fractions (Fig. 3). The Na<sup>+</sup>/H<sup>+</sup> exchanger was found in low density fractions that were isolated by two independent techniques. Low density fractions were made by either detergent-free [11] or detergent-containing techniques [15,16]. In addition, we confirmed that the low density Na<sup>+</sup>/H<sup>+</sup> exchanger containing fractions isolated by detergent-free techniques were resistant to extraction by Triton X-100 (Fig. 3D). As another independent method of determining the Na<sup>+</sup>/H<sup>+</sup> exchanger localization we used

immunocytochemistry. We found that the Na<sup>+</sup>/H<sup>+</sup> exchanger colocalized with GM1 as indicated by cholera toxin staining (Fig. 5). While there was clearly a colocalization with these low density fractions, there was also clearly a significant fraction of the Na<sup>+</sup>/H<sup>+</sup> exchanger protein that did not colocalize with these fractions. This was evident by both subcellular fractionation and immunocytochemistry. Recently, the NHE3 isoform of the Na<sup>+</sup>/H<sup>+</sup> exchanger has been shown to be present in both raft and nonraft membrane fractions [36]. Our results show that the NHE1 isoform also has a similar distribution in these membrane fractions. While the bulk of the Na<sup>+</sup>/H<sup>+</sup> exchanger was generally found in low density fractions, significant amounts were present in fractions of higher density.

We also examined whether an intact cytoskeleton was significant in localization of NHE1 to the low density lipid fractions. Cells were treated with cytochalasin D to disrupt the cytoskeleton and to disrupt interactions that have recently been suggested to occur between NHE1 and the cytoskeleton [37]. Subcellular fractionation showed that NHE1 was still contained within both low and high density membranes. Surprisingly, the distribution of NHE1 did not vary greatly suggesting that an intact cytoskeleton was not an important factor in its localization to lipid fractions. These results suggest that the localization of NHE1 is probably due to an intrinsic property of the membrane region of the protein and its interaction with surrounding lipids.

To examine if specific membrane localization is required for NHE1 activity we depleted cholesterol using methyl  $\beta$ -cyclodextrin treatment. Figure 4D shows that cholesterol depletion altered the membrane distribution of the Na<sup>+</sup>/H<sup>+</sup> exchanger and caveolin, confirming that methyl  $\beta$ -cyclodextrin was active. Surprisingly, we found that this treatment slightly stimulated NHE1 activity and was not inhibitory. We have recently shown that the Na<sup>+</sup>/H<sup>+</sup> exchanger is regulated by MEK-dependent (mitogen-activated protein kinase/extracellular signal-regulated kinase kinase-dependent) specific signaling and that the cytoplasmic domain is phosphorylated by ERK (extracellular signal-related protein kinase) [10,38]. A recent report [39] has also shown depletion of cholesterol from the plasma membrane by methyl  $\beta$ -cyclodextrin results in activation of mitogen-activated protein kinase pathways including activation of ERK. Thus it is possible that the activation of NHE1 we see is due to activation through ERK-dependent phosphorylation. Further experiments are necessary to confirm the mechanism of this slight stimulation of activity, though it is clear that there is no inhibitory effect of methyl  $\beta$ -cyclodextrin treatment on NHE1 activity.

In summary, our results demonstrate both the previously uncharacterized membrane behavior of the Na<sup>+</sup>/H<sup>+</sup> exchanger and its location within membranes. We show that this hydrophobic protein is sensitive to temperature induced aggregation and suggest that lower temperatures of incubation for SDS/PAGE will improve detection and characterization of expression of this low abundance protein. We also demonstrate that the Na<sup>+</sup>/H<sup>+</sup> exchanger is also present in relatively large amounts low density membrane fractions. The results are the first that illustrate that the NHE1 isoform of the Na<sup>+</sup>/H<sup>+</sup> exchanger is present in low density microdomains in the plasma membrane.

## ACKNOWLEDGMENTS

This work was supported by a grant from the Canadian Institute of Health Research to LF. LF is a Scientist of the Alberta Heritage Foundation for Medical Research.

## REFERENCES

- Sardet, C., Franchi, A. & Pouyssegur, J. (1989) Molecular cloning, primary structure, and expression of the human growth factor-activatable Na<sup>+</sup>/H<sup>+</sup> antiporter. *Cell* **56**, 271–280.
- Counillon, L. & Pouyssegur, J. (2000) The expanding family of eukaryotic Na<sup>+</sup>/H<sup>+</sup> exchangers. *J. Biol. Chem.* **275**, 1–4.
- Sardet, C., Counillon, L., Franchi, A. & Pouyssegur, J. (1990) Growth factors induce phosphorylation of the Na<sup>+</sup>/H<sup>+</sup> antiporter, glycoprotein of 110 kD. *Science* **247**, 723–726.
- Wakabayashi, S., Pang, T., Su, X. & Shigekawa, M. (2000) A novel topology model of the human Na<sup>+</sup>/H<sup>+</sup> exchanger isoform 1. *J. Biol. Chem.* **275**, 7942–7949.
- Fafournoux, P., Noel, J. & Pouyssegur, J. (1994) Evidence that Na<sup>+</sup>/H<sup>+</sup> exchanger isoforms NHE1 and NHE3 exist as stable dimers in membranes with a high degree of specificity for homodimers. *J. Biol. Chem.* **269**, 2589–2596.
- Silva, N.L.C.L., Haworth, R.S., Singh, D. & Fliegel, L. (1995) The carboxyl-terminal region of the Na<sup>+</sup>/H<sup>+</sup> exchanger interacts with mammalian heat shock protein. *Biochemistry* **34**, 10412–10420.
- Grinstein, S., Woodside, M., Waddell, T.K., Downey, G.P., Orlowski, J., Pouyssegur, J., Wong, D.C. & Foskett, J.K. (1993) Focal localization of the NHE-1 isoform of the Na<sup>+</sup>/H<sup>+</sup> antiporter: assessment of effects on intracellular pH. *EMBO J.* **12**, 5209–5218.
- Barry, D.M., Trimmer, J.S., Merlie, J.P. & Nerbonne, J.M. (1995) Differential expression of voltage-gated K<sup>+</sup> channel subunits in adult rat heart. Relation to functional K<sup>+</sup> channels? *Circ. Res.* **77**, 361–369.
- Wang, H., Singh, D. & Fliegel, L. (1998) Functional role of cysteine residues in the Na<sup>+</sup>/H<sup>+</sup> exchanger. *Arch. Biochem. Biophys.* **358**, 116–124.
- Moor, A.N. & Fliegel, L. (1999) Protein kinase mediated regulation of the Na<sup>+</sup>/H<sup>+</sup> exchanger in the rat myocardium by MAP-kinase-dependent pathways. *J. Biol. Chem.* **274**, 22985–22992.
- Song, K.S., Li, S., Okamoto, T., Quilliam, L.A., Sargiacomo, M. & Lisanti, M.P. (1996) Co-purification and direct interaction of Ras with caveolin, an integral membrane protein of caveolae microdomains. *J. Biol. Chem.* **271**, 9690–9697.
- Ricci, V., Galimiche, A., Doye, A., Necchi, V., Solcia, E. & Boquet, P. (2000) High cell sensitivity to *Helicobacter pylori* VacA toxin depends on a GPI-anchored protein and is not blocked by inhibition of the clathrin-mediated pathway of endocytosis. *Mol. Biol. Cell* **11**, 3897–3909.
- Manes, S., Mira, E., Gomez-Mouton, C., Lacalle, R.A., Keller, P., Labrador, J.P., Martinez, A. & C. (1999) Membrane raft microdomains mediate front-rear polarity in migrating cells. *EMBO J.* **18**, 6211–6220.
- Keller, P. & Simons, K. (1998) Cholesterol is required for surface transport of influenza virus hemagglutinin. *J. Cell. Biol.* **140**, 1357–1367.
- McCabe, J.B. & Berthiaume, L.G. (2001) N-terminal protein acylation confers localization to cholesterol, sphingolipid-enriched membranes but not to lipid rafts/caveolae. *Mol. Biol. Cell.* **12**, 3601–3617.
- Navarrete Santos, A., Roentsch, J., Danielsen, E.M., Langner, J. & Riemann, D. (2000) Aminopeptidase N/CD13 is associated with raft membrane microdomains in monocytes. *Biochem. Biophys. Res. Commun.* **269**, 143–148.
- Scheiffele, P., Rietveld, A., Wilk, T. & Simons, K. (1999) Influenza viruses select ordered lipid domains during budding from the plasma membrane. *J. Biol. Chem.* **274**, 2038–2044.
- Wang, H., Singh, D. & Fliegel, L. (1997) The Na<sup>+</sup>/H<sup>+</sup> antiporter potentiates growth and retinoic acid induced differentiation of P19 embryonal carcinoma cells. *J. Biol. Chem.* **272**, 26545–26549.
- Rieder, C.V. & Fliegel, L. (2002) Developmental regulation of Na<sup>+</sup>/H<sup>+</sup> exchanger expression in the fetal and neonatal mouse. *Am. J. Physiol.* **283**, H273–H283.
- Bertrand, B., Wakabayashi, S., Ikeda, T., Pouyssegur, J. & Shigekawa, M. (1994) The Na<sup>+</sup>/H<sup>+</sup> exchanger isoform 1 (NHE1) is a novel member of the calmodulin-binding proteins. *J. Biol. Chem.* **269**, 13703–13709.
- Anderson, R.G.W. (1998) The caveolae membrane system. *Annu. Rev. Biochem.* **67**, 199–225.
- Brown, D.A. & London, E. (1998) Functions of lipid rafts in biological membranes. *Annu. Rev. Cell Dev. Biol.* **14**, 111–136.
- Simons, K. & Ikonen, E. (1997) Functional rafts in cell membranes. *Nature* **387**, 569–572.
- Denker, S.P., Huang, D.C., Orlowski, J., Furthmayr, H. & Barber, D.L. (2000) Direct binding of the Na-H exchanger NHE1 to ERM proteins regulates the cortical cytoskeleton and cell shape independently of H(+) translocation. *Mol. Cell.* **6**, 1425–1436.
- Fliegel, L. (1999) Functional and cellular regulation of the myocardial Na<sup>+</sup>/H<sup>+</sup> exchanger. *J. Thrombosis Thrombolysis* **8**, 9–14.
- Sagne, C., Isambert, M.-F., Henry, J.-P. & Gasnier, B. (1996) SDS-Resistant aggregation of membrane protein: application to the purification of the vesicular monoamine transporter. *Biochem. J.* **316**, 825–831.
- Manley, D.M., McComb, M.E., Perreault, H., Donald, L.J., Duckworth, H.W. & O'Neil, J.D. (2000) Secondary structure and oligomerization of the *E. coli* glycerol facilitator. *Biochemistry* **39**, 12303–12311.
- Cowling, R.T. & Birnboim, H.C. (1998) Preliminary characterization of the protein encoded by human testis-enhanced gene transcript. *Mol Membrane Biol.* **15**, 177–187.
- Huang, K.S., Bayley, H., Liao, M.J., London, E. & Khorana, H.G. (1981) Refolding of an integral membrane protein. Denaturation, renaturation, and reconstitution of intact bacteriorhodopsin and two proteolytic fragments. *J. Biol. Chem.* **256**, 3802–3809.
- Douglas, R.M., Schmitt, B.M., Xia, Y., Bevenssee, M.O., Biemesderfer, D., Boron, W.F. & Haddad, G.G. (2001) Sodium-hydrogen exchangers and sodium-bicarbonate co-transporters: ontogeny of protein expression in the rat brain. *Neuroscience* **102**, 271–228.
- Sandmann, S., Yu M., Kaschina, E., Blume, A., Bouzinova, E., Aalkjaer, C. & Unger, T. (2001) Differential effects of angiotensin AT1 and AT2 receptors on the expression, translation and function of the Na<sup>+</sup>-H<sup>+</sup> exchanger and Na<sup>+</sup>-HCO<sub>3</sub><sup>-</sup> symporter in the rat heart after myocardial infarction. *J. Am. Coll. Cardiol.* **37**, 2154–2165.
- Gan, X.T., Chakrabarti, S. & Karmazyn, M. (1999) Modulation of Na<sup>+</sup>/H<sup>+</sup> exchange isoform 1 mRNA expression in isolated rat hearts. *Am. J. Physiol.* **277**, H993–H998.
- Hyman, M.R. & Arp, D.J. (1993) An electrophoretic study of the thermal- and reductant-dependent aggregation of the 27 kDa component of ammonia monooxygenase from *Nitrosomonas europaea*. *Electrophoresis* **14**, 619–627.
- Harder, T. & Simons, K. (1997) Caveolae, DIGs, and the dynamics of sphingolipid-cholesterol microdomains. *Curr. Opin. Cell Biol.* **9**, 534–542.
- Fridriksson, E.K., Shipkova, P.A., Sheets, E.D., Holowka, D., Baird, B. & McLafferty, F.W. (1999) Quantitative analysis of phospholipids in functionally important membrane domains from RBL-2H3 mast cells using tandem high-resolution mass spectrometry. *Biochemistry* **38**, 8056–8063.
- Li, X., Galli, T., Leu, S., Wade, J.B., Weinman, E.J., Leung, G., Cheong, A., Louvard, D. & Donowitz, M. (2001) Na<sup>+</sup>-H<sup>+</sup> exchanger 3 (NHE3) is present in lipid rafts in the rabbit ileal

- brush border: a role for rafts in trafficking and rapid stimulation of NHE3. *J. Physiol.* **537**, 537–552.
37. Denker, S.P., Huang, D.C., Orlowski, J., Furthmayr, H. & Barber, D.L. (2000) Direct binding of the Na-H exchanger NHE1 to ERM proteins regulates the cortical cytoskeleton and cell shape independently of H<sup>+</sup> translocation. *Mol Cell* **8**, 1425–1436.
38. Moor, A., Xiaohong, T.G., Karmazyn, M. & Fliegel, L. (2001) Protein kinase mediated regulation of the Na<sup>+</sup>/H<sup>+</sup> exchanger isoform 1 (NHE1) in ischemic and ischemic-reperfused Rat Heart. *J. Biol. Chem.* **27**, 16113–16122.
39. Chen, X. & Resh, M.D. (2001) Activation of mitogen-activated protein kinase by membrane-targeted Raf chimeras is independent of raft localization. *J. Biol. Chem.* **276**, 34617–34623.