The Carboxyl-Terminal Region of the Na^+/H^+ Exchanger Interacts with Mammalian Heat Shock Protein†

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ABSTRACT: We expressed the carboxyl-terminal 178 amino acids of the rabbit cardiac Na^+/H^+ exchanger as a fusion protein with glutathione-S-transferase. The fusion protein (PCR178) was found in the supernatant of extracts of *E. coli* and was purified using Glutathione-Sepharose affinity chromatography. Affinity-purified antibodies raised against the carboxyl-terminal region of the Na^+/H^+ exchanger identified the resultant protein. PCR178 copurified with a 70 kDa protein. Amino-terminal sequencing of the 70 kDa protein identified it as dnaK, the bacterial equivalent of the mammalian 70 kDa heat shock protein (hsp70). DnaK was dissociated from the Na^+/H^+ exchanger fusion protein by the addition of MgATP. When purified PCR178 was coupled to a cyanogen bromide-activated Sepharose column, bovine hsp70 bound to the column and was eluted with MgATP. Nondenaturing polyacrylamide gel electrophoresis showed that, in the absence of MgATP, hsp70 formed a complex with PCR178. The complex was dissociated by the addition of MgATP. GST alone did not form a complex with hsp70. Immunoprecipitation of the Na^+/H^+ exchanger with antiexchanger antibodies resulted in coprecipitation of hsp70 protein from antiporter containing cells. Cells that overexpress the Na^+/H^+ exchanger had increased amounts of hsp70 which coprecipitated with antiexchanger antibody. The results show that heat shock protein complexes with the mammalian Na^+/H^+ exchanger.

The Na^+/H^+ exchanger is an almost universally distributed protein in mammalian cells. It has been implicated in a number of cellular functions, including pH regulation, cell volume regulation and cell proliferation [for review, see Grinstein et al. (1989)]. There are several tissue-specific isoforms of the exchanger (NHE1-NHE4), which vary in molecular weight and in their sensitivity to the inhibitor amiloride [reviewed in Fliegel and Frohlich (1993)]. The amiloride-sensitive isoform (NHE1) is inhibited by nanomolar concentrations of amiloride analogs such as ethylisopropylamiloride. It extrudes one proton in exchange for one sodium ion when decreases in intracellular pH occur. The amino acid sequence of NHE1 has been deduced from the nucleotide sequence of a cDNA clone (Sardet et al., 1989). The protein is predicted to have a molecular mass of 91 kDa (Sardet et al., 1990) and is a glycoprotein with N- and O-linked glycosylation sites (Haworth et al., 1993; Counillon et al., 1994).

The cDNA encoding the NHE1 isoform of the Na^+/H^+ exchanger has been cloned from a number of different mammalian species and tissues (Sardet et al., 1989; Fliegel et al., 1991; Hildebrandt et al., 1991; Reilly et al., 1991; Tse et al., 1991; Takaichi et al., 1992). The predicted sequence of the protein is highly conserved. The NHE1 isoform is often referred to as the ubiquitous or "housekeeping" form (NHE1) and appears to exist in the plasma membrane of most cells. NHE1 is found in the basolateral membrane of most epithelial cells, and amiloride-resistant isoforms are usually found in brush border membranes. The human placenta appears to vary from this norm, carrying the NHE1 isoform of the exchanger in relatively enriched amounts in the brush border membrane (Balkovetz et al., 1986; Kulanthaivel et al., 1990; Fliegel et al., 1993).

A great deal of effort has been made to determine the mechanisms of regulation of this essential protein. Such studies have generally been done with intact cells, where a number of different modulators of cellular activity have been shown to affect the activity of the Na^+/H^+ exchanger (Grinstein et al., 1989). These changes are thought to be caused by phosphorylation of the exchanger, and, indeed, phosphorylation has been shown to occur *in vivo* (Bianchini et al., 1991; Livine et al., 1991; Sardet et al., 1991). The large carboxyl-terminal domain of the protein is the site of such regulation, being cytoplasmic and therefore accessible to the action of intracellular kinases (Sardet et al., 1989; Wakabayashi et al., 1992). Residues 567−635 of the cytoplasmic domain are required for growth factor activation of the protein (Wakabayashi et al., 1994a).

Another mechanism by which proteins can be regulated is through interactions with other proteins. The renal brush border membrane Na^+/H^+ exchanger interacts with (or is regulated by) a 42−43 kDa protein (Weinman et al., 1988; Morell et al., 1990). This suggests that at least one isoform of the Na^+/H^+ exchanger can interact with putative regulatory proteins. Wakabayashi et al. (1992) also suggested that the cytoplasmic domain of the NHE1 isoform could interact with cytoskeletal proteins. More recent evidence has shown that the cytoplasmic domain of the NHE1 isoform of the antiporter can interact with calmodulin in a calcium dependent manner (Bertrand et al., 1994; Wakabayashi et al., 1994b). In addition, it has recently been demonstrated by

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ourselves (Fliegel et al., 1993) and others (Fafournoux et al., 1994) that the Na^+/H^+ exchanger is a dimer and is therefore capable of intermolecular protein−protein interaction with itself.

The heat shock proteins are known to interact with and act as chaperones for a variety of mammalian proteins (Schlesinger et al., 1990; Craig & Gross, 1991). The 70 kDa family of heat shock proteins (hsp70)[1] has been implicated in posttranslational protein folding and translocation (Beckman et al., 1990; Kang et al., 1990). The proteins in this family are related in both structure and in their ability to bind and hydrolyze ATP (Beckman et al., 1990; Kang et al., 1990; Schlesinger et al., 1990; Craig & Gross, 1991). The heat shock proteins are very highly conserved among widely divergent species. The major mammalian heat shock protein, hsp70, has conserved domains with over 96% homology to *E. coli* dnaK and an overall homology of about 50% (Schlesinger et al., 1990). Members of this group have been found in a variety of cellular locations, including the endoplasmic reticulum, the nucleus, and the cytoplasm (Ellis & Van der Vies, 1991). In this study we present evidence that hsp70 can interact with the carboxyl-terminal cytoplasmic region of the NHE-1 isoform of the Na^+/H^+ exchanger. We demonstrate that the cytoplasmic region of the protein interacts with both dnaK and hsp70 in an ATP-dependent manner. In addition, we show that antibodies against the Na^+/H^+ exchanger coimmunoprecipitate hsp70 from intact cells. The results show that hsp70 interacts with the cytoplasmic domain of the Na^+/H^+ exchanger.

MATERIALS AND METHODS

Materials. Bovine brain hsp72/73 and anti-hsp72/73 mouse monoclonal antibody were from StressGen Biotechnologies Corp., Victoria, B.C. The hsp72/73 consists primarily of constitutive hsp72 and smaller amounts of inducible hsp 73. The heat shock proteins will subsequently be referred to as hsp70. Cyanogen bromide-activated Sepharose 4B, plasmid pGEX-3X, and the Glutathione Sepharose 4B affinity column were from Pharmacia LKB. Horseradish peroxidase-conjugated goat anti-rabbit and goat anti-mouse IgG and goat anti-mouse IgG were obtained from Bio/Can (Mississauga, ON). Sodium dodecyl sulfate−polyacrylamide gels were prepared with reagents from Bio-Rad or Boehringer Mannheim. Detergent compatible protein assay kit and prestained SDS−PAGE standards were from Bio-Rad Laboratories. Nitrocellulose membranes were from Schleicher and Schuell (Keene, NH). Protein A insoluble extract from *Staphylococcus aureus* was obtained from Sigma. All other chemicals were obtained commercially and were of the highest available grade.

Preparation of Purified Membranes. Human placental syncytiotrophoblast brush border membrane vesicles were prepared by the method of Balkovetz et al. (1986) from normal term human placenta, essentially as described earlier (Fliegel et al., 1993; Haworth et al., 1993).

Polyacrylamide Gels and Immunostaining. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) was on 9% polyacrylamide gels as described earlier (Fliegel et al., 1992). Gels were silver stained utilizing the Bio-Rad Silver Stain kit, as described by the manufacturer, or were stained with Coomassie blue. For immunostaining, proteins were transferred electrophoretically onto nitrocellulose membranes. Immunostaining of nitrocellulose membranes was carried out in the presence of 1% skim milk powder using affinity-purified antibodies essentially as described earlier (Fliegel et al., 1992) or by using the Amersham Enhanced Chemiluminescence Western Blotting and Detection System as described by the manufacturer. All antibodies against the Na^+/H^+ exchanger were against a carboxyl-terminal fusion protein or against a synthetic peptide consisting of the carboxyl-terminal 13 amino acids. Antibodies were affinity-purified before use as described earlier (Fliegel et al., 1992, 1993).

Preparation of Glutathione-S-transferase-Na^+/H^+ Exchanger Fusion Protein. For production of a carboxyl-terminal Na^+/H^+ exchanger fusion protein, we expressed a portion of the rabbit Na^+/H^+ exchanger cDNA clone. cDNA of the carboxyl-terminal 178 amino acids of the rabbit cardiac Na^+/H^+ exchanger was prepared by polymerase chain reaction (PCR), using the primers 5′ GCG GAT CCT GCA GAA GAC CCG GCA GCG GCT 3′ and 5′ AAG AAT TCT ACT GCCC (C/T)TT GGG GA(A/T) GAA, designed from bp 510−533 and 1027−1047 of the rabbit cardiac Na^+/H^+ exchanger clone described earlier (Fliegel et al., 1991). The redundancies present in the second primer are for use with the human Na^+/H^+ exchanger cDNA. The fragment generated was cloned into the *Bam*H1−*Eco*R1 sites of expression plasmid pGEX-3X. The glutathione-S-transferase-Na^+/H^+ exchanger fusion protein (PCR178) was expressed in *Escherichia coli* DH5-α, after induction with 0.2 mM IPTG. Cells were lysed with a French-press and the supernatant fraction (10000*g* × 10 min) containing PCR178 was analyzed by SDS−PAGE as described above. The separated proteins were transferred to nitrocellulose, and the fusion protein was identified using an affinity-purified antibody raised against a synthetic peptide consisting of the carboxyl-terminal 13 amino acids of the rabbit cardiac Na^+/H^+ exchanger [described in Fliegel et al. (1992)].

Purification of PCR178. Purification of PCR 178 was via glutathione Sepharose 4B affinity chromatography. All steps were performed at 4 °C in the presence of a cocktail of protease inhibitors (Michalak et al., 1990). Samples were dialyzed against phosphate buffered saline (PBS) containing 1% Triton X-100 and protease inhibitors and were applied to the column at least twice. The column was washed with several bed volumes of PBS, and the samples were eluted with 5 mM glutathione. On some occasions, the column was prewashed with several volumes of buffer containing 3 mM MgATP before elution with glutathione. Protein electroblotted to Immobilon polyvinylidene difluoride membrane was used for amino-terminal sequence analysis (Matsudaira et al., 1987).

Preparation of PCR178 Affinity Column. A PCR178-affinity column was prepared by linking PCR178 to CNBr-activated Sepharose 4B. PCR178 was first purified as described above. Further purification of PCR178 was achieved using preparative SDS−PAGE with a Bio-Rad Prep Cell model 491. A 6 cm 9% acrylamide separating gel was

[1] Abbreviations: GST, glutathione-S-transferase; PCR178, GST fusion protein with carboxyl-terminal 178 amino acids from human Na^+/H^+ exchanger; hsp70, mammalian 70 kDa heat shock protein; dnaK, *E. coli* 70 kDa heat shock protein; PCR, polymerase chain reaction; PBS, phosphate buffered saline; IPTG, isopropyl β-D-thiogalactopyranoside.

used, with the same composition as that described above. Fractions containing PCR178 were dialyzed overnight against 0.1 M $NaHCO_3$ and 0.5 M NaCl, pH 8.3. Purified protein (300 μg) was coupled to CNBr-activated Sepharose 4B (400 mg) using the procedure described by the manufacturer. Unused sites were blocked after coupling.

Interaction of hsp70 with PCR178 by Column Chromatography. The PCR178 affinity column was washed with 50 mL of buffer containing 50 mM Tris, pH 8.0 (flow rate 0.5 mL/min). Bovine brain hsp70 (10 mg in 4 mL of buffer) was loaded onto the column, which was then washed with buffer (20 mL), followed by buffer containing 3 mM $MgCl_2$ (10 mL). Bound hsp70 was eluted from the column with buffer (50 mL) containing 3 mM MgATP. Fractions (4 mL) were collected throughout and assayed for hsp70 by dot blotting onto nitrocellulose followed by immunoblotting, essentially as described above, using anti-hsp70 antibody and peroxidase-conjugated goat anti-mouse antibody. Immunoreactive proteins were visualized using the Amersham Enhanced Chemiluminescence kit, as described by the manufacturer.

Interaction of hsp70 with PCR178 by Nondenaturing Polyacrylamide Gel Electrophoresis. Column-purified PCR178 (1 mg) and GST (1 mg) were incubated with bovine hsp70 (10 mg) for 5 min at room temperature (total volume 15 mL). MgATP (final concentration 3 mM) was added to some samples, and incubation continued for a further 2 min. The samples were analyzed by nondenaturing gel electrophoresis. Sample buffer (5×) contained 50% glycerol, 0.05% bromophenol blue, and 312.5 mM Tris, pH 6.8. The separating gel contained 9% acrylamide, 1% ammonium persulfate, and 375 mM Tris, pH 8.8. The stacking gel contained 5% acrylamide, 1% ammonium persulfate, and 125 mM Tris, pH 6.8. Polymerization was initiated by the addition of 0.1% *N,N,N′,N′*-tetramethylethylenediamine. The electrophoresis buffer contained 192 mM glycine and 25 mM Tris, pH 8.8.

Cell Culture and Immunoprecipitation of hsp70 and NHE1. PS127A fibroblasts overexpressing the Na^+/H^+ exchanger were a generous gift of Dr. B. Berk (University of Seattle) and Dr. J. Pouyssegur (University de Nice). Na^+/H^+ exchanger deficient AP-1 cells were a generous gift of Dr. S. Grinstein (University of Toronto). PS127A and AP-1 cells were maintained in Dulbecco's modified Eagle's medium D-MEM or α-MEM, respectively, supplemented with 10% fetal bovine serum, penicillin, and streptomycin as described earlier (Sardet et al., 1990). PS127A cells underwent regular ammonium chloride selection to ensure expression of the Na^+/H^+ exchanger. Examination of Na^+/H^+ exchanger activity using intracellular pH measurements with 2′,7′-bis (2-carboxyethyl)- 5(6)-carboxyfluorescein (BCECF) confirmed the presence of a Na^+/H^+ exchanger in PS127A cells and its absence or very low level of activity in AP-1 cells (not shown). Cells were harvested from a 100 mm culture flask using trypsin EDTA solution and washed two times with PBS buffer. Cells were resuspended in STE buffer (20 mM Tris, pH 7.8, 1 mM EDTA, 150 mM NaCl) and sonicated at 5 W for 40 s. The cellular extract was centrifuged at 100000*g* for 30 min. The membrane proteins were extracted by resuspending the pellet in IPB (20 mM Tris, pH 7.8, 1 mM EDTA, 650 mM NaCl, 0.5% NP40, 0.1% azide, 0.1% SDS) containing an additional 2% SDS. The membrane−detergent mixture was incubated at 37 °C for 30 min and then centrifuged at 100000*g* for 30 min. The solubilized membrane proteins were diluted 100× with STE buffer. For immunoprecipitation, affinity-purified anti-NHE1 antibody was used (Fliegel et al., 1992). Antiexchanger antibody was prebound to *S. aureus* insoluble protein A extract for 4 h at 4 °C and then washed three times with IPB buffer to remove unbound antibody before addition to the membrane protein sample. Immunoprecipitation was carried out by incubating equal amounts of the solubilized membrane proteins with *S. aureus* insoluble protein A extract−exchanger antibody complex. After incubation samples rotated at 4 °C overnight and were washed three times with IPB buffer prior to SDS−PAGE electrophoresis. STE and IPB buffers were supplemented with a cocktail of protease inhibitors as described above. For some experiments immunoprecipitation was in the presence of up to 10 mM ATP. This concentration of ATP was maintained throughout the immunoprecipitation procedure where indicated. In some experiments cells were subjected to heat shock prior to harvesting. Cells grown to confluency were incubated at 42 °C for 30 min and then returned to 37 °C for either 20 h or for the time indicated.

Measurement of pH_i. The fluorescent pH indicator 2′,7′-bis(2-carboxyethyl)- 5(6)-carboxyfluorescein was used to monitor changes in cytosolic pH essentially as described earlier (Fliegel et al., 1995). Fibroblasts cultured on glass coverslips were placed into a holder device and inserted into a 1 cm × 1 cm fluorescence cuvette at 37 °C and perfused with normal buffer (135 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgSO_4$, 5.5 mM glucose, 10 mM Hepes-Tris, pH 7.4). Intracellular acidosis was induced by exposing the cells at 37 °C to 15 mM NH_4Cl in a Na^+ free buffer (135 mM *N*-methylglucamine, 5 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgSO_4$, 5.5 mM glucose, 10 mM Hepes-Tris, pH 7.4) for 5 min prior its rapid withdrawal. pH recovery was obtained by transferring the cells to normal buffer at 37 °C. Fluorescence determinations were carried out using a Shimadzu RF5000U spectrofluorophotometer. The excitation wavelength was varied between 452 and 500 nm, and the emission wavelength remained at 520 nm.

Immunocytochemistry and Immunofluorescence. Polyclonal antibodies against the Na^+/H^+ exchanger were raised in rabbits and were used at a dilution of 1:100 in PBS. Anti hsp70 antibodies raised in mice (StressGen) were used at a dilution of 1:100. Fluorescein conjugated goat anti-rabbit IgG and rhodamine conjugated goat anti-mouse IgG were from BioCan scientific. For double-labeling fluorescence microscopy PS127A cells were grown on sterile glass coverslips as described above and were routinely treated with ammonium chloride selection prior to use to ensure expression of the Na^+/H^+ exchanger. Cells were washed in PBS followed by fixation in 3% paraformaldehyde for 12 min. After fixation cells were washed for 15 min in 100 mM glycine in PBS (pH 7.3) followed by permeabilization with 0.1% Triton X-100 in PBS containing 1% (w/v) bovine serum albumin (TA-PBS). Nonspecific binding was blocked by incubating cells in 5% goat serum for 20 min. The cells were washed three times with TA-PBS and incubated with anti- Na^+/H^+ antibody at 4 °C overnight. After being washed for 1 h in TA-PBS, the cells were incubated with anti-hsp70 antibody for 10 h at 4 °C. Cells were washed for 1 h with TA-PBS followed by incubation with fluorescein conjugated antibody for 1 h (1:200). After washing the cells were

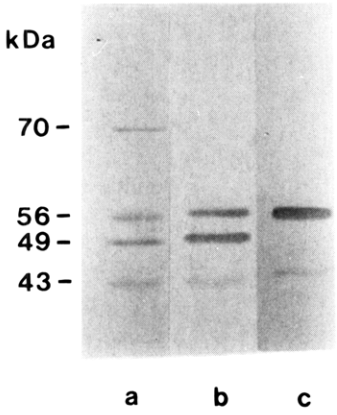

FIGURE 1: SDS−PAGE and immunoblotting of purified PCR178 in *E. coli*. Na^+/H^+ exchanger−GST fusion protein was produced and purified as described in Materials and Methods. Lane a shows the protein purified by affinity chromatography with a glutathione sepharose column. Lane b shows the results of immunoblotting with affinity-purified antibody made against the carboxyl-terminal region of the protein. Lane c shows results of immunoblotting with affinity-purified antibody made against a synthetic peptide based on the carboxyl-terminal 13 amino acids of the protein. The positions and sizes of the Bio-Rad prestained markers are as indicated.

incubated with rhodamine-labeled antibody (1:200) for 1 h. The cells were washed in TA-PBS and mounted in 50% glycerol containing 1% *n*-propyl gallete. Control experiments contained either preimmune sera, no primary antibody, or primary antibody blocked with PCR178 as indicated. A Zeiss microscope with a fluorescence condenser and selective filter combinations was used for observation and photography. Photographs were recorded to TMax-400 (Kodak) film.

RESULTS

Expression of PCR178 in E. coli. To characterize the cytoplasmic domain of the Na^+/H^+ exchanger, this region of the protein was expressed as a fusion protein with GST in *E. coli*. The purified product is shown in Figure 1. Lane a shows Coomassie blue staining of the fusion protein product purified by glutathione Sepharose 4B affinity chromatography. Four major bands are apparent of molecular masses 70, 56, 49, and 43 kDa, together with some minor products of smaller size. To confirm the identity of the major products as a fusion protein of the Na^+/H^+ exchanger with GST, we used an affinity-purified antibody raised against a carboxyl-terminal region fusion protein (Fliegel et al., 1993). Lane b shows that this antibody reacts strongly with the 56, 49, and 43 kDa proteins. It showed no cross-reactivity with the 70 kDa protein and only weak reactivity with the smaller products. To confirm the identity of the expressed protein further, we used a second affinity-purified antibody made against a synthetic peptide coding for the carboxyl-terminal 13 amino acids of the Na^+/H^+ exchanger (Fliegel et al., 1992). Lane c shows that this antibody reacts with the 56 and 43 kDa proteins but not with the 49 kDa protein. This suggests that the smaller proteins that copurify with the 56 kDa protein are degradation products. The most likely explanation for the lack of immunoreactivity of the 49 kDa protein to the synthetic peptide antibody is that proteolysis removes the carboxyl-terminal amino acids which the antibody recognizes. We observed proteolysis of the fusion protein despite inclusion of a cocktail of protease inhibitors (Michalak et al., 1990) throughout the purification procedure. Production of the protein in a strain of *E. coli* that is deficient in some proteases showed only a minor decrease in the degree of proteolysis (not shown).

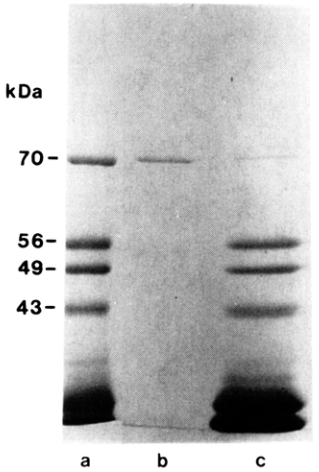

FIGURE 2: ATP-dependent elution of dnaK from GST affinity column. PCR178 was purified using a Glutathione Sepharose 4B affinity column as described in Materials and Methods. Lane a shows the product eluted by glutathione. After removal of glutathione, the purified product was reapplied to the column and washed thoroughly. Lane b shows the result of elution with buffer containing 3 mM MgATP in the absence of glutathione. Lane c shows the products of subsequent elution with glutathione.

We observed that a 70 kDa protein always copurified with PCR178 (Figure 1, lane a). GST alone did not copurify with this protein (not shown). The 70 kDa protein was not immunoreactive with the Na^+/H^+ exchanger antibodies tested (lanes b and c) but was always present in significant amounts. To reveal the identity of this protein, we used amino-terminal sequence analysis. The amino-terminal sequence of the protein was Gly-Lys-Ile-Ile-Gly-Ile-Asp-Leu-Gly-Thr. A search of protein databases revealed that this was identical to the amino-terminal sequence of the *E. coli* protein encoded by the dnaK gene (Bardwell & Craig, 1984). To determine whether the association of dnaK with PCR178 was ATP-dependent, we purified the fusion protein as described above and reapplied it to the glutathione Sepharose column. The column was then subjected to an ATP wash (3 mM MgATP, 50 mM Tris-HCl, pH 8.0). The results are shown in Figure 2. Sample applied to the column contained relatively large amounts of dnaK (lane a). Washing the column with MgATP selectively eluted dnaK (lane b). The remaining sample was eluted with glutathione. Subsequent analysis by SDS−PAGE confirmed that dnaK had been depleted in the fraction containing PCR178 (lane c). Washing the column with $MgCl_2$ alone did not remove dnaK. Washing the column with ATP for longer periods of time could further reduce the amount of dnaK remaining (data not shown).

Further experiments explored the possibility that mammalian hsp70 interacts with the carboxyl-terminal region of the Na^+/H^+ exchanger. We purified the full length fusion protein from the PCR178 degradation products by a combination of Glutathione Sepharose 4B affinity chromatography and preparative SDS−PAGE, as described in Materials and Methods. Overnight dialysis was used to remove SDS and allow renaturation of the protein. The purified full length protein was free from all proteolytic fragments and was immunoreactive with antibodies against the carboxyl-terminal region of the Na^+/H^+ exchanger (not shown). It was subsequently coupled to CNBr-activated Sepharose 4B as

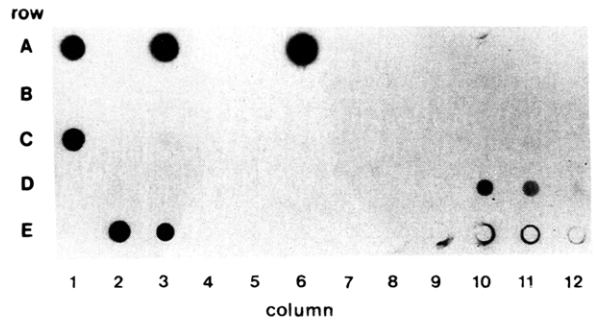

FIGURE 3: Dot blot analysis of interactions of hsp70 with column-bound PCR178. Purified PCR178 was covalently coupled to a CNBr-activated Sephrose column as described in Materials and Methods. Bovine brain hsp70 (10 mg in 4 mL) was loaded by repeated passage through the column. A-1, A-3, and A-6 are 50, 100, and 150 mL of the flow through containing hsp70 which was loaded onto the column. Excess hsp70 was removed by washing the column with Tris buffer, pH 8.0 (fractions C-1 to D-6) and Tris buffer containing 3 mM $MgCl_2$ (D-9 to D-12). The column was then washed with 3 mM MgATP (E-1 to E-12). Fractions were collected as 4 mL aliquots. A volume of 150 mL of sample was added to each well, and the nitrocellulose membrane was reacted with antibody against hsp70 as described in Materials and Methods.

described in Materials and Methods. Samples of hsp70 were loaded onto the column, which was then washed thoroughly with buffer and with buffer containing 3 mM MgATP. Samples of eluent were analyzed by dot blotting and immunoblotting with antibody against hsp70. The results are shown in Figure 3. Row A consists of positive controls. Different volumes (50, 100, and 150 mL) of the flow-through recovered during the loading of hsp70 were spotted onto the nitrocellulose, and the hsp70 protein was detected by immunostaining. Rows C and D show the flow-through from the various washes of the column prior to the addition of ATP. Very little hsp70 was detected in these fractions, although the addition of $MgCl_2$ alone (3 mM) caused the loss of some hsp70 (fractions D9−D12). The inclusion of 3 mM MgATP caused an initial dramatic release of hsp70 from the column (row E) that ended rapidly.

To explore the interaction of hsp70 with PCR178 in more detail, we used nondenaturing gel electrophoresis. Aliquots of hsp70 were incubated for 10 min with different volumes of buffer containing PCR178 or GST. MgATP (3 mM) was added to some samples 2 min before the addition of sample buffer. Figure 4 shows the typical results of such an experiment with a silver-stained 9% acrylamide gel. Lanes 1−3 were loaded with samples containing hsp70 (1 mg), PCR178 (1 mg), and GST (1 mg), respectively. Hsp70 was not observed with silver staining on 9% (Figure 4), 6% (not shown), or 3−6% (not shown) gels. This is because the protein does not migrate into the separating phase of the nondenaturing gel. Hsp70 has a more acidic isoelectric point than either PCR178 or GST, which may account for this observation. PCR178 is observed near the top of a 9% gel (Figure 4, lane 2), while GST migrates much farther (lane 3). When PCR178 and hsp70 are combined (1:10 or 1:5 ratio), neither protein is observed (lanes 4 and 6). This suggests that hsp70 has bound to PCR178, preventing it from entering the gel. To test if the binding of PCR178 to hsp70 is reversible, we incubated the complex with 3 mM MgATP. Under these conditions, PCR178 is again visible (Figure 4, lanes 5 and 7). This suggests that a complex is formed between the two proteins that can be dissociated by the addition of ATP. Similar experiments showed that purified

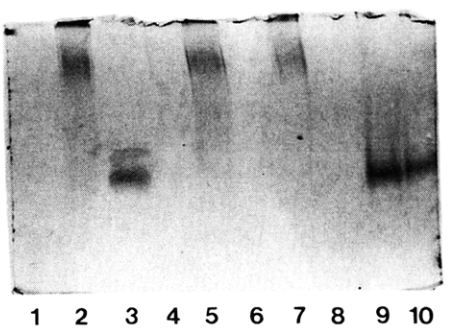

FIGURE 4: Nondenaturing polyacrylamide gel electrophoresis of hsp70 with PCR178 and GST. Nondenaturing polyacrylamide gel electrophoresis was performed using a 9% acrylamide gel, as described in Materials and Methods. Samples were loaded in 15 mL aliquots in nondenaturing sample buffer. Samples are as follows: lane 1, hsp70 (1 mg); lane 2, PCR178 (1 mg); lane 3, GST (1 mg); lane 4, hsp70 (10 mg) + PCR178 (1 mg); lane 5, as lane 4, plus 3 mM MgATP; lane 6, hsp70 (10 mg) + PCR178 (2 mg); lane 7, as lane 6, plus 3 mM MgATP; lane 9, hsp70 (10 mg) + GST (1 mg); lane 10, as lane 9, plus 3 mM MgATP.

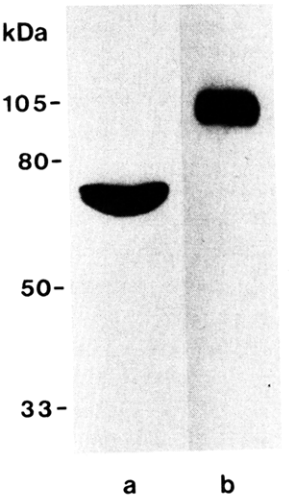

FIGURE 5: Immunoblotting of human placental brush border membranes with anti-hsp70 antibody or anti-Na^+/H^+ exchanger antibody. (Lane a) Immunoblotting with anti-hsp70 antibody. (Lane b) Immunoblotting with anti-Na^+/H^+ exchanger antibody directed against the carboxyl terminal region of the protein.

GST alone did not bind hsp70 and did not show complex formation (lane 9) or ATP-dependent band shifts (lane 10).

Interactions of hsp70 with the Na^+/H^+ Exchanger in Intact Mammalian Cells. To determine whether the Na^+/H^+ exchanger could colocalize with hsp70 *in vivo*, we examined human placental brush border membrane vesicles. Vesicles were isolated as described earlier (Haworth et al., 1993). Both the Na^+/H^+ exchanger and hsp70 were present in this membrane fraction. Immunoblotting of the vesicles with anti-hsp70 antibody showed that hsp70 was apparent as a single 70 kDa band (Figure 5, lane a). In the presence of β-mercaptoethanol the Na^+/H^+ exchanger also appeared as a single immunoreactive band of apparent molecular mass of 105 kDa (Figure 5, lane b).

To examine the possible interactions of the Na^+/H^+ exchanger with hsp70 *in vivo,* we used affinity purified antibodies to immunoprecipitate the Na^+/H^+ exchanger from intact mammalian cells. This was followed by Western blotting to examine for the presence of hsp70. The results are shown in Figure 6a. Lane 1 is a negative control with only protein A−antibody complex present. After a Western

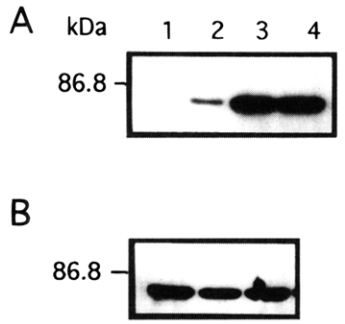

FIGURE 6: Immunoprecipitation of hsp70 with anti-Na^+/H^+ exchanger antibodies and hsp70 content of AP-1 and PS127A cells. (A) Cell extracts were immunoprecipitated with anti-Na^+/H^+ exchanger antibody. Samples were then run on SDS−PAGE gels and immunoblotted with hsp70 antibody. Lane 1, control without cell membrane extracts. Lane 2, cell extract of AP-1 cells was used. Lanes 3 and 4 used cell extracts of PS127A cells which were not treated (lane 3) or treated (lane 4) with a half hour heat shock as described in Materials and Methods. (B) Cell extracts of AP-1 (lane 2) and PS127A cells (lanes 2 and 3). PS127A cells in lane 3 were subjected to a half hour heat shock.

blot of the resulting sample was probed, no immunoreactivity with hsp70 antibody is shown. Lanes 2−4 are immunoprecipitation of cell extracts with NHE1 antibody present followed by Western blotting. Lane 2 is an immunoprecipitation from AP-1 cells. A small band of approximately 70 kDa was apparent with the Western blotting of the immunoprecipitate. Lane 3 shows the same experiment using the Na^+/H^+ exchanger overexpressing cells PS127A. A much larger amount of hsp70 was present in the cell extract. Lane 4 shows the same experiment with PS127A cells that were subjected to a half hour heat shock followed by 20 h of recovery. Approximately the same amount of hsp70 was recovered as in lane 3. To ensure that the differences noted between the AP-1 cells and PS127A cells was not due to different endogenous levels of hsp70, we examined the levels of hsp70 present in cellular extracts of the two cells types. The results (Figure 6B) show that AP-1 cells have similar or higher levels of hsp70 than PS127A. Lane 2 shows that significant hsp70 was present in AP-1 cells. Lane 3 shows that a somewhat reduced amount of hsp70 is present in PS127A cells. Treatment of PS127A cells with a half hour heat shock resulted in a small increase in the amount of hsp70 present in PS127A cells.

We examined the stability of the hsp70−Na^+/H^+ antiporter interaction in the presence of various concentrations of ATP. PS127A cultures were grown and harvested as described above, and immunoprecipitation was in the presence of added ATP. Figure 7 shows the results. Lanes 1 and 2 contain no added ATP and 1 mM added ATP, respectively. The level of hsp70 protein present after immunoprecipitation declines slightly in lane 2. Lanes 3 and 4 contain 3 and 10 mM added ATP, respectively. The amount of hsp70 protein decreased to less than half the amount present in lanes 1 and 2. There was no apparent difference between the amount of hsp70 present in lanes 3 and 4.

To determine whether heat shock can induce synthesis of the Na^+/H^+ antiporter, we treated PS127A cells with a half hour heat shock and examined levels of hsp70 and the Na^+/H^+ exchanger. PS127A cultures were harvested at time 0, 2, 4, 10, 24, and 30 h after the heat shock treatment. Hsp70 levels were examined by Western blotting of the cytosolic fraction, and Na^+/H^+ exchanger levels were examined by

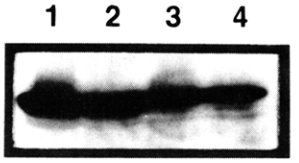

FIGURE 7: Immunoprecipitation of hsp70 with anti-Na^+/H^+ exchanger antibodies in the presence or absence of ATP. Cell extracts were immunoprecipitated with anti-Na^+/H^+ exchanger antibody. Samples were then run on SDS−PAGE gels and immunoblotted with hsp70 antibody. Lane 1, control immunoprecipitation in the absence of ATP, Lanes 2, 3, and 4, immunoprecipitation of cell extracts in the presence of 1, 3, and 10 mM ATP, respectively. Samples were then run on SDS−PAGE gels and immunoblotted with anti-hsp70 antibody.

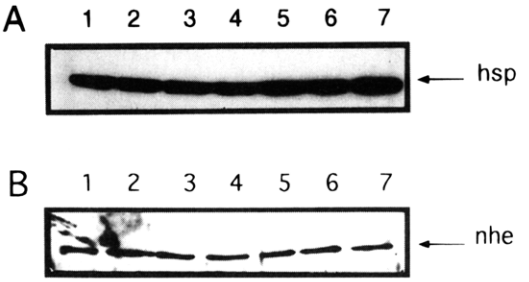

FIGURE 8: Immunoblot analysis of hsp70 and NHE1 content of PS127A cells subjected to heat shock. (A) Membrane extracts were harvested, and NHE1 protein was examined by Western Blotting using affinity-purified anti-NHE1 antibody. Lane 1, cells were harvested prior to heat shock. Lanes 2−7, cells were subjected to heat shock and harvested at times 0, 2, 4, 10, 24, and 30 h (respectively). (B) Cells were treated and harvested at times shown in panel A. Cytosolic fractions were analyzed by Western blotting with anti-hsp70 antibody.

immunoprecipitation of the Na^+/H^+ antiporter from membrane fractions using anti-NHE1 antibody followed by Western blotting (Figure 8). Ten hours after heat shock there was a small and steadily increasing amount of hsp70 present (Figure 8A). There was, however, no increase in the apparent amount of Na^+/H^+ antiporter present even 30 h after the initial heat shock.

We also examined whether heat shock can induce increased activity of the Na^+/H^+ antiporter in PS127A cells. Cells were treated with a half hour heat shock and allowed to recover for 24 h. We measured the initial rate of recovery from an acid load induced by ammonium chloride prepulse. The results (Figure 9) show that there was no difference in the initial rate of recovery between cells treated with or without heat shock. There was also no difference in the rate of recovery from acidosis in cells which were allowed to recover for 12 h after heat shock (not shown).

To determine whether the Na^+/H^+ exchanger and hsp70 colocalize in intact cells, we used double labeling of PS127A cells immunostained with antibody against hsp70 and the Na^+/H^+ exchanger. The results are shown in Figure 10. The Na^+/H^+ exchanger showed a weak diffuse plasma membrane localization plus a stronger focal localization at the edge of fusiform processes and along the border of lamillipodia. This focal pattern of staining was typical of many preparations of cells (Figure 10A). Hsp70 showed a much stronger and widespread cellular distribution; however, it was also found in the same regions as the Na^+/H^+ exchanger including the foci where the Na^+/H^+ exchanger was concentrated (Figure 10B). Blocking the Na^+/H^+ exchanger antibody with a fusion protein of the carboxyl-terminal region of the protein

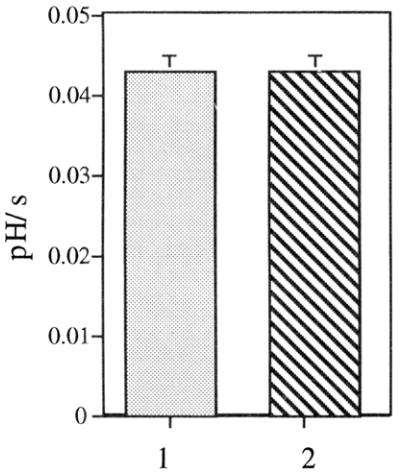

FIGURE 9: Initial rates of recovery from an acute acid load by PS127A cells. Control cells and cells treated with heat shock were examined after a 24 h recovery period. Cells were prepared and the intracellular pH was measured as described in Materials and Methods. The rate of recovery was in the presence of 35 mM NaCl. Each group represents the mean of at least nine independent experiments. Error bars = SEM.

eliminated the Na^+/H^+ exchanger signal in the intact cells (Figure 10C). Preimmune sera or reactions without primary antibody also showed no exchanger signal (not shown).

DISCUSSION

The large cytoplasmic domain of the Na^+/H^+ exchanger is believed to be an important regulatory region of the protein (Sardet et al., 1990, 1991; Wakabayashi et al., 1992). Recent evidence has shown that it can interact with calmodulin and it has been suggested to be linked to other cytoskeletal proteins (Wakabayashi et al., 1992, 1994a). In this study we examined the interactions of this domain of the protein with heat shock proteins. Heat shock proteins have been shown to associate with many proteins, facilitating their transport across biological membranes and promoting their proper folding (Langer et al., 1992). In some cases it is suggested that heat shock proteins may function to protect proteins from inactivation before transport to their final intracellular locations (Pelham, 1989; Langer et al., 1992). They are released from target proteins after the hydrolysis of ATP, a process that induces conformational changes in the attached protein (Langer et al., 1992).

Initial experiments in this study identified the interaction of the mammalian Na^+/H^+ exchanger with *E. coli* heat shock protein dnaK. DnaK has an overall 50% homology with the major mammalian heat shock protein, hsp70, and has conserved domains with over 96% homology (Schlesinger et al., 1990). This fusion protein, PCR178, consists of GST fused to the carboxyl-terminal 178 amino acids of the mammalian Na^+/H^+ exchanger. We found that PCR178 copurifies with *E. coli* dnaK. We could dissociate dnaK from the Na^+/H^+ exchanger by the addition of ATP (Figure 2). This behavior is consistent with the known properties of mammalian hsp70, which binds to target proteins and requires MgATP for release (Pelham, 1986, 1989; Margulis & Welsh, 1991).

Due to the observed interactions between dnaK and PCR178, and to the high degree of homology between dnaK and mammalian equivalents (Schlesinger et al., 1990), we

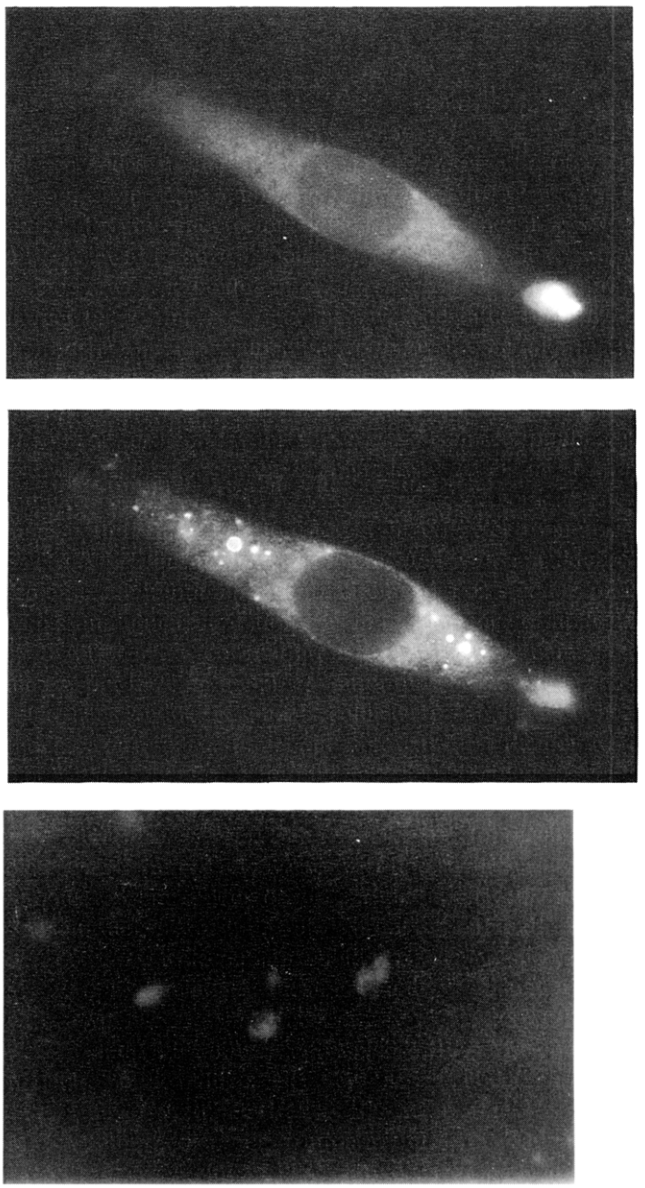

FIGURE 10: Double labeling of PS127A cells with anti Na^+/H^+ exchanger (a and c) and anti-hsp70 (b) antibodies. PS127A cells were grown and immunostained with hsp70 and Na^+/H^+ exchanger antibodies as described in Materials and Methods. (a) Immunofluoresence of PS127A cells with Na^+/H^+ exchanger antibody. (b) Immunofluoresence of corresponding cells with hsp70 antibody. (c) Immunoflouresence of PS127A cells with Na^+/H^+ exchanger antibody which was blocked by preincubation with Na^+/H^+ exchanger fusion protein prior to application.

tested for interactions between PCR178 and hsp70. Highly purified PCR178 was coupled to a CNBr-activated Sepharose column. We found that this column bound mammalian hsp70 in an ATP-dependent manner (Figure 3). These experiments showed that PCR178 interacts with bovine hsp70. The binding appears to be specific for the Na^+/H^+ exchanger part of the fusion protein, as GST alone does not interact with hsp70. One possible interpretation of our results is that the fusion protein is simply not properly folded. Hsp70 and various other molecular chaperones have been shown to function to prevent the formation of incorrect structures and to unscramble those that occur (Ellis & Hemmingsen, 1989; Rothman, 1989). Any exposed hydrophobic regions would be recognized by the hsp70 protein, which normally binds to partially denatured proteins. How-

ever, the region of the protein we have expressed is a hydrophilic region of the protein that is known to be cytosolic (Sardet et al., 1989, 1990). An alternative explanation is therefore that the binding of hsp70 to the carboxyl terminal of the Na^+/H^+ exchanger in PCR178 may be representative of binding to the native Na^+/H^+ exchanger protein *in vivo*.

To examine whether hsp70 could interact with the Na^+/H^+ exchanger in intact cells, we used two approaches. First, we examined the possibility that the two proteins could coexist in purified membrane fractions from the same cell type. Second, we used dual labeling immunocytochemistry of PS127A cells. The results are shown in Figures 5 and 10. We have earlier shown that the human placenta is a highly enriched source of the NHE1 isoform of the Na^+/H^+ exchanger (Fliegel et al., 1993; Haworth et al., 1993). Western blot analysis showed that human placental brush border membranes contained the Na^+/H^+ exchanger (Figure 5). In addition, hsp70 was present in surprisingly large amounts in this membrane. Our second approach used dual labeling to examine PS127A cells. The Na^+/H^+ exchanger had a diffuse cellular distribution and a focal concentration along the border of lamillipodia. This result was similar to that described earlier (Grinstein et al., 1993). Hsp70 had a stronger signal and a wide cellular distribution. It was also found in the same location as the Na^+/H^+ exchanger including the focal concentrations (Figure 10). Overall, these two sets of experiments suggest that the two proteins can exist in the same cellular location in these cell types.

As a more direct examination of the possible interactions of the two proteins *in vivo*, we used anti-NHE1 antibody to precipitate the Na^+/H^+ exchanger. We initially used two cell lines for this purpose, the AP-1 cell line and PS127A cells. AP-1 cells are deficient in Na^+/H^+ antiporter activity. The exact defect in Na^+/H^+ exchanger activity in these cells is not known although it is known that activity is greatly reduced or nearly absent. PS127A cells are Na^+/H^+ exchanger overproducers and have been shown to have highly active Na^+/H^+ exchanger activity and increased protein levels (Sardet et al., 1990). We found that with immunoprecipitation of the Na^+/H^+ antiporter we were able to coprecipitate hsp70. In PS127A cells the amount of hsp70 coprecipitating with the antiporter was quite large. In AP-1 cells that amount was very greatly reduced in comparison to PS127A (Figure 6A). The difference between the two cell types was not due to different endogenous levels of hsp70 present since Western blot analysis showed that AP-1 cells contain more hsp70 than PS127A cells (Figure 6B).

To examine the effect of ATP on the interaction between hsp70 and the Na^+/H^+ antiporter, we used immunoprecipitation in the presence of varying concentrations of ATP. The results (Figure 7) showed that the presence of 1 mM ATP had only a small effect on the interaction between hsp70 and the antiporter. However, 3 and 10 mM ATP resulted in a dramatic reduction in the association. It is noteworthy that both concentrations did not totally eliminate the association. The reason for the remaining association is not yet clear; however, similar results have been reported recently with hsp70 association with hsp40 (Sugito et al., 1995). These results show that most of the association between the two proteins is ATP-dependent though there appears to be an ATP-resistant component to the association, at least under the present conditions. It is likely that ATP causes a conformational shift in hsp70 which resulted in disruption of most of the association between the two proteins (Sugito et al., 1995).

Because of the demonstrated interaction between hsp70 and the antiporter, we examined whether Na^+/H^+ antiporter levels were increased in response to heat shock. The results are shown in Figures 6B and 8. Heat shock of PS127A cells produced a gradual increase in the amount of hsp70 protein which was quite evident after 24−30 h (Figure 8). In contrast, the amount of NHE1 protein and the activity of the protein (Figure 9) did not increase during this time, suggesting that heat shock does not upregulate the amount of NHE1 protein. Together, these results show that the NHE1 protein is associated with hsp70 and that the amount of Na^+/H^+ exchanger is not elevated with increases in hsp70. It appears likely that hsp70 is involved in regulation of constitutive expression of the Na^+/H^+ exchanger.

The exact function of the association between hsp70 and the Na^+/H^+ exchanger is not yet known. However, molecular chaperones such as hsp70 have been shown to prevent the formation of incorrect protein to protein structures (Ellis & Hemmingsen, 1989). Because the exchanger has been suggested to interact with several other proteins including itself, hsp70 could function to protect and regulate these interactions during biosynthesis. For example, experiments have shown that the Na^+/H^+ exchanger forms a complex with both itself and with calmodulin (Haworth et al., 1993; Wakabayashi et al., 1994b). It is possible that the binding of hsp70 to the exchanger protein during biosynthesis would protect the exchanger from inappropriate interactions until the correct association can be made. A similar situation occurs when hsp70 binds to the carboxy terminal of tubulin (Sanchez et al., 1994).

It is intriguing that hsp70 and the Na^+/H^+ exchanger were both found in human placental brush border membranes and that they colocalize in PS127A cells. However, whether hsp70 plays a more active role other than in biosynthesis is not yet known at this time. In several cases heat shock proteins are known to associate with proteins, possibly playing a role in their normal function. Hsp70 is found associated with the glucocorticoid receptor and may influence DNA-binding and/or transcriptional activities (Srinivasan et al., 1994). The androgen receptor (Veldscholte et al., 1994) and the progesterone receptor (Johnson et al., 1994) are also associated with hsp70. Whether this can occur with the Na^+/H^+ exchanger is not yet known at this time. Further experiments are necessary to explore this possibility.

It is known that the activity of the Na^+/H^+ exchanger is elevated in many cell types in hypertension (Berk et al., 1988; Foster et al., 1992). The defect may be in translation or posttranslational regulation of the protein (Lifton et al., 1991; Rosskopf et al., 1993). It is therefore noteworthy that the hsp70 gene is associated with hypertension in some animal models and in humans (Kunes et al., 1992; Hamet et al., 1992). If hsp70 regulates expression or activity of the Na^+/H^+ exchanger *in vivo*, it is possible that it could be involved with the abnormal activity of the Na^+/H^+ exchanger in this disease. Future experiments may explore this possibility.

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