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Identification of the protein and cDNA of the cardiac Na^+/H^+ exchanger

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We examined the myocardial form of the Na^+/H^+ exchanger. A partial length cDNA clone was isolated from a rabbit cardiac library and it encoded for a Na^+/H^+ exchange protein. In comparison with the human Na^+/H^+ exchanger, the sequence of the 5' end of the cDNA was highly conserved, much more than the 3' region, while the deduced amino acid sequence was also highly conserved. To further characterize the myocardial Na^+/H^+ exchange protein, we examined Western blots of isolated sarcolemma with antibody produced against a fusion protein of the Na^+/H^+ exchanger. The antibodies reacted with a sarcolemma protein of 50 kDa and with a protein of 70 kDa. The results show that the rabbit myocardium does possess a Na^+/H^+ exchanger protein homologous to the known human Na^+/H^+ exchanger.

Na^+/H^+ exchanger; Sarcolemma; cDNA cloning

1. INTRODUCTION

The heart cell plasma membrane, the sarcolemma (SL), is responsible for the regulation of cytosolic ionic concentrations and intracellular pH. The Na^+/H^+ exchanger is the protein that regulates intracellular pH by extruding one H^+ in exchange for Na^+ when decreases in intracellular pH occur. It is responsible for recovery of intracellular pH following acidification and the Cl^-/HCO_3^- exchange system is probably only involved in special circumstances [1–6]. Only some basic characterization studies exist on the Na^+/H^+ exchanger in the heart SL [7–10] and its activity has been shown to exist there and has some similarities to that of other tissues [11,12]. It has recently been shown that there may be more than one form of the Na^+/H^+ exchanger in the myocardium [10] and in other tissues [13–16]. Pharmacologically different antiporters exist on the apical and basolateral surfaces of cultured porcine kidney cells [13].

Attempts have been made to identify the particular protein(s) responsible for Na^+/H^+ exchange in various cell lines using antibodies or the Na^+/H^+ exchange inhibitor amiloride and its analogues. One study on the renal exchanger showed that amiloride blocked specifically the labelling of a 100-kDa protein by *N,N'*dicyclohexyl-carbodiimide suggesting this protein may be the exchanger or part of it [11]. However, others [12] have found similar results in the same tissue type with a protein of M_r 65 000. Huot et al. [19] also suggested that the rabbit renal exchanger was a 25-kDa protein. Wu and Lever [20] photoaffinity labelled renal brush border membrane proteins of M_r 81 000 and 107 000 and these cofractionate with Na^+/H^+ antiport activity. An antibody has recently been made against a fusion protein of the human Na^+/H^+ exchanger and it reacted with a 110 000 kDa protein in fibroblasts [21]. Another recent study [22] has suggested that the low amiloride analogue affinity form of the exchanger is a 66-kDa protein in bovine brush border membranes.

In this study we identify for the first time protein and cDNA encoding for the cardiac Na^+/H^+ exchanger. We have isolated a partial length cDNA clone encoding for the carboxyl-terminal of the rabbit cardiac form of the protein and compare it with the human form of the Na^+/H^+ exchanger [23]. We also expressed the human form of the Na^+/H^+ exchanger as a fusion protein in *E. coli* and have used antibodies against the expressed protein and a synthetic peptide to identify the exchanger in isolated SL vesicles.

2. MATERIALS AND METHODS

2.1. *Materials*

Restriction endonuclease and DNA-modifying enzymes were obtained from Boehringer Mannheim and Bethesda Research Laboratories. The plasmids pTZ18R and 19R were from Pharmacia LKB Biotechnology Inc. [^{32}P]ATP was obtained from New England Nuclear. Nitrocellulose membrane filters were from Schleicher and Schuell and nylon membranes were from Amersham. Sequencing gels were prepared using reagents from Bio-Rad or Boehringer Mannheim. SDS-polyacrylamide gel electrophoresis reagents and protein standards were purchased from Bio-Rad, horseradish peroxidase-conjugated goat anti-rabbit IgG was obtained from Bio/Can. All chemicals were of the highest grade available.

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2.2. *Screening and sequencing of cDNA clones*

The λgt 10 cDNA expression library was constructed from poly(A)$^+$ RNA of rabbit cardiac muscle and was a generous gift of Dr J. Lyton (Brigham and Womens Hospital, Harvard Medical School). Screening of the library was with ^{32}P-labeled nick-translated cDNA of the human Na^+/H^+ exchanger [23] as described earlier [24]. This resulted in the isolation of a cDNA clone of 2235 bp which was subcloned into the plasmids pTZ18R and 19R (Pharmacia) and characterized by restriction endonuclease mapping. Single-stranded DNA templates were made of overlapping cDNA fragments subcloned into pTZ18R and 19R in both directions, and were sequenced by the dideoxy method of Sanger et al. [25].

2.3. *Preparation of SL vesicles*

SL membranes were isolated from bovine or canine hearts as described by Jones [26] in the presence of the protease inhibitors PMSF and benzamidine as described earlier [27]. Vesicles were suspended at a concentration of 2–5 mg/ml in 250 mM sucrose and 20 mM Hepes, pH 7.4, frozen in liquid nitrogen and stored at −85°C. Protein was determined by the method of Bradford [28].

2.4. *Polyacrylamide gels and immunostaining*

SDS-polyacrylamide gel electrophoresis was on polyacrylamide gels (10%) as described earlier [23], essentially by the method of Laemmli [29]. After separation proteins were transferred electrophoretically onto nitrocellulose membrane [30]. Immunostaining of nitrocellulose membranes was carried out in the presence of 1% skim milk powder essentially as described earlier [31].

2.5. *Expression of Na^+/H^+ exchanger protein and antibody production*

A portion of the Na^+/H^+ exchanger cDNA clone corresponding to amino acids 109-676 [23] of the human Na^+/H^+ exchanger was expressed in *E. coli* using the pRIT 2T (Pharmacia) vector which expresses proteins as a fusion protein with protein A. The plasmid was transformed into host cells N4830-1 and the protein was induced by temperature shift from 30 to 42°C. The induced cells were harvested by centrifugation and lysed by passage through a french press. A 20000 × *g* supernatant of the lysed cells was passed through an IgG Sepharose column (Pharmacia) and the purified fusion protein was eluted with pH 3.4 acetic acid as described by the manufacturer. For immunization studies the isolated protein was separated by SDS-PAGE as described above and briefly stained with 0.05% Coomassie brilliant blue R-250 in water (without methanol or acetic acid). The gel was destained with water and the largest band (≈70 kDa) was excised and used for immunization of rabbits with Freund's adjuvant followed by 3 booster injections at 2 week intervals.

A synthetic peptide encoding for the amino terminal region of the Na^+/H^+ exchanger [23] RSSEPPRERS was synthesized and coupled to Keyhole Limpet Hemocyanin. It was used to raise polyclonal sera as described above.

2.6. *RNA isolation and analysis*

Poly(A)$^+$ RNA was isolated from 8-day-old rabbit skeletal muscle as described earlier [24]. The Northern blot was probed with ^{32}P-labelled random primed cDNA (BRL) encoding for bp 511–1264 of the rabbit cardiac cDNA clone.

3. RESULTS AND DISCUSSION

We isolated the rabbit cardiac cDNA encoding for Na^+/H^+ exchanger. The cDNA clone was 2235 bp and encoded for a portion of the Na^+/H^+ exchanger corresponding to the carboxyl-terminal of the human Na^+/H^+ exchanger. Fig. 1 shows a homology matrix analysis of the nucleotide sequence of the entire rabbit cardiac cDNA clone and a portion of the human cDNA

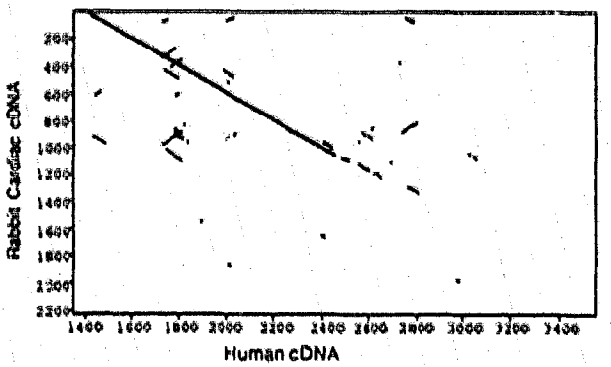

Fig. 1. Comparison of nucleotide sequence of human Na^+/H^+ exchanger cDNA clone [24] and rabbit cardiac cDNA clone. The nucleotide sequences were compared using the Pustell DNA Matrix analysis program of MacVector (IBI). The search element was 30 bp in length and the minimum score was 65%. The entire length of the cardiac cDNA clone (*y*-axis) was compared with the human clone (bp 1388–3560, *x*-axis).

clone. It is evident that the two cDNA clones are closely related especially in the more 5′ regions. In the 3′ untranslated regions the homology largely disappears, possibly representing either species or tissue differences in the splicing of this region. The results show that the myocardium does possess a Na^+/H^+ exchanger protein that is homologous to the reported human Na^+/H^+ exchanger [23]. We have recently isolated several human myocardial Na^+/H^+ exchanger cDNA clones and they show a greater homology in the untranslated region to the rabbit cardiac sequence than to the human exchanger (unpublished observation). The 3′ untranslated region may thus serve as an important tissue specific function. Fig. 2 shows the nucleotide and deduced amino acid sequence of this clone. A putative polyadenylation signal is noted at nucleotide 2205. The last nucleotide was followed by a poly(A)$^+$ tail. The amino acid homology between the human amino acid sequence and the rabbit cardiac clone is striking. Only in the carboxyl terminal region of the protein do these two types diverge somewhat and some of these changes are quite conservative. This region is hypothesized to be a regulatory region of the protein and may be where the exchanger is subject to protein kinase-mediated phosphorylation [21,23]. The sequence does however terminate in the same location as the human Na^+/H^+ exchanger (see [21]). Studies are currently in progress to examine the importance of this region in regulation of the Na^+/H^+ exchanger.

To identify the Na^+/H^+ exchange protein in the myocardium we cloned a portion of the human Na^+/H^+ exchanger into the expression vector pRIT2T and produced a fusion protein with protein A. Because of the protein A portion of the molecule we could detect the fusion protein with conjugated antibody directly (Fig. 3). The resultant protein product was a mixture of fusion proteins ranging from 70 kDa and decreasing in size. The smaller protein bands shown (Fig. 3) were probably

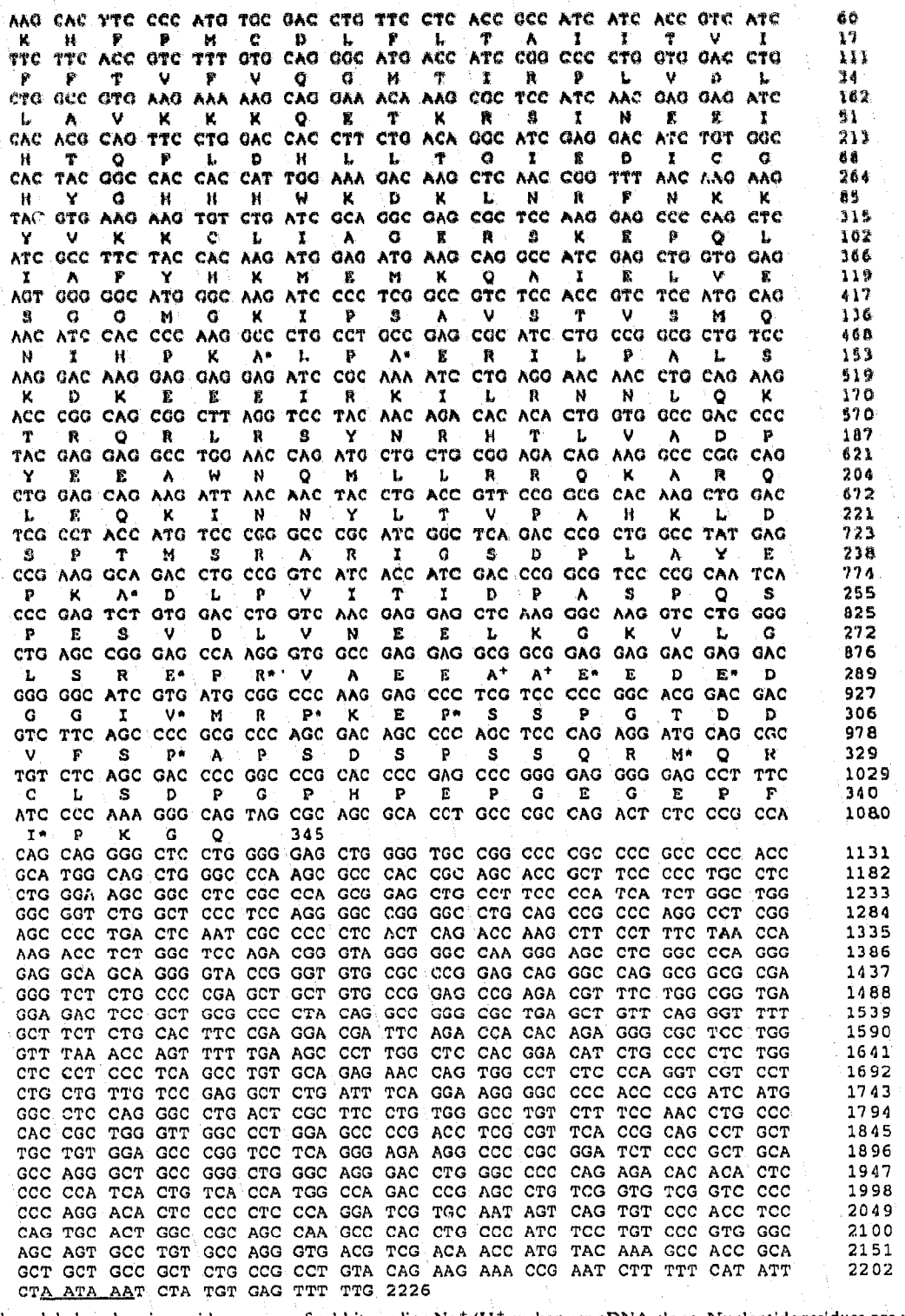

Fig. 2. Nucleotide and deduced amino acid sequence of rabbit cardiac Na^+/H^+ exchanger cDNA clone. Nucleotide residues are numbered positively from the beginning of the cDNA clone excluding the first 9 bp which included a polylinker. The deduced amino acid sequence is below and differences between the human [21] and rabbit cardiac sequence are noted with: (*) changes, (–) deletions, and (+) for insertions.

the results of proteolysis in vivo in *E. coli*, since we used several protease inhibitors throughout the isolation procedure. We used the largest fusion protein excised from SDS-PAGE to immunize rabbits and produce antibodies against the Na^+/H^+ exchanger. We tested the resultant antibodies against highly purified heart SL isolated by the procedure developed by Jones [26]. The vesicles were characterized as described earlier [27] and are highly

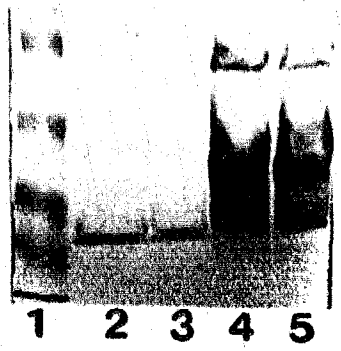

Fig. 3. Expression of the human Na^+/H^+ exchanger in *E. coli*. A portion of the human Na^+/H^+ exchanger corresponding to amino acids 109–676, was cloned into the expression vector pRIT2T (Pharmacia) which expresses proteins as a fusion protein with protein A. Control and fusion protein plasmids were transfected into *E. coli* cells and protein production was induced by temperature shift from 30 to 42°C. The cells were pelleted and protein separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. The fusion product could be detected by incubating the blots with peroxidase conjugated second antibody alone (rabbit). (Lane 1) Bio-Rad prestained markers of size 75, 50, 39 and 27 kDa. (Lanes 2 and 3) Controls of expressed pRIT2T alone with no Na^+/H^+ exchanger insert; (lanes 4 and 5) the fusion protein with Na^+/H^+ exchanger insert.

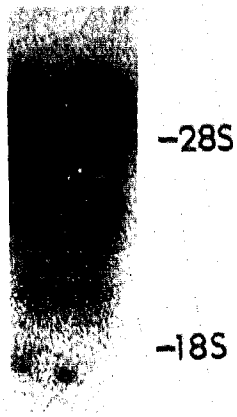

Fig. 5. RNA blot analysis of poly(A)$^+$ RNA from 8-day-old rabbit skeletal muscle. Residues 511–1265 of the rabbit cardiac cDNA clone were used as a probe as described in Materials and Methods. Sizes were estimated based on the positions of the ribosomal subunits run simultaneously on the same gel.

enriched in plasma membrane markers. Fig. 4A shows that antibodies produced against the Na^+/H^+ exchanger fusion protein reacted weakly with a protein of 70 kDa and more strongly with a 50-kDa protein of bovine SL. Preimmune sera showed no reactivity at all with these bands. Fig. 4B shows similar results with the antibody against the synthetic peptide but with a stronger reaction against the 70-kDa protein. The results suggest that the Na^+/H^+ exchanger exists in the heart SL as proteins of 70 and 50 kDa. They are in agreement with studies which suggest that the protein is 65–70 kDa [12]; however, they disagree with results which suggest that the protein is over 100 kDa in SDS-polyacrylamide gels [21]. Also, the full-length clone of the human protein encodes for a protein of about 90–100 kDa [21,23]. The discrepancies can be due to a number of reasons. Proteolysis may account for the smaller proteins observed in the present study, and the sum of the two polypeptides observed in this study is similar to that larger size observed earlier [21,23]. However, we have used several protease inhibitors to reduce this possibility. An alternative explanation is that different forms of the Na^+/H^+ exchanger are expressed in various tissues, possibly due to differential splicing or some post-translational modifications. This could also explain the various types of Na^+/H^+ exchanger suggested by pharmacological data [10,13–16]. To investigate this possibility we performed Northern blot analysis to examine 'forms' of the protein present in muscle tissue. Fig. 5 shows the analysis of 8-day-old rabbit muscle poly(A)$^+$ RNA with the cardiac cDNA probe as described in Materials and Methods. The results suggest that a smaller form of the Na^+/H^+ exchanger does exist which is 3.8 kb in size and may correspond to the smaller form observed in this study and by Ross et al. [22]. The larger form of the protein (about 5–5.5 kb) apparently corresponds to that reported earlier [21]. An alternative possibility is that the differences in size of the message are due to a longer or shorter 3′ noncoding region. Experiments are now in progress to determine which alternative is correct.

Overall our results show that the Na^+/H^+ exchanger is present in the myocardium in a form that is closely

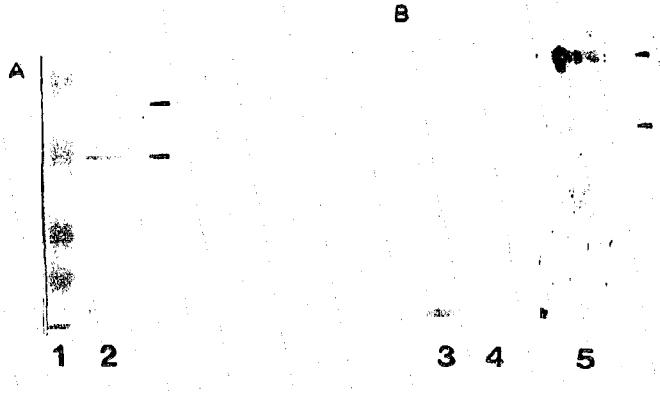

Fig. 4. (A) Immunoreactivity of antibodies against the Na^+/H^+ exchanger fusion protein. Antibodies produced against the fusion protein of the Na^+/H^+ exchanger were reacted with bovine SL (15 μg) separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose as described in Materials and Methods. (Lane 1) Bio-Rad prestained markers as described in Fig. 2. (Lane 2) SL reacted with the antibody. (B) Immunoreactivity of synthetic peptide antibody against the Na^+/H^+ exchanger. Samples are as described above (A) except that canine SL was used. (Lane 3) Bio-Rad prestained markers; (lane 4) SL with preimmune sera; (lane 5) SL reacted with the antibody.

related to the previously known human form [23]. In the myocardium it will certainly function to control intracellular pH, and aside from this role, it has recently been shown [17] that the Na^+/H^+ exchanger is important in modulating the cardiac response to reperfusion. Also intracellular acidosis is suggested to be important during contractile failure associated with ischemia [18] and the Na^+/H^+ exchanger likely plays a role in modulating this effect. Further experiments will determine the exact nature of this protein and its regulation and expression in this tissue.

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