Site-Directed Mutagenesis of the Na,K-ATPase: Consequences of Substitutions of Negatively-Charged Amino Acids Localized in the Transmembrane Domains†

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ABSTRACT: Site-directed mutagenesis was used to examine the importance of five carboxyl-containing amino acids localized in the putative membrane-spanning regions of the Na,K-ATPase (i.e., E327, E778, D803, D807, and D925 of the rat α2 isoform). The substitutions were introduced into a cDNA encoding a ouabain-resistant isoform (i.e., rat α2* which was mutated to encode a ouabain-resistant isoform), and the effect of these substitutions on Na,K-ATPase function was assessed by screening the altered enzymes for their ability to confer ouabain resistance when expressed in otherwise ouabain-sensitive cells. The expression of the α isoform containing certain substitutions at positions 327 and 925 was able to confer ouabain resistance to HeLa cells while the expression of rat α2* containing substitutions at positions 778, 803, and 807 was not. In particular, amino acids in each of these positions were substituted with leucine to evaluate the importance of the carboxyl-containing side chain. The ability of rat α2* containing E327L and D925L to confer ouabain resistance to HeLa cells indicates that neither the negative charge nor the oxygen-containing side chain is absolutely essential for overall function in this position. In contrast, the inability of rat α2* carrying E778L, D803L, and D807L to confer ouabain resistance suggests that the naturally occurring amino acid may be more critical structurally and/or functionally for the Na,K-ATPase. Other more conservative substitutions introduced to further characterize the role of particular amino acid side chains include E327D, E327Q, D803N, D803E, and D925N. Substitutions E327Q and D925N allowed the enzyme to retain function, in agreement with the effects of the leucine substitutions, while E327D, D803E, and D803N caused inactivation of the enzyme. The observation that Na,K-ATPase retains function when E327 is substituted with leucine and glutamine, but not aspartic acid, suggests that the size or the nature of the side chain may be important in this position for overall function. Cation dependence of Na,K-ATPase activity was assessed in enzyme containing substitutions which retained function. These results reveal that the Na^+ and K^+ stimulation of Na,K-ATPase activity is clearly different for rat α2* than for rat α2* containing the substitutions E327L, E327Q, D925L, and D925N. This study represents the first report of the effects of amino acid substitutions to five carboxyl-containing transmembrane residues in the Na,K-ATPase.

The Na,K-ATPase is an integral membrane protein essential for the survival of mammalian cells due to its role in the establishment and maintenance of the electrochemical gradient across the cell membrane. Na,K-ATPase belongs to a family of transport ATPases known as P-type ATPases, all of which contain a phosphorylated intermediate during their reaction cycle. The enzyme functions to catalyze the exchange of 2 mol of K^+ for 3 mol of Na^+ at the expense of 1 mol of ATP. Despite extensive studies performed on the Na,K-ATPase, the mechanism by which Na,K-ATPase effects cation exchange remains unclear [for reviews, see Glynn and Karlish (1990), Pedemonte and Kaplan (1990), and Karlish et al. (1991)].

To better understand this process, it is essential to identify functionally significant amino acids. It is reasonable to assume that amino acids with negatively-charged and/or oxygen-containing side chains are important for binding or translocating Na^+ and K^+ ions due to the positive charge of the ions transported by the Na,K-ATPase. Chemical modification utilizing reagents which react specifically with carboxyl groups has been used in an attempt to localize potential cation binding sites (Robinson, 1974; Yamaguchi et al., 1983; Shani-Sekler et al., 1988; Arguello & Kaplan, 1991). On the basis of the 10-transmembrane model predicted for the α subunit of the Na,K-ATPase (Karlish et al., 1993), there are 7 negatively-charged residues localized in the transmembrane regions. By combining chemical modification with proteolytic digestion, Goldshleger et al. (1992) suggested the involvement of E953 and possibly E954 of the pig kidney enzyme in cation binding. However, using a site-specific mutagenesis/expression strategy, Van Huysse et al. (1993) demonstrated that the carboxyl groups of these amino acids are not essential for function of the Na,K-ATPase and, therefore, are not likely to be directly involved in cation binding.

The goal of the present study was to assess the functional consequences of altering the remaining five negatively-charged amino acids localized in the transmembrane domains (i.e., E327, E778, D803, D807, and D925 of the rat α2 isoform). Since functionally important amino acids are often assumed to be conserved among related proteins, it is informative to compare results of effects of substitutions in conserved amino acids in related transport ATPases. Previously, the sarcoplasmic reticulum Ca-ATPase has been studied extensively using a similar approach [for a review, see MacLennan (1990)]. In particular, four of the five negatively-charged residues presented in this study are conserved between the Na,K-ATPase and the Ca-ATPase and have been postulated

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to be involved in cation binding in the Ca-ATPase (Clarke et al., 1989, 1990). A fifth negatively-charged transmembrane amino acid examined in this study, D925, is not conserved in the Ca-ATPase.

A site-directed mutagenesis/expression strategy was used to introduce substitutions into a cDNA which encodes a ouabain-resistant isoform of the Na,K-ATPase. By expressing these cDNAs in HeLa cells (which normally express only a ouabain-sensitive Na,K-ATPase) and assaying for their ability to survive in the presence of 1 μM ouabain, we were able to determine the effect of various amino acid substitutions in these carboxyl-containing transmembrane amino acids on overall Na,K-ATPase function. Specifically, each of the five carboxyl-containing transmembrane residues was substituted with leucine (i.e., E327L, E778L, D803L, D807L, and D925L) to assess the role of the carboxyl group in Na,K-ATPase function. Other substitutions were also introduced (i.e., E327D, E327Q, D803E, D803N, and D925N) to further characterize the role of particular amino acid side chains. Finally, cation-dependence properties of Na,K-ATPase containing various amino acid substitutions which did not eliminate enzyme activity were assessed.

EXPERIMENTAL PROCEDURES

Mutagenesis and Cloning. All substitutions were introduced into the rat α2 isoform because of the availability of an antibody (generously provided by Dr. K. Sweadner) which would distinguish between the transfected α isoform containing the substitution and the endogenous HeLa α isoform. The rat α2 cDNA was previously modified to encode a ouabain-resistant isoform (Jewell & Lingrel, 1991) by substituting L111R and N122D at the borders of the first extracellular domain. This modified rat α2 cDNA (referred to as rat α2*) was cloned into M13mp18 for site-directed mutagenesis (Kunkel, 1985), and restriction sites were simultaneously introduced which created cassettes to facilitate the site-directed mutagenesis procedure. *Hin*dIII recognition sequences were created 5′ of the ATG start codon as well as 3′ of the stop codon. *Eco*RI, *Bss*HII, *Xba*I, and *Mlu*I recognition sequences were introduced at positions 780, 1146, 2340, and 2904, respectively, within the rat α2* cDNA (Shull et al., 1986). To create the desired restriction sites, frequently used alternate codons were substituted such that the amino acids encoded were not altered. Mutations were then introduced in the modified rat α2* cDNA at positions corresponding to nucleotides encoding E327, E778, D803, D807, and D925 via site-directed mutagenesis (Kunkel, 1985). Following identification of the desired mutation using single-nucleotide tracking, the entire cassette was sequenced (Sanger et al., 1987) and subcloned into the context of the rat α2* cDNA contained in the pKC4 expression vector (Jewell & Lingrel, 1991). cDNAs containing substitutions corresponding to E327D, E327Q, E778L, D803L, D803E, D803N, D807L, and D925L were then subcloned into pRC CMV (Invitrogen) which contains both a neomycin resistance gene and elements required to promote expression of the cloned cDNA. Final constructs were analyzed by restriction analysis as well as by sequencing across the mutation site to confirm the presence of the substitution introduced.

Tissue Culture and Transfection. HeLa cells were maintained essentially as described (Jewell & Lingrel, 1991). Transfections were carried out using calcium phosphate precipitation (Chen & Okayama, 1987, 1988) as follows. Briefly, HeLa cells were seeded at an approximate density of 5×10^5 cells per plate and allowed to incubate at 37 °C, 5% CO_2, for 24 h: 25 μg of supercoiled plasmid DNA in 0.12 M $CaCl_2$/BES-buffered saline was then added dropwise to the media. Following 24-h incubation at 37 °C, 5% CO_2, the plates were washed several times with phosphate-buffered saline, the medium was replaced, and either ouabain (1 μM) or G418 (600 μg/mL) was added. Following selection for 2–3 weeks, colonies were isolated and expanded into stable cell lines.

Northern and Western Analyses. Total RNA was isolated by a modification of a procedure by Chomczynski and Sacchi (1987) which is described in detail in Jewell and Lingrel (1991). Northern blots were performed exactly as described (Jewell & Lingrel, 1991) except blots were hybridized with DNA fragments excised from the 3′-untranslated region of either the pKC4 or the pRC CMV transcription unit depending on the vector in which the mutated cDNA was carried.

Proteins contained in the NaI-treated plasma membrane fraction were separated on a 7.5% SDS–polyacrylamide gel (Laemmli, 1970) and electroblotted onto nitrocellulose at 350 mA for 90 min in 25 mM Tris-HCl, pH 8.3. The filter was incubated with a 1:100 dilution of MCB2, an α2-specific antibody (generously provided by Dr. K. Sweadner), followed by an anti-mouse horseradish peroxidase conjugated second antibody. The blots were developed using Enhanced Chemiluminescence (Amersham).

Isolation of Crude Plasma Membranes. Plasma membrane preparations were isolated as described previously (Jewell & Lingrel, 1991). Protein was determined using a modification of the Bradford procedure (Bradford, 1976) using bovine serum albumin as a standard.

Determination of Na,K-ATPase Activity and Data Analysis. ATPase activity was measured using a modification of the assay described by Brown (1982) which is based on the release of $[^{32}P]P_i$ from $[\gamma\text{-}^{32}P]ATP$. The assay was performed exactly as described in Jewell and Lingrel (1991). Less than 20% of the total ATP was hydrolyzed in each experiment, and the rate of ATP hydrolysis was linear over the specified periods of incubation. Na,K-ATPase activity contributed by the enzyme containing the transfected ouabain-resistant isoform was distinguished from the relatively ouabain-sensitive endogenous activity by conducting the assays in the presence of 5 μM ouabain (which inhibits the endogenous HeLa Na,K-ATPase) and in the presence of 10 mM ouabain (which inhibits all Na,K-ATPase activity). The activity measured in 10 mM ouabain was then subtracted from that measured in 5 μM ouabain to yield the activity of Na,K-ATPase molecules containing the transfected isoform. It has been established that the activities corresponding to ATPases other than Na,K-ATPase (i.e., those not inhibited by 10 mM ouabain) present in the membrane preparation do not exhibit a dependence on NaCl or KCl (data not shown). Therefore, the activity at 0 mM NaCl or KCl in 5 μM ouabain, which was equal to that at all cation concentrations in 10 mM ouabain, was subtracted from each ATPase activity in 5 μM ouabain. ATPase activity not inhibited by 10 mM ouabain comprised 30–70% of the total ATPase activity in the membrane preparation. Also, nonenzymatic ATP hydrolysis was analyzed for each experiment and subtracted from enzymatic ATP hydrolysis. Samples from separate enzyme preparations were assayed in triplicate for the analysis of ouabain-inhibitable Na,K-ATPase activity, and NaCl and KCl stimulation. Specific conditions for these experiments are as follows: for ouabain-inhibitable Na,K-ATPase activity, 100 mM NaCl, 10 mM KCl, 40 mM choline chloride, 3 mM Tris–ATP, 3 mM $MgCl_2$, 0.1 M EGTA, 5 mM histidine (pH 7.4) and varying ouabain concentrations; for NaCl stimulation, 10 mM KCl, 3 mM

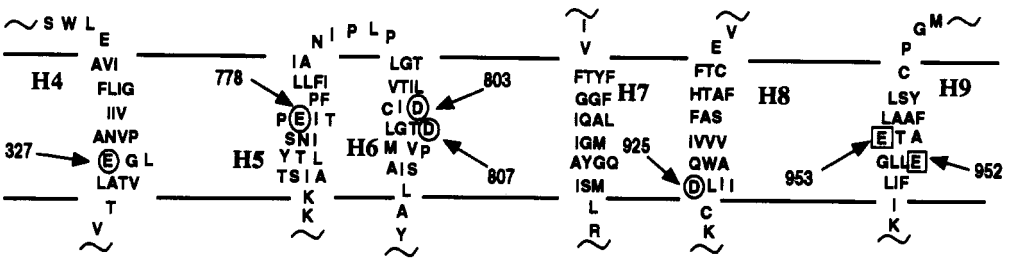

FIGURE 1: Membrane-spanning domains H4–H9 of the 10-transmembrane model of the rat Na,K-ATPase $\alpha 2^*$ subunit. Shown are six transmembrane regions in a hypothetical model of the organization of the membrane-spanning domains (Karlish et al., 1993) of the rat $\alpha 2$ subunit of the Na,K-ATPase. The extracellular and intracellular sides of the plasma membrane as well as six transmembrane passes (H4–H9) are indicated. The circled residues, E327, E778, D803, D807, and D925, are the amino acids which have been substituted in this study. The amino acids shown in squares, E952 and E953, were focused on in a previous study (Van Huysse et al., 1993).

Tris–ATP, 3 mM $MgCl_2$, 0.1 M EGTA, 5 mM histidine (pH 7.4), and varying NaCl concentrations; for KCl stimulation, 30 mM NaCl, 3 mM Tris–ATP, 3 mM M gCl_2, 0.1 M EGTA, 5 mM histidine (pH 7.4), and varying KCl concentrations. For both NaCl and KCl stimulation experiments, the concentration of NaCl + KCl + choline chloride = 150 mM.

Data were analyzed as described in Jewell and Lingrel (1991) by nonlinear regression using the Sigma Plot Scientific Graph System (Jandel Scientific). All NaCl and KCl stimulation data were analyzed by a noncooperative model for ligand binding (Garay & Garrahan, 1973) as well as by a highly cooperative model for ligand binding.

RESULTS

The molecular cloning of cDNAs for the α subunit of the Na,K-ATPase (Shull et al., 1986) has provided us with the opportunity to utilize a site-directed mutagenesis/expression strategy as a way in which to identify functionally significant amino acids. It has been previously shown that transfection of a ouabain-resistant isoform of Na,K-ATPase into otherwise ouabain-sensitive HeLa cells allows the cells to become ouabain-resistant (Price & Lingrel, 1988; Jewell & Lingrel, 1991). Therefore, by introducing a substitution into a cDNA which encodes a ouabain-resistant isoform and assaying for its ability to confer ouabain resistance to HeLa cells, it is possible to assess whether a particular substitution has a significant effect on Na,K-ATPase function. For example, if a substitution is introduced which only mildly affects or has no effect on Na,K-ATPase function, ouabain resistance will be observed following transfection of the substituted cDNA into HeLa cells. Alternatively, if a substitution is introduced at a critical site in the enzyme and it eliminates Na,K-ATPase function, ouabain resistance will not be conferred, and, therefore, the cells will not survive following treatment with ouabain.

Our objective in this study was to examine the effects of substitutions to negatively-charged transmembrane amino acids on Na,K-ATPase function. Figure 1 highlights the amino acids focused on in this study including E327, E778, D803, D807, and D925 of the rat $\alpha 2$ isoform (shown in circles). These amino acids are conserved across all isoforms and species of the Na,K-ATPase, and several of them are also conserved in other transport ATPases. Various substitutions were introduced into the five negatively-charged transmembrane residues in order to characterize the role of specific amino acid side chains in several of these positions (see Table I). In particular, the importance of the carboxyl side chain was examined by substituting each residue with leucine.

Table I: Summary of the Ability of Cells Expressing Rat $\alpha 2^*$ Containing Various Amino Acid Substitutions To Survive Growth in 1 μM Ouabain

rat $\alpha 2^*$ isoform substitutions	growth in ouabain[a]
rat $\alpha 2^*$	+
E327L	+
E327Q	+
E327D	–
E778L	–
D803L	–
D803N	–
D803E	–
D807L	–
D925L	+
D925N	+

[a] The (+) indicates growth in the presence of 1 μM ouabain whereas the (–) indicates no growth.

The rat $\alpha 2$ isoform was chosen for these studies due to the availability of an $\alpha 2$-specific antibody (provided by Dr. K. Sweadner) which was necessary to confirm expression of the cDNAs at the protein level. This antibody is specific for rat $\alpha 2$ and therefore does not interact with the endogenous HeLa α isoform. However, as this isoform is sensitive to ouabain, it was previously modified to encode a ouabain-resistant isoform (designated rat $\alpha 2^*$) by substituting uncharged residues with charged residues at the borders of the first extracellular region (i.e., L111R and N122D). These residues have previously been shown to alter the ouabain sensitivity of the rat $\alpha 2$ isoform approximately 1000-fold (Emanuel et al., 1989; Jewell & Lingrel, 1991). Following site-directed mutagenesis to introduce the desired mutation, cDNAs containing each substitution were subcloned into a eukaryotic expression vector and transfected into HeLa cells. Successfully transfected cells were then selected in 1 μM ouabain. After approximately 2–3 weeks, ouabain-resistant colonies appeared for cells transfected with the positive control, rat $\alpha 2^*$, as well as with rat $\alpha 2^*$ carrying substitutions at positions 327 and 925 (Table I). In contrast, colonies were not obtained for cells transfected with rat $\alpha 2^*$ containing substitutions at 778, 803, or 807. As expected, ouabain-resistant colonies were never obtained with mock-transfected cells or with cells transfected with a ouabain-sensitive isoform. These results suggest that while the naturally occurring amino acids are not

absolutely essential for Na,K-ATPase function at positions 327 and 925, the amino acids in positions 778, 803, and 807 may be more critical for overall activity.

The clones expressing rat $\alpha 2^*$ containing substitutions E327L, E327Q, and D925N were obtained following transfection into HeLa cells and selection in 1 μM ouabain. Ouabain-resistant colonies appeared after 2–3 weeks, and these were isolated and expanded into stable cell lines. Clones expressing the D925L substitution were obtained in a different manner since, for a reason which is unclear, we were unable to obtain ouabain-resistant colonies following transfection and selection in 1 μM ouabain. Therefore, the cDNA containing the substitution was cloned into an expression vector containing a neomycin resistance gene in addition to elements required to promote expression of the cloned cDNA (pRC CMV). This vector was transfected into HeLa cells, and successfully transfected cells were selected in G418 (a neomycin analog). Cell lines expressing mRNA corresponding to the substituted cDNA were then screened by Northern analysis (data not shown). Finally, lines positive for mRNA expression were treated with 1 μM ouabain, and, surprisingly, these cells survived. Therefore, cell lines expressing rat $\alpha 2^*$ containing the D925L substitution were, in fact, able to survive in 1 μM ouabain. Several of these lines were expanded in ouabain and analyzed further (see below). Again, the reason ouabain-resistant colonies were not obtained directly is unclear; perhaps poor transfection efficiency was a factor in those experiments. Although only a qualitative comparison, it is interesting to mention that the ouabain-resistant cell lines expressing rat $\alpha 2^*$ containing E327L, E327Q, D925L, and D925N substitutions seemed to grow slower than cells expressing rat $\alpha 2^*$. Quantitative comparisons in growth rate are difficult to assess due to differences in expression levels even among clones derived from the same cDNA.

Those rat $\alpha 2^*$ substitutions which did not allow HeLa cells to survive in ouabain following transfection include E327D, E778L, D803L, D803E, D803N, and D807L. Possible explanations for these results include the inability of the protein containing the substitution to be synthesized or inserted into the plasma membrane, or, alternatively, these results could indicate that these substitutions in the α subunit have inactivated the enzyme and, therefore, the substituted amino acid must be essential for enzyme function. To test these possibilities, cDNAs containing each substitution were subcloned into pRC CMV and transfected into HeLa cells. Successfully transfected cells were selected in G418, which does not require the expression of functional rat $\alpha 2^*$ to obtain colony formation, and Northern analysis was used to screen for cells expressing the substituted rat $\alpha 2^*$ cDNA (data not shown). At this point, we treated cells positive for mRNA expression with 1 μM ouabain to confirm the inability of these substitutions to confer ouabain resistance to otherwise sensitive cells. Cell lines expressing the mRNA for rat $\alpha 2^*$ containing substitutions E327D, E778L, D803N, D803L, D803E, and D807L were not able to survive in ouabain. Therefore, despite the fact that the DNA was successfully transfected (as judged by mRNA expression), rat $\alpha 2^*$ containing these substitutions was unable to confer ouabain resistance to HeLa cells.

To confirm the rat $\alpha 2^*$ subunit containing the introduced substitutions is present at the protein level, plasma membranes were isolated, and Western analysis was performed. In particular, it was important to analyze the G418-selected clones which were positive for the expression of mRNA, but which were unable to survive in 1 μM ouabain. Immunoblots of plasma membranes isolated from ouabain-resistant clones

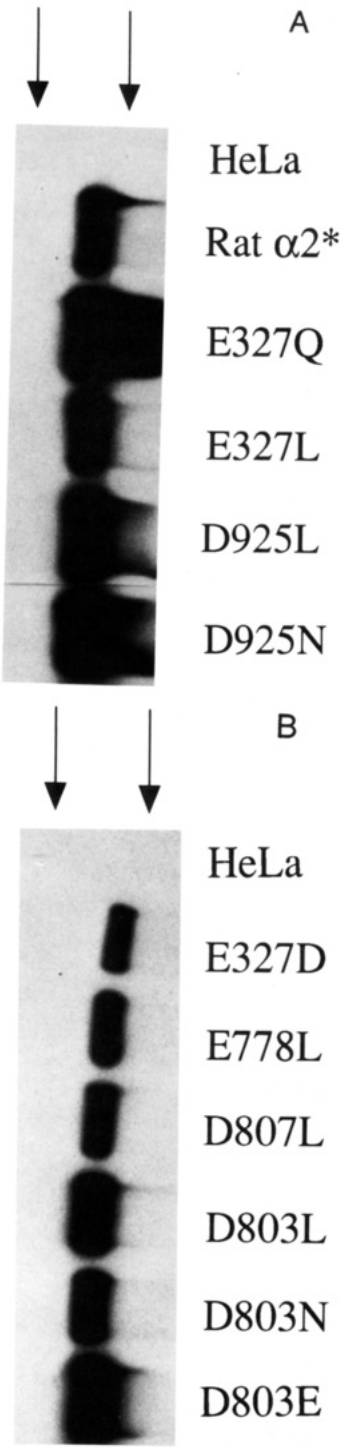

FIGURE 2: Western analysis of rat $\alpha 2^*$ cDNA transfectants. Both panels A and B contain autoradiographs of immunoblots developed using the ECL system (Amersham). The arrows indicate the positions of the prestained molecular weight markers, phosphorylase *b* and bovine serum albumin (Bio-Rad), which were used as a reference for molecular weight. Panel a contains rat $\alpha 2^*$ substitutions which resulted in functional enzyme (i.e., E327L, E327Q, D925L, and D925N) as well as samples from untransfected HeLa cells and from rat $\alpha 2^*$ transfected cells. Cell lines expressing each of these substitutions were selected in ouabain. Each lane contains 20 μg of protein. Panel b contains rat $\alpha 2^*$ substitutions which inactivated the Na,K-ATPase (i.e., E327D, E778L, D803E, D803L, D803N, and D807L). Therefore, clones expressing each of these substitutions were selected in G418 (except the untransfected HeLa cell control). Each lane contains 80 μg of protein.

derived from cells transfected with rat $\alpha 2^*$ containing E327L, E327Q, D925L, and D925N substitutions (Figure 2A) confirm the presence of protein at levels comparable to rat $\alpha 2^*$. There

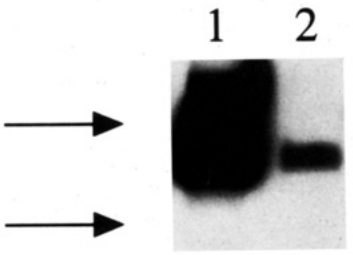

FIGURE 3: Western analysis of a G418-selected clone maintained in G418 or ouabain. Shown is an autoradiograph of an immunoblot developed using the ECL system (Amersham). The arrows indicate the positions of the prestained molecular weight markers, phosphorylase *b* and bovine serum albumin (Bio-Rad), which were used as a reference for molecular weight. Both lanes were loaded with 80 μg of protein. A functional rat α2* cDNA in pRC CMV was transfected into HeLa cells and selected in G418. Lane 1 shows the Western blot of this clone following growth in ouabain. Lane 2 shows the same clone which was selected and maintained in G418.

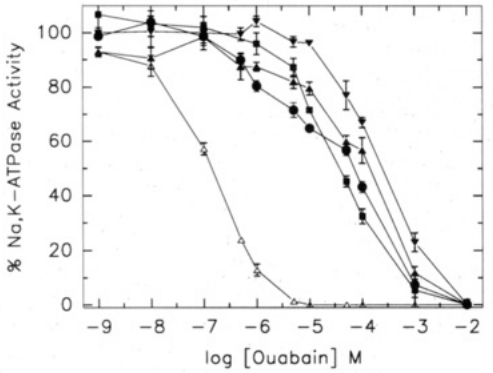

FIGURE 4: Ouabain-inhibitable Na,K-ATPase activity. The data are presented as the percent of total Na,K-ATPase activity versus increasing ouabain concentration. This plot represents the results of one experiment performed in triplicate using a single membrane preparation. Error bars represent the standard error of the mean of the triplicate velocity determinations and are as indicated. Na,K-ATPase activity at 10 mM ouabain was equal to Na,K-ATPase activity under Na^+-free conditions. Therefore, ATPase activity at 10 mM ouabain was defined as zero Na,K-ATPase activity. The specific activity of Na,K-ATPase in 0 mM ouabain is as follows (units are micromoles of P_i released per milligram of protein per hour): untransfected HeLa = 8.93; E327L = 1.27; E327Q = 1.48; D925L = 1.48; and D925N = 2.09. Symbols are as follows: (○) untransfected HeLa; (▲) E327L; (■) E327Q; (●) D925L; (▼) D925N.

is no signal corresponding to the presence of the rat α2* isoform in membrane preparations isolated from untransfected HeLa cells. The lower panel (Figure 2B) shows immunoblots of plasma membranes isolated from G418-selected clones which were positive for mRNA expression but were unable to confer ouabain resistance to HeLa cells (i.e., those transfected with rat α2* containing E327D, E778L, D803L, D803E, D803N, and D807L). As expected, there is no signal present in the untransfected HeLa cells. However, it is clear that protein is expressed in G418-selected clones derived from cells transfected with substitutions not able to confer ouabain resistance to HeLa cells. Since more protein was loaded in each lane in Figure 2B (80 μg) compared to Figure 2A (20 μg), it is obvious that there is less protein expressed for the G418-selected clones (Figure 2B) than for those selected and maintained in ouabain (Figure 2A). We believe this difference is attributable to the inability to directly select for enzyme since it is not functional. This is supported by the observation that functional enzyme (i.e., that which is able to confer ouabain resistance to HeLa cells) is present at levels comparable to that seen in Figure 2B when selected in G418 (Figure 3, lane 2). However, the level increases substantially when

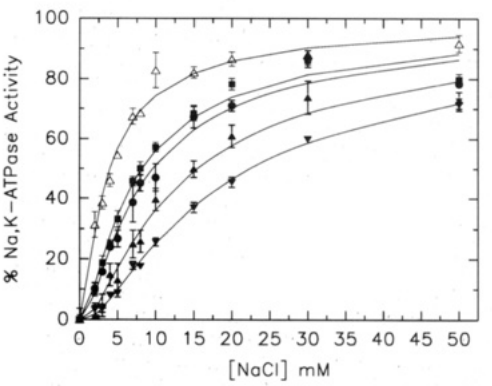

FIGURE 5: [NaCl]dependence of Na,K-ATPase activity. Determination of ATPase activity and definition of Na,K-ATPase activity contributed by the transfected isoform are as described under Experimental Procedures. Data are expressed as the percent of total Na,K-ATPase activity [defined as the percentage of activity at each [NaCl] relative to the V_{max}, which was determined from nonlinear regression analysis using the equation for noncooperative ligand binding which is described in Jewell and Lingrel (1991)] versus [NaCl] (0–50 mM). Separate enzyme preparations isolated from more than one clone for each substitution were assayed in triplicate. This plot represents the results of one of those experiments. Error bars represent the standard error of the mean of the triplicate velocity measurements. Data were also analyzed according to a cooperative model for ligand binding described in Jewell and Lingrel (1991). Kinetic constants are as follows [K_{Na^+} (mM) ± SD; $K_{0.5}$ (mM) ± SD; and {number of trials (one trial represents an individual experiment conducted in triplicate using different enzyme preparations)}, respectively]: rat α2* = 1.06 ± 0.08; 3.42 ± 0.23; {5}; E327L = 3.87 ± 0.42; 9.48 ± 0.99; {4}; E327Q = 2.06 ± 0.17; 5.71 ± 0.33; {3}; D925L = 2.06 ± 0.32; 6.09 ± 0.80; {4}; D925N = 5.50 ± 0.31; 12.63 ± 1.56; {4}. The V_{max} for each preparation used in this experiment is as follows (units are micromoles of P_i released per milligram of protein per hour): rat α2* = 4.27; E327L = 1.08; E327Q = 2.62; D925L = 1.09; and D925N = 2.97. Symbols are as follows: (△) rat α2*; (▲) E327L; (■) E327Q; (●) D925L; (▼) D925N.

the same clone is exposed to ouabain (Figure 3, lane 1). Therefore, even functional enzyme is expressed at lower levels in the absence of direct selection. This suggests that rat α2* isoforms containing substitutions E327D, E778L, D803L, D803E, D803N, and D807L are expressed at levels sufficient to confer ouabain resistance if, in fact, they were functional.

Thus, these results confirm the expression of the transfected rat α2* isoform containing various substitutions at the protein level. The presence of protein containing substitutions which were not able to confer ouabain resistance when expressed in HeLa cells suggest that the introduced substitution has inactivated overall Na,K-ATPase function. Enzymes with substitutions which resulted in inactivation were not analyzed further.

Cell lines expressing functional enzyme with amino acid substitutions E327Q, E327L, D925L, and D925N were characterized in terms of ouabain-inhibitable Na,K-ATPase activity (Figure 4). Na,K-ATPase isolated from untransfected HeLa cells exhibited a profile for ouabain inhibition which was approximately 1000-fold more sensitive to ouabin than Na,K-ATPase containing the substitutions. This result confirms the presence of the activity of the ouabain-resistant isoforms containing the various amino acid substitutions. As shown in Figure 4, it appears that slight differences may exist between enzyme containing the various substitutions. This may imply that the substitution introduced has some effect on ouabain affinity. However, this possibility does not affect the cation dependence properties shown in Figures 5 and 6 as under the experimental conditions, the endogenous HeLa enzyme is completely inhibited, while the transfected activity is measured.

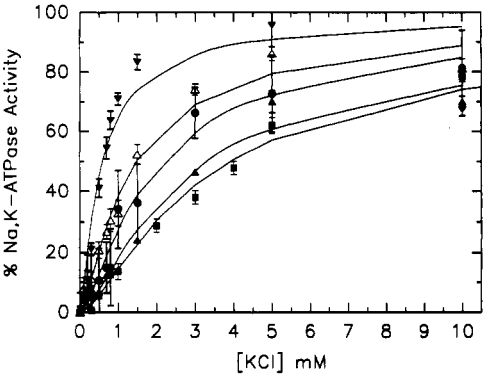

FIGURE 6: [KCl] dependence of Na,K-ATPase activity. Determination of ATPase activity and definition of Na,K-ATPase activity contributed by the transfected isoform are described under Experimental Procedures. Data are expressed as the percent of total Na,K-ATPase activity [defined as percentage of activity at each [KCl] relative to V_{max}, which was determined from nonlinear regression analysis using the equation for noncooperative ligand binding which is described in Jewell and Lingrel (1991)] versus [KCl] (0–10 mM). Separate membrane preparations isolated from more than one clone for each substitution were assayed in triplicate. This plot represents one of those experiments. Error bars represent the standard error of the mean of triplicate velocity determinations. Data were also analyzed according to a cooperative model for ligand binding described in Jewell and Lingrel (1991). Kinetic constants are as follows [K_{K^+} (mM) ± SD; $K_{0.5}$ (mM) ± SD; and {number of trials (one trial represents an individual experiment conducted in triplicate using different enzyme preparations)}, respectively]: rat $\alpha2^*$ = 0.59 ± 0.16; 1.03 ± 0.22; {7}; E327L = 1.25 ± 0.13; 1.95 ± 0.18; {4}; E327Q = 1.65 ● 0.30; 3.18 ± 0.45; {3}; D925L = 1.28 ● 0.39; 1.91 ± 0.46; {4}; D925N = 0.26 ± 0.02; 0.56 ± 0.06; {3}. The V_{max} for each preparation used in this experiment is as follows (units are micromoles of P_i released per milligram of protein per hour): rat $\alpha2^*$ = 2.16; E327L = 1.61; E327Q = 1.84; D925L = 0.47; and D925N = 1.94. Symbols are as follows: (△) rat $\alpha2^*$; (▲) E327L; (■) E327Q; (●) D925L; (▼) D925N.

Since these negatively-charged transmembrane amino acids could be involved in cation binding, Na,K-ATPases containing each of the substitutions able to confer ouabain resistance to HeLa cells were characterized in terms of the dependence of their activity on NaCl and KCl concentration. From these data, apparent affinities for Na^+ and K^+ can be calculated. Although the apparent affinity is not a direct measure of cation affinity and therefore does not directly assess the involvement of the substituted amino acid in cation binding, it does provide a means by which to assess the effect of the substitution on cation stimulation of overall enzyme activity.

NaCl stimulation of Na,K-ATPase activity (shown in Figure 5) containing substitutions at position 327 revealed a 3-fold decrease in the apparent affinity for the leucine substitution ($K_{0.5}$ = 9.48 ± 0.99) relative to rat $\alpha2^*$ ($K_{0.5}$ = 3.42 ± 0.23), while the substitution to a glutamine resulted in less than 2-fold difference ($K_{0.5}$ = 5.71 ± 0.33). Additionally, as discussed earlier, the E327D substitution inactivated enzymatic activity. It is clear from these results that neither the negative charge nor the presence of an oxygen-containing side chain is required in this position for overall function of the enzyme (definied as the ability of the transfected enzyme to support growth of HeLa cells in the presence of 1 μM ouabain). It is possible that it is the bulkiness of the side chain which is important in this position for overall function since glutamic acid, glutamine, and leucine are larger than aspartic acid which, when substituted for E327, inactivated enzymatic activity. We obviously have not excluded the possibility that some functional parameter of the E327L- and E327Q-substituted enzyme has been altered compared to the rat $\alpha2^*$ enzyme. Indeed, the altered cation dependence properties may be indicative of this amino acid being part of or near to a cation binding site(s).

NaCl stimulation of Na,K-ATPase activity for enzyme containing substitutions in position 925 revealed an approximate 2-fold increase in the $K_{0.5}$ for Na^+ when substituted with leucine ($K_{0.5}$ = 6.09 ± 0.80) and a 4-fold increase when substituted with asparagine ($K_{0.5}$ = 12.63 ± 1.56) as compared to rat $\alpha2^*$ ($K_{0.5}$ = 3.42 ± 0.23). Again, it is clear that neither the negative charge nor the presence of an oxygen-containing side chain is essential in this position for overall function. The data for a representative experiment conducted in triplicate have been fit to the noncooperative model for ligand binding (Figure 5), and constants derived from both the cooperative and noncooperative models are summarized in the legend to Figure 5.

KCl dependence properties were also analyzed for each of these substitutions. Results of an experiment performed in triplicate are shown in graphical form in Figure 6, and the constants are summarized in the figure legend. Substitutions at position 327 resulted in an apparent affinity for KCl which was more significantly altered (approximately 3-fold) for the glutamine substitution ($K_{0.5}$ = 3.18 ± 0.45) than for the leucine substitution ($K_{0.5}$ = 1.95 ± 0.18) relative to rat $\alpha2^*$ ($K_{0.5}$ = 1.03 ± 0.22). The apparent affinity for K^+ at position 925 increased slightly for the asparagine substitution ($K_{0.5}$ = 0.56 ± 0.06), while the apparent affinity slightly decreased for the leucine substitution ($K_{0.5}$ = 1.91 ± 0.46). The D925N substitution exhibited interesting effects on cation stimulation in that the apparent affinity for Na^+ was decreased approximately 4-fold (the largest change measured with any substitution) while the apparent affinity for K^+ was increased slightly.

DISCUSSION

The identification of amino acid residues important structurally and/or functionally for ion-transporting ATPases represents an important step in the elucidation of a mechanism for ion translocation. The P-type ATPases are relatively homologous, and it is assumed that conserved amino acids may be important for overall structure and function in each of these pumps. Previous studies (Clarke et al., 1989, 1990; Andersen & Vilsen, 1992; Vilsen & Andersen, 1992) have suggested that transmembrane carboxyl-containing amino acids are critical for the function of cation-translocating ATPases. This study represents the first report of the effects of amino acid substitutions at many of these residues in the Na,K-ATPase.

In an initial attempt to identify functionally important amino acids in the Na,K-ATPase, we have used site-directed mutagenesis to substitute carboxyl-containing amino acids localized in the membrane-spanning regions. We have assessed the functional importance of these amino acids by expressing enzyme containing various amino acid substitutions in HeLa cells, which are naturally ouabain-sensitive, and then assaying for the ability of the exogenously expressed protein to confer ouabain resistance to these cells. MacLennan and colleagues (Maruyama et al., 1988; Clarke et al., 1989) have shown that substitutions made in amino acids critical for function inactivate the overall enzymatic cycle, measured by calcium transport. On the basis of this criterion, it is reasonable to assume that if a substitution is made in a critical residue in the Na,K-ATPase, the enzyme will be inactivated and ouabain resistance will not be conferred when expressed in sensitive cells. Alternatively, if a substituted enzyme is able to confer ouabain resistance to HeLa cells, the substitution must have

allowed the enzyme to retain function. However, while the ability of the enzyme to confer ouabain resistance does not exclude the possibility that the substituted amino acid may serve some functionally important role, it does indicate that the enzyme containing the substitution is able to perform the essential functional role of the Na,K-ATPase in HeLa cells.

Our results indicate that several carboxyl-containing amino acids localized in the membrane-spanning regions (see Figure 1) are critical for overall enzymatic function (defined as the ability of the enzyme to support growth of HeLa cells in 1 μM ouabain). On the basis of the 10-transmembrane model predicted for the Na,K-ATPase (Karlish et al., 1993), there are 7 negatively-charged amino acids localized in the transmembrane domains. It has been previously demonstrated that the side chains of two of these residues, E952 and E953 (in the rat α2 isoform), are not required for function (Van Huysse et al., 1993). In this study, we have examined the role of the remaining negatively-charged amino acids. It is clear from these experiments that while the naturally occurring amino acids are not absolutely essential for overall function in positions 327 and 925, they may be important in positions 778, 803, and 807.

To further examine the role of these amino acids in Na,K-ATPase function, additional amino acid substitutions were made in several of these positions. For example, E327 was replaced with a leucine (as discussed above), as well as with more conservative amino acids, aspartic acid and glutamine. Since the E327L substitution was functional, it is clear that a carboxyl-containing side chain is not required. In addition, replacing the carboxyl with an amide group (glutamine) was not deleterious to enzyme function while shortening the amino acid side chain by one carbon (aspartic acid) was sufficient to abolish activity. This may indicate that it is the size of the amino acid side chain that is important in this position since glutamine and leucine are somewhat larger than aspartic acid, and substitution with both of the larger amino acids permitted the enzyme to function to confer ouabain resistance to otherwise ouabain-sensitive HeLa cells.

The residue analogous to E327 in the Na,K-ATPase is E309 in the Ca-ATPase. Extensive analyses have been carried out on the role of this amino acid in calcium transport as well as the role of surrounding amino acids localized in the fourth transmembrane domain. This region seems to be central to conformational transitions involved in the translocation event (Vilsen et al., 1989; Andersen et al., 1992; Andersen & Vilsen, 1992; Vilsen & Andersen, 1992b). In addition, E309 has been shown to be involved in calcium occlusion (Vilsen & Andersen, 1992a). Therefore, since amino acids in this region are absolutely conserved in the Na,K-ATPase and the Ca-ATPase, it is reasonable to assume the fourth transmembrane domain is also important in the Na,K-ATPase. However, while the Ca-ATPase was not able to tolerate substitutions in position 309 without loss of calcium transport activity, the analogous amino acid in the Na,K-ATPase was able to be substituted with leucine and glutamine while retaining the ability to confer ouabain resistance to HeLa cells (indicating the maintenance of overall enzymatic function). Therefore, this represents a fundamental difference between the Na,K-ATPase and the Ca-ATPase. It is possible that these discrepancies provide the basis for the differences in cation specificity.

To further characterize the enzymes containing substitutions which retained function, rat α2* isoforms containing E327Q and E327L were analyzed in terms of their cation dependence properties. While the E327Q substitution had only a modest effect on the apparent Na^+ affinity (i.e., less than a 2-fold decrease compared to rat α2*), a more significant effect was seen on the apparent affinity for K^+ (i.e., an approximate 3-fold decrease compared to rat α2*). In contrast, the E327L substitution had a more significant effect on the apparent Na^+ affinity (i.e., an approximate 3-fold decrease) than on the apparent K^+ affinity (i.e., less than a 2-fold decrease). While these changes in apparent affinity could be due to a decreased affinity for cations as a result of the substitution introduced, they could also be due to effects on conformational transitions in the enzyme. Although the enzyme does retain function with these substitutions in positon 327, it is clear that neither enzyme containing substitutions in position 327 exhibits the same cation dependence properties as rat α2*. Therefore, this could be indicative of this amino acid being part of or near to a cation binding site(s).

Another negatively-charged amino acid, D925, which is localized in the eighth transmembrane domain, is not conserved between the Na,K-ATPase and the Ca-ATPase, and, therefore, the negative charge at this position is unique to the Na,K-ATPase. Substitution of this amino acid with a leucine or an asparagine did not result in loss of enzyme activity. However, examination of the cation dependence of ATPase activity revealed clear differences from rat α2*. While only modest differences were observed in the $K_{0.5}$ for Na^+ and K^+ stimulation for D925L as compared to rat α2* (i.e., less than a 2-fold decrease in apparent affinity for Na^+ and K^+), the differences for D925N were more remarkable. In fact, while the apparent affinity for Na^+ for D925N decreased approximately 4-fold compared to rat α2*, the apparent affinity for K^+ actually increased slightly (i.e., an approximate 2-fold decrease in the apparent affinity of rat α2* compared to D925N). These results may reflect a stabilization of the E2 form (K^+ form) of the enzyme relative to the E1 form (Na^+ form). Alternatively, the effect could be due to perturbations at the actual cation binding site(s) introduced by the substituted amino acid side chain. However, the precise reason for this effect is unclear.

The remaining amino acids, E778, D803, and D807 of the Na,K-ATPase, are all conserved in the Ca-ATPase (i.e., E771, N796, and D800, respectively). Substitution of each of these amino acids in the Na,K-ATPase as well as in the Ca-ATPase had profound effects on enzyme function (Clarke et al., 1989, 1990; Andersen & Vilsen, 1992; Vilsen & Andersen, 1992a). In the Ca-ATPase, two of these amino acids, E771 and D800, have been shown to be involved in calcium occlusion (Vilsen & Andersen, 1992a), and, in addition, E771 may be involved in energy transduction events (Andersen & Vilsen, 1992). On the basis of our result of complete loss of overall enzyme function upon substitution of E778, D803, and D807 in the Na,K-ATPase, it is possible that these amino acids may serve an important functional role similar to the conserved residues in the Ca-ATPase.

This study represents an initial characterization of the functional importance of carboxyl-containing transmembrane amino acids in the Na,K-ATPase. According to the 10-transmembrane model predicted for the Na,K-ATPase, there are 7 carboxyl-containing residues localized in the membrane-spanning regions (Karlish et al., 1993). On the basis of the assumption that the substitution of critically important amino acids results in enzyme inactivation, E952 and E953 (Van Huysse et al., 1993) and D925 (as shown in this study) can be eliminated as being essential for overall Na,K-ATPase function. On the other hand, E778, D803, and D807 appear to be more critical for enzyme activity. Additionally, since

enzyme is able to be substituted with certain amino acids (i.e., leucine and glutamine) at position 327 with the maintenance of enzymatic function while substitution with aspartic acid abolishes enzymatic activity, it is clear that the carboxyl side chain is not essential for overall function. However, this does not exclude the possibility that some functional parameter has been altered in enzyme containing substitutions E327L and E327Q. Further analysis is required to define the specific role of these amino acids in Na,K-ATPase function.

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