**Abstract.** The γ subunit of the Na,K-ATPase is a small membrane protein that copurifies with the α and β subunits of the enzyme. Strong evidence that the γ subunit is a component of the Na,K-ATPase comes from studies indicating that the subunit is involved in forming the site for cardiac glycoside binding. We have isolated and characterized the cDNAs coding the γ subunit from several species. The γ subunit is a highly conserved protein consisting of 58 amino acids with a molecular weight of 6500. Hydrophathy analysis revealed the presence of a single hydrophobic domain that is sufficient to cross the membrane. There are no sites for N-linked glycosylation. Northern blot analysis revealed that the γ subunit mRNA is expressed in a tissue-specific fashion and is present in all tissues characterized. γ-specific antibodies have been used to verify that the sequenced protein is the same protein labeled with \([\text{H}]\)nitroazidobenzoyl-ouabain (NAB-ouabain), and that this protein, the γ subunit of the Na,K-ATPase, has a distribution pattern along nephron segments that is identical with the α subunit. In addition, coimmunoprecipitation of the α, β and γ subunits demonstrate specific association of the subunits. These results are consistent with the notion that the γ subunit is specifically associated with and may be an important component of the Na,K-ATPase.

**The Na,K-ATPase** is a membrane protein that uses the energy from the hydrolysis of ATP to transport Na and K across the plasma membrane. The enzyme consists of at least two polypeptides, a catalytic α subunit of ~110 kD and a smaller glycosylated β subunit of ~55 kD. The α subunit contains the binding site for ATP and the cardiac glycoside inhibitor ouabain; it is phosphorylated and undergoes ligand-dependent conformation changes accompanying the binding and translocation of cations across the plasma membrane. All catalytic functions have been assigned to the α subunit, however, the β subunit may play an essential role in either stabilization, maturation, or enzymatic activity of functional Na,K-ATPase molecules (Gerking, 1991). The amino acid sequences of the α and β subunits of the Na,K-ATPase from several species have been determined from their cDNAs (reviewed in Lingrel et al., 1990; Mercer et al., 1989).

Distinct forms of the rat Na,K-ATPase have been described which can be distinguished by SDS-PAGE (Sweedner, 1979), antigenic determinants (Sweedner and Gilkeson, 1985), and differences in affinity for ouabain (Sweedner, 1979). These differences have been ascribed to the presence of two isoforms of the α subunit (α and α'). The cDNAs for the α(+) isoform (termed α2) and an α subunit previously unidentified, designated α3, have been isolated and sequenced (Shull et al., 1986; Takeyasu et al., 1990). In addition to the α isoforms, a putative β subunit isoform cDNA, termed β2, has been identified in mammals (Martin-Vasallo et al., 1989). In glia cells this polypeptide functions as an adhesion molecule and is associated with the α2 subunit of the Na,K-ATPase (Gloor et al., 1990). Characterization of α, α isoform, and β mRNAs has demonstrated that the subunits are expressed in a tissue-specific and developmentally regulated fashion (Mercer et al., 1986; Young and Lingrel, 1987; Schneider et al., 1988; Orlowski and Lingrel, 1988; Martin-Vasallo et al., 1989). Thus, the diversity in subunits and their differential expression and regulation suggests that the Na,K-ATPase may have several specialized functional roles.

Besides the other subunits, a small peptide of ~10 kD, termed the γ subunit (previously "proteolipid component"), has also been identified in purified preparations of the enzyme. Strong evidence that the γ subunit is a component of the Na,K-ATPase and not a contaminant of purification has come from studies of the cardiac glycoside binding site. These studies have shown that the binding site consists of not only the α and β subunits, but the γ subunit as well (Forbush et al., 1978; Rogers and Lazdunski, 1979; Lowndes et al., 1984). For example, the α and γ subunits are labeled by photoaffinity compounds with approximately equal probability when the reactive groups are 23–24 Å from the lactone ring of the cardiotonic steroid (Forbush, 1983). Although small hydrophobic proteins are associated with the H-ATPase of mitochondria (Blondin, 1979), bacteria (Sierra and Tza-
Materials and Methods

Isolation of cDNA Clones

The cDNA cloning strategy is described in the figure legends. The procedure used to isolate the cDNA clones was modified from that of Frohman et al. (1988). Briefly, poly(A)$^+$ sheep kidney RNA (Davis et al., 1986) was reverse transcribed according to the supplier's instructions (GIBCO BRL, Gaithersburg, MD) using a 35-base oligonucleotide containing 17 dT residues and an 18-base adaptor sequence. The reverse transcription reaction was denatured at 94°C for 2 min, followed by a final extension at 72°C for 15 min. The reaction was electrophoresed and a band consisting of ~200 bp excised from the gel and sequenced using M13mp18 or M13mp19 vectors. Several clones were isolated and sequenced using the dideoxy chain termination method of Sanger et al. (1977) as modified by Biggin et al. (1983). One clone, corresponding to the sheep kidney $\gamma$ subunit, was identified and used to screen a nylon plaque library (Collins and Leszyk, 1987). Three clones were obtained, and the corresponding mRNA was reverse transcribed as before using oligo dT as a primer. This reaction was diluted to 0.5 ml with TE buffer and used for PCR. The reactions were as described except the 5' primer consisted of 5'-ATGTAACGACGACGACGACGAGA(A,G)(A,C)(C,T)GA(A,G)G coding the first eight amino acids and a primer from the 3' untranslated region consisting of 5'-GCATTGCGTCGCCTTC. The PCR reaction was electrophoresed and a band consisting of ~400 bp subcloned into pHBluescript II (Stratagene, La Jolla, CA) and sequenced.

RNA Blot Hybridization

RNA was isolated from adult rat tissues, and fractionated by electrophoresis through a 1% agarose formaldehyde gel. The RNA was transferred to nylon (BioTrans; ICN Biomedicals, Costa Mesa, CA) by vacuum (Hoeffer Scientific Instruments, San Francisco, CA) and prehybridized and hybridized as described (Schneider et al., 1985). cDNA probes were labeled employing the Klenow fragment of E. coli DNA polymerase I and oligonucleotide primers by the method of Feinberg and Vogelstein (1984).

In Vitro Translation

RNA was prepared using SP6 RNA polymerase as described by the supplier (Promega Corp., Madison, WI). For translations, 3 $\mu$L of a 20-ml transcription reaction were added directly to a 30-$\mu$L rabbit reticulocyte lysate translation reaction (Promega Corp.), supplemented with 1 ml of dog pancreatic microsomes (Promega Corp.) and containing [3H]leucine (Amersham Corp., Arlington Heights, IL). After 30 min, the reaction was centrifuged in a Beckman Airfuge (Beckman Instruments, Inc., Fullerton, CA), the pellet washed once in 100 $\mu$L of 150 mM NaCl, 25 mM Hepes, pH 7.4, and analyzed by SDS-PAGE (Schägger and Jagow, 1987) and fluorography.

Immunological Reagents

The preparation of a mAb (mAb C62.4) to the $\gamma$ subunit of the dog Na,K-ATPase has been previously described (Kashgarian et al., 1985). A polyclonal antibody (Ab-gam) against the $\gamma$ subunit was raised in rabbits by immunization with dog kidney $\gamma$ polypeptide obtained by elution from SDS-PAGE gels of purified Na,K-ATPase. Another antibody (Ab-017) was prepared by immunizing rabbits with hemocyanin-conjugated synthetic peptide prepared to amino acids 6-22 of the sheep $\gamma$ subunit.

Immunoprecipitation and Immunoblots

Dog renal medullary membranes, enriched in Na,K-ATPase, were prepared as previously described (Torbush and Palfrey, 1983). Membranes were solubilized on ice for 15 min at a concentration of 500 g/ml in buffer containing 1% detergent (Triton X-100, CHAPS, or n-dodecylmaltoside), 50 mM Tris-Cl, pH 7.3, and 100 mM NaCl. Samples were cleared of insoluble material by centrifugation in a microtube for 10 min at 15,000 g at 4°C. 10 $\mu$L of control IgG or 10 $\mu$L of mAb C62.4 ascites fluid were added to the cleared sample and allowed to incubate on a Nutator (Clay Adams, Parsippany, NJ) for 1 h at room temperature. Because mouse antibodies have a low affinity for Protein A, 10 $\mu$L of goat anti-mouse IgG was added, and the complex precipitated by centrifugation. The precipitate was washed three times in solubilization buffer, once in the same buffer containing 750 mM NaCl, and once in solubilization buffer diluted 1:10 in water. Samples were boiled for 3 min in 50 $\mu$L of sample buffer, separated by SDS-PAGE (Schägger and Jagow, 1987) and electrophoretically transferred to nitrocellulose. Sections the nitrocellulose were probed using antibodies to the $\alpha$, $\beta$, or $\gamma$ subunits of the Na,K-ATPase as described in the figure legends.
results

isolation and characterization of γ subunit cDNA clones

The strategy for the isolation of the cDNA for the γ subunit is based on the procedure of Frohman et al. (1988). mRNA from sheep kidney was isolated and reverse transcribed using a 35-base oligonucleotide with 17 dT residues and an 18-base adapter sequence containing three endonuclease sites. This reaction creates a single-stranded antisense DNA strand. Next, a 38-base oligonucleotide containing deoxyinosine in two positions (Ohtsuka et al., 1985), and derived from the 13-amino acid NH2-terminal γ peptide sequence (Collins and Leszky, 1987) using preferred codons, was annealed to the cDNA strand and used as a primer to generate the complementary second strand. Repetitive PCR cycles using the γ subunit primer and the 3' adaptor primer resulted in the specific amplification of the double-stranded γ subunit cDNA. Using this technique a cDNA clone corresponding to a portion of the sheep kidney γ subunit was isolated. This clone was subsequently used to identify and isolate a cDNA containing the complete coding region for the rat kidney γ subunit. Primers based on the sheep and rat DNA sequences corresponding to the PCR primers are underlined, above the deduced amino acid sequences for the sheep, mouse and bovine γ subunits. The portion of the cDNA nucleotide sequences corresponding to the PCR primers are underlined. These sequences are available from EMBL under accession numbers X70059 (bovine), X70060 (mouse), 70061 (sheep), and X70062 (rat).

expression of γ subunit mRNAs in rat tissues

Rat kidney γ subunit cDNA was used to analyze the RNA products of γ subunit gene expression in several rat tissues by hybridization to total cellular RNA. As shown in Fig. 3 B the γ subunit gene encoded two RNA species of ~1.5 and 0.8 kb in length. The smaller transcript was more abundant in the kidney, with lower levels in the spleen, lung, and heart. In contrast, the larger transcript was more abundant in the spleen and lung. Thus, the expression of γ subunit mRNA appears to be regulated in a tissue-specific fashion. Moreover, in contrast to α subunit mRNA expression shown in Fig. 3 A, total γ mRNA levels are higher in the spleen and lower in the brain. In the kidney, however, it appears that the level of α1, β1, and γ mRNA expressed is similar, suggesting that the subunits may be coordinately regulated (data not shown).
Hydropathy profiles were determined using the procedure of Kyte-Doolittle (1982) and Eisenberg et al. (1984). Hydrophobic regions are above the x-axis and hydrophilic regions are below. Plots were generated using Geneworks from IntelliGenetics, Inc. (Mountain View, CA).

**Immunological Characterization of the γ Polypeptide**

The γ subunit was characterized using two polyclonal antibodies to the γ polypeptide; the first of these (Ab-gam) was raised by immunization with dog kidney γ peptide obtained by elution from SDS polyacrylamide gels of purified Na,K-ATPase. The second antibody (Ab-G17) was prepared by immunization with hemocyanin-conjugated synthetic peptide prepared to amino acids 6-22 (G-17) of the sheep γ subunit. For some purposes the antibodies were purified by adsorption to nitrocellulose-immobilized dog kidney γ peptide.

To characterize the specificity of the γ reactive antisera, renal membranes were analyzed by SDS-PAGE (Fig. 4). As shown in Fig. 4 B (Ab-gam) and C (Ab-G17), the antisera react to two polypeptides of ~8.8 and ~9.9 kD (described in recent reports as 6.7 and 7.6 kD because of an error in commercial molecular weight standards; Kratzin et al., 1989).

The antisera do not recognize either the α and β subunits. It is not known why there is a difference in the molecular weight as deduced from the cDNA from that determined by SDS-PAGE. In addition, the reason γ migrates as a doublet is not clear, but importantly the same protein doublet is seen when synthetic γ subunit RNA is translated in vitro. Using rabbit reticulocyte lysate, synthetic RNA prepared from the γ cDNA was translated in the presence of dog pancreatic microsomes. Microsomal membranes were isolated and analyzed by SDS-PAGE. As shown in Fig. 4 D two proteins are translated in the presence of the single message. These bands correspond in size to the immunoreactive proteins identified by the γ antisera. Consequently, it appears that the two immunoreactive γ polypeptides are most likely products of a single RNA message.
Identification and Immunoprecipitation of [H]Nitroazidobenzoyl-ouabain-Labeled γ Polypeptide

The photoaffinity derivative of ouabain, nitroazidobenzoyl- (NAB)l-ouabain, specifically inhibits and covalently labels the Na,K-ATPase (Forbush et al., 1978). The results of an experiment designed to test if the γ antisera recognize polypeptides that have been specifically labeled with [H]NAB-ouabain are shown in Fig. 5. [H]NAB-ouabain–labeled polypeptides were separated by SDS-PAGE, transferred to nitrocellulose and analyzed for reactivity to the G-17 antiserum. As shown, the major polypeptides labeled by NAB-ouabain correspond to the α and γ subunits of the Na,K-ATPase, however, only the polypeptides that correspond to the labeled γ subunit of the Na,K-ATPase are immunoreactive. Additional evidence that the antisera identifies polypeptides that are specifically labeled by [H]NAB-ouabain is shown in Fig. 6. Labeled γ polypeptides were immunoprecipitated with either Ab-gam or Ab-G17 antisera. When compared with preimmune sera, both antisera immunoprecipitate solubilized NAB-ouabain–labeled polypeptides. Moreover, the immunoprecipitation of labeled γ polypeptide is specific since it can be partially (Ab-gam) or completely blocked (Ab-G17) by the presence of the G-17 peptide.

The γ and α subunits of the Na,K-ATPase Co localize in the Sheep Kidney

To immunolocalize both γ and α subunits of the Na,K-ATPase in the kidney, double-label immunofluorescence experiments were carried out using antibodies specific for each subunit. As shown in Fig. 7, antibodies to both proteins colocalized to the basolateral membranes of the tubular epithelial cells in sheep kidney. In addition, staining of both antibodies varies axially along the nephron with cells of the distal convoluted tubule, connecting tubule cells and principal cells having the strongest staining while the proximal segments were stained less intensely. Both antibodies failed to stain a subpopulation of cells in connecting tubules and collecting ducts presumed to be intercalated cells. Similar results were obtained in rat kidney using the Ab-gam antibody. These results are in agreement with previous studies showing that the expression of the Na,K-ATPase is restricted to the basolateral membrane in renal epithelial cells and varies in its level of expression along the nephron (Kashgarian et al., 1985). As shown in Fig. 7, the γ subunit specifically codistributes with the α subunit and is present or absent only in conjunction with the α subunit, supporting the notion that the polypeptide is specifically associated with the Na,K-ATPase.

The γ subunit Coimmunoprecipitates with the α and β subunits of the Na,K-ATPase

Having demonstrated that γ colocalized with the Na,K-ATPase in renal epithelial cells, biochemical experiments were carried out in order to establish direct molecular interaction of the γ with the Na,K-ATPase. For this purpose the subunit composition of immunopurified enzyme was analyzed following solubilization in various detergents (Fig. 8). The Na,K-ATPase was immunoprecipitated using a mAb to the α subunit (C62.4) and the resulting immunoprecipitate probed on nitrocellulose with anti-α, -β and -γ antibodies. Fig. 8 shows that α, β, and γ subunits were coprecipitated after solubilization in CHAPS. However, when the enzyme was solubilized using n-octylglucoside only the α and β subunits were detected. Only the α and β subunits were detected when membranes were solubilized with Triton X-100, C12E8 or n-dodecylmaltoside (data not shown). These results demonstrate that the γ subunit forms a molecular assembly with the α and β subunits of the Na,K-ATPase via interactions that can be disrupted upon detergent solubilization.

Discussion

We have isolated the cDNAs from several species coding the
γ subunits of the Na,K-ATPase. As deduced from the nucleotide sequence the primary structure of the subunit consists of 58 amino acids with a molecular mass of 6,500. The deduced sheep amino acid sequence is identical with the partial sequence obtained by peptide analysis of the sheep γ subunit. Hydropathy analysis of the amino acid sequence suggests the presence of one transmembrane domain. In the rat, the γ subunit gene encodes two mRNA species that are expressed in a tissue-specific manner. The tissue-specific pattern of expression of the two mRNAs suggests that their noncoding sequences may contain functional information important in the regulation of enzyme biosynthesis. As has been shown for the β subunit (Noguchi et al., 1986), the heterogeneity of γ subunit mRNAs may result from multiple transcription initiation or polyadenylation sites. Though the levels vary considerably, RNAs encoding the γ subunit are present in all tissues examined. Highest levels of γ RNA were expressed in the kidney and spleen. However, in marked contrast to the α and β subunits, there is little γ mRNA expression in the brain. This implies that translational or posttranslational regulation of the subunits maintains the necessary quantities of the Na,K-ATPase polypeptides. Alternatively, the possibility that an isoform of the γ subunit may be present cannot be excluded.

Comparison of the γ amino acid sequences demonstrates a high degree of homology (93% amino acid similarity, 72% identity) between the subunits. The high degree of homology is consistent with the view that the γ subunit may be an essential component of Na,K-ATPase structure or function. Strong evidence that the identified and sequenced γ polypeptide is specifically associated with the Na,K-ATPase is provided by the immunological characterization of the subunit. Antibodies raised against either the purified γ subunit from dog kidney or to a synthetic peptide from the sheep γ sequence react with only two kidney membrane proteins of ~8.8 and ~9.9 kD. Moreover, similar size polypeptides are produced by the in vitro expression of synthetic γ subunit.
mRNA. Also, the γ subunit antisera is immunoreactive with the in vitro synthesized γ protein and both antisera immunoprecipitate solubilized [3H]NAB-ouabain–labeled γ subunit.

In the kidney the Na,K-ATPase is confined to the basolateral membrane of the renal tubular epithelial cells, and varies in density and distribution along the nephron. The strongest staining of the Na,K-ATPase α subunit is found in the thick ascending limb of Henle's loop and in the distal convoluted tubule (Kashgarian et al., 1985). Immunofluorescence microscopy using purified antibody to the dog kidney γ subunit indicates that the subunit is found only on the basolateral membrane of the tubular epithelium in rat kidney. In addition, double immunofluorescence using the γ subunit antisera and an antiserum specific to the α subunit demonstrates that the γ subunit has a distribution pattern identical with the α subunit. Coimmunoprecipitation experiments demonstrated a direct association of γ with the α and β subunits. These results strongly suggest that the γ subunit is an integral component of the Na,K-ATPase.

There are a growing number of proteins associated with, or a component of, the Na,K-ATPase. For example, ankyrin, a linker protein that in erythrocytes mediates the attachment of the spectrin-actin cytoskeleton to the anion transporter in the plasma membrane, specifically binds to the α subunit of the Na,K-ATPase (Nelson and Veshnock, 1987; Morrow et al., 1989; Koob et al., 1990). The colocalization of ankyrin and spectrin homologues with the Na,K-ATPase at the basolateral surface of kidney epithelial cells has suggested a mechanism for the maintenance of the polarized distribution of this enzyme (Nelson and Veshnock, 1987; Hammerton et al., 1991). Moreover, a putative isof orm of the β subunit, termed β2, has been identified as an adhesion molecule of glial (AMOG) cells (Martin-Vasallo et al., 1989; Gloo et al., 1990). AMOG is a Ca2+-independent adhesion molecule, which in glia cells, mediates neuron–astrocyte adhesion. Consequently, it appears that the Na,K-ATPase may function as a linkage between extracellular adhesion and the intracellular cytoskeleton. In addition, two other proteins have been shown to be associated with the Na,K-ATPase (Kraemer et al., 1990). Pasin 1 and pasin 2 are peripheral membrane proteins with apparent molecular masses of 77 and 73 kD, respectively, which purified and immunoprecipitate with the Na,K-ATPase. In tissue sections, pasin 1 is colocalized with the Na,K-ATPase to the basolateral surface of columnar epithelial cells (Kraemer et al., 1990). The role of these peripheral proteins in Na,K-ATPase function is unknown.

The exact role of small membrane proteins in the regulation of cation transport is unclear. However, the Ca-ATPase of the sarcoplasmic reticulum is under regulation by phospholamban, a small membrane protein with a molecular weight of 6,000. In reconstituted vesicles, inclusion of phospholamban with the Ca-ATPase inhibits Ca uptake. Phosphorylation of phospholamban by the catalytic subunit of cAMP-dependent protein kinase reverses the inhibitory effect of phospholamban (James et al., 1989). Studies using a cross-linking agent (James et al., 1989) and a synthetic peptide derived from phospholamban (Kim et al., 1990) suggest that phospholamban interacts directly with the Ca-ATPase. The mechanism of regulation of the Ca-ATPase by phospholamban remains unknown. Although phospholamban and the γ subunit are of similar size they have no sequence homology (Fujii et al., 1987), therefore, it seems likely that the γ subunit has a function separate from the role of phospholamban. Interestingly, another small membrane protein with a structure similar to phospholamban and containing a region homologous to the γ subunit has been recently identified (Palmer et al., 1991). This 8.4-kD protein, called phospholemman, is a membrane protein that in myocardium is the major plasma membrane substrate for cAMP-dependent protein kinase and protein kinase C. Recently, phospholemman has been shown to function as a chloride channel (Moorman et al., 1992). A stretch of 50 amino acids that contains the transmembrane domain of the polypeptides exhibits 48% sequence similarity with the γ subunit (Fig. 9). The region of homology excludes the phosphorylation sites of phospholemman suggesting that γ subunit has a function not requiring regulation by multisite phosphorylation.

Although the function of the γ subunit must await further investigation, our results are consistent with the notion that the polypeptide is a component of the Na,K-ATPase. The γ subunit is specifically labeled by photoreactive inhibitors of the Na,K-ATPase, it is evolutionarily conserved across species, and it colocalizes with the α subunit in the kidney. The availability of γ cDNA and antibodies should be useful for studies designed to understand the possible roles of the γ subunit in Na,K-ATPase structure, function and assembly.

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