The Na,K-ATPase belongs to a family of P-type ion-translocating ATPases sharing homologous catalytic subunits (α) that traverse the membrane several times and contain the binding sites for ATP and cations. In this family, only Na,K- and H,K-ATPases have been shown to have a second subunit, a single-span glycoprotein called β. Recently a new isoform (β3) has been identified in mammals. Here we describe structural features and tissue distribution of the β3 protein, utilizing an antisem specific for its N terminus. β3 was the only β detected in Na,K-ATPase purified from C6 glioma. Treatment with N-glycosidase F confirmed that β3 is a glycoprotein containing N-linked carbohydrate chains. Molecular masses of the glycosylated protein and core protein were estimated to be 42 and 35 kDa, respectively, which are different from those of the β1 and β2 subunits. Detection of β subunits has historically been difficult in certain tissues. Sensitivity was improved by deglycosylating, and expression was evaluated by obtaining estimates of β3/α ratio. The proportion of β3 protein in the rat was highest in lung and testis. It was also present in liver and skeletal muscle, whereas kidney, heart, and brain contained it only as a minor component of the Na,K-ATPase. In P7 rats, we found skeletal muscle and lung Na,K-ATPase to be the most enriched in β3 subunit, whereas expression in liver was very low, illustrating developmentally regulated changes in expression. The substantial expression in lung and adult liver very likely explains long-standing puzzles about an apparent paucity of β subunit in membranes or in discrete cellular or subcellular structures.

The Na,K-ATPase catalyzes the active uptake of K⁺ and efflux of Na⁺ ions, thus controlling ionic gradients through the enzymatic hydrolysis of ATP. It is a heterodimer of two different kinds of subunit, a large α subunit with multiple membrane spans and a smaller glycoprotein subunit, β, with just one span and the bulk of its mass in the extracellular space. Both subunits of Na,K-ATPase are encoded by multigene families. In the rat, three isoforms of α subunit and two isoforms of β subunit have been identified and substantially characterized (1, 2). Although the major ATPase characteristics are assigned to the α subunit, the β subunit is required for folding and transport of α,β-heterodimers to the plasma membrane (2). Nonetheless, there have been a number of reports in which little or no β1 or β2 subunit or mRNA was detected despite significant levels of α or its mRNA (3–6), or alternatively, in which α and β mRNA or protein did not appear to colocalize in tissue sections (7–9).

Recently several cDNA fragments having homology with Na,K-ATPase β subunits were identified in the GenBank™ expressed sequence tag library. The full-length human clone revealed 59% sequence identity with the β3 subunit of Xenopus laevis and 38 and 48% identity with human β1 and β2 subunits, respectively, and was named β3 (10). Full-length clones were independently isolated from cDNA libraries from C6 rat glioma cells (GenBank™ D84450) and mouse retina (11). Northern blot analysis and representation in the expressed sequence tag data bank has revealed β3 message in a wide variety of tissues. Expression of the β3 subunit at the protein level and its association with the Na,K-ATPase has not been demonstrated yet however. Here we accomplish these aims with an antibody, specific for the rat β3 subunit, that will be a useful addition to an existing panel of isof orm-specific antibodies to the Na,K-ATPase.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—Antipeptide-directed antiserum RNTβ3 (rat N terminus of β3) was raised at Quality Control Biochemicals, Inc. (Hopkinton, MA), against the 13-mer peptide TKTEKKSFHQSLAC-(NH₂), corresponding to the N-terminal sequence of the β3 isoform (GenBank™ D84450) plus a terminal cysteine residue. The peptide was conjugated to keyhole limpet hemocyanin as a carrier, and two rabbits were immunized. The immune response was tested with an enzyme-linked immunosorbent assay with bovine serum albumin-coupled peptide on solid phase. The titer of crude sera against the peptide was >200,000 for the better rabbit. Other antibodies were generous gifts of other investigators: antiserum against the KETKY peptide representing the C terminus of all α subunits of Na,K-ATPase from R. Bayer and J. Kyte (University of California, San Diego); antipeptide antiserum 757 against sheep β1 from W. J. Ball, Jr. (University of Cincinnati); antipeptide protein antiserum FPβ1 against rat β1 from A. McDonough (University of Southern California); antiserum SpETβ2 against human β2 from P. Martin-Vasallo (University de la Laguna, Tenerife, Spain); and monoclonal antibody SM-GF50 for β2 from J. Gurd (University of Toronto) and P. Beesley (Royal Holloway and Bedford New College, Egham, United Kingdom).

**Cell Culture**—The C6 glioma cell line was obtained from the American Type Culture Collection. Cells were maintained in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum and penicillin/streptomycin.

**Membrane Isolation and Gel Electrophoresis**—Membrane preparations from rat tissues were made as follows. Brain, lung, liver, kidney, and testis were minced and homogenized with a motor-driven Teflon homogenizer in 10 volumes of buffer containing 250 mM sucrose, 30 mM imidazole, pH 7.2, 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride. Unhomogenized material and nuclei were removed by low-speed centrifugation at 7,000 rpm for 15 min, and membranes were sedimented at 33,000 rpm for 30 min. For cardiac and skeletal muscle preparations, the tissues were minced and homogenized in 40 volumes of buffer containing 250 mM sucrose, 20 mM Tris-HCl, pH 7.2, 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride, followed by further disruption with an Omni homogenizer for 30 s at the maximal setting. The homogenates were centrifuged at 14,000 rpm for 30 min. Sarco-
RESULTS

\textbf{β3 Protein Detection and Gel Mobility}—To detect \( \beta_3 \) protein, we generated an antisera based on the N-terminal sequence deduced from the rat cDNA sequence. To test its reactivity with \( \beta_3 \), rat C6 glioma cells were used, since the clone had been isolated from a C6 cDNA library. The antisera stained C6 cell plasma membranes by immunofluorescence (data not shown).

To determine whether \( \beta_3 \) was associated with the Na,K-ATPase, the enzyme was partially purified from C6 crude membranes using SDS treatment followed by equilibrium centrifugation on a sucrose density gradient. During such treatment most other membrane proteins are solubilized, while Na,K-ATPase remains associated with plasma membrane. As illustrated in Fig. 1A, the RNT\( \beta_3 \) antibody stained a fuzzy band

\begin{itemize}
  \item with a molecular mass of about 42–45 kDa in preparations that had a Na,K-ATPase specific activity of 100–150 μmol of P/ mg/h (equivalent to 30–50-fold purification).
  \item Since both \( \beta_1 \) and \( \beta_2 \) subunits have been shown to be glycoproteins containing several N-linked oligosaccharide chains (14, 15), N-glycosidase F treatment was performed to determine whether \( \beta_3 \) was also glycosylated. As seen in Fig. 1A, this resulted in a decrease in apparent molecular mass to 35 kDa. Fig. 1B demonstrates that \( \beta_3 \) is the only known \( \beta \) isoform of the Na,K-ATPase purified from C6 glioma cells, contrasting with the \( \beta_1 \) and \( \beta_2 \) isoforms found in Na,K-ATPase purified from rat brain. Different dilutions of sample were added to facilitate comparisons. The content of \( \alpha \) (\( \alpha_1 \) only for \( \beta_3 \); all three \( \alpha \) subunits for rat brain) was determined by staining the blots with the KETYY antibody against the C-terminal peptide of all \( \alpha \) subunits. Equal amounts of the sodium pump \( \alpha \) subunits were loaded for rat brain and C6 samples, but the C6 membranes had no \( \beta_1 \) or \( \beta_2 \) and were more than four times more enriched in \( \beta_3 \) than rat brain enzyme.
  \item Removal of oligosaccharide chains often facilitates glycoprotein detection on gels. As seen in Fig. 2, fully glycosylated \( \beta_3 \) could be detected in rat brain microsomes only when 25–50 μg of protein were loaded. In deglycosylated samples, the binding was almost linear within a 5–50 μg range, however. Consequently, in further experiments we routinely used glycosidase treatment.
  \item Molecular masses of the mature and core \( \beta_3 \) protein calculated from their mobility on SDS gels were different from those of \( \beta_1 \) and \( \beta_2 \) subunits. As seen in Fig. 3A, the mature \( \beta_3 \) protein migrated faster than the mature \( \beta_2 \) subunit. This was not surprising since rat \( \beta_3 \) has only two potential N-linked glycosylation sites in the extracellular domain, whereas rat \( \beta_2 \) has seven. As shown in Fig. 3B (in an experiment where no compensation was made for the smaller amount of \( \beta_3 \) than \( \beta_1 \) or \( \beta_2 \)), the core \( \beta_3 \) protein migrated significantly slower than the core \( \beta_2 \) protein and slightly slower than the core \( \beta_1 \) subunit. Average values for glycosylated proteins were 46–50 kDa for \( \beta_3 \) and 42–45 kDa for \( \beta_3 \). Average values for core proteins were 34–37 kDa for \( \beta_1 \), 32–34 kDa for \( \beta_2 \), and 35–38 kDa for \( \beta_3 \). The apparent size of core \( \beta_3 \) is larger than predicted from the cDNA clone (30 kDa); the biochemical basis for the discrepancy remains to be determined.
  \item Fig. 3B also illustrates the inherent polarity of glycoprotein staining. \( \beta_1 \) and \( \beta_2 \) were each stained with two different antibodies, and certain antibodies were substantially less sensitive to the glycosylated protein than to the core. This is likely to reflect differences in the proximity of their epitopes to glycosylation sites.
  \item \textbf{Species Specificity of the Antibody}—The species specificity of the RNT\( \beta_3 \) antibody was examined with blots of 50 μg of deglycosylated brain microsomes from several species. \( \beta_3 \) pro-
tein was detected readily in rat and mouse brain samples and more weakly in guinea pig samples (data not shown). All other samples, including human, chicken, rabbit, dog, cat, sheep, calf, monkey (Macaca), frog (Rana), and goldfish brain membranes, were negative. There is only 69% identity within the first 13 amino acids among mammalian β3 subunits, and even less for other vertebrates.2

We propose that the dominant epitope includes the amino acids TKTE and that substitution of serine for threonine in guinea pig β3 is weakly permissive, but substitution of asparagine in human β3 prevents RNTβ3 binding. RNTβ3 has also been observed to be negative on human β3 expressed in insect cells.3

Expression of the β3 Isoform in Rat Tissues—A panel of adult rat tissues was examined for the presence of the Na,K-ATPase β3 subunit on blots. In Fig. 4A, concentrations of samples were adjusted to ensure reasonable levels of the α subunit, as detected with anti-KETYY. β3 staining was strong in testis, lung, and liver when as much as 150 μg of microsomes were analyzed. A strong signal was also obtained with kidney outer medulla microsomes, whereas much less was detected in rat brain microsomes. Only a little β3 was seen in heart and skeletal muscle samples. Blots were scanned, and Fig. 4B shows the relative abundance of the β3 protein within the Na,K-ATPase complex estimated by taking the ratios between β3 and α subunit staining. We found lung and testis to have the highest proportion of β3 isoform expression per Na,K-ATPase unit among the adult rat tissues tested. β3 content was lower in skeletal muscle, liver, and kidney outer medulla, and in heart and brain Na,K-ATPase it comprised only a minor fraction. It should be stressed that the data are expressed relative to the β3/α ratio in lung. It is known that lung also expresses β1 protein (18), and the proportion of the two isoforms has not yet been determined.2

2 Although named β2, this isofrm of β from the chicken is grouped with β3 sequences by dendrogram analysis of evolutionary relationships (2).

3 N. N. Modyanov, personal communication.

The same tissues (except testis) were analyzed at day 7 after birth. Fig. 4C illustrates that as in the adult, the expression of β3 protein in lung was high, but it was almost undetectable in the liver. Surprisingly, the strongest signal for β3 was in sarcolema-enriched membranes from hind limb skeletal muscle. Since there was almost no difference in the amount of α subunit between lung and skeletal muscle samples as judged by anti-KETYY staining, this indicates that skeletal muscle has the highest proportion of β3 in the P7 rat of the tissues studied (Fig. 4D). Comparison of Fig. 4, B and D, illustrates that brain and heart from newborn rat, like skeletal muscle, were more enriched in the β3 protein per Na,K-ATPase unit than those tissues from adult rat. Thus expression of the β3 subunit of the Na,K-ATPase is subject to developmental regulation in several tissues.

DISCUSSION

In several experimental systems it has been possible to form active combinations of Na,K-ATPase or H,K-ATPase α subunits with different β subunits, even ostensibly mismatched pairs (i.e. Na/K/αHK β). The outcome is that exchange of β subunits has effects on the enzymatic properties of the complex, most notably on the affinities for K+ or Na+ (19–25). This implies that tissue- or developmentally controlled differences in α-β combination may have direct physiological consequences mediated by alteration of substrate affinity. β2 has also been implicated in intercellular adhesion in the developing nervous system (26). Since the β isoforms differ in the number and structure of their carbohydrate groups (14, 15), such differences (reflected here in differences in gel mobility) may be important in early development.

The observation that β3 is expressed at the highest level (relative to total α subunit) in liver and lung may resolve some long-standing controversies. In the liver, β1 subunit has been detected only after density gradient purification (18); in prior studies it was not seen (6, 27). Even mRNA for β3 has been frequently undetectable or low (3, 4, 28, 29), although it was high in liver from young animals and was specifically increased by thyroid hormone and by resection-induced proliferation (4,
but the greater sensitivity. Rat liver RNA (10, 11), but this is likely to be due to the low fluidity of the adjacent hepatocyte (7). Oddly, a less well characterized anti-Na,K-ATPase immunocytochemistry by the same investigators documented a canalicular (apical) distribution. The polarized distribution of reactivity for β1 correlated with the prior histochemistry, suggesting that Na,K-ATPase activity and immunoreactivity (reviewed in Ref. 32). The interpretation was complicated by evidence that membrane lipid fluidization of the membrane increased Na,K-ATPase activity and immunoreactivity (reviewed in Ref. 32). Most of the negative results could in principle be attributed to insufficient sensitivity, but an analysis of the subcellular distribution of histochemical reaction for Na,K-ATPase in various tissues presents a more complete picture of this enzyme’s isofrom composition and points to possible functional differences important for the physiology of several tissues. It has not been ruled out that β1 may also associate with H,K-ATPases, a possibility supported by the interchangeability of β subunits in expression systems. The effect of β1 subunits on substrate affinities, and now potentially on the presence or absence of measurable activity, supports the idea that β has a regulatory role.

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The literature on Na,K-ATPase expression in lung also has some relevant peculiarities. Na,K-ATPase activity increases sharply during the perinatal period because of its role in fluid reabsorption immediately after birth. In whole lung, a1 and β1 mRNA levels peaked at birth and then fell to a lower level maintained in the adult (9, 34, 35). Both type II pneumocytes in alveoli and bronchiolar epithelial cells have high levels of Na,K-ATPase (36, 37). The most intriguing results were obtained by examining mRNA levels with in situ hybridization (9, 37). During the perinatal period, a1 mRNA increased dramatically in bronchiolar epithelium, but not so much in alveoli, while β1 mRNA increased dramatically in both loci. Beginning at day 8 postnatally, β1 signal developed a punctate pattern in alveoli, while a1 signal remained broadly distributed. We can speculate that the some of the a1 may be associated with β3, since β1 appeared to be distributed in specific cells of the adult alveolar epithelium. mRNA for β3 has been detected in adult rat and mouse lung (10, 11). In conclusion, the presence of β3 associated with Na,K-ATPase in various tissues presents a more complete picture of this enzyme’s isofrom composition and points to possible functional differences important for the physiology of several tissues. It has not been ruled out that β3 may also associate with H,K-ATPases, a possibility supported by the interchangeability of β subunits in expression systems. The effect of β subunits on substrate affinities, and now potentially on the presence or absence of measurable activity, supports the idea that β has a regulatory role.