Subunit isoform selectivity in assembly of Na,K-ATPase α-β heterodimers

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Running Title: Isoform selectivity of the Na,K-ATPase αβ heterodimers

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Background: The Na,K-ATPase consists of one α (four isoforms) and one β (three isoforms) subunits. Result: The α1 preferentially assembles with β1, whereas α2 preferentially binds to β2 isoform. Conclusion: Assembly of α-β complexes is isoform-selective. Significance: This selectivity is crucial for cell- and tissue-specific functions of the Na,K-ATPase.

To catalyze ion transport, the Na,K-ATPase must contain one α and one β subunit. When expressed by transfection in various expression systems, each of the four α subunit isoforms can assemble with each of the three β subunit isoforms and form an active enzyme, suggesting the absence of selective α-β isoform assembly. However, it is unknown whether in vivo conditions the α-β assembly is random or isoform-specific. The α2-β2 complex was selectively immunoprecipitated by both anti-α2 and anti-β2 antibodies from extracts of mouse brain, which contains cells co-expressing multiple Na,K-ATPase isoforms. Neither α1-β2 nor α2-β1 complexes were detected in the immunoprecipitates. Further, in MDCK cells co-expressing α1, β1 and β2 isoforms, a greater fraction of the β2 subunits was unassembled with α1 as compared to that of the β1 subunits, indicating preferential association of the α1 isoform with the β1 isoform. In addition, the α1-β2 complex was less resistant to various detergents than the α1-β1 complex isolated from MDCK cells or the α2-β2 complex isolated from mouse brain. Therefore, the diversity of the α-β Na,K-ATPase heterodimers in vivo is determined not only by cell-specific co-expression of particular isoforms, but also by selective association of the α and β subunit isoforms.

The Na,K-ATPase is universally expressed in all animal cells where it generates electrochemical ion gradients that are critical for many cellular processes (1-3). The Na,K-ATPase is composed of two essential subunits, α and β. There are four isoforms of the α subunits and three isoforms of the β subunits. In some tissues, the α-β heterodimer is associated with one of the seven members of the FXYD protein family (4,5) that modulate kinetic properties of the enzyme.

The α1 and β1 isoforms are ubiquitously expressed, suggesting a house-keeping role for the α1-β1 Na,K-ATPase in most cells. In contrast, other Na,K-ATPase subunit isoforms are expressed in a tissue-specific manner. The α2 isoform is expressed mainly in muscle and nervous system (1-3,6,7); the α3 isoform is expressed mainly in neurons (8,9), whereas the α4 isoform is found only in testis (10,11). The β2 isoform is expressed predominantly in brain and muscle (3,7), while the β3 isoform is mainly expressed in lung, testis, skeletal muscle and liver (12,13). The α2 isoform regulates contractility of cardiac, smooth and skeletal
muscle and plays a key role in the modulation of blood pressure in response to stress (3,14,15). In vivo human mutations in the α2 and α3 isoforms are associated with neurological diseases, familial hemiplegic migraine type 2 and rapid-onset dystonia-parkinsonism (16). The α4 isoform is required for sperm motility and fertility (10,11). The β1 subunit plays an important role in intercellular adhesion in epithelia (17,18), and the β2 subunit, or AMOG (adhesion molecule on glia), is important for adhesion and migration of neurons on glia (19). Decreased expression of the β1 subunit is associated with cancer (reviewed in (20)), whereas abnormalities in expression and distribution of the β2 subunit are linked to glioma and epilepsy (21-24).

Therefore, it is clear that both α and β isoforms of the Na,K-ATPase have organ- and tissue-specific functions. However, very little is known about particular α-β heterodimers responsible for these roles. Transfection studies indicate that each of the four α subunit isoforms can assemble with each of the three β subunit isoforms and form a functional pump (6,7,25). These data imply that in cells co-expressing multiple Na,K-ATPase subunits isoforms, various α and β isoforms also assemble in different combinations, dependent on their relative cellular content. However, selective co-immunoprecipitation of the α2 subunit, but not of the ubiquitously expressed α1 subunit, with the β2 subunit from mouse and rat brain (19,26), as well as from heart and adrenal medullary cells of guinea-pigs and rats (26,27) suggest that the α2 subunit is the preferred binding partner of the β2 subunit. In support of this hypothesis, the tissue expression pattern of the β2 subunit, mainly in muscle and nervous system, is similar to that of the α2 subunit (3,7).

Here we show that not only does the β2 subunit preferentially assemble with the α2 subunit, but also the α2 subunit is mostly associated with the β2 subunit in mouse brain. In addition, by analyzing the competition of the β1 or β2 subunits for binding to the α1 subunit in MDCK cells, we demonstrate that the β1 subunit is a greatly preferred binding partner of the α1 subunit compared to the β2 subunit. The results of co-immunoprecipitation of α and β subunits from various detergent extracts of native tissues and cultured cells indicate that α1-β1 and α2-β2 heterodimers are more stable than α1-β2 heterodimers. Therefore, there is selective assembly of the different α and β subunit isoforms with likely tissue-specific functional consequences.

**EXPERIMENTAL PROCEDURES**

**Cell lines**- The Na,K-ATPase dog β1 or human β2 subunits linked with their N-termini to YFP were constructed as described previously (28,29). Stable MDCK cell lines expressing YFP-β1** and YFP-β2 were obtained and maintained as described previously (30). Confocal microscopy- Confocal microscopy images were acquired using the Zeiss LSM 510 laser scanning confocal microscope and LSM 510 software, version 3.2.

**Primary antibodies used for immunofluorescent staining and Western blot analysis**- For immunofluorescent staining, the monoclonal antibodies against the Na,K-ATPase α1 subunit, clone C464.6 (Millipore) and against the Na,K-ATPase β1 subunit, clone M17-P5-F11 (Affinity Bioreagents) and polyclonal antibodies against the Na,K-ATPase α2 subunit, (Millipore) and against the Na,K-ATPase β2 subunit (Millipore) were used. The polyclonal antibody against the Na,K-ATPase β1 subunit (31), which was a generous gift of Dr. W. James Ball Jr. (University of Cincinnati), was used for Western blot analysis. Also, the following monoclonal antibodies were used for Western blot analysis: against GFP, clones 7.1 and 13.1, which also recognizes YFP (Roche Diagnostics), against the Na,K-ATPase α1 subunit, clone C464.6 (Millipore), against the Na,K-ATPase α3 subunit (Upstate), against the Na,K-ATPase β2 subunit, clone 35 (BD Bioscience Pharmingen), and against the Na,K-ATPase β3 subunit (Santa Cruz).

**Extraction of proteins from MDCK cells and mouse brain homogenates**- Confluent MDCK cell monolayers grown in 6-well plates were rinsed twice with ice cold PBS and incubated with 200 μl/well of the extraction buffer at 4°C for 30 min followed by scraping cells. Mouse brain homogenates containing 600 μg protein in 500 μl of 150 mM NaCl in 50 mM Tris pH 7.5 were incubated with 500 μl of the extraction buffer (affinity reagents).
buffer at 4°C for 30 min. The extraction buffer contained 150 mM NaCl in 50 mM Tris pH 7.5 and the 2x concentration of the indicated detergent(s). When DOC was used alone, no NaCl was added to the extraction buffer. Prior to using, the extraction buffer was mixed with Complete Protease Inhibitor Cocktail (Roche Diagnostics), 1 tablet/50 ml. Cell extracts were clarified by centrifugation (15,000 g, 10 min) at 4°C. Where indicated, protein extracts were treated by PNGase F from Flavobacterium meningosepticum (New England BioLabs) or by Endo H from Streptomyces plicatus (GlycoProzyme Inc.) according to manufacturers’ instructions prior to loading on SDS-PAGE.

**Immunoprecipitation**- Protein extracts from MDCK cells or from mouse brain homogenates (100-300 µg protein) were incubated with 30 µl of the protein A-agarose suspension (Roche Diagnostics) in a total volume 1 ml of the extraction buffer at 4°C with continuous rotation for at least 3 hours (or overnight) to remove the components that non-specifically bind to protein A. The pre-cleared cell extract was mixed with 2 µl of polyclonal antibodies against GFP, which also recognize YFP (Clontech), or 10 µl of polyclonal antibodies against the Na,K-ATPase α subunit (CHEMICON International), or 10 µl of polyclonal antibodies against the Na,K-ATPase α subunit (32) and incubated with continuous rotation at 4°C for 60 min. After addition of 30 µl of the Protein A-agarose suspension, the mixture was incubated at 4°C with continuous rotation overnight. The bead-adherent complexes were washed 3 times on the beads and then eluted as described previously (33).

Multi-round immunoprecipitation from MDCK cells expressing either YFP-β₁ or YFP-β₂ was performed as described above with the exception that, after the first round of immunoprecipitation using 30 µg protein in 1% CHAPS and 5 µl anti-α antibody, the unbound proteins in the supernatant were collected and incubated again with 5 µl anti-α antibody in a second round of immunoprecipitation. A third round of immunoprecipitation was performed by using 4 µl anti GFP/YFP antibody from the supernatant after the second round of immunoprecipitation.

Where indicated, the bead-adherent proteins were treated with PNGase F or with Endo H. Deglycosylation by PNGase F was performed by incubation of the bead-adherent proteins with 1 µl PNGase F in 30 µl of 50 mM sodium phosphate, pH 7.5 containing 1% NP-40 and at 37°C for 1 hour. Digestion by Endo H was performed by incubation of the bead-adherent proteins with 3 µl of Endo H in 30 µl of 50 mM sodium citrate/phosphate, pH 5.5 containing 1% NP-40 at 37°C for 3 hours. After incubation with glycosidases, the reaction mixture was separated from the beads. The adherent proteins were eluted from the beads by incubation in 30 µl of 2x SDS-PAGE sample buffer for 5 min at 80°C. To account for possible dissociation of immunoprecipitated proteins from the beads during deglycosylation, the eluted proteins were combined with the reaction mixture. After separation by SDS-PAGE, the immunoprecipitated and co-immunoprecipitated proteins were analyzed by Western blot by using appropriate antibodies.

**Isolation of basolateral plasma membrane proteins of MDCK cells using surface-specific biotinylation**- Cells were maintained for 6 days after becoming confluent in transwell inserts. Biotinylation and isolations of basolateral surface proteins was performed according to previously described procedures (34-36).

**Western blot analysis**- 1-10 µg of proteins extracted from MDCK cells, microsomal membranes isolated from animal tissues in SDS-PAGE sample buffer, or 5-20 µl of proteins eluted from the Protein A-conjugated agarose beads were loaded onto 4-12% gradient SDS-PAGE gels (Invitrogen). Proteins were separated by SDS-PAGE, transferred onto a nitrocellulose membrane (BioRad) and detected by Western blot analysis as described previously (33). Immunoblots were quantified by densitometry using Zeiss LSM 510 software, version 3.2.

**Immunofluorescent staining**- MDCK cells were fixed by incubation with 3.75% formaldehyde in PBS for 15 min at 37°C and permeabilized by incubation with 0.1 % Triton X-100 for 5 min. Fixed cells or frozen tissue sections on FDA Standard frozen tissue rat or human arrays (BioChain) were incubated with Dako Protein Block Serum-Free solution (Dako Corporation) for 30 min. Immunostaining was performed by
1hr-incubation with the monoclonal antibodies followed by 1hr-incubation with Alexa Fluor 633 or Alexa Fluor 488 conjugated anti-mouse or anti-rabbit antibodies (Invitrogen).

Statistical analysis was performed using Student’s t-test (GraphPad Prism 4 software and Microsoft Excel). Statistical significance and number of experiments are specified in the figure legends.

RESULTS

Distribution of the Na,K-ATPase α₁, α₂, β₁ and β₂ subunits in rat and human tissues. Normal rat or human frozen tissue arrays (BioChain) that contained sections of adrenal gland, brain, breast, colon, esophagus, heart, kidney, liver, skeletal muscle, nerve and ovary were used to perform immunofluorescent double staining of the Na,K-ATPase α₁ and α₂ subunits. The Na,K-ATPase α₁ and α₂ subunits were differentially distributed in several organs, particularly in peripheral nerves and kidney, which are shown as examples (Fig. 1 and Fig. 2A). The α₂ subunit was found predominantly in perineurium that surrounds fascicles, while the α₁ subunit was also present in epineurium that surrounds nerves (Fig. 1). In the kidney, the α₁ subunit was detected in tubules, while the α₂ subunit was seen predominantly in renal arteriole and glomerulus (Fig. 2A). Double staining of the α₁ and β₂ subunits showed that tissue distribution of the β₂ subunit is similar to that of the α₁, but not of the α₂ subunit in both peripheral nerve and kidney sections (Fig. 1 and Fig. 2A). Also, double staining of the β₁ and β₂ subunits showed that distribution of the β₁ subunit in the nerve is similar to that of the α₁, but not of the β₂ subunit (Fig. 1B). Staining of the α₁, α₂ and β₂ subunits in rat brain cortex showed both co-expression of these isoforms in the same cells and cell-specific expression (Fig. 2B). Co-expression of α₁ and α₂ or α₁ and β₂ is evident from co-localization of red and green fluorescence, producing a yellow color on the merged images, while the differential expression of these isoforms is seen from separated green and red spots on the merged images. Co-expression of the β₁ isoform with both α₂ and β₂ isoforms in the same cells was also seen in human brain sections (not shown).

Subunit composition of the Na,K-ATPase α-β heterodimers in mouse brain. Western blot analysis detected the presence of six different isoforms of the Na,K-ATPase subunits in mouse brain extracts (Fig. 3), consistent with previously published results for rat brain (37). To validate the antibodies used to detect β subunits isoforms, we performed Western blot analysis of mouse brain extracts incubated with or without PNGase F that cleaves N-glycans attached to the β subunits. The bands at 33-35 kDa, which were expected for deglycosylated β₁, β₂ and β₃ subunits were detected in samples treated with PNGase F by using the antibodies listed in Experimental Procedures (Fig. 3A). In control samples, these antibodies detected bands at 45-50 kDa (Fig. 3A).

Immunoprecipitation of the β₂ subunit from 1% NP40/0.5% DOC extracts of mouse brain homogenates resulted in co-precipitation of the α₂ isoform and of a minor amount of the α₃ isoform, but not of the α₁ subunit (Fig. 3B). These results are consistent with detection of the α₂ subunit, but not of the α₁ subunit, in the fraction isolated from mouse brain by immunoaffinity chromatography using the antibody against the β₂ subunit (38).

Immunoprecipitation of the Na,K-ATPase α₂ subunit resulted in co-precipitation of the β₂ subunit, but not of the β₁ subunit (Fig. 3C). A minor amount of the β₃ subunit was detected in the immunoprecipitated fraction (Fig. 3C). No α₁ or α₃ subunits were detected in the immunoprecipitated fraction, confirming specificity of the anti-α₂ antibody. In contrast, the antibody raised against the 4/5 loop of the Na,K-ATPase α₁ subunit that is relatively homologous in other α isoforms (32) immunoprecipitated all three α isoforms, α₁, α₂ and α₃ and all three β subunit isoforms, from mouse brain extracts (Fig. 3C) confirming its non-selectivity, as expected.

To evaluate stability of the various Na,K-ATPase α-β complexes, we compared co-immunoprecipitates of the β subunits with α subunits from mouse brain extracts obtained by using increasing concentrations of the non-ionic detergent, DDM. The amount of the α₂ subunit immunoprecipitated with specific anti-α₂ antibodies was similar in 0.5%, 1% and 2% DDM (Fig. 4A, left panel). In contrast, the
amount of the β₂ subunits, which were co-precipitated with the α₂ subunit, gradually decreased with increasing detergent concentration (Fig. 4A, left panel and Fig. 4B), showing a partial dissociation of the α₂-β₂ complex by DDM. No co-precipitation of the β₁ or β₃ subunit was found at any DDM concentration tested.

The total amount of all three α subunit isoforms immunoprecipitated by non-specific anti-α antibodies was similar in 0.5%, 1% and 2% DDM extracts (Fig. 4A, right panel). The amount of the β₂ subunit co-precipitated by non-specific anti-α antibodies gradually decreased with increasing detergent concentration (Fig. 4A, right panel and Fig. 4B). This decrease was similar to that observed with the specific anti-α₂ antibodies (Fig. 4A, left panel and Fig. 4B), strongly suggesting that precipitation of the β₂ subunit by non-specific anti-α antibodies reflects α₂-β₂ complexes. This conclusion is consistent with co-precipitation of the α₂ but not of the α₁ subunit, with the β₂ subunit (Fig. 3C). The amount of the β₃ subunit that was co-precipitated with α subunits by the non-specific anti-α antibodies was not affected by detergent concentration (Fig. 4A, right panel). Only a slight decrease in the amount of the β₁ subunit co-precipitated with α subunits was observed with an increase in DDM concentration (Fig. 4A, right panel and Fig. 4B). These results indicate that complexes of the α₁ or α₃ subunit with either β₁ or β₃ subunit are preserved at all tested DDM concentrations. Therefore, both α₂ and β₂ subunits predominantly associate with each other, but not with other partner subunits in mouse brain.

Plasma membrane and intracellular distribution of the Na,K-ATPase α₁, β₁ and β₂ in MDCK cells. To test whether the α₁ subunit preferentially assembles with the β₁ or β₂ isoform, the content of α₁-assembled and α₁-unbound β subunits was compared in stable MDCK cell lines expressing either YFP-β₁ or YFP-β₂. The major endogenous Na,K-ATPase isoforms in these cells are α₁ and β₁. Both YFP-β₁ and YFP-β₂ were co-localized with the endogenous α₁ subunit in the lateral membranes, as detected by immunofluorescence (Fig. 5A), consistent with previously reported results (29). In addition, both YFP-β₁ and YFP-β₂, but not the α₁ subunit, were found inside the cells (Fig. 5A), indicating these intracellular forms of YFP-β₁ and YFP-β₂ are not assembled with the α₁ subunit. This intracellular retention was greater in dispersed cells than in confluent monolayers (Fig. 5B), and it showed co-localization with the ER marker (Fig. 5C). The fraction of YFP-β₂ localized in the ER was greater than that of YFP-β₁ (Fig. 5A-B).

To quantify the levels of the endogenous and exogenous Na,K-ATPase subunits in the ER and the plasma membrane in the two cell lines, we analyzed total cell lysates and plasma membrane fractions by Western blot analysis. In both cell lines, the YFP-linked β subunit was detected as two bands in total lysates (Fig. 6A). These two bands represent differentially N-glycosylated species of fusion proteins. Only a single band, which corresponded to the upper band in cell lysate, was found in the basolateral membrane (Fig. 6A), indicating that the lower band in cell lysate corresponds to the intracellular fraction of YFP-β₁ or YFP-β₂. As shown previously, this intracellular form of either YFP-β₁ or YFP-β₂ mostly represents the ER-resident fraction of each fusion protein (34). The relative amount of this ER-resident fraction appears to be greater for YFP-β₂ than for YFP-β₁ (Fig. 6A, left lanes). However, direct densitometric quantification of the two fractions of YFP-β₂ was not possible because of a significant overlap between two bands on SDS-PAGE (Fig. 6A, top panel, left lane).

To separate these two bands, we used Endo H, which is known to remove high-mannose- and hybrid- but not the complex-type N-glycans, from glycoproteins. Treatment of total cell lysates or biotinylated proteins with Endo H resulted in a slight increase in electrophoretic mobility of the plasma membrane fraction of YFP-β₂, but not of YFP-β₁ (Fig. 6A). The ER-resident form in cell lysates was completely deglycosylated by Endo H, producing a band at ~60 kDa that corresponds to the protein core molecular mass of YFP-β (Fig. 6A). As a result, a better separation of the plasma membrane and ER fractions on SDS-PAGE was observed in Endo H treated cell lysates, allowing densitometric quantification (Fig. 6B).

This quantification confirmed the greater ER retention of YFP-β₂ (49% of total cellular
content) as compared to YFP-β1 (31% of total cellular content) in mature cell monolayers. The difference in the relative content of the ER form of YFP-β2 and YFP-β1 was more prominent in immature cell monolayers, 80% and 38% of total cellular content, respectively (Fig. 6A-B). In contrast, the levels of YFP-β1 and YFP-β2 in the basolateral membrane were similar in the two transfected cell lines (Fig. 6C). Also, the levels of the endogenous α1 and β1 subunits were similar in YFP-β1- and YFP-β2-expressing MDCK cell lines (Fig. 6C).

Therefore, the amount of α1-unassembled YFP-β2 in the ER is significantly greater than that of YFP-β1, while the quantities of α1-assembled YFP-β1 and YFP-β2 in the plasma membrane are similar (Fig. 5 and 8), suggesting preferential assembly of the α1 subunit with the β1 isofrom rather than with the β2 isofrom.

**Stability of the Na,K-ATPase α1-β1 and α1-β2 complexes isolated from MDCK cells**. Co-immunoprecipitation of the Na,K-ATPase α1 subunit with YFP-β1 was detected in 1% Triton X-100, 1% DDM, 1% NP-40, 1% digitonin and also in a mixture of 1% NP-40 and 0.5% DOC (Fig. 7A). The amount of co-precipitated α1 subunit was similar in all tested detergents. In contrast, co-immunoprecipitation of the Na,K-ATPase α1 subunit with YFP-β2 was observed only in a few selected detergents, 1% digitonin, 1% DOC, 1% CHAPS and a mixture of 1% NP-40 and 0.5% DOC (Fig. 7A). The maximal amount of the Na,K-ATPase α1 subunit was co-immunoprecipitated with YFP-β2 in digitonin, DOC and CHAPS. No co-precipitation was seen in 1% Triton X-100, 1% DDM, or 1% NP-40 (Fig. 7A). These results indicate that the Na,K-ATPase α1-β2 complexes are less stable than the α1-β1 complexes.

Alignment of the β1 subunit with the β2 subunit shows that ten α1-interacting residues in the β1 subunit predicted by the high resolution structure of the α1-β1 Na,K-ATPase (39) differ from the corresponding residues in the β2 subunit (Fig. 7B). These differences are expected to weaken the α-β interaction. For example, the presence of an asparagine residue in the β2 subunit instead of Arg86 would not allow effective interaction with Glu122, Glu124 and Trp894 of the α1 subunit (Fig. 7B), consistent with the lower stability of α1-β2 complexes as compared to that of the α1-β1 complexes.

To determine whether co-precipitation of α1 subunits with YFP-β1 and YFP-β2 was specific, YFP-linked NTCP (sodium-taurocholate co-transporting polypeptide), the integral basolateral membrane protein that does not interact with the Na,K-ATPase, was used as a negative control. YFP-NTCP stably expressed in MDCK cells (28) was immunoprecipitated from 1% digitonin cell extracts under the same conditions that were used to immunoprecipitate YFP-β1 and YFP-β2 (Fig. 8A). The Na,K-ATPase α1 subunits were co-precipitated with YFP-β1 and YFP-β2, but not with YFP-NTCP, indicating that co-precipitation of α1 subunits with YFP-β1 and YFP-β2 was exclusively due to specific α-β interactions.

The α1 subunits were predominantly located in the plasma membrane, whereas both YFP-β1 and YFP-β2 were also found in the ER (Fig. 5A). Therefore, the amount of co-precipitated α1 subunits should be compared to the amount of the plasma membrane fraction of YFP-β proteins, but not to the total amount of immunoprecipitated YFP-β. To better separate the plasma membrane and ER-resident fractions, the immunoprecipitated proteins were treated with Endo H prior to SDS-PAGE (Fig. 8A). Western blot analysis followed by densitometric quantification, which allowed calculation of the amount of co-precipitated α1 subunits relative to the amount of the plasma membrane fractions of immunoprecipitated YFP-β1 or YFP-β2. This quantification showed that the amount of the YFP-β2-bound α1 subunits was 15% less than the amount of YFP-β1-bound α1 subunits (Fig. 8B). Similar results were obtained in 1% CHAPS (not shown).

If the lower amount of YFP-β2-bound α1 subunit was related to partial disruption of the α1-β2 complex by detergent, the detergent extract must contain the α1-unbound plasma membrane forms of YFP-β2 subunits. To determine the presence of α1-unbound β subunits in 1% CHAPS extracts of both YFP-β1- and YFP-β2-expressing MDCK cells, multi-round immunoprecipitation using anti-α antibody was performed. The first round of immunoprecipitation pulled down a vast majority of the α1 subunits. Co-precipitated
fractions of YFP-β₁ or YFP-β₂ contained predominantly mature forms and minor amounts of the ER-resident immature forms (Fig. 8C-D). The second round of immunoprecipitation using anti-α antibody precipitated the rest of the α₁ subunits and mature YFP-β₁ or YFP-β₂. The third round of immunoprecipitation using anti-GFP antibody pulled down almost exclusively the immature forms of YFP-β₁ and YFP-β₂, while the unassembled subunits are bound to ER chaperones (33,41), which facilitate normal folding of the subunits and possibly the assembly process per se. The β₂ subunit, but not the β₁ subunit, persistently binds calnexin in the ER, suggesting that it undergoes repeated calnexin-assisted folding prior to its assembly with the α subunit (29,33). Persistent calnexin binding to glycoproteins is dependent on repeated cycles of de- and re-glucosylation of glycoprotein N-glycans by the ER glucosidase and UGGT1, respectively (42). It is possible that the β₂ subunit is not completely folded by the time when calnexin is dissociated from deglycosylated β₁ subunit and thus is recognized by the folding sensing enzyme, UGGT1 (43). UGGT1 re-glucosylates the β₂ subunit N-glycans, which results in repeated calnexin binding. However, it cannot be excluded that calnexin-free β₂ subunits bind UGGT1 not because they are misfolded, but because they fail to assemble with the α₁ subunits due to their lower α₁-binding affinity as compared to that of the β₁ subunits. The UGGT1-mediated re-glucosylation of the β₂ subunit would then induce its re-binding to calnexin. Therefore, both longer association with calnexin and greater accumulation in the ER of the β₂ subunit as compared to those of the β₁ subunit could result from either the longer time required for folding of the β₂ subunit or its lower affinity to the α₁ subunit. The lower binding affinity of the β₂ subunit toward the α₁ subunit is anticipated from the differences between the α₁-interacting residues of the β₁ subunit and the corresponding residues of the β₂ subunit (Fig. 7B).

The α₁-β₂ heterodimers are less resistant to the disruptive effect of various detergents than the α₁-β₁ complexes. Co-immunoprecipitation of the Na,K-ATPase α₁ subunit with YFP-β₂ was observed only in selected detergents, and the amount of co-precipitated α₁ subunit varied in

DISCUSSION

The β₁, but not the β₂ isoform, is a preferred binding partner of the α₁ subunit. YFP-β₁ and YFP-β₂ stably expressed in MDCK cells are predominantly distributed between the basolateral plasma membrane and ER (Fig. 5). All the β subunits present in the basolateral membrane are α₁-assembled, as we showed previously for the endogenous β₁ subunits (40) and now for both exogenous YFP-β₁ and YFP-β₂ (Fig. 8). On the other hand, the majority of the ER-resident YFP-β₁ and YFP-β₂ are not assembled with the α₁ subunits (34,40), (Fig. 5B and Fig. 8C-D). These results are consistent with the previous finding showing that both YFP-β₁ and YFP-β₂ compete with the endogenous β₁ subunits for binding to the limited amount of the endogenous α₁ subunits (34). Only the α₁-assembled β subunits exit the ER, while the unassembled subunits are retained in the ER and rapidly degraded (34). As a result of this competition, a fraction of α₁-β₁ heterodimers exported from the ER is replaced by α₁-(YFP-β) heterodimers, explaining the decrease in the amount of the mature endogenous β₁ subunit in the basolateral membrane in both YFP-β₁- and YFP-β₂-expressing cell lines as compared to non-transfected MDCK cells (Fig. 6C). YFP-β₁- and YFP-β₂-expressing cell lines have similar quantities of exogenous and endogenous Na,K-ATPase subunits in the plasma membrane (Fig. 6C), whereas the abundance of the ER-located α₁-unbound YFP-β₂ is greater than that of YFP-β₁ (Fig. 5 and Fig. 6A-B). Since the ER retention of YFP-β₁ or YFP-β₂ is due to their competition with the same number of endogenous β₁ subunits for binding to the α₁ subunit, these results imply that the β₂ subunit has lower affinity for the α₁ subunit than does the β₁ subunit.

This interpretation, however, is complicated by the fact that α₁-unassembled β subunits and β-unassembled α₁ subunits are not freely floating in the ER. Instead, the orphan α and β subunits are bound to ER chaperones (33,41), which facilitate normal folding of the subunits and possibly the assembly process per se. The β₂ subunit, but not the β₁ subunit, persistently binds calnexin in the ER, suggesting that it undergoes repeated calnexin-assisted folding prior to its assembly with the α subunit (29,33). Persistent calnexin binding to glycoproteins is dependent on repeated cycles of de- and re-glucosylation of glycoprotein N-glycans by the ER glucosidase and UGGT1, respectively (42). It is possible that the β₂ subunit is not completely folded by the time when calnexin is dissociated from deglycosylated β₁ subunit and thus is recognized by the folding sensing enzyme, UGGT1 (43). UGGT1 re-glucosylates the β₂ subunit N-glycans, which results in repeated calnexin binding. However, it cannot be included that calnexin-free β₂ subunits bind UGGT1 not because they are misfolded, but because they fail to assemble with the α₁ subunits due to their lower α₁-binding affinity as compared to that of the β₁ subunits. The UGGT1-mediated re-glucosylation of the β₂ subunit would then induce its re-binding to calnexin. Therefore, both longer association with calnexin and greater accumulation in the ER of the β₂ subunit as compared to those of the β₁ subunit could result from either the longer time required for folding of the β₂ subunit or its lower affinity to the α₁ subunit. The lower binding affinity of the β₂ subunit toward the α₁ subunit is anticipated from the differences between the α₁-interacting residues of the β₁ subunit and the corresponding residues of the β₂ subunit (Fig. 7B).
of these detergents (Fig. 7), showing that the α₁-β₂ complex is partially or completely disrupted by the majority of tested detergents. On the other hand, the amount of the Na,K-ATPase α₁ subunit that co-precipitated with YFP-β₁ is the same in all tested detergents, indicating that the α₁-β₁ complex is fully preserved in these detergents (Fig. 7). These results are in agreement with previously reported disruption of α₁-β₂, but not of the α₁-β₁ or α₂-β₂ complexes formed in Xenopus oocytes by Triton X-100 (44,45).

Selective formation of the α₂-β₂ Na,K-ATPase in mouse brain. The β₂ subunit of the Na,K-ATPase was first discovered as AMOG (19). Immunoaffinity purification of AMOG from mouse brain by using AMOG-specific antibody resulted in co-purification of a 100 kDa protein that later was identified as the Na,K-ATPase α₂ subunit (and possibly α₃ subunit), but not the α₁ subunit (38). Consistent with these data, we found that immunoprecipitation of the β₂ subunit from mouse brain resulted in co-immunoprecipitation of the α₂, but not of the α₁ subunit (Fig. 3B). Conversely, immunoprecipitation of the α₂ subunit selectively co-precipitated the β₂ subunit (Fig. 3C and Fig. 4). Six isoforms of the Na,K-ATPase are expressed in the brain (9,37). Both α₂ and β₂ subunits are predominantly expressed in glial cells (37), so the formation of the α₂-β₂ complexes is, in part, due to the cell-specific co-expression of the two isoforms. However, both α₂ and β₂ subunits are also found in subsets of neurons (37). Similarly, the α₁ and β₁ isoforms are expressed in both glial cells and neurons. With the exception of the neuron-specific α₃ subunit, other isoforms are expressed in both neurons and glial cells (37,46-48). Even though there are cell- and region-specific differences in expression of various isoforms, many cell types in brain contain multiple Na,K-ATPase subunit isoforms (9,37,46). Accordingly, co-expression of the α₁, α₂ and β₂ subunits in the same cells in rat brain cortex is detected here by immunofluorescence (Fig. 2B). Therefore, the preferential formation of the α₂-β₂ complexes in the brain is determined not only by cell-specific co-expression of these isoforms, but also by their binding preferences. Preferential formation of the α₂-β₂ was also detected in heart and adrenal medullary cells, where the α₁ subunit is more abundant than the α₂ subunit (26,27), emphasizing preferential β₂ subunit binding to the α₂ subunit.

Interestingly, the α₂-β₂ complexes are less stable than complexes of the α₁ or α₃ subunit with either β₁ or β₃ subunit (Fig. 4). Recent studies have demonstrated that α₂-β₂ complexes are less stable to heat and detergents than α₁-β₁ or α₃-β₁ complexes perhaps due to weaker interactions of the α₂ subunit with phosphatidylserine, which stabilizes the protein (49). Thus, it is possible that detergent-mediated disruption of α₂-β₂ complexes (Fig. 4) is the result of displacement of selectively bound phosphatidylserine.

Preferential assembly of α₂ and β₂ isoforms in the brain may have several implications. The β subunits are known to modify the kinetic properties of the α isoforms. The α₂-β₂ heterodimer has the lowest K⁺ affinity among nine different α-β heterodimers formed by each of the three α subunit isoforms (α₁-α₃) and each of the three β subunit isoforms (β₁-β₃) (25). Thus, assembly of the α₂ subunit preferentially with the β₂ isoform may be crucial to restore external K⁺ homeostasis after a series of action potentials in the nervous system, since the α₂-β₂ heterodimer would respond to an increase in external K⁺ because of its low K⁺ affinity (25). In astrocytes, the Na,K-ATPase α₂ subunits form complexes with different glutamate transporters, and glutamate inward transport is inhibited by ouabain, suggesting a specific role of the Na,K-ATPase α₂ subunit in reuptake of glutamate from the synaptic cleft (50). Since the activity of the least K⁺-sensitive α₂-β₂ isoform would increase more at the elevated external K⁺ concentration, assembly with the β₂ subunit may be important for the specific role of the Na,K-ATPase α₂ subunit in glutamate clearance.

Considerable evidence exists for the presence of endogenous ouabain-like molecules in mammalian tissues that may serve to regulate Na,K-ATPase activity (3,14,51). Particularly, a signaling role of the Na⁺/K⁺-ATPase has been demonstrated in regulating synaptic plasticity and dendritic growth in cortical neurons (52). The human α₁, α₂ and α₃ isoforms have similar ouabain affinities (25). However, the lowest K⁺ affinity of the α₂-β₂ isoform implies that this
heterodimer has the lowest \( K^+ \)/ouabain antagonism as compared to other \( \alpha-\beta \) heterodimer isoforms (25). So, at physiological \( K^+ \) concentrations, ouabain and endogenous ouabain-like compounds may predominantly bind to the \( \alpha_2-\beta_2 \) isomer and to a lesser extent to other complexes and thus specifically regulate the \( \alpha_2 \)-dependent signaling pathways.

Natural in vivo mutations in the \( \alpha_2 \) subunit are associated with familial hemiplegic migraine and epilepsy (16). Most of these mutations cause functional defects in active \( Na^+ \) and \( K^+ \) transport and impaired clearance of extracellular \( K^+ \) or glutamate due to either the impairment of maturation and hence plasma membrane delivery of the enzyme, or the loss of the catalytic activity (16,53). It is known that neurological diseases, particularly epilepsy, are closely associated with the ER stress-related retention of essential ion transporters in the ER (54-57). We showed recently that the \( \beta_2 \) isomer is much more sensitive to the ER stress than the \( \beta_1 \) isomer (33). Since the \( \beta \) subunit is essential for maturation of the Na,K-ATPase \( \alpha-\beta \) heterodimers (58) and the \( \alpha_2 \) selectively forms a complex with the \( \beta_2 \) isomer in the brain, it is possible that stress-induced impairment of the \( \beta_2 \) subunit folding in the ER increases the ER retention of the \( \alpha_2 \) subunit, which decreases the Na,K-ATPase ion transport activity of the \( \alpha_2\beta_2 \) Na,K-ATPase and thus contributes to epilepsy. Consistent with this hypothesis, abnormalities in distribution of the \( \beta_2 \) subunit are linked to epilepsy (21,22).

Therefore, the selectivity of \( \alpha-\beta \) assembly, which is determined both by cell-specific expression and by isoform-specific binding preferences of \( \alpha \) and \( \beta \) subunits, is crucial for cell- and tissue-specific functions of the Na,K-ATPase.

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**FOOTNOTES**

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**The abbreviations used are: YFP-β1 and YFP-β2, the fusion proteins between the yellow fluorescent protein and the Na,K-ATPase β1 subunit and β2 subunit, respectively; GFP, green fluorescent protein; PNGase F, Peptide:N-Glycosidase F; Endo H, Endo-β-N-acetylglucosaminidase H; DOC, Sodium Deoxycholate; DDM, n-dodecyl β-D-maltoside; NP-40, Nonidet P-40; CHAPS, 3-{(3-Cholamidopropyl)dimethylammonio}-1-propanesulfonate; NTCP, Sodium-taurocholate cotransporting polypeptide.**

**FIGURE LEGENDS**

Fig. 1. Localization of the Na,K-ATPase β2 subunit in rat sciatic nerve and kidney sections is similar to that of the Na,K-ATPase α1 subunit, but not of the Na,K-ATPase α1 subunit or β1 subunit. A, Frozen sections of rat sciatic nerve were double stained by using mouse antibodies against α1 subunit
(green) and either rabbit antibodies against α subunit (red) or rabbit antibodies against β subunit (red). B, Frozen sections of human trigeminal nerve were double stained by using either mouse antibodies against α1 subunit (green) or mouse antibodies against β1 subunit (green) and rabbit antibodies against β2 subunit (red). Anti-mouse Alexa Fluor 488 conjugated secondary antibodies were used to detect anti-α1 primary antibodies and anti-rabbit Alexa Fluor 633 conjugated secondary antibodies were used to detect anti-β2 and anti-α2 primary antibodies.

Fig. 2. Localization of the Na,K-ATPase α1, α2 and β2 subunits in rat kidney and brain sections. Frozen sections of rat kidney (A) and rat brain (B) were double stained by using mouse antibodies against α1 subunit (green) and either rabbit antibodies against α2 subunit (red) or rabbit antibodies against β2 subunit (red). Anti-mouse Alexa Fluor 488 conjugated secondary antibodies were used to detect anti-α1 primary antibodies and anti-rabbit Alexa Fluor 633 conjugated secondary antibodies were used to detect anti-β2 and anti-α2 primary antibodies. Insets in the right panels (B) show 5-fold zoomed images.

Fig. 3. The Na,K-ATPase α2 and β2 subunits are selectively co-immunoprecipitated from mouse brain extracts. Proteins were extracted from mouse brain homogenate by using 1% NP40/0.5% DOC. A, The antibodies against the Na,K-ATPase β1, β2 and β3 isoforms were validated by Western blot analysis of mouse brain extracts pre-incubated with or without PNGase F that cleaves N-glycans from the β isoforms subunits and, hence, results in an increase in electrophoretic mobility of the subunits. B, Western blot analysis of the immunoprecipitated β2 subunit and co-immunoprecipitated α subunit isoforms shows that the α2 subunit is preferentially co-precipitated with the β2 subunit. Input lanes contain 4% and 10% of the extract used for immunoprecipitation on α and β blots, respectively. C, Western blot analysis of proteins immunoprecipitated and co-immunoprecipitated by using either the α2-specific antibodies (left panels) or the α-non-specific antibodies (right panels) shows selective co-immunoprecipitation of the β2 subunit with the α2 subunit. Input lanes contain 10% of the extract used for immunoprecipitation. To prevent an overlap of the β subunit bands with the heavy chain band of the antibodies used for immunoprecipitation, the immunoprecipitated proteins were treated with PNGase F prior to SDS-PAGE. Inputs are HCh, heavy chain; IP, immunoprecipitation; WB – Western blot; DG – deglycosylated.

Fig. 4. The Na,K-ATPase α1-β2 complex is less stable than the Na,K-ATPase α-β1 or α-β3 complexes in detergent extracts obtained from mouse brain membranes. A, Various concentrations of n-dodecyl β-D-maltoside (DDM) were used to extract proteins from mouse brain homogenate. Western blot analysis of proteins immunoprecipitated and co-immunoprecipitated by using either the α2-specific antibodies (left panels) or the α-non-specific antibodies (right panels) shows a stepwise decrease in the amount of β2 subunits co-immunoprecipitated by using both antibodies, but not in the amount of β1 or β3 subunits co-immunoprecipitated by using α-non-specific antibodies, with increasing detergent concentrations. B, Densitometric quantification of the results shown in A was performed by dividing the signal from the β antibody by the corresponding signal of the α antibody. A comparative graph shows these ratios as a percentage of the ratio obtained in 0.5% DDM. IP, immunoprecipitation; WB – Western blot; DG – deglycosylated by PNGase F prior to SDS-PAGE.

Fig. 5. The amount of α1-unbound YFP-β2 retained in the ER of MDCK cells is greater than that of YFP-β1. Horizontal confocal microscopy sections of confluent monolayers (A) or dispersed colonies (B-C) of MDCK cells expressing either YFP-β1 or YFP-β2. Both YFP-β1 and YFP-β2 (green) are co-localized with the endogenous α1 subunit (red) in the lateral membranes, but not inside the cells as detected by immunostaining of fixed cells using the monoclonal antibody against the Na,K-ATPase α1 subunit (A-B). The intracellular retention of α1-unassembled YFP-β2 is more prominent than that of YFP-β1 and more...
evident in dispersed colonies than in confluent monolayers. This intracellular fraction of YFP-β₂ (green) shows co-localization with the ER (red) as detected by transient expression of the fluorescent ER marker, DsRed2-ER (C). N-nucleus; PM – plasma membrane.

**Fig. 6.** The greater intracellular retention of YFP-β₂ than YFP-β₁ is not associated with its higher level in the plasma membrane. A-B, The comparative Western blot analysis (A) of total cell lysates and basolateral biotinylated proteins (BL membrane) of MDCK cells stably expressing either YFP-β₁ or YFP-β₂ shows that the upper band found in either cell lysate represents the mature plasma membrane fraction (BLM), while the lower band corresponds to the intracellular fraction ER-resident fraction (ER). Treatment with endoglycosidase H (*Endo H*) resulted in a slight increase in electrophoretic mobility of BLM YFP-β₂, but not of BLM YFP-β₁, and a major increase in electrophoretic mobility of ER YFP-β₁ and ER YFP-β₂. This allows a better separation of BLM and IC fractions of YFP-β₁ or YFP-β₂ on SDS-PAGE and their densitometric quantification (B). C, Western blot analysis of proteins isolated by basolateral surface-selective biotinylation show that stable expression of either YFP-β₁ or YFP-β₂ in MDCK cells resulted in a significant decrease in the amount of the endogenous Na,K-ATPase β₁ subunits in the basolateral membranes, but did not change the level of the α₁ subunits, as compared to non-transfected cells (NT cells). YFP-β₁ and YFP-β₂ are present in the basolateral membrane at similar levels in the two transfected cell lines. N – nucleus; PM – basolateral plasma membrane.

**Fig. 7.** The Na,K-ATPase α₁-β₁ complex is more stable than the Na,K-ATPase α₁-β₂ complex in detergent extracts from MDCK cells. A, MDCK cells stably expressing either YFP-β₁ or YFP-β₂ were lysed by incubation with the extraction buffer containing an appropriate detergent (as indicated). After scraping the cells and removing non-extracted material by centrifugation, YFP-linked β₁ or β₂ subunits were immunoprecipitated. Immunoprecipitated YFP-β₁ or YFP-β₂ and co-immunoprecipitated α₁ subunits were analyzed by Western blot. Co-immunoprecipitation of the Na,K-ATPase α₁ subunit with YFP-β₁ was detected in all tested detergents. In contrast, co-immunoprecipitation of the Na,K-ATPase α₁ subunit with YFP-β₂ was observed only in selected detergents. B, A model of the Na,K-ATPase α₁ and β₁ subunits based on the crystal structure of the sodium-potassium pump at 2.4 Å resolution (2ZXE) (39) shows the α₁-interacting residues in the β₁ subunit that are different in the β₂ subunit (yellow labels and yellow halos). The corresponding β₁-interacting residues in the α₁ subunit are indicated in light-blue. A close-up view of Arg86 of the β₁ subunit and its interacting residues in the α₁ subunit is shown in the right panel.

**Fig. 8.** The Na,K-ATPase α₁-β₂ complex is preserved in digitonin and CHAPS extracts from MDCK cells. A, Proteins were extracted from MDCK cells expressing YFP-β₁, or YFP-β₂, or YFP-linked bile acid transporter (YFP-NTCP), with the extraction buffer containing 1% digitonin. YFP-linked β₁ or β₂ subunits were immunoprecipitated and treated with Endo H followed by elution of proteins from the beads. Co-immunoprecipitation of the Na,K-ATPase α₁ subunit was detected with YFP-β₁ and with YFP-β₂, but not with YFP-NTCP, indicating that there is no non-specific precipitation of the α₁ subunit. B, Densitometric quantification of the results presented in A was performed by dividing the density of the α₁ subunit band by the density of the corresponding PM YFP-β band. C-D, Proteins were extracted from MDCK cells expressing either YFP-β₁ (C) or YFP-β₂ (D) using extraction buffer containing 1% CHAPS and subject to successive rounds of immunoprecipitation using anti-α antibody followed by a final round of immunoprecipitation using anti-GFP/YFP antibody. Both extracted and immunoprecipitated proteins were treated with Endo H prior to SDS-PAGE. The majority of YFP-β subunits co-immunoprecipitated with α₁ were complex-type glycosylated. Almost all of the β subunits not assembled with α₁ were Endo H sensitive and immunoprecipitated in the final anti-GFP/YFP immunoprecipitation. PM – plasma membrane, Super2 – supernatant after the second round of immunoprecipitation.
Fig. 1
Fig. 3
Fig. 4
Fig. 5
Fig. 6
A

| MDCK cell line | YFP-β₁ | YFP-β₂ | YFP-β₁ |
|----------------|--------|--------|--------|
| Detagent       |        |        |        |
| 1% Triton X-100| 100    |        | 100    |
| 1% DDM         |        |        |        |
| 1% NP40        |        |        |        |
| 1% Digitonin   |        |        |        |

IP: anti-YFP

B

α, subunit

(W1016)K35
(F867)L55
(E875)L62
(F902)F65E66
(K86)W894,E122, E124
(L890)Q892
(L131)Q905

β₁ subunit

E124
W894
K86 (N in β₂)

Fig. 7
Fig. 8

**A**

Detergent: 1% Digitonin  
IP: anti-YFP → Endo H

**B**

Co-IP of Na,K-\(\alpha_1\) with YFP-\(\beta_2\)

**C**

Cell line: YFP-\(\beta_1\)  
Detergent: 1% CHAPS + Endo H

**D**

Cell line: YFP-\(\beta_2\)  
Detergent: 1% CHAPS + Endo H
Subunit isoform selectivity in assembly of Na,K-ATPase α-β heterodimers
Elmira Tokhtaeva, Rebecca J. Clifford, Jack H. Kaplan, George Sachs and Olga Vagin

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