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Isoform-specific interactions of Na,K-ATPase subunits are mediated via extracellular domains and carbohydrates

(assembly/detergents/Na,K-pump isozymes/N-glycosylation)

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ABSTRACT **The functional unit of the Na,K-ATPase consists of a catalytic α subunit noncovalently linked with a glycoprotein subunit, β. Using ouabain binding assays and immunoprecipitation of rodent α/β complexes, we show here that all six possible isozymes between three α and two β isoforms can be formed in *Xenopus* oocytes. Two isoform-specific differences in α/β interactions are observed: (*i*) $\alpha1/\beta1$ and $\alpha2/\beta2$ complexes, in contrast to $\alpha1/\beta2$ complexes, are stable against Triton X-100-mediated dissociation, and (*ii*) $\beta2$ subunits must carry *N*-glycans to combine with $\alpha1$ but not with $\alpha2$. The interacting surfaces are mainly exposed to the extracellular side because coexpression of a truncated $\beta1$ subunit comprising the ectodomain results in assembly with $\alpha1$ and $\alpha2$, but not with $\alpha3$; the $\beta2$ ectodomain combines with $\alpha2$ only. A chimera consisting of 81% and 19% of the $\alpha1$ N terminus and $\alpha2$ C terminus, respectively, behaves like $\alpha2$ and coprecipitates with the $\beta2$ ectodomain. In contrast, the reciprocal chimera does not coprecipitate with the $\beta2$ ectodomain. These results provide evidence for a selective interaction of Na,K-ATPase α and β subunits.**

The Na,K-ATPase is a heterodimer consisting of a 110-kDa α subunit that is noncovalently linked with a glycoprotein β subunit (protein core, 33–35 kDa). The α subunit is the catalytic active part and bears the site for ATP hydrolysis, the binding sites for Na^+, K^+, and cardiac glycosides (1). The α subunit spans the membrane several times. The β subunit exposes a short N-terminal tail to the cytoplasm and the majority of its mass at the extracellular surface. It confers conformational stability to the α subunit during or after assembly in the endoplasmic reticulum and is needed for routing of the enzyme to the plasma membrane (2–5).

Three α isoforms ($\alpha1$, $\alpha2$, and $\alpha3$) and two β isoforms ($\beta1$ and $\beta2$) have been identified in mammals and birds, each encoded by a separate gene (6). The α isoforms show more than 80% homology even for very diverse species. The two β isoforms have only about 40% amino acid sequence identity within the same species. Sequences encoding a mammalian $\beta3$ isoform have been deposited in the GenBank database. Both α and β isoforms display complex patterns of tissue-specific and developmental expression (7, 8).

All six possible α/β isozymes are formed from the three α and two β isoforms of birds (9) and mammals (this work) *in vitro*, supporting the assumption that different isozymes exist *in vivo* (10, 11). Hybrid Na,K-pumps are readily formed from $\alpha1$ and $\beta1$ subunits of virtually all species investigated (for a review, see ref. 12). The $\beta1$ isoform can even be replaced by the β subunit of the H,K-pump to yield a functional Na,K-pump (13).

Protein domains involved in $\alpha1/\beta1$ interactions have only partially been identified. Deletion of the cytoplasmic domain and part of the transmembrane domain of chicken $\beta1$ does not disturb assembly as long as the truncated mutants are inserted into the membrane (14). Chimeric $\beta1$ subunits containing the N terminus and the membrane spanning domain from unrelated type II membrane proteins also assemble with the $\alpha1$ subunit (15, 16). Evidence for a role of the β subunit ectodomain in assembly is controversial. Whereas a $\beta1$ mutant lacking 146 C-terminal amino acids was capable of combining with $\alpha1$, (15), point mutations at the extreme C terminus of the $\beta1$ subunit interfered with assembly (17). Using chimeric subunits between the rat Na,K-pump and H,K-pump (18) or the chicken Na,K-pump and Ca-pump (9, 19), domains that determine assembly on the α subunit were localized to 26 extracellular amino acids of the $\alpha1$ subunit.

Two important issues have not yet been resolved. First, do all α and β isoforms assemble with equal efficiency? One might anticipate that there is some preference since, for example, no hybrid ATPases have been found in epithelial cells producing both H,K-ATPase and Na,K-ATPase (18). Second, does glycosylation of β subunits play a role in subunit assembly? To address these questions, we have examined the stability of each isozyme against detergent-assisted dissociation. Our data indicate that some isozymes are preferentially formed, that *N*-linked glycosylation is essential for assembly of the $\beta2$ subunit with $\alpha1$ and that assembly involves extracellular domains of either subunit.

MATERIALS AND METHODS

cDNA Constructs and Transcripts. Complete coding regions for the α subunits were subcloned into the *Sma*I site of pNKS2 (20). pNKS2 contains a 110 poly(A) region downstream of the multiple cloning site. For the construction of cDNAs encoding chimeras between rat $\alpha1$ and rat $\alpha2$ subunits, a conserved *Bsp*HI site was used as an exchange point allowing in-frame fusion of complementary parts of the two cDNAs. This exchange occurred at codon 831 on the rat $\alpha1$ cDNA and at codon 828 on the rat $\alpha2$ cDNA. cDNAs for the $\beta1$ subunit (21) and $\beta2$ subunit of the Na,K-pump of the mouse (22) in pGEM2 (Promega) have been described (23). cDNAs encoding ectodomains of the mouse $\beta1$ subunit (233 aa) and $\beta2$ subunit (228 aa) were described (24). Both cDNAs were subcloned into pSP64polyA (Promega). Capped cRNAs were synthesized with SP6 RNA polymerase, gel purified, and quantified as described (20).

Injection and Maintenance of Oocytes. *Xenopus laevis* females were imported from South Africa. Follicle cell-free oocytes of oogenesis stages V or VI were obtained as described (23) and injected with 46 nl aliquots of cRNA. Injected oocytes

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and noninjected controls of the same batch were cultured in sterile oocyte Ringer's solution (90 mM NaCl/1 mM KCl/1 mM $CaCl_2$/1 mM $MgCl_2$/10 mM Hepes, pH 7.4) supplemented with 50 mg/liter gentamycin. The culture medium was changed daily.

Quantitation of the Number of Surface Na,K-Pumps. The number of Na,K-pump molecules of the cell surface was assessed using [^{3}H]ouabain as a ligand and [^{14}C]sucrose as an extracellular marker (23). Oocytes were first loaded with Na^+ to direct Na,K-pumps into an ouabain-sensitive conformation and then exposed for 20 min to 1 μM [^{3}H]ouabain and 1 mM [^{14}C]sucrose in K^+-free oocyte Ringer's solution at ambient temperature. Nonspecific binding was measured in the presence of 1 mM unlabeled ouabain and represented <5% of total binding.

Antibodies. The rat hybridoma clone producing mAb BSP/3 to the β1 subunit of the mouse Na,K-pump and the rat mAb 426 to the mouse β2 subunit have been described (22, 25). IgGs were isolated from hybridoma culture media by protein G–Sepharose CL 4B chromatography (Pharmacia) and covalently coupled via their carbohydrate moieties with Sephadex beads according to the protocol of Bio-Rad.

Inhibition of N-Glycosylation. Oocytes were injected with 40 μg/ml of tunicamycin (Boehringer Mannheim) and then maintained overnight in oocyte Ringer's solution containing 2 μg/ml of tunicamycin before the cRNA was injected. All subsequent incubations were also performed in the presence of tunicamycin. Tunicamycin from Boehringer Mannheim did not reduce total protein synthesis in *Xenopus* oocytes by >6%, as assessed from the incorporation of [^{35}S]methionine into protein, and was used throughout the study.

Metabolic Labeling, Immunoprecipitation, SDS/PAGE, and Fluorography. Oocytes were labeled with L-[^{35}S]methionine as described (20). After a 3-h pulse at 19°C, oocytes were either immediately extracted with the indicated detergent in extraction buffer (0.1 M NaCl/1 mM EDTA/0.2% BSA/20 mM Tris·HCl, pH 7.6/1 mM phenylmethylsulfonyl fluoride) supplemented with 5 μg/ml of each antipain, pepstatin A, and leupeptin, or first chased with 10 mM unlabeled methionine in oocyte Ringer's solution. Extracts from 5 oocytes (100 μl) were diluted 5-fold with extraction buffer supplemented with the indicated detergent (1% digitonin or 0.5% Triton X-100) and 20 μl of packed immunobeads corresponding to 5–10 μg of covalently bound mAb BSP/3 or mAb 426. Following end-over-end mixing for 4 h at 5°C, Sepharose beads were washed four times with extraction buffer containing the same detergent as for extraction. Protein was eluted by two subsequent incubations with sample buffer, each for 10 min at 37°C. Immunoprecipitates were electrophoresed on 8 to 10% SDS polyacrylamide gels. For fluorography, gels were fixed, soaked in Amplify (Amersham), dried, and exposed to preflashed X-Omat-AR film (Eastman Kodak) at −80°C.

RESULTS

Mouse β1 and β2 subunits can combine with α1 subunits of *Torpedo californica* to yield functional Na,K-pumps (26). To examine whether β1 and β2 subunits also combine with the α2 and α3 isoforms, *Xenopus* oocytes were injected with the respective cRNAs and assayed for the number of surface ouabain binding sites. Our data indicate that all six theoretically possible Na,K-pump isozymes can be formed: $\alpha1/\beta1$, $\alpha2/\beta1$, $\alpha3/\beta1$, $\alpha1/\beta2$, $\alpha2/\beta2$, and $\alpha3/\beta2$. Coinjection of cRNA for one of the β isoforms together with cRNA for either of the rat isoforms caused a 1.5- to 3-fold increase in the ouabain binding capacity (Fig. 1*A*). The low affinity of the rat α1 isoform for ouabain underestimates the actual number of enzymes formed. The capability to bind ouabain indicates that all Na,K-pump isozymes are functional because they must be able to undergo characteristic changes in conformation and to adopt, at least, the E_2P conformation. Ouabain binding capability always coincides with transport activity (23, 26). All α isoforms failed to bind ouabain when synthesized in the absence of either of the two β isoforms (results not shown). The 10–20% increase in the number of surface Na,K-pumps after injection of β1 or β2 subunit cRNA without α subunit cRNA (Fig. 1*A*) is consistent with previous observations showing that exogenous β subunits combine with endogenous α subunits to form interspecies hybrid Na,K-pumps (23, 26). Endogenous α1 subunits are constitutively synthesized in *Xenopus* oocytes in excess over endogenous β subunits (4). Further evidence for assembly was provided by coimmunoprecipitation experiments. Fig. 1*B* shows comparable amounts of coprecipitated 110-kDa α subunits in all digitonin immunoprecipitates from cells synthesizing one of the rat α or the

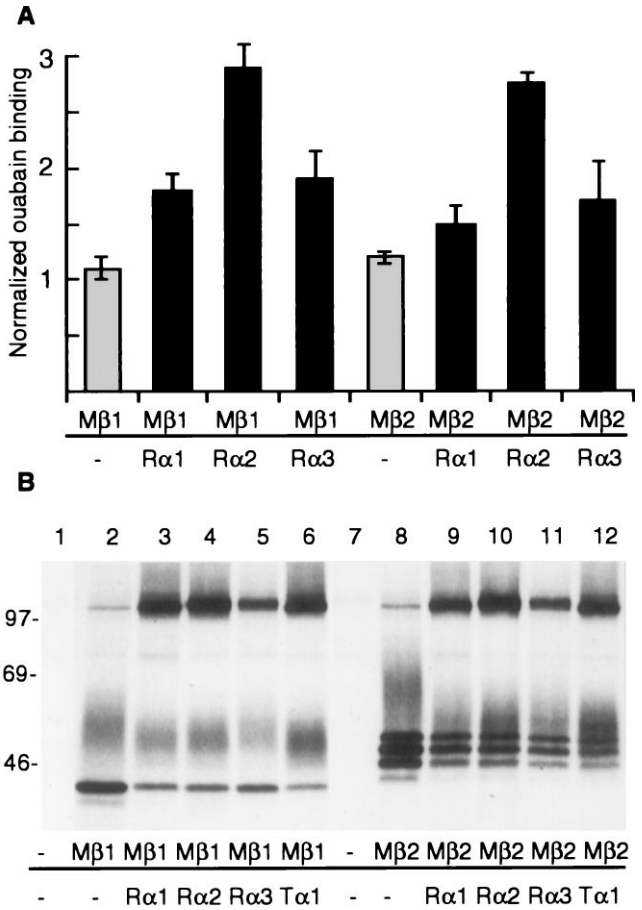

FIG. 1. All α isoforms are able to assemble with both β isoforms. (*A*) Oocytes injected with 4 ng of one of the indicated β cRNAs either alone (shaded bars) or together with 20 ng (Tα1, Rα3) or 40 ng (Rα1, Rα2) of one of the indicated α cRNAs (filled bars). After 3 days at 19°C, cells were assayed for the number of surface Na,K-pumps. The number of Na,K-pumps comprising the rat α1 subunit is significantly underestimated, because such Na,K-pumps exhibit low affinity for ouabain. Data on ouabain binding are given as means ± SE of at least 10 separate determinations in single oocytes of one experiment. The experiments have been repeated at least three times. Binding values are normalized to the value of noninjected control oocytes, which is set to the arbitrary unit 1 [$P < 0.01$ (with the exception of $\alpha1/\beta2$ and $\alpha3/\beta2$, $P < 0.05$)]. (*B*) Subsets of the same cRNA-injected oocytes were labeled with [^{35}S]methionine for 3 h, chased for 20 h, and then extracted with 1% digitonin. Polypeptides isolated from the detergent extracts by immunoprecipitation with mAb BSP/3 (lanes 1–6) or mAb 426 (lanes 7–12) were analyzed by SDS/PAGE and fluorography. Positions of relative molecular mass markers (in kDa) are indicated on the left margin. Mβ1 and Mβ2, mouse β1 and β2 subunit isoform, respectively; Tα1, *Torpedo* α1 subunit; Rα1, Rα2, and Rα3, rat α1, α2, and α3 subunits, respectively. Lanes 1 and 7, noninjected controls.

Torpedo α1 isoform together with the mouse β2 subunit. Likewise, each of the rat α isoforms and *Torpedo* α1 could be coprecipitated with the mouse β1 subunit (Fig. 1*B*). The faint 110-kDa band in immunoprecipitates from oocytes injected solely with one of the β subunit-specific cRNAs represents coprecipitated subunits synthesized from host α1 mRNA (26).

The previously shown disintegration of $\alpha1/\beta2$ complexes but not of $\alpha1/\beta1$ complexes in Triton X-100 (26) suggests that probing detergent resistance may be suitable to elucidate isoform-specific differences of α/β complexes. To probe the stability of complexes of β2 with each α isoform, we used digitonin and Triton X-100 as prototypes of mild and harsh detergents, respectively. $\alpha2/\beta2$ complexes, in contrast to $\alpha1/\beta2$ are stable in Triton X-100 (a small fraction of $\alpha3/\beta2$ complexes is also stable in Triton X-100; Fig. 2). This suggests that the subunits of $\alpha2/\beta2$ complexes behave similar as $\alpha1/\beta1$ complexes and are held together by stronger forces than the subunits in $\alpha1/\beta2$ and $\alpha3/\beta2$ complexes. All complexes are preserved in digitonin (Fig. 2). The *Torpedo* α1 subunit behaves similar to the rat α1 isoform.

Additional evidence for isoform-dependent differences in Na,K-pump subunit assembly comes from experiments in which N-glycosylation of β subunits was completely blocked by tunicamycin. First, surface appearance was studied on intact oocytes using [^{3}H]ouabain. Because of the high affinity for ouabain, *Torpedo* α1 subunits were studied instead of rat α1, which have a low affinity for ouabain and hence are difficult to titrate. *Torpedo* α1 shares 93% amino acid identity with the rat α1 subunit. Fig. 3*A* shows that tunicamycin had no effect on the cell surface expression of the $\alpha1/\beta1$ and $\alpha2/\beta2$ isozymes, but completely suppressed the appearance of $\alpha1/\beta2$ at the plasma membrane. Because N-glycans may play a role in the assembly with α1 subunits or in the routing of the holoenzyme through the cell, we examined whether $\alpha1/\beta2$ Na,K-pumps remain in internal membranes of tunicamycin-treated oocytes. Usually, about half of the Na,K-pumps synthesized in *Xenopus* oocytes reside in the cell interior (23, 27). Two days after cRNA injection, oocytes were permeabilized with detergents and incubated with [^{3}H]ouabain to estimate the total number of Na,K-pumps (surface plus internal). In the absence of tunicamycin, oocytes synthesizing mouse β2 subunits together with *Torpedo* α1 subunits had about three times more Na,K-pumps in their membranes than controls (Fig. 3*B*).

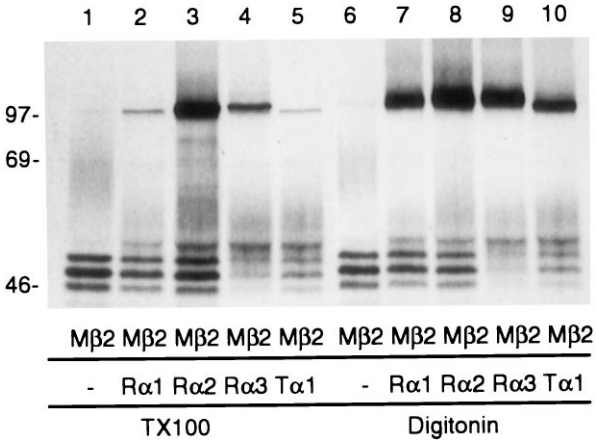

FIG. 2. Stability of α/β complexes in various detergents. Oocytes injected with the indicated cRNAs (Tα1, 20 ng of cRNA per cell; Rα1, Rα2, and Rα3, 40 ng of cRNA per cell; Mβ2, 4 ng of cRNA per cell) were [^{35}S]methionine-labeled for 3 h and chased for 20 h. Extracts of subsets of these cells were prepared using 0.5% Triton X-100 (lanes 1–5) or 1% digitonin (lanes 6–10) for extraction and mAb 426 for immunoprecipitation. Immunoprecipitates were analyzed by SDS/PAGE and fluorography. Positions of relative molecular mass markers (in kDa) are indicated on the left. Abbreviations are as in Fig. 1.

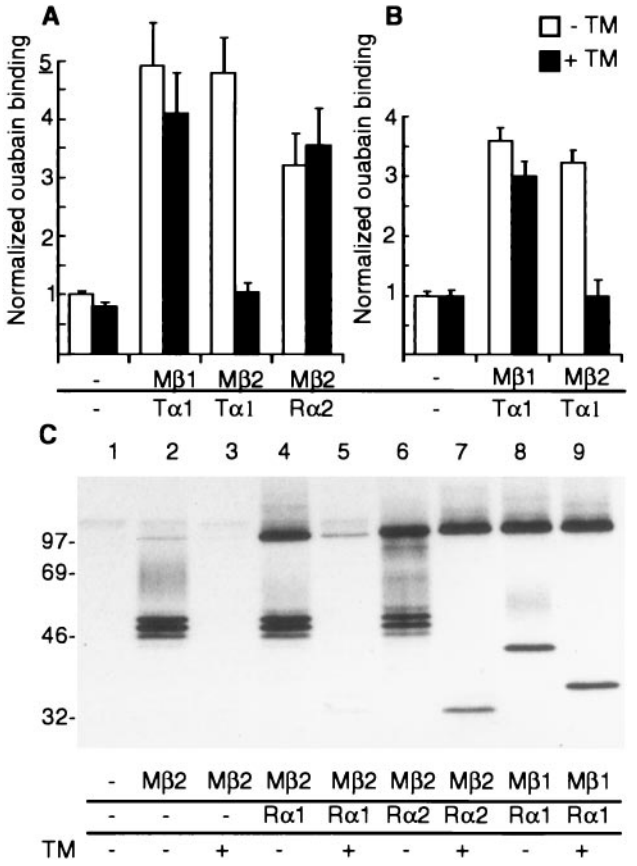

FIG. 3. β2 subunits need *N*-glycans for assembly with α1 subunits, but not with α2 subunits. Oocytes pretreated or not with tunicamycin for 16 h were coinjected with the indicated α and β cRNAs (Tα1, 20 ng of cRNA per cell; Rα2, 40 ng of cRNA per cell; Mβ1 and Mβ2, 4 ng of cRNA per cell). (*A*) Following 2 days of incubation in the absence or presence of 2 μg/ml of tunicamycin, cells were assayed for the surface number of ouabain binding sites. (*B*) In a separate experiment cells treated as in *A* were permeabilized with 1% digitonin and assayed for ouabain binding in the presence of 0.02% SDS to determine the total number of Na,K-pumps per cell (surface plus internal). Data were normalized to those of control oocytes of the same cell batch cultured and assayed under identical conditions. Open bars, without tunicamycin; solid bars, tunicamycin treated. Ouabain binding determination was done exactly as described in Fig. 1. Binding values are normalized to the value of noninjected control oocytes (in the absence of tunicamycin), which is set to the arbitrary unit 1 ($P < 0.01$). (*C*) Oocytes injected with the indicated cRNAs were labeled for 3 h with [^{35}S]methionine. After an additional 20-h chase, 1% digitonin extracts were prepared and subjected to immunoprecipitation with mAb 426 (lanes 1–7) or mAb BSP/3 (lanes 8 and 9). Immunoprecipitates were analyzed by SDS/PAGE and fluorography. Lane 1, noninjected controls. Abbreviations are as in Fig. 1.

When N-glycosylation was blocked, the total number of Na,K-pumps of the cRNA-injected oocytes was the same as in the noninjected controls, indicating that tunicamycin acts already at the level of subunit assembly.

Ouabain binding data are corroborated by immunoprecipitation experiments. The mouse β1 subunit is synthesized in oocytes as a 43-kDa polypeptide (Fig. 3*C*, lane 8), which carries three N-glycan side chains (23). In tunicamycin-treated oocytes the apparent molecular mass of synthesized mouse β1 subunits is reduced to 35 kDa (lane 9)—i.e., the molecular mass predicted by the cDNA sequence (21). The amount of coprecipitated α1 subunits was not reduced in the presence of tunicamycin (Fig. 3*C*, lanes 8 and 9). These results indicate that mouse β1 subunits do not need their N-glycans for assembly with α1 subunits or as a protection against rapid degradation.

Mouse β2 subunits are represented by three major polypeptides of 47, 50, and 53 kDa (Fig. 3*C* lanes 2, 4, and 6). Since the 3-kDa difference between the bands is of about the size expected for a single oligosaccharide side chain, we suppose that β2 subunits are synthesized in *Xenopus* oocytes with a variable number of N-glycans. When synthesized in the presence of tunicamycin β2 subunits had an apparent molecular weight of 33 kDa in SDS gels (Fig. 3*C*, lane 7) corresponding to the protein core (22). In contrast to nonglycosylated mouse β1 subunits, nonglycosylated β2 subunits synthesized alone or cosynthesized with α1 subunits were rapidly degraded and could hardly be detected after a 20-h chase (Fig. 3*C*, lanes 3 and 5). Only a small amount of α1 subunits could be coprecipitated from tunicamycin-treated oocytes (Fig. 3*C*, lane 5). Nonglycosylated β2 subunits behaved differently when synthesized together with rat α2 subunits. Nonglycosylated β2 subunits and coprecipitated α2 subunits could easily be detected in the oocytes (Fig. 3*C*, lane 7) in amounts similar to those observed in controls not treated with tunicamycin (lane 6). Thus, the absence of N-glycans does not grossly impair the efficiency of association with α2 subunits. Moreover, as a result of this association, nonglycosylated β2 subunits adopt a stable configuration. These results indicate that N-glycans of β2 subunits are absolutely required for the formation of the $\alpha1/\beta2$ isozyme, but unnecessary for the formation of the $\alpha1/\beta1$ and the $\alpha2/\beta2$ isozyme.

The intracellular N terminus and a part of the transmembrane region of the chicken β1 subunit are unessential for assembly with α1 subunits (14). We therefore examined whether soluble β1 or β2 subunit ectodomains combine with one of the α isoforms to form a detergent-resistant complex. *Xenopus* oocytes injected with appropriate cRNAs (24, 25) secreted the fully glycosylated form of the mouse β1 and β2 ectodomains into the medium (not shown). Oocytes were then injected with cRNA for the β1 ectodomain together with cRNA for the *Torpedo* α1 subunit or for one of the rat α isoforms. Both α1 and α2 subunits, but not α3 subunits were coprecipitated with the β1 ectodomain in digitonin extracts (Fig. 4*A*). In contrast, only α2 subunits but neither α1 nor α3 subunits coprecipitated with the β2 ectodomain (Fig. 4*B*). These results provide direct evidence for isoform-specific interactions between α and β subunits.

Because β ectodomains are sufficient for interaction with particular α isoforms, one or several ectodomain(s) on the α subunits must be involved in this type of assembly. To identify regions on the α subunit that are responsible for isoform-specific interactions, we constructed a cDNA for a chimeric $\alpha1\alpha2$ subunit consisting of the N-terminal 831 aa of the α1 isoform and the C-terminal 191 aa of the α2 isoform (Fig. 5*A*). As shown in Fig. 5*B* (lane 10), this chimera could be coprecipitated with cosynthesized β2 ectodomains and conferred Triton X-100 resistance to complexes formed with the wild-type β2 subunit (Fig. 5*C*, lanes 3 and 4). In contrast, the reciprocal chimera (Fig. 5*A*) did not assemble with the β2 ectodomain (Fig. 5*B*, lane 11) and conferred only minimal Triton X-100 resistance to complexes formed with the wild-type β2 subunit (Fig. 5*C*, lanes 1 and 2). These results suggest that amino acid residues responsible for isoform-specific $\alpha2/\beta2$ interactions probably reside on the ectodomains between the 7th and 8th and/or 9th and 10th transmembrane segment of the α2 subunit (based on the 10-transmembrane domain model). Because the predicted loop between transmembrane segment 9 and 10 is identical in α1 and α2, the specificity determining residues must reside in the 45- to 50-aa loop between domains 7 and 8 (Fig. 5). In support of an indiscriminate interaction of the β1 ectodomain with α1 and α2 subunits, both chimeras combined with the β1 ectodomain (Fig. 5*B*, lanes 2–6).

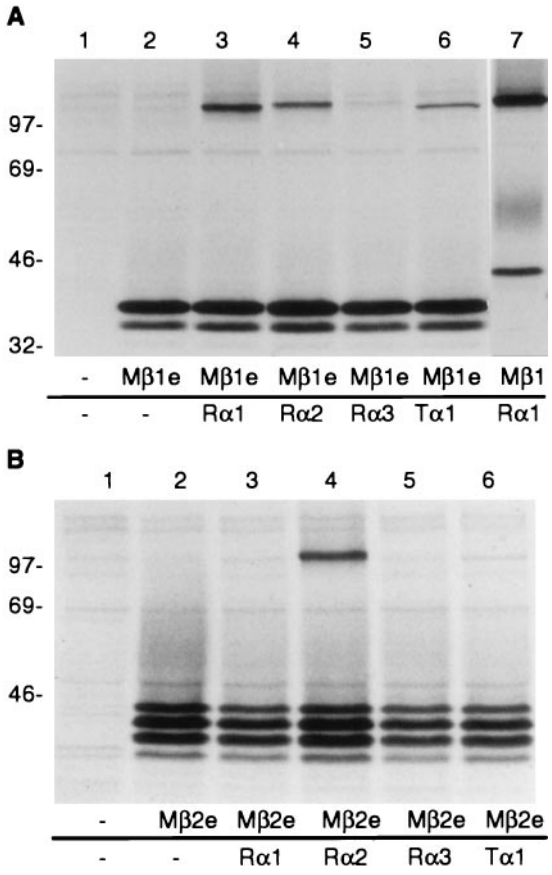

FIG. 4. Detergent-stable association of β ectodomains with α subunits. Oocytes were injected with 4 ng cRNA for the β1 or β2 ectodomain either alone or together with cRNA for one of the indicated α subunits (Tα1, 20 ng of cRNA per cell; Rα1, Rα2, and Rα3, 40 ng of cRNA per cell each). After a 3-h [^{35}S]methionine pulse and a 1-h chase, 1% digitonin extracts were prepared and subjected to immunoprecipitation using mAb BSP/3 or mAb 426 as appropriate. Isolated proteins were analyzed by SDS/PAGE and fluorography. The multiple bands represent (high-manose)-glycosylated forms of the ectodomains. Lane 1 in *A* and *B*, noninjected controls. (*A*) Selective precipitation of α1 and α2 subunits together with the β1 ectodomain. (*B*) Selective precipitation of α2 subunits together with the β2 ectodomain. Abbreviations are as in Fig. 1; Mβ1e, ectodomain of β1 subunit; Mβ2e, ectodomain of β2 subunit.

DISCUSSION

We have presented evidence for isoform-specific interactions between Na,K-pump α and β subunits. They are mainly mediated by extracellular amino acids of both proteins and, in the case of the assembly of β2 with α1, also involve N-glycans of β2. These results suggest that marked differences in the affinities of α isoforms for a particular β isoform exist. The observations differ from data demonstrating nonselective pairing of virtually any β isoform with any α isoform in birds (9). However, these authors did not examine detergent-specific effects or glycosylation.

The question of isoform-specific assembly is a central issue, since random combination of Na,K-pump subunits is difficult to reconcile with the proposed physiological significance of isozyme diversity (28–30). *In vivo*, formation of unwanted isozymes may be bypassed by temporal and spatial separation of the synthesis of distinct Na,K-pump isozymes. However, colocalization of three α isoforms in the same cell has been demonstrated in neurones and astrocytes on the mRNA (10) and protein level (11, 31, 32). It is unknown which α-β dimers are actually formed in these cells. From our observations we deduce that the formation of a particular Na,K-pump isozyme

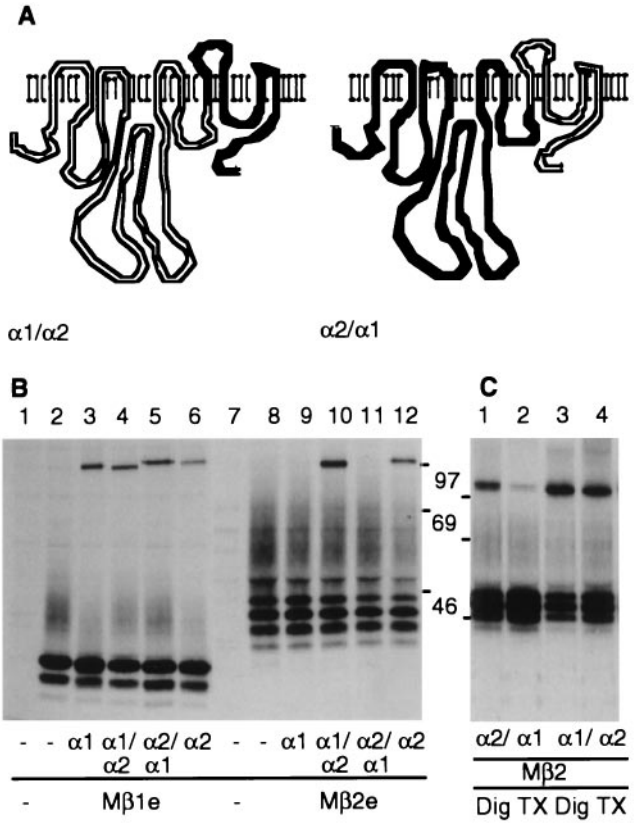

FIG. 5. The C terminus of the rat $\alpha 2$ isoform mediates isoform-specific and 0.5% Triton X-100-resistant assembly with the $\beta 2$ ectodomain. Oocytes were injected with 4 ng cRNA for the $\beta 1$ ectodomain or the $\beta 2$ ectodomain either alone or together with 20 ng cRNA for one of the indicated wild-type or chimeric α subunits. (*A*) Cartoon of the topology of the chimeric α subunits. Open lines, $\alpha 1$; shaded lines, $\alpha 2$. (*B*) Assembly with ectodomains. After a 3-h [^{35}S]methionine pulse and a 16-h chase, 1% digitonin extracts were prepared and subjected to immunoprecipitation using mAb BSP/3 or mAb 426 as appropriate. (*C*) Assembly with wild-type $\beta 2$. After a 3-h [^{35}S]methionine pulse and a 16-h chase, detergent extracts were prepared with 1% digitonin (Dig) or 0.5% Triton X-100 (TX) as indicated and supplemented with mAb 426. $\alpha 1$ and $\alpha 2$, rat $\alpha 1$ and $\alpha 2$ subunits, respectively; $\alpha 1/\alpha 2$ ($\alpha 2/\alpha 1$), chimera comprising the N-terminal 831 (828) of the rat $\alpha 1$ ($\alpha 2$) subunit and the remainder 191 aa of the rat $\alpha 2$ or $\alpha 1$ subunit, as illustrated in the model; Mβ1e, ectodomain of $\beta 1$ subunit, Mβ2e, ectodomain of $\beta 2$ subunit.

is governed by preferential formation of the most stable α/β isozymes—i.e., $\alpha 1/\beta 1$, $\alpha 2/\beta 2$, and presumably $\alpha 2/\beta 1$—perhaps in combination with regulated gene expression. The $\alpha 1/\beta 2$ isozyme may be formed only when there are no $\alpha 2$ subunits competing for assembly with $\beta 2$ subunits as is the case when $\alpha 1$ and $\beta 2$ are expressed in *Xenopus* oocytes (26). The finding that immunoaffinity-purified native $\beta 2$ subunits from mouse brain are primarily associated with $\alpha 2$ but not at all with $\alpha 1$ and barely with $\alpha 3$ (22) is compatible with this view.

A preference for its own β subunit has been observed for the H,K-pump α subunit (18). When expressed in epithelial cells that synthesize endogenous Na,K-pump $\beta 1$ subunits, no stable hybrids composed of the exogenous H,K-pump α subunit and the Na,K-pump $\beta 1$ subunit of the host were seen (18). The selectivity of subunit assembly in epithelial cells was attributed to a preferential assembly of H,K-pump α subunits with cosynthesized H,K-pump β subunits. In a converse situation, however, the H,K-pump β subunit combined with the Na,K-pump α subunit to form functional hybrid dimers in *Xenopus* oocytes and yeast (13, 33, 34). Because both fully grown *Xenopus* oocytes and yeast synthesize little or no endogenous Na,K-pump β subunits, Na,K-pump α subunits are perhaps free for less-specific interactions with the H,K-pump β subunits.

The data presented in Fig. 3 provide evidence that β subunit glycosylation can play an important role during subunit assembly. Na,K-pumps are not formed from nonglycosylated $\beta 2$ subunits and cosynthesized $\alpha 1$ subunits. This result may be attributed to a possibly rapid degradation of nonglycosylated $\beta 2$ subunits, preventing the accumulation of nonglycosylated $\beta 2$ subunits to concentrations sufficient for pairing with $\alpha 1$ subunits. However, nonglycosylated $\beta 2$ subunits efficiently combine with cosynthesized $\alpha 2$ subunits, suggesting that N-glycan-driven conformational maturation of $\beta 2$ subunits is perhaps required for the induction of a conformation capable of combining with $\alpha 1$ subunits. It remains to be shown, whether $\beta 2$ carbohydrates directly participate in interactions with $\alpha 1$. In contrast, the more stable $\alpha 2/\beta 2$ interaction occurs independently of N-glycan-driven conformational maturation, indicating that the interaction with $\alpha 2$ is sufficient to direct nonglycosylated $\beta 2$ subunits in an assembly-competent conformation. Such a conformational change of nonglycosylated $\beta 2$ subunits by the assembly reaction itself is indicated by the observation that binding to $\alpha 2$ subunits confers metabolic stability to the nonglycosylated $\beta 2$ subunits (data not shown).

The involvement of N-glycans and the fact that a soluble β subunit ectodomain assembles with α provide the most direct evidence for mainly extracellular interactions of both subunits, but participation of the transmembrane and/or intracellular domain cannot be strictly excluded (see Fig. 5). Therefore, the knowledge of the α subunit membrane topology is of central importance to further characterize the interacting sites. The α subunit has been proposed to have either 8 (35) or 10 transmembrane domains (36). All models agree in proposing four transmembrane segments in the N-terminal third and a large cytoplasmic loop in the central portion of the molecule. Both the N- and C-terminal segments are cytoplasmic (37–39). The number of transmembrane segments in the C-terminal half of the molecule remained controversial until recently. Epitope mapping experiments were consistent with an 8-transmembrane model (40). However, alternative approaches provide good evidence for a model with 10 transmembrane segments (39, 40–43). The next paragraph is based on a structural model with 10 transmembrane segments.

The rat $\alpha 1$, $\alpha 2$, and $\alpha 3$ isoforms comprise 1023, 1020, and 1013 aa, respectively, and share $\approx$85% sequence identity (35, 44). The C-terminal portions are highly conserved. Only 12 residues among the $\alpha 2$ C-terminal 196 aa differ from both $\alpha 1$ and $\alpha 3$. Five of these $\alpha 2$-specific residues map to the fourth extracellular loop comprising about 49 aa (Glu-872–Phe-920), suggesting that these residues could contribute to specificity. This is also supported by experiments using chimeras between the sodium and calcium ATPase (9) or using the gastric H,K-ATPase (45). However, two intracellular $\alpha 2$-specific residues, (Ser-836, Gln-837), located just in front of the seventh transmembrane segment, could also play a role, for example, by modulating the conformation of the extracellular loop. The fifth $\alpha 2$ ectodomain (Leu-983–Tyr-1001) may also be involved in association, but cannot be responsible for $\alpha 2/\beta 2$-specific pairing since the amino acids of this region are identical among the three rat α isoforms. Thus, to account for isoform-specific differences in assembly, probably as little as 7 aa unique to the $\alpha 2$ subunit determine the contact strength between subunit isoforms directly and/or indirectly in the case of assembly of $\beta 2$ with $\alpha 2$ and $\beta 1$ with $\alpha 1$ or $\alpha 2$, respectively. For the interaction with $\alpha 3$ more interacting sites appear to be needed, suggesting that both specific amino acid residues and conformational changes direct isoform-specific assembly.

Do these findings have physiological implications? For example, brain microvessels express all six transcripts (10) and $\alpha 1$, $\beta 1$, and $\beta 2$ proteins are detected in the choroid plexus (10, 46). These interface structures play important roles in con-

trolling the molecular composition of plasma and cerebrospinal fluid, respectively. The nervous system depends on a such a tight control and a role of the Na,K-ATPase in potassium homeostsis appears likely. Hence the possibility to preferentially assemble different ATPase isozymes, perhaps combined with delivery to defined cellular domains, would allow a cell to respond to different demands. In support of this, ATPase isozymes display slightly different enzymatic properties (26, 47, 48). and compartmentalization has been shown in endothelial cells (49) and polarized epithelial cells (50).

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