

# $\beta$ m, a Structural Member of the X,K-ATPase $\beta$ Subunit Family, Resides in the ER and Does Not Associate with Any Known X,K-ATPase $\alpha$ Subunit<sup>†</sup>

Gilles Crambert,<sup>‡</sup> Pascal Béguin,<sup>‡</sup> Nikolay B. Pestov,<sup>§,||</sup> Nikolai N. Modyanov,<sup>||</sup> and Käthi Geering<sup>\*,‡</sup>

*Institute of Pharmacology and Toxicology, University of Lausanne, rue du Bugnon 27, CH-1005 Lausanne, Switzerland, Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, Moscow 117871, Russia, and Department of Pharmacology, Medical College of Ohio, Toledo, Ohio 43699-0008*

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**ABSTRACT:**  $\beta$ m, a muscle-specific protein, is structurally closely related to the X,K-ATPase  $\beta$  subunits, but its intrinsic function is not known. In this study, we have expressed  $\beta$ m in *Xenopus* oocytes and have investigated its biosynthesis and processing as well as its putative role as a chaperone of X,K-ATPase  $\alpha$  subunits, as a regulator of sarcoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase (SERCA), or as a  $\text{Ca}^{2+}$ -sensing protein. Our results show that  $\beta$ m is stably expressed in the endoplasmic reticulum (ER) in its core glycosylated, partially trimmed form. Both full-length  $\beta$ m, initiated at Met<sup>1</sup>, and short  $\beta$ m species, initiated at Met<sup>89</sup>, are detected in in vitro translations as well as in *Xenopus* oocytes.  $\beta$ m cannot associate with and stabilize Na,K-ATPase (NK), or gastric and nongastric H,K-ATPase (HK)  $\alpha$  isoforms.  $\beta$ m neither assembles stably with SERCA nor is its trypsin sensitivity or electrophoretic mobility influenced by  $\text{Ca}^{2+}$ . A mutant, in which the distinctive Glu-rich regions in the  $\beta$ m N-terminus are deleted, remains stably expressed in the ER and can associate with, but not stabilize X,K-ATPase  $\alpha$  subunits. On the other hand, a chimera in which the ectodomain of  $\beta$ m is replaced with that of  $\beta$ 1 NK associates efficiently with  $\alpha$  NK isoforms and produces functional Na,K-pumps at the plasma membrane. In conclusion, our results indicate that  $\beta$ m exhibits a cellular location and functional role clearly distinct from the typical X,K-ATPase  $\beta$  subunits.

The P-type ATPases represent a highly diverse protein family which are responsible for cation homeostasis of various cellular compartments. P-type ATPases can be divided into three different groups according to the ions transported and their structural similarity (1). The P2-ATPase subgroup is characterized by a catalytic  $\alpha$  subunit which has 10 transmembrane segments and comprises the  $\text{H}^{+}$ -ATPases, the  $\text{Ca}^{2+}$ -ATPases, and the X,K-ATPases. These latter ATPases are located in the plasma membrane and exchange external  $\text{K}^{+}$  for internal  $\text{H}^{+}$  and/or  $\text{Na}^{+}$  and are represented by various isozymes of the Na,K-ATPase and the gastric and nongastric H,K-ATPases. Significantly, among all P-type ATPases, only X,K-ATPases contain, in addition to the catalytic  $\alpha$  subunit, a  $\beta$  subunit which is indispensable for the structural and functional maturation of the  $\alpha$  subunit (for review, see 2, 3). Indeed, in contrast to other P-type ATPases, the catalytic  $\alpha$  subunits of X,K-ATPases are not able by themselves to adopt a correct membrane topology. C-terminal membrane domains contain specific sequence information that impedes efficient insertion into the lipid bilayer which

in consequence leads to exposure of degradation signals that mediate  $\alpha$  subunit degradation by the cytosolic proteasome (4, 5). Interaction of the  $\beta$  subunit with the extracytoplasmic loop between M7 and M8 of the nascent  $\alpha$  subunit favors membrane insertion of its C-terminal membrane domains and its ultimate, correct packing (5). The stable, functionally active  $\alpha/\beta$  complexes are then able to leave the endoplasmic reticulum (ER)<sup>1</sup> and are routed to the plasma membrane.

So far, four members of the  $\beta$  subunit protein family have been characterized. Three  $\beta$  isoforms can be attributed to Na,K-ATPase ( $\beta$ 1 NK,  $\beta$ 2 NK,  $\beta$ 3 NK) and one to gastric H,K-ATPase ( $\beta$  HK). Significantly, for the nongastric H,K-ATPases, no specific  $\beta$  subunit has been identified. X,K-ATPase  $\beta$  isoforms exhibit only about 20–30% overall sequence identity, but they share several structural features. They are type II glycoproteins which contain, in the ectodomain, three consecutive disulfide bonds and a highly conserved Y-Y/F-P-Y-Y-G-K sequence which can be considered as signature motifs of the  $\beta$  subunit family. As X,K-ATPase  $\alpha$  isoforms, X,K-ATPase  $\beta$  isoforms exhibit a tissue-specific distribution (for review, see 6) with  $\beta$ 1 NK being expressed ubiquitously,  $\beta$ 2 NK mainly in the heart, skeletal muscles, and glial cells,  $\beta$ 3 NK in many tissues, and  $\beta$  HK mainly in the stomach. So far, little is known about the determinants that govern the specificity of X,K-ATPase  $\alpha$ – $\beta$  interactions in cells expressing multiple  $\alpha$  and  $\beta$  isoforms. In heterologous expression systems, all  $\beta$  NK isoforms can

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<sup>\*</sup> To whom correspondence should be addressed at the Institute of Pharmacology and Toxicology, rue du Bugnon 27, CH-1005 Lausanne, Switzerland. Tel: 41 21 692 54 10, Fax: 41 21 692 53 55, E-mail: kaethi.geering@ipharm.unil.ch.

<sup>‡</sup> University of Lausanne.

<sup>§</sup> Russian Academy of Sciences.

<sup>||</sup> Medical College of Ohio.

<sup>1</sup> Abbreviations: SERCA, sarcoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase; ER, endoplasmic reticulum; NK, Na,K-ATPase; HK, H,K-ATPase;  $\beta$ m,  $\beta$ muscle; Endo H, endoglycosidase H.

associate with all  $\alpha$  NK isoforms and produce functionally active  $\alpha/\beta$  complexes with slightly different transport and pharmacological properties (6–8). Moreover,  $\alpha$  NK can produce stable complexes with  $\beta$  HK which are, however, partially inactive (9–11). On the other hand,  $\alpha$  HK does not associate with  $\beta$ 1 NK (12, 13). In addition to discriminating structural properties of  $\alpha$  and  $\beta$  subunits, it is possible that tissue-specific factors prevent promiscuous association of different X,K-ATPase  $\alpha$  and  $\beta$  isoforms in situ.

Recently, a novel member of the  $\beta$  subunit family has been identified which shares common structural features and signature motifs with X,K-ATPase  $\beta$  isoforms (14). The overall sequence identity with X,K-ATPase  $\beta$  subunits is 30–40%. The new protein, termed  $\beta$ muscle ( $\beta$ m), is predominantly expressed in skeletal muscles where it is developmentally regulated (14–16). Despite the similarities with X,K-ATPase  $\beta$  isoforms,  $\beta$ m has also some atypical characteristics. First, it contains two long glutamate-rich regions in the cytoplasmic N-terminus which are not present in X,K-ATPase  $\beta$  subunits (15). Second, based on results of cell fractionation experiments (16) and on its state of glycosylation (15),  $\beta$ m appears to be concentrated in the sarcoplasmic reticulum, in contrast to X,K-ATPase  $\beta$  subunits which are predominantly found at the plasma membrane. Finally, a specific feature of  $\beta$ m is the existence of splice variants lacking four amino acids adjacent to the start of the transmembrane domain (14).

The unique characteristics of  $\beta$ m, compared to other members of the  $\beta$  subunit family, raise the question whether  $\beta$ m is also a X,K-ATPase  $\beta$  subunit that interacts and regulates the expression of one of the X,K-ATPase in a tissue-specific manner. In this study, we used the *Xenopus* oocyte, a well-defined expression system for X,K-ATPases, to further characterize the biosynthesis and processing of  $\beta$ m and to investigate its putative functional role. Our results reveal that  $\beta$ m is an ER-resident protein which does not act as a chaperone for the maturation of any of the known X,K-ATPase  $\alpha$  subunits and thus represents a protein functionally distinct from other members of the  $\beta$  subunit family.

## MATERIALS AND METHODS

**cDNA Constructs and Mutagenesis.** Cloning and insertion of human Na,K-ATPase  $\alpha$ 1,  $\alpha$ 2,  $\alpha$ 3 ( $\alpha$ 1 NK,  $\alpha$ 2 NK,  $\alpha$ 3 NK), and  $\beta$ 1 cDNAs into pSD5 or pNKS2 (kindly provided by G. Schmalzing) vectors have been described previously (8). Human full-length  $\beta$ m (14) was inserted into the pSD5 vector between *Nco*I and *Sma*I restriction sites. ATP1a1 (the human nongastric H,K-ATPase  $\alpha$  subunit, AL1) (17) and the rabbit gastric H,K-ATPase  $\alpha$  subunit ( $\alpha$  HK, kindly provided by G. Sachs) have been introduced into pSD3 and pSD5 vectors as previously reported (18). cDNAs coding for the human sarcoplasmic  $\text{Ca}^{2+}$ -ATPases 2a and 2b (SERCA 2a and SERCA 2b, kindly provided by D. H. McLennan) were inserted into the pSD5 vector between *Sac*I and *Not*I restriction sites. Mouse calmodulin was obtained by PCR using a brain library and was subcloned into the pSD5 vector.

The  $\beta$ 1 $\beta$ m chimera (Figure 1) consisting of the human  $\beta$ 1 N-terminus (Met<sup>1</sup>–Lys<sup>34</sup>) and the  $\beta$ m transmembrane and ectodomain regions (Leu<sup>111</sup>–Thr<sup>357</sup>) was produced by introducing a *Bam*HI restriction site into the pSD5/ $\beta$ 1 vector at

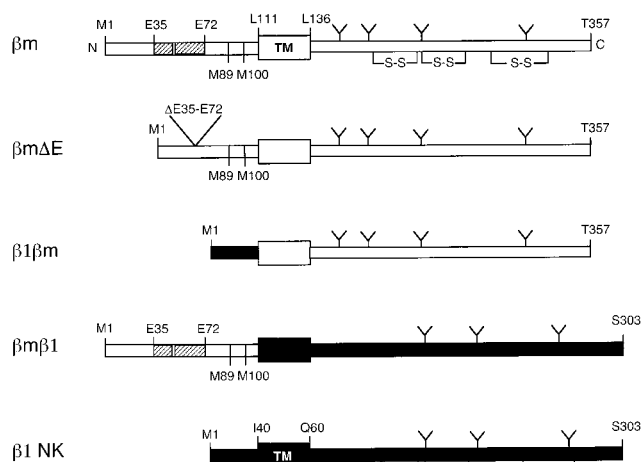


FIGURE 1: Linear models of human, wt  $\beta$ m (white), wt  $\beta$ 1 NK (black) and mutants. Indicated are the positions of the three methionines in the N-terminus of human  $\beta$ m (M1, M89, M100), the glutamine-rich regions in  $\beta$ m (hatched), and the transmembrane domain (TM) in  $\beta$ m and  $\beta$ 1 NK. The positions of sugar chains (Y) and of disulfide bonds (S–S) in the ectodomain are shown.  $\beta$ m $\Delta$ E =  $\beta$ m lacking the glutamine-rich regions (E35–E72).

nucleotide 101 and a *Bgl*II site into the pSD5/ $\beta$ m vector at nucleotide 321. A *Bgl*II fragment containing the coding sequence for the  $\beta$ m transmembrane and ectodomain regions was introduced into the pSD5/ $\beta$ 1 vector previously digested with *Bam*HI and *Bgl*II. For the construction of the reverse chimera,  $\beta$ m $\beta$ 1 (Figure 1), consisting of the  $\beta$ m N-terminal domain (Met<sup>1</sup>–Leu<sup>111</sup>) and the transmembrane and ectodomain regions of  $\beta$ 1 (Ile<sup>36</sup>–Ser<sup>303</sup>), a fragment encoding all 111 amino acid residues of the  $\beta$ m N-terminus was amplified using a sense oligonucleotide encoding part of the pSD5 vector, and an antisense oligonucleotide coding for amino acids Thr<sup>105</sup>–Leu<sup>111</sup> of  $\beta$ m. Another fragment encompassing the transmembrane and ectodomain regions of  $\beta$ 1 was amplified using a sense oligonucleotide coding for amino acids Ile<sup>35</sup>–Ile<sup>42</sup> of  $\beta$ 1 tailed with nucleotides coding for amino acids Thr<sup>105</sup>–Leu<sup>111</sup> of  $\beta$ m, and an antisense oligonucleotide coding for the Asn<sup>158</sup>–Asn<sup>163</sup> region. The two amplified fragments were then joined by recombinant PCR, and the final fragment obtained was subcloned into the pSD5/ $\beta$ 1 vector between *Nhe*I and *Hind*III restriction sites. The  $\beta$ m mutant lacking the two glutamate-rich regions ( $\beta$ m $\Delta$ E, Figure 1) was constructed from two PCR-amplified fragments, one encoding the  $\beta$ m region from Met<sup>1</sup> to Ala<sup>33</sup> and the other encoding the region from Glu<sup>72</sup> to Asn<sup>160</sup> and containing a 5' tail coding for Glu<sup>26</sup>–Ala<sup>33</sup> of  $\beta$ m. The two amplified fragments were joined by recombinant PCR, and the final fragment obtained was subcloned into the pSD5/ $\beta$ m vector between *Nhe*I and *Bam*HI restriction sites.

The  $\beta$ m M89A mutant was prepared by first PCR-amplifying a fragment of  $\beta$ m using an antisense oligonucleotide consisting of nucleotides 538–553 tailed by primer D of Nelson and Long (19) and a sense oligonucleotide containing the mutated sequence. The mutated fragment was used as a primer to elongate the inverse DNA strand and was amplified using a sense oligonucleotide encoding part of the pSD5 vector and primer D of Nelson and Long (19). The mutated fragment was introduced into the pSD5 vector between *Nhe*I and *Bam*HI restriction sites. The cDNA of the  $\beta$ m M89A mutant served as a template for the preparation

of the double M89A/M100A mutant which was introduced into the pSD5  $\beta$ m M89A vector between *Bam*HI and *Sma*I restriction sites.

**Test of  $\alpha/\beta$  Association.** Oocytes were obtained from *Xenopus* females (Noerhoeck, South Africa) as described (20). Routinely, oocytes were injected with 10 ng of  $\alpha$  and 1–1.5 ng of  $\beta$  cRNA. To investigate the ability of wt  $\beta$ m, mutant  $\beta$ m, and  $\beta$ m/ $\beta$ 1 chimeras to associate with Na,K- or H,K-ATPase  $\alpha$  subunits or SERCA, oocytes were incubated in a modified Barth's medium containing 0.7 mCi/mL  $^{35}$ S Easytag expressed protein mix (New England Nuclear) for 24 h and then subjected to a chase period of 48 h in the presence of 10 mM cold methionine. Microsomal fractions were prepared as previously described (20). Aliquots of oocyte microsomes were directly loaded onto SDS gels, or  $\alpha/\beta$  complexes or individual subunits were immunoprecipitated under nondenaturing conditions (0.5% digitonin), which preserves  $\alpha/\beta$  interactions, or under denaturing conditions after heating samples 7 min at 56 °C ( $\alpha$  subunits) or 5 min at 95 °C ( $\beta$ m) in the presence of 3% SDS. In some instances, nonimmunoprecipitated or immunoprecipitated proteins were treated with endoglycosidase H (Endo H) as described (21). The three human  $\alpha$  isoforms were immunoprecipitated with a rabbit polyclonal Bufo  $\alpha$ 1 antibody (22). AL1 was immunoprecipitated with a rabbit polyclonal antibody against a specific N-terminal region (23). The rabbit gastric H,K-ATPase antibody was kindly provided by D. Clays (24). The human  $\beta$ m was immunoprecipitated with a polyclonal rabbit antibody against a recombinant protein lacking the transmembrane domain (15). Commercially available mouse IgG2a antibodies (Affinity Bioreagents) were used to immunoprecipitate SERCA 2a and 2b. The dissociated immune complexes were resolved by SDS–PAGE, and labeled proteins were detected by fluorography.

**Controlled Proteolysis.** Microsomes were prepared in the absence of protease inhibitors as described (20) from metabolically labeled oocytes expressing  $\beta$ m,  $\beta$ 1 NK,  $\beta$  HK, or SERCA 2a. Microsomes were resuspended in 10 mM Tris-HCl (pH 7.4) and subjected to two freeze/thaw cycles in liquid nitrogen. Aliquots (12  $\mu$ g) were preincubated for 30 min at 25 °C in the presence of either 10 mM Tris-HCl, 1 mM CaCl<sub>2</sub> or 0.5 mM EGTA. The osmolarity was adjusted to 60 mM with choline chloride. Trypsin (Type XI, Sigma) was added at a trypsin-to-protein ratio of 0.001 or 0.05, and the samples were incubated for 1 h at 25 °C. Trypsin digestion was stopped by the addition of a 5-fold excess (w/w) of soybean trypsin inhibitor (Sigma), and left at 25 °C for 10 min before addition of SDS, immunoprecipitation with protein-specific antibodies (see above), gel electrophoresis, and revelation of the proteins by fluorography.

**In Vitro Translation and Proteinase K Digestion.** In vitro translations in rabbit reticulocyte lysates of wt, mutant, or chimeric  $\beta$ m/ $\beta$ 1 cRNAs (60 ng/reaction) were performed in the presence or absence of canine pancreatic microsomal membranes according to the manufacturer's instructions (Promega). The [ $^{35}$ S]methionine-labeled proteins were treated or not treated with Endo H (see above) or proteinase K (Sigma) and directly subjected to SDS–PAGE. For proteinase K treatment, microsomes were diluted 40 times in a buffer containing 250 mM sucrose, 50 mM K<sup>+</sup>-acetate, 5 mM MgCl<sub>2</sub>, 10 mM dithiothreitol, 10 mM CaCl<sub>2</sub>, 50 mM Tris-HCl, pH 7.4, in the presence or absence of 1% Triton

X-100. Proteinase K (200  $\mu$ g/mL) was added, and the samples were incubated for 1 h at 4 °C. The reaction was stopped by addition of phenylmethylsulfonyl fluoride (10 mM), and the samples were left on ice for 15 min before addition of sample buffer.

**Western Blot Analysis of  $\beta$ m from *Xenopus* Oocyte and Piglet Skeletal Muscle Microsomes.** Microsomes were prepared from *Xenopus* oocytes expressing  $\beta$ m as previously described (20) and were resuspended in a solution containing 30 mM DL-histidine, 5 mM EDTA, 18 mM Tris, pH 7.4. Microsomes from piglet skeletal muscle were obtained with the approval of Animal Care and Use Committee of the Medical College of Ohio. Male piglets were supplied within 24 h after parturition and were euthanized by intramuscular injection of 4–6 mg/kg of Telazol followed by intraperitoneal pentobarbital (100 mg/kg). Skeletal muscle was collected and either used immediately or frozen at –80 °C. Upon use, tissue samples were homogenized with a blender in cold buffer A [250 mM sucrose, 20 mM Na-HEPES (pH 7.2), 5 mM EDTA, 5 mM benzamidine, 200 mM PMSF, and protease inhibitor cocktail (Sigma) containing 0.5 M KCl]. The homogenate was centrifuged at 6000g at 4 °C for 20 min, the supernatant was collected, and the microsomal fraction was pelleted at 100000g at 4 °C for 1 h. The pellet was washed with buffer A, centrifuged, and stored at –80 °C.

Two hundred micrograms of protein from piglet muscle and 50  $\mu$ g of protein from oocyte microsomes were treated or not with Endo H after denaturing proteins with 3.7% SDS and precipitation with acetone for 2 h at –20 °C. After overnight incubation, samples were reprecipitated with acetone and taken up in sample buffer before loading on a 5–13% SDS–polyacrylamide gel. After migration, proteins were transferred onto a nitrocellulose membrane. The membrane was blocked with a solution containing 0.1% Tween 20 and 3% lowfat milk overnight at 4 °C and then incubated with an anti- $\beta$ m antibody diluted 1/1000 in the above-mentioned solution for 2 h at room temperature (15). After thorough washing of the membrane with a solution containing 0.1% Tween 20, proteins were revealed by a peroxidase-conjugated anti-rabbit antibody (Amersham) and ECL technology (Pharmacia).

**Ca<sup>2+</sup>-Binding Assay.** Aliquots of microsomes of metabolically labeled oocytes expressing  $\beta$ m,  $\beta$ 1 $\beta$ m, calmodulin, SERCA 2a, or  $\alpha$ 1 NK were loaded on SDS gels or, in the case of calmodulin, on SDS–Tricine gels (prepared according to the manufacturer's instructions) containing either 1 mM CaCl<sub>2</sub> or 0.1 mM EGTA. Samples containing  $\beta$ m or  $\beta$ 1 $\beta$ m were treated with Endo H before gel migration.

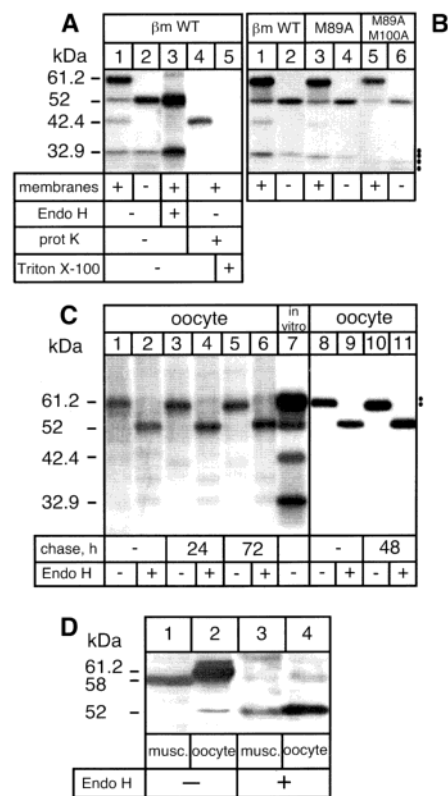
**Pump Current Measurements.** Na,K-pump currents were measured as the K<sup>+</sup>-induced outward current using the two-electrode voltage-clamp technique as described (25). Briefly, current measurements were performed 3 days after injection of oocytes with human  $\alpha$ 1 NK cRNAs together with  $\beta$ 1 NK,  $\beta$ m,  $\beta$ m $\Delta$ E,  $\beta$ m $\beta$ 1, or  $\beta$ 1 $\beta$ m. To determine the maximal K<sup>+</sup>-induced current, injected and noninjected oocytes were loaded with Na<sup>+</sup> by incubation in a K<sup>+</sup>-free solution 1 day before measurements. The current induced by addition of 10 mM K<sup>+</sup> was recorded at –50 mV in a Na<sup>+</sup>-containing solution [80 mM sodium gluconate, 0.82 mM MgCl<sub>2</sub>, 0.41 mM CaCl<sub>2</sub>, 5 mM BaCl<sub>2</sub>, 10 mM tetraethylammonium chloride, 10 mM N-methyl-D-glucamine (NMDG)–HEPES,

pH 7.4]. To determine the kinetics of  $K^+$ -activation of Na,K-pumps, the pump current was measured in the presence of 0.3, 1, 3, and 10 mM  $K^+$ . The Hill equation,  $I_K = I_{max}/[1 + (K_{1/2}^{K^+}/[K])^{n_H}]$ , was fitted to the current ( $I_K$ ) induced at different  $K^+$  concentrations ( $[K]$ ) to yield least-squares estimates of the maximal current ( $I_{max}$ ) and of the half-activation constant for  $K^+$  ( $K_{1/2}^{K^+}$ ). A Hill coefficient ( $n_H$ ) of 1.6 was used as previously described (25). The  $K^+$  activation of pump currents mediated by Na,K-pumps expressed in oocytes was determined after subtracting the currents due to endogenous Na,K-pumps, i.e., the mean currents measured under the same conditions in noninjected oocytes.

## RESULTS

**Biosynthesis and Processing of  $\beta m$  Proteins Expressed in Vitro and in Vivo.** By using a specific  $\beta m$  antibody, Western blot analysis previously revealed a  $\beta m$  species in human skeletal muscle which, after deglycosylation with endoglycosidase H (Endo H), exhibited an electrophoretic mobility corresponding to a molecular mass of 52 kDa that was much higher than that predicted from the cDNAs of the two identified  $\beta m$  variants (41.6 and 41 kDa) [Pestov et al. (15)]. The atypical electrophoretic mobility of  $\beta m$  may be due to the presence, in the N-terminus, of the Glu-rich regions that may interact abnormally with SDS, or to co-translational modifications other than N-linked glycosylation. To distinguish between these two possibilities, we synthesized wt or mutant  $\beta m$  in an in vitro translation system in the absence of ER microsomes, which prevents most co-translational processing, or in the presence of microsomes, which allows for co-translational modifications such as Endo H-sensitive core N-glycosylation. In the presence of microsomes, in vitro translation of  $\beta m$  cRNA led to the production of a major  $\beta m$  species with a molecular mass of 61.2 kDa and three minor species of 52, 42.4, and 32.9 kDa, respectively (Figure 2, lane 1). Both the 61.2 and the 42.4 kDa species represent N-glycosylated forms of  $\beta m$  since they disappeared after Endo H treatment (lane 3) and were missing after translations in the absence of microsomes (lane 2). Moreover, our results show that the 52 and 32.9 kDa species are the nonglycosylated forms of the 61.2 and 42.4 kDa  $\beta m$  species, respectively, since they correspond to the species produced after Endo H treatment (lane 3) or after translation in the absence of microsomes (lane 2). Thus, these results confirm that  $\beta m$  is N-glycosylated, consistent with its four putative consensus glycosylation sites (Figure 1), and that the 61.2 kDa  $\beta m$  species migrates abnormally on SDS gels independent of N-glycosylation.

The presence of two  $\beta m$  species in the absence of microsomes suggested either the presence of two methionines which can act as initiators of translation or a partial co-translational cleavage of  $\beta m$ , most likely of its N-terminus. To distinguish between these two possibilities, we produced  $\beta m$  mutants lacking the additional methionines (Met<sup>89</sup> and Met<sup>100</sup>), present in the  $\beta m$  N-terminus, of which only Met<sup>89</sup> is encoded by a consensus Kozak sequence. The M89A mutant synthesized in vitro in the absence or presence of microsomes still produced two nonglycosylated  $\beta m$  species (Figure 2B, lanes 3 and 4), but the lower molecular mass species migrated with the lower band of the doublet observed in wt  $\beta m$  around 33 kDa (lanes 1 and 2). In



**FIGURE 2:** Expression of  $\beta m$  in vitro and in *Xenopus* oocytes. (A)  $\beta m$  cRNA was translated in a reticulocyte lysate in the absence (lane 2) or presence (lanes 1, 3, 4, and 5) of microsomes. The [<sup>35</sup>S]-methionine-labeled proteins were treated or not with Endo H or proteinase K (prot K)  $\pm$  Triton X-100 as described under Materials and Methods and subjected to SDS-PAGE. Indicated are the molecular masses of the nonglycosylated and core glycosylated species of  $\beta m$ . (B) In vitro translation of wt and mutant  $\beta m$ . Indicated are the molecular masses of nonglycosylated and core glycosylated  $\beta m$  species. Dots represent the nonglycosylated  $\beta m$  species that are initiated on methionines other than Met<sup>1</sup>. (C)  $\beta m$  was expressed in *Xenopus* oocytes by cRNA injection (lanes 1–6, lanes 8–11), metabolically labeled during a 24 h pulse period, and subjected to a 24 and a 72 h chase period. Oocyte microsomes were prepared after the pulse and chase periods, and  $\beta m$  was directly loaded on gels (lanes 1–6), or first immunoprecipitated with a  $\beta m$  antibody (lanes 8–11). Nonimmunoprecipitated and immunoprecipitated samples were treated or not with Endo H. Indicated are the molecular masses of the nonglycosylated and core glycosylated  $\beta m$  species synthesized in *Xenopus* oocytes. For comparison, the pattern of  $\beta m$  species translated in a reticulocyte lysate is shown in lane 7. Dots represent the core glycosylated untrimmed and the partially trimmed  $\beta m$  species observed during the pulse (lane 8) and the chase (lane 10) period, respectively. One of two similar experiments is shown. (D) Comparison between  $\beta m$  expressed in muscle or in *Xenopus* oocytes. Microsomes from piglet skeletal muscle (lanes 1 and 3) or from oocytes expressing  $\beta m$  (lanes 2 and 4) were treated (lanes 3 and 4) or not (lanes 1 and 2) with Endo H and submitted to gel electrophoresis and Western blot analysis as described under Materials and Methods. Indicated are the apparent molecular masses of core-glycosylated and nonglycosylated  $\beta m$ . musc. = piglet skeletal muscle.

addition, the double mutant M89A/M100A produced still faster migrating bands (lanes 5 and 6) corresponding to bands occasionally observed in wt  $\beta m$  (Figure 2A, lanes 1 and 2). These results suggest that  $\beta m$  synthesized in vitro can indeed initiate its synthesis not only at Met<sup>1</sup>, to produce the nonglycosylated 52 kDa species, but also at Met<sup>89</sup> and following methionines, to produce the major and minor bands observed in wt  $\beta m$  around and below 33 kDa. In conse-

quence, the presence of two nonglycosylated and two glycosylated  $\beta$ m species in in vitro translations is probably not due to cleavage of the N-terminus. Significantly, nonglycosylated  $\beta$ m species initiated at Met<sup>89</sup> or Met<sup>100</sup>, which lack most of the  $\beta$ m N-terminus and thus the Glu-rich regions, exhibited a molecular mass that closely corresponds to that expected of such a protein (34 kDa). This supports that the presence of the Glu-rich regions is responsible for the aberrant electrophoretic migration of the major population of 61.2 kDa  $\beta$ m.

As previously done for Na,K-ATPase  $\beta$  subunits (26), we verified the type II membrane topology of  $\beta$ m by performing a protease protection assay on  $\beta$ m synthesized in vitro in the presence of sealed, right-side-out ER microsomes. Proteinase K produced a  $\beta$ m species which corresponded to the glycosylated form of  $\beta$ m initiated at Met<sup>89</sup> and lacking the N-terminus (Figure 2A, compare lane 4 to lane 1). In the presence of Triton X-100 which opens membrane vesicles, proteinase K completely digested  $\beta$ m. These results indicate that, in the absence of detergent, proteinase K removes the cytosolic N-terminus of  $\beta$ m and thus confirms its type II membrane topology.

Comparison of the protein pattern of  $\beta$ m synthesized in vitro (Figure 2C, lane 7) to that of  $\beta$ m synthesized in *Xenopus* oocytes (lanes 1–6) showed that, in intact cells,  $\beta$ m is mainly synthesized in its glycosylated, Endo H-sensitive form initiated at Met<sup>1</sup>, to produce the 61.2 kDa species (lanes 1, 3, and 5). However, in nonimmunoprecipitated samples, we also detected a small fraction of the total  $\beta$ m population which appeared as the glycosylated, Endo H-sensitive 42.4 kDa species initiated at Met<sup>89</sup> (Figure 2C, lanes 1–6, compare to lane 7). This species was no longer apparent after immunoprecipitation with  $\beta$ m antibodies (lanes 8–11), possibly due to a weaker interaction of the antibodies with the  $\beta$ m ectodomain than with the N-terminus. As shown in nonimmunoprecipitated and immunoprecipitated samples, metabolically labeled  $\beta$ m, expressed in *Xenopus* oocytes, was stably expressed in or close to the ER (lanes 1, 3, 5, 8, and 10) since it remained in its core glycosylated Endo H-sensitive form over prolonged chase periods (lane 2, 4, 6, 9, and 11). Interestingly, in both nonimmunoprecipitated and immunoprecipitated samples, we observed a small decrease in the apparent molecular mass of the glycosylated  $\beta$ m band during chase periods (Figure 2C, compare lane 1 to 3 and lane 8 to 10). This shift was absent in Endo H-treated samples (compare lane 2 to 5 and lane 9 to 11), indicating that the protein per se is not truncated or modified but rather that high mannose sugar chains may be partially trimmed by ER mannosidases (27).

Comparison of the  $\beta$ m protein synthesized in *Xenopus* oocytes and in piglet skeletal muscles by Western blot analysis (Figure 2D) revealed a slightly faster gel migration of  $\beta$ m expressed in situ. Since  $\beta$ m of both sources migrated with a similar molecular mass after Endo H treatment, it can be concluded that the difference in electrophoretic mobility is due to a differential processing of core sugars in different cells.

**Association of  $\beta$ m with P2-ATPase  $\alpha$  Subunits.** To investigate the role of  $\beta$ m as a chaperone for X,K-ATPases, we expressed  $\beta$ m together with  $\alpha$ 1 NK,  $\alpha$ 2 NK,  $\alpha$ 3 NK, AL1, or  $\alpha$  HK in *Xenopus* oocytes, probed the  $\beta$ m- $\alpha$  association by nondenaturing immunoprecipitations with

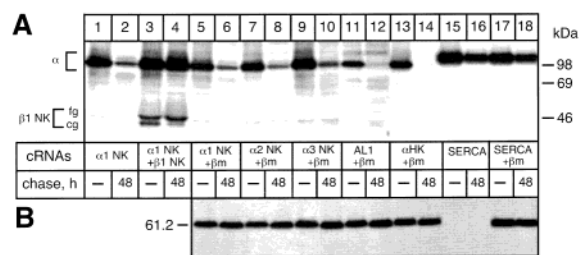
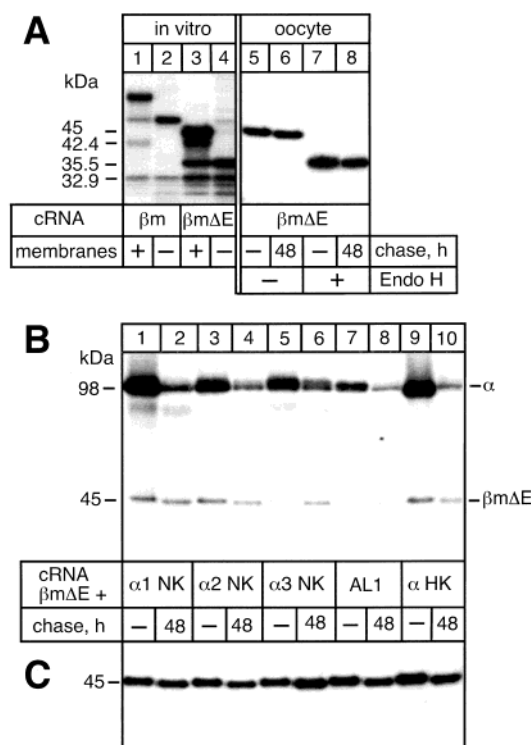


FIGURE 3:  $\beta$ m does not associate with X,K-ATPase  $\alpha$  subunits or SERCA 2. (A)  $\alpha$ 1 NK was expressed alone or together with  $\beta$ 1 NK or  $\beta$ m, and  $\alpha$ 2 NK,  $\alpha$ 3 NK, AL1,  $\alpha$  HK, or SERCA 2a were expressed together with  $\beta$ m in *Xenopus* oocytes, metabolically labeled for 24 h, and subjected to a 48 h chase period. Oocyte microsomes were prepared after the pulse and chase periods and subjected to nondenaturing immunoprecipitations with specific  $\alpha$  NK (lanes 1–10), AL1 (lanes 11 and 12),  $\alpha$  HK (lanes 13 and 14), or SERCA (lanes 15–18) antibodies. Indicated are the positions on SDS gels of  $\alpha$  subunits and of the  $\alpha$ 1 NK-associated core (cg) and fully glycosylated (fg)  $\beta$ 1 NK. Fully glycosylated  $\beta$ 1 NK migrates at a position similar to actin (21) which co-immunoprecipitates with  $\alpha$  NK (lanes 1, 2, 5–10). On the right side, the migration of proteins with known molecular masses is shown. (B) Denaturing immunoprecipitations with a  $\beta$ m antibody of samples shown in (A), lanes 5–14. Indicated is the molecular mass of the immunoprecipitated  $\beta$ m. One of two similar experiments is shown.

specific  $\alpha$  antibodies after a pulse and a chase period, and compared the stabilizing effect of  $\beta$ m on  $\alpha$  subunits to the established stabilizing effect of  $\beta$ 1 NK on  $\alpha$  NK isoforms and of  $\beta$  HK on AL1 and  $\alpha$  HK. As a representative example for all  $\alpha$  subunits, Figure 3A shows that  $\alpha$ 1 NK, expressed alone in oocytes, is degraded after a chase period (Figure 3A, lanes 1 and 2), and that coexpressed  $\beta$ 1 NK stabilizes  $\alpha$ 1 NK and permits for the routing of  $\alpha$ 1 NK/ $\beta$ 1 NK complexes to the plasma membrane as reflected by the full glycosylation of  $\beta$ 1 NK (lanes 3 and 4). Significantly, none of the Na,K-ATPase  $\alpha$  isoforms (Figure 3A, lanes 5–10) nor AL1 or  $\alpha$  HK was able to associate with or to be stabilized by the 61.2 kDa  $\beta$ m (lanes 5–14) despite its high and stable expression (Figure 3B). This result indicates that  $\beta$ m is not able to act like a X,K-ATPase  $\beta$  subunit and to associate with and stabilize any of the known X,K-ATPase  $\alpha$  subunits. Since, similar to  $\beta$ m expressed alone in oocytes (Figure 2C),  $\beta$ m coexpressed with various X,K-ATPase  $\alpha$  subunits remained in its core glycosylated ER form during prolonged chase periods, we wondered whether  $\beta$ m may associate with other P2-ATPases, namely, SERCA 2a or 2b, which are present in the endo/sarcoplasmic reticulum of various tissues including skeletal muscle. Though SERCA is a monomeric P-type ATPase, it is regulated by transient interaction with small proteins such as phospholamban (for review, see 28). As expected, SERCA 2a was stably expressed in oocytes independent of the presence of a  $\beta$  subunit (Figure 3A, lanes 15 and 16). Coexpressed  $\beta$ m did not co-immunoprecipitate with SERCA 2a nor did it change its stability (lanes 17 and 18). Similar results were obtained with SERCA 2b (data not shown). Thus, these results indicate that, at least under the experimental conditions used,  $\beta$ m does not interact with SERCA 2.

**Structure–Function Analysis of  $\beta$ m.** To understand the structural basis for the inability of  $\beta$ m to associate with X,K-ATPase  $\alpha$  subunits and for its permanent residence in the ER, we first produced a mutant lacking the Glu-rich domain



**FIGURE 4:** In vitro translation and expression in *Xenopus* oocytes of  $\beta m$  lacking the Glu-rich regions. (A)  $\beta m$  or  $\beta m\Delta E$ , lacking the Glu-rich regions in the  $\beta m$  N-terminus (see Figure 1), were either translated in a reticulocyte lysate in the presence or absence of microsomes (lanes 1–4) or expressed in *Xenopus* oocytes (lanes 5–8). Oocytes were metabolically labeled for 24 h and subjected to a 48 h chase before microsomes were prepared.  $\beta m\Delta E$  was immunoprecipitated under denaturing conditions, treated or not with Endo H, and subjected to SDS–PAGE. Indicated are the molecular masses of the nonglycosylated and core glycosylated  $\beta m\Delta E$  species. (B)  $\beta m\Delta E$  was coexpressed in *Xenopus* oocytes with  $\alpha$  NK isoforms, AL1 or  $\alpha$  HK. Oocytes were metabolically labeled for 24 h and subjected to a 48 h chase period before microsomes were prepared. Samples were immunoprecipitated under nondenaturing conditions with specific  $\alpha$  antibodies, and immunoprecipitates were subjected to SDS–PAGE. Indicated are the positions and the molecular mass of the  $\alpha$  subunits and the  $\alpha$ -associated  $\beta m\Delta E$ . (C) Denaturing immunoprecipitations with a  $\beta m$  antibody of samples shown in (B). Indicated is the molecular mass of the immunoprecipitated  $\beta m\Delta E$ . One of two similar experiments is shown.

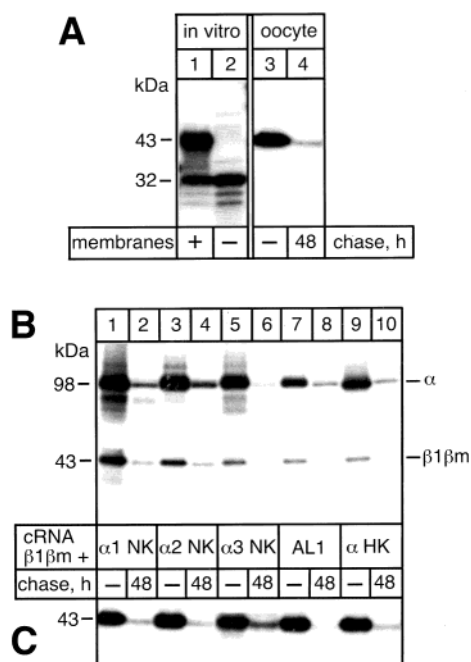
( $\beta m\Delta E$ , Figure 1), which is the most important structural difference between  $\beta m$  and X,K-ATPase  $\beta$  subunits.

In vitro, in the presence of microsomal membranes,  $\beta m\Delta E$  was mainly synthesized as a glycosylated 45 kDa species (Figure 4A, lane 3), which in the absence of membranes (lane 4) or after Endo H treatment (data not shown) migrated as a band of 35.5 kDa, a molecular mass close to that expected for this mutant. This result confirms that the aberrant electrophoretic migration of wt  $\beta m$  (lanes 1 and 2) is due to the presence of the Glu-rich regions in the  $\beta m$  N-terminus. Similar to wt  $\beta m$  (lanes 1 and 2), part of  $\beta m\Delta E$  synthesized in vitro appeared as a glycosylated 42.4 kDa and a nonglycosylated 32.9 kDa band (lanes 3 and 4), indicating that Met<sup>89</sup>, still present in the mutant, can also be used as an initiator methionine. Synthesized in oocytes, immunoprecipitated  $\beta m\Delta E$  appeared as a single species with a molecular mass of 45 kDa in its glycosylated form (lanes 5 and 6), and of 35.5 kDa in its deglycosylated form (lanes 7 and 8), corresponding to  $\beta m\Delta E$  initiated at Met<sup>1</sup>. Similar to wt  $\beta m$  (Figure 3C), core glycosylated  $\beta m\Delta E$  was stably expressed

in oocytes, did not become fully glycosylated, and underwent a small shift in its molecular mass during a 48 h chase period (Figure 4A, lanes 5 and 6). These results indicate that the Glu-rich region is not crucially involved in the structural maturation and the ER retention of  $\beta m$ . Significantly, removal of the Glu-rich region permitted co-immunoprecipitation and thus association of  $\beta m\Delta E$  with all Na,K-ATPase  $\alpha$  isoforms, AL1 and  $\alpha$  HK (Figure 4B, lanes 1–10). However, association of  $\beta m\Delta E$  with X,K-ATPase  $\alpha$  subunits was not efficient since, when taking into account the number of methionines available for metabolic labeling in  $\alpha$  subunits (22–26) and  $\beta m\Delta E$  (7), it becomes apparent that the stoichiometry between immunoprecipitated  $\alpha$  subunits and co-immunoprecipitated  $\beta m\Delta E$  after the pulse period is far from 1. Inefficient association of  $\beta m\Delta E$  may at least partially be responsible for the lack of stabilization of X,K-ATPase  $\alpha$  subunits observed after a 48 h chase period (lanes 2, 4, 6, 8, and 10). Also, despite the persistent association of  $\beta m\Delta E$  with residual  $\alpha$  subunits observed after the chase period,  $\beta m\Delta E$  remained in its core glycosylated, partially trimmed form, indicating that  $\alpha/\beta m\Delta E$  complexes are not correctly folded to permit routing to the plasma membrane. Together, these results indicate that the removal of the Glu-rich region influences  $\beta m$ 's conformation which allows for some, though highly inefficient, association with X,K-ATPase  $\alpha$  subunits.

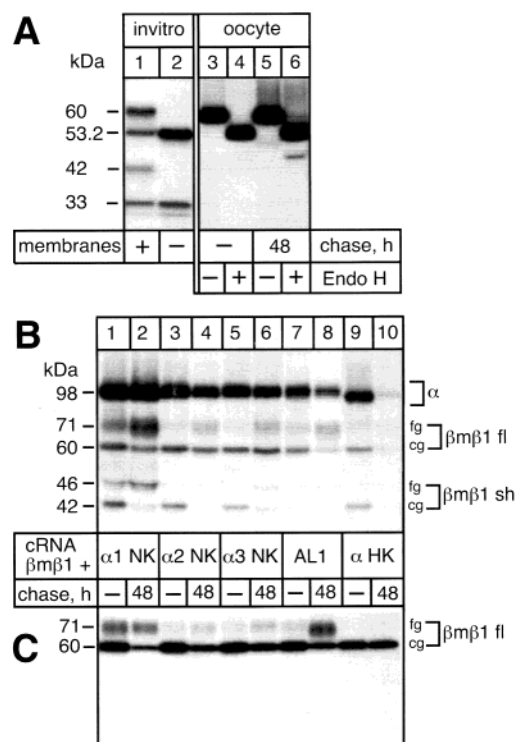
The molecular determinants that retain  $\beta m$  in the ER or impede efficient association with X,K-ATPase  $\alpha$  subunits are not easily predictable from sequence comparison. Therefore, we produced chimeras between  $\beta m$  and  $\beta 1$  NK. In vitro translation of a  $\beta 1\beta m$  chimera, containing the cytoplasmic N-terminus of  $\beta 1$  NK and the transmembrane and the extracytoplasmic domains of  $\beta m$  (Figure 1), produced a nonglycosylated species with a molecular mass of 32 kDa, as expected for this chimera, and a glycosylated species of 43 kDa (Figure 5A, lanes 1 and 2). Consistent with the only presence of Met<sup>1</sup> in the  $\beta 1$  NK N-terminus, no additional glycosylated species were observed resulting from alternative protein initiation. As in the in vitro translation system in the presence of membranes,  $\beta 1\beta m$  cRNA injected into *Xenopus* oocytes produced a glycosylated 43 kDa species (lane 3). In contrast to wt  $\beta m$  or  $\beta m\Delta E$ ,  $\beta 1\beta m$  was degraded during a 48 h chase period (lane 4). Coexpression of  $\beta 1\beta m$  with X,K-ATPase  $\alpha$  subunits permitted the formation of  $\alpha/\beta 1\beta m$  complexes during the pulse period, but  $\beta 1\beta m$  was not able to stabilize  $\alpha$  subunits or become itself stabilized during the chase period (Figure 5B,C, lanes 1–10). Thus, when the Glu-rich regions of the  $\beta m$  N-terminus are deleted or when the  $\beta m$  N-terminus is replaced with that of an authentic X,K-ATPase  $\beta$  subunit,  $\beta m$  becomes partially competent for association with X,K-ATPases  $\alpha$  subunits but not sufficiently to ensure their structural maturation.

To confirm the implication of the ectodomain in the association process of  $\beta$  subunits with X,K-ATPase  $\alpha$  subunits (5), we finally produced a  $\beta m\beta 1$  chimera containing the  $\beta m$  cytoplasmic N-terminus and the  $\beta 1$  transmembrane and extracytoplasmic domains (Figure 1). Due to the presence of the  $\beta m$  N-terminus, the in vitro translated  $\beta m\beta 1$  chimera produced two glycosylated species in the presence of microsomes, an aberrantly migrating species at 60 kDa initiated at Met<sup>1</sup> and a normally migrating species at 42 kDa initiated at Met<sup>89</sup> (Figure 6A, lane 1). The nonglycosylated forms observed in the absence and presence of microsomes



**FIGURE 5:** In vitro translation and expression in *Xenopus* oocytes of the  $\beta$ 1 $\beta$ m chimera. (A)  $\beta$ 1 $\beta$ m chimeras (see Figure 1) were either translated in a reticulocyte lysate in the presence or absence of microsomes (lanes 1 and 2) or expressed in *Xenopus* oocytes (lanes 3 and 4). Oocytes were metabolically labeled for 24 h and subjected to a 48 h chase before microsomes were prepared.  $\beta$ 1 $\beta$ m was immunoprecipitated under denaturing conditions before gel electrophoresis. Indicated are the molecular masses of the nonglycosylated and core glycosylated  $\beta$ 1 $\beta$ m species. (B)  $\beta$ 1 $\beta$ m was coexpressed in *Xenopus* oocytes with  $\alpha$  NK isoforms, AL1 or  $\alpha$  HK. Oocytes were metabolically labeled for 24 h and subjected to a 48 h chase period before microsomes were prepared. Samples were immunoprecipitated under nondenaturing conditions with specific  $\alpha$  antibodies, and immunoprecipitates were subjected to SDS-PAGE. Indicated are the positions and the molecular mass of the  $\alpha$  subunits and the  $\alpha$ -associated  $\beta$ 1 $\beta$ m. (C) Denaturing immunoprecipitations with a  $\beta$ m antibody of samples shown in (B). Indicated is the molecular mass of the immunoprecipitated  $\beta$ 1 $\beta$ m. One of two similar experiments is shown.

exhibited molecular masses of 53.2 and 33 kDa (lane 2). The immunoprecipitated  $\beta$ m $\beta$ 1 expressed in oocytes corresponded to the full-length, glycosylated  $\beta$ m $\beta$ 1 which was stably expressed and remained in its core glycosylated, Endo H-sensitive form during a 48 h chase period (lanes 3–6).  $\beta$ m $\beta$ 1 (60 kDa) had a lower molecular mass than  $\beta$ m (61.2 kDa). Since  $\beta$ 1 NK has a longer ectodomain and three glycosylation sites which are all used (29), the higher molecular mass of  $\beta$ m than of  $\beta$ m $\beta$ 1 could reflect the glycosylation of  $\beta$ m on all of its four glycosylation sites. The presence of the transmembrane and extracytoplasmic domains of  $\beta$ 1 in the  $\beta$ m $\beta$ 1 chimera permitted for the association with all X,K-ATPase  $\alpha$  subunits during the pulse period. Interestingly, two main species of  $\beta$ m $\beta$ 1 were co-immunoprecipitated with X,K-ATPase  $\alpha$  subunits (Figure 6B, lanes 1, 3, 5, 7, 9) that corresponded to the core glycosylated species of 60 and 42 kDa observed in in vitro translations (Figure 6A, lane 1), and which are initiated at Met<sup>1</sup> and Met<sup>89</sup>, respectively. The expression in *Xenopus* oocytes of the 60 kDa species could be revealed by denaturing immunoprecipitations with  $\beta$ m antibodies (Figure 6C). On the other hand, similar to the short 42.4 kDa form of  $\beta$ m which was visible in nonimmunoprecipitated samples (Figure 2B, lanes 1, 3, 5) but not after immunoprecipitations



**FIGURE 6:** In vitro translation and expression in *Xenopus* oocytes of  $\beta$ m $\beta$ 1. (A)  $\beta$ m $\beta$ 1 chimeras (see Figure 1) were either translated in a reticulocyte lysate in the presence or absence of microsomes (lanes 1 and 2) or expressed in *Xenopus* oocytes (lanes 3–6). Oocytes were metabolically labeled for 24 h and subjected to a 48 h chase before microsomes were prepared.  $\beta$ m $\beta$ 1 was immunoprecipitated under denaturing conditions, treated or not with Endo H, and subjected to SDS-PAGE. Indicated are the molecular masses of the nonglycosylated and core glycosylated  $\beta$ m $\beta$ 1 species. (B)  $\beta$ m $\beta$ 1 was coexpressed in *Xenopus* oocytes with  $\alpha$  NK isoforms, AL1 or  $\alpha$  HK. Oocytes were metabolically labeled for 24 h and subjected to a 48 h chase period before microsomes were prepared. Samples were immunoprecipitated under nondenaturing conditions with specific  $\alpha$  antibodies, and immunoprecipitates were subjected to gel electrophoresis. Indicated are the positions and the molecular masses of the  $\alpha$  subunits and the  $\alpha$ -associated  $\beta$ m $\beta$ 1 species. fl = full length, sh = short, cg = core glycosylated, fg = fully glycosylated  $\beta$ m $\beta$ 1. (C) Denaturing immunoprecipitations with a  $\beta$ m antibody of samples shown in (B). Indicated are the molecular masses of the immunoprecipitated  $\beta$ m $\beta$ 1 species. cg = core glycosylated, fg = fully glycosylated  $\beta$ m $\beta$ 1. One of two similar experiments is shown.

(Figure 2B, lanes 8 and 10), the short 42 kDa form of  $\beta$ m $\beta$ 1 associated with X,K-ATPase  $\alpha$  subunits was not recognized in denaturing immunoprecipitations with  $\beta$ m antibodies (Figure 6C). Despite the considerable fraction of short  $\beta$ m $\beta$ 1 associated with  $\alpha$  NK in oocytes (Figure 6B), the overall expression of short  $\beta$ m $\beta$ 1 was not more important than that of  $\beta$ m (Figure 2A) as visualized in nonimmunoprecipitated samples (data not shown). This result may indicate that, compared to the full-length  $\beta$ m $\beta$ 1, the short  $\beta$ m $\beta$ 1 species is preferentially associated with  $\alpha$  NK.

Significantly,  $\beta$ m $\beta$ 1 association permitted efficient stabilization of all Na,K-ATPase  $\alpha$  isoforms during the chase period (Figure 6B, lanes 1–6). On the other hand, AL1 was less (lanes 7 and 8) and  $\alpha$  HK (lanes 9 and 10) was not at all stabilized by  $\beta$ m $\beta$ 1, in agreement with the lower assembly efficiency of  $\beta$ 1 NK with these X,K-ATPase  $\alpha$  subunits (12, 30). Both full-length and short  $\beta$ m $\beta$ 1 species contributed to the efficient maturation of  $\alpha$  NK isoforms and the partial

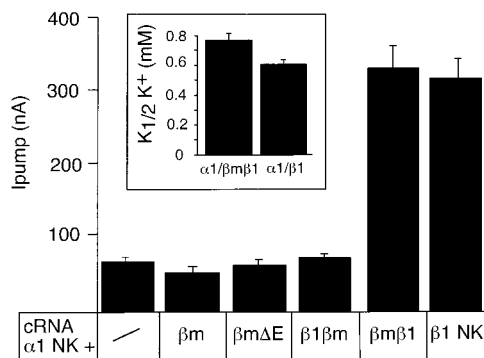


FIGURE 7: Functional expression and apparent  $\text{K}^+$ -affinity of  $\alpha 1 \text{ NK}/\beta \text{m}\beta 1$  complexes.  $\alpha 1 \text{ NK}$  was coexpressed with  $\beta \text{m}$ ,  $\beta \text{m}\Delta \text{E}$ ,  $\beta 1\beta \text{m}$ ,  $\beta \text{m}\beta 1$ , or  $\beta 1 \text{ NK}$  in *Xenopus* oocytes. Three days after cRNA injection, the maximal Na,K-pump currents ( $I_{\text{pump}}$ ) were measured by electrophysiological means as described under Materials and Methods. Inset: The  $K_{1/2}$  values for  $\text{K}^+$  of  $\alpha 1 \text{ NK}/\beta \text{m}\beta 1$  or  $\alpha 1 \text{ NK}/\beta 1 \text{ NK}$  complexes, expressed in *Xenopus* oocytes, were determined as described under Materials and Methods. The represented values are means  $\pm$  SE of 8–12 oocytes from 2 different *Xenopus* females.

maturation of nongastric H,K-ATPase  $\alpha$  subunits. Indeed, both core glycosylated species, associated during the pulse period with  $\alpha \text{ NK}$  isoforms or AL1, became fully glycosylated during the chase period, resulting in the progressive appearance of a 71 and a 46 kDa species (lanes 2, 4, 6, and 8) which were Endo H-resistant (data not shown). These results indicate that these  $\alpha/\beta \text{m}\beta 1$  complexes can leave the ER and are routed to the plasma membrane.

**Functional Expression of Na,K-ATPase  $\alpha/\beta \text{m}\beta 1$  Complexes.** To verify whether  $\alpha/\beta \text{m}\beta 1$  complexes are expressed at the cell surface in a transport-competent form, we compared the  $\text{K}^+$ -activated Na,K-pump transport activity by electrophysiological means, in noninjected oocytes and in oocytes injected with  $\alpha 1 \text{ NK}$  cRNA together with wt  $\beta \text{m}$ , mutant  $\beta \text{m}\Delta \text{E}$ , or chimeric  $\beta 1\beta \text{m}$  or  $\beta \text{m}\beta 1$  cRNA. As expected from their inefficient association with  $\alpha 1 \text{ NK}$  (Figures 3A, 4B, 5B),  $\beta \text{m}$ ,  $\beta \text{m}\Delta \text{E}$ , and  $\beta 1\beta \text{m}$ , coexpressed with  $\alpha 1 \text{ NK}$ , did not produce an increase in the Na,K-pump current compared to that observed in noninjected oocytes (Figure 7). On the other hand, coexpression of  $\alpha 1 \text{ NK}$  with  $\beta \text{m}\beta 1$  increased the Na,K-pump current in oocytes by more than 3-fold, similar to oocytes expressing  $\alpha 1 \text{ NK}$  with  $\beta 1 \text{ NK}$  (Figure 7). Together these results indicate that the  $\beta 1$  transmembrane and/or ectodomain is responsible for the formation of functional  $\alpha 1 \text{ NK}/\beta \text{m}\beta 1$  complexes at the cell surface.

X,K-ATPase  $\beta$  subunits not only act as chaperones for the maturation of X,K-ATPase  $\alpha$  subunits but also influence the  $\text{K}^+$ -activation of X,K-ATPases (21, 25, 32). The  $\text{K}^+$ -effect of X,K-ATPase  $\beta$  subunits is mainly mediated by the ectodomain (21, 25, 31, 32) and the transmembrane domain (33). Indeed, replacement of the  $\beta 1 \text{ NK}$  N-terminus with that of  $\beta \text{ HK}$  does not influence the  $\text{K}^+$ -affinity of Na,K-pumps (32). On the other hand, complete truncation of the  $\beta 1 \text{ NK}$  N-terminus produces a 3-fold decrease in the  $\text{K}^+$ -affinity of Na,K-pumps which can, however, be overcome by the addition of a few alanine residues at the N-terminus (11). Measurements of the  $\text{K}^+$ -activation of  $\alpha 1 \text{ NK}/\beta \text{m}\beta 1$ , containing both full-length and short forms of  $\beta \text{m}\beta 1$ , showed that the  $K_{1/2}$  value for  $\text{K}^+$  was only slightly higher for Na,K-pumps associated with  $\beta \text{m}\beta 1$  than for Na,K-pumps associ-

ated with  $\beta 1 \text{ NK}$  (Figure 7, inset), consistent with previous observations that the N-terminus is not essentially involved in the  $\text{K}^+$ -effect of X,K-ATPase  $\beta$  subunits.

**$\text{Ca}^{2+}$ -Sensitivity of  $\beta \text{m}$ .** The present study indicates that  $\beta \text{m}$  does not function as a chaperone for X,K-ATPase  $\alpha$  subunits. In view of the presence of Glu-rich regions in the cytoplasmic N-terminus, which occur in  $\text{Ca}^{2+}$ -binding or  $\text{Ca}^{2+}$ -transporting proteins, we considered, as an alternative function, that  $\beta \text{m}$  might be a  $\text{Ca}^{2+}$ -sensing protein. To assess the capacity of the  $\beta \text{m}$  N-terminus to bind  $\text{Ca}^{2+}$ , we performed a controlled trypsinolysis assay on microsomes of oocytes expressing  $\beta \text{m}$  in the presence of 1 mM  $\text{CaCl}_2$  or 0.1 mM EGTA. We reasoned that potential  $\text{Ca}^{2+}$  binding by  $\beta \text{m}$  may influence the accessibility and thus sensitivity to trypsin of the  $\beta \text{m}$  N-terminus.

As seen in Figure 8A,  $\beta \text{m}$  (lanes 1–7) exhibited a much higher overall trypsin sensitivity than  $\beta 1 \text{ NK}$  (lanes 8–11) or  $\beta \text{ HK}$  (lanes 12–15). At a trypsin-to-protein ratio of 0.05, intact  $\beta 1 \text{ NK}$  and  $\beta \text{ HK}$  were revealed as well as a digestion product which corresponds to the N-terminally cleaved  $\beta 1 \text{ NK}$  (20) (lanes 9–11) or  $\beta \text{ HK}$  (lanes 13–15). Together, these  $\beta$  species observed in trypsinized samples represented about 15% and 100% of the total population of  $\beta 1 \text{ NK}$  (lane 8) and  $\beta \text{ HK}$  (lane 12), respectively. In contrast, under the same conditions,  $\beta \text{m}$  was nearly completely digested (lanes 5–7), and a tryptic fragment of about 46 kDa was already observed at a trypsin-to-protein ratio of 0.001 (lanes 2–4). If, similar to  $\beta 1 \text{ NK}$ , the initial tryptic cleavage of  $\beta \text{m}$  occurred in the N-terminus, the 6 kDa shift of the trypsinized  $\beta \text{m}$  could result from cleavage at Lys<sup>50</sup> located in the loop between the two  $\alpha$  helices formed by the two Glu-rich regions (14). Interestingly, a short  $\beta \text{m}$  species showing a similar shift to that observed after trypsinolysis could occasionally be observed in nontrypsinized  $\beta \text{m}$  (lane 1).

The tryptic pattern of  $\beta \text{m}$  was similar in the presence of 10 mM Tris-HCl, 1 mM  $\text{CaCl}_2$ , or 0.1 mM EGTA (compare lanes 2–4 and 5–7) whereas N-terminal cleavage of  $\beta 1 \text{ NK}$  and  $\beta \text{ HK}$  was slightly impeded in the presence of EGTA (compare lanes 9–11 and 13–15). As a positive control for the controlled trypsinolysis assay, we show that the trypsin resistance of SERCA was increased in the presence of  $\text{CaCl}_2$ , compared to that observed in the presence of Tris-HCl or EGTA (lanes 15–18), reflecting a  $\text{Ca}^{2+}$ -dependent conformational change of SERCA.

As a second approach to test whether  $\beta \text{m}$  binds  $\text{Ca}^{2+}$ , we looked at whether  $\beta \text{m}$  changes its electrophoretic mobility in  $\text{Ca}^{2+}$ -containing SDS gels, a technique which has frequently been used for the detection of  $\text{Ca}^{2+}$ -binding proteins (for references, see 34). Figure 8B shows the fractional changes in the migration of several proteins in gels containing 1 mM  $\text{CaCl}_2$  compared to gels containing 0.1 mM EGTA. It can be seen that calmodulin migrated about 20% and SERCA about 10% faster in the presence than in the absence of  $\text{Ca}^{2+}$ . On the other hand, the electrophoretic mobility of  $\alpha 1 \text{ NK}$  as well as of  $\beta \text{m}$  or of the  $\beta 1\beta \text{m}$  chimera was not affected by the presence of  $\text{Ca}^{2+}$ . Altogether these results speak against a role of  $\beta \text{m}$  as a  $\text{Ca}^{2+}$ -binding protein.

## DISCUSSION

Based on several common structural features, the recently identified  $\beta \text{m}$  protein (14) has been assigned to the family

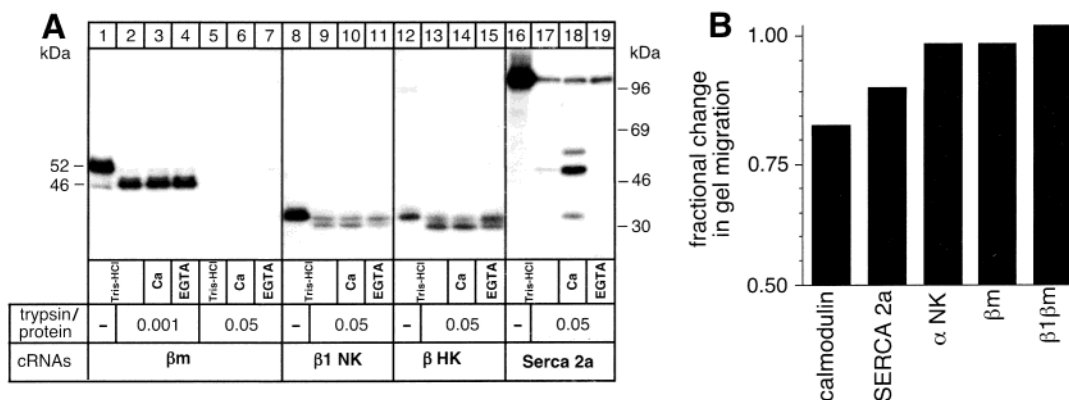


FIGURE 8:  $\text{Ca}^{2+}$  does not influence the protease sensitivity or the electrophoretic mobility of  $\beta$ m. (A) Controlled trypsinolysis of  $\beta$ m in the presence of  $\text{Ca}^{2+}$  or EGTA.  $\beta$ m,  $\beta$ 1 NK,  $\beta$  HK, or SERCA 2a were expressed in *Xenopus* oocytes and metabolically labeled during a 24 h pulse. Microsomes were prepared, and aliquots were subjected or not to trypsinolysis at trypsin/protein ratios of 0.001 and/or 0.05 in the presence of 10 mM Tris-HCl, 1 mM  $\text{CaCl}_2$ , or 0.1 mM EGTA as described under Materials and Methods. Samples were immunoprecipitated with specific antibodies before SDS-PAGE. On the left side, molecular masses of nontrypsinized, Endo H-treated  $\beta$ m and of trypsinized  $\beta$ m is shown. On the right side, the migration of proteins with known molecular masses is indicated. (B) Electrophoretic mobility of  $\beta$ m and other proteins migrated on gels containing 1 mM  $\text{CaCl}_2$  or 0.1 mM EGTA.  $\beta$ m,  $\beta$ 1  $\beta$ m, SERCA 2a,  $\alpha$ 1 NK, or calmodulin were expressed in *Xenopus* oocytes and labeled during a 24 h pulse period. Microsomes were prepared, and aliquots were loaded on gels containing either 1 mM  $\text{CaCl}_2$  or 0.1 mM EGTA. Shown are the fractional changes of gel migration of the various proteins on gels containing  $\text{CaCl}_2$  compared to gels containing EGTA. Gel migration in the presence of EGTA was arbitrarily set to 1.

of X,K-ATPase  $\beta$  subunits, which are all well characterized chaperones necessary for the maturation of X,K-ATPase  $\alpha$  subunits (for review, see 3). In this study, we characterized the biosynthesis and processing of  $\beta$ m and investigated whether  $\beta$ m has a similar functional role as the other members of the  $\beta$  subunit family. Our results show that  $\beta$ m does not associate with any known X,K-ATPase  $\alpha$  subunit and permanently remains in the ER, indicating that  $\beta$ m represents a novel member of the  $\beta$  subunit family with a distinct function from that of X,K-ATPase  $\beta$  subunits.

*$\beta$ m Is a Type II Glycoprotein Stably Expressed in the ER.* Sequence analysis predicts that  $\beta$ m is a type II glycoprotein similar to X,K-ATPase  $\beta$  subunits. In this study, we confirm the cytoplasmic exposure of the  $\beta$ m N-terminus by controlled proteolysis assays and the presence of sugars by Endo H treatment of  $\beta$ m expressed in vitro or in intact cells. On the other hand, we reveal that  $\beta$ m may structurally be distinct from X,K-ATPase  $\beta$  subunits since it exhibits a much higher trypsin sensitivity than  $\beta$  NK or  $\beta$  HK. We also show that, in vitro and in *Xenopus* oocytes,  $\beta$ m is mainly synthesized in a full-length form which shows an aberrant gel migration due to the presence of the N-terminal Glu-rich regions, that distinguishes  $\beta$ m from other members of the  $\beta$  subunit family. However, human  $\beta$ m is also synthesized, though to a lesser extent, as a short, correctly migrating form, initiated at a second methionine, which is located in a Kozak consensus sequence distally from the Glu-rich regions. Since the short form of  $\beta$ m is a minor population of  $\beta$ m synthesized in oocytes and possibly is less well recognized by  $\beta$ m antibodies, it is so far not known whether this form exists in situ where only full-length  $\beta$ m was detected by Western blotting (this study and 15).

In situ,  $\beta$ m was identified as a Endo H-sensitive protein (15), mainly located in the endo/sarcoplasmic reticulum of skeletal muscle (16). Our results support that  $\beta$ m is indeed permanently retained in or close to the ER in its core glycosylated, probably partially trimmed form since after expression in *Xenopus* oocytes,  $\beta$ m expression persists over prolonged chase periods in a Endo H-sensitive form.

Interestingly,  $\beta$ m synthesized in *Xenopus* oocytes has a slightly lower electrophoretic mobility than  $\beta$ m expressed in situ in piglet skeletal muscle. Since the molecular mass of the deglycosylated forms of both  $\beta$ m proteins is similar, it is likely that the protein itself is not modified differently but that processing of core sugars, and in particular of ER mannosidase trimming, may differ in situ and in *Xenopus* oocytes.

The intracellular localization distinguishes  $\beta$ m from all other known members of the  $\beta$  subunit family which are mainly expressed at the cell surface in association with X,K-ATPase  $\alpha$  subunits. Some but not all X,K-ATPase  $\beta$  subunits may remain in the ER when they are expressed in cells without a X,K-ATPase  $\alpha$  subunit. Indeed, *Xenopus*  $\beta$ 1 NK and  $\beta$ 3 NK, expressed in *Xenopus* oocytes without  $\alpha$  NK, remain in the ER in their core glycosylated form and are slowly degraded (35, 36). On the other hand, individual  $\beta$  HK are routed to the plasma membrane in various cell types as reflected by their acquisition of complex-type sugars which are added in the distal Golgi compartment (9, 12, 21, 37). The reason for this different behavior of X,K-ATPase  $\beta$  subunits is not known, but results obtained with chimeras between  $\beta$ 1 NK and  $\beta$  HK indicate that the ectodomain determines the ER retention or exit of X,K-ATPase  $\beta$  subunits (21). This may also be true for  $\beta$ m. Indeed, deletion of the Glu-rich regions in the  $\beta$ m N-terminus, which potentially could play a role in protein-protein interactions necessary for the ER location of  $\beta$ m, did not change the glycosylation pattern and thus the cellular routing of  $\beta$ m. Moreover, the short  $\beta$ m species observed in nonimmunoprecipitated samples apparently remained in its core glycosylated form, indicating that not only the Glu-rich regions but also the whole N-terminus is not sufficient for ER retention of  $\beta$ m. This is also supported by the observation that the  $\beta$ m $\beta$ 1 chimera, in which the  $\beta$ m N-terminus is added to the transmembrane and ectodomain of  $\beta$ 1 NK, can leave the ER when it is coexpressed and stably associated with  $\alpha$  NK. Whether, in situ, diarginine motifs which have been implicated in ER retention (for review, see 38), and which are

present in the  $\beta$ m N-terminus, may contribute to the  $\beta$ m ER location is so far not known.

Not only ER retention but also the stable cellular expression appears to be independent of the  $\beta$ m N-terminus. Deletion of the Glu-rich regions still permits accumulation of  $\beta$ m in the ER, and short  $\beta$ m forms, initiated at Met<sup>89</sup>, appear to be stably expressed over prolonged chase periods. Only replacement of the  $\beta$ m N-terminus by that of  $\beta$ 1 NK destabilizes  $\beta$ m and leads to its rapid degradation, indicating that the  $\beta$ 1 N-terminus itself provides a degradation signal or that it has a global destabilizing effect on  $\beta$ m.

*$\beta$ m Is Functionally Distinct from X,K-ATPase  $\beta$  Subunits.* Whereas coexpression of X,K-ATPase  $\beta$  subunits with an appropriate X,K-ATPase  $\alpha$  subunit in *Xenopus* oocytes permits assembly and structural maturation of both  $\alpha$  and  $\beta$  subunits and allows for ER exit of  $\alpha$ - $\beta$  complexes (for review, see 3), our results show that  $\beta$ m cannot function as a chaperone for any known X,K-ATPase  $\alpha$  subunit and remains in the ER. These observations raise the following questions: (1) what are the structural differences in  $\beta$ m which prevent its association with X,K-ATPase  $\alpha$  subunits and (2) what is the intrinsic physiological role of  $\beta$ m?

Interestingly, removal of the Glu-rich regions in the  $\beta$ m N-terminus or replacement of the  $\beta$ m N-terminus by that of  $\beta$ 1 NK permits a transient association of  $\beta$ m with X,K-ATPase  $\alpha$  subunits which is, however, not sufficient to stabilize  $\alpha$  subunits. This result could indicate that certain association determinants present in X,K-ATPase  $\beta$  subunits may also be present in  $\beta$ m but that their exposure may be prevented by a particular, global conformation imposed by the  $\beta$ m N-terminus. A role of the N-terminus in the overall conformation of X,K-ATPase  $\beta$  subunits (11, 32) or of other type II proteins (39, 40) is indeed well established. In  $\beta$  NK, truncation of the N-terminus leads to a repositioning of the transmembrane domain and adjacent ectodomain regions (32).

So far, little is known on the structural identity of X,K-ATPase  $\beta$  domains that interact with X,K-ATPase  $\alpha$  subunits. Though  $\alpha$ - $\beta$  interactions in the transmembrane and the extracytoplasmic domains appear to participate in the overall maturation of X,K-ATPase  $\alpha$  subunits (11, 32), only interactions between the extracellular loop between M7 and M8 of X,K-ATPase  $\alpha$  subunits and the  $\beta$  ectodomain, possibly adjacent to the transmembrane domain, appear to be important for an initial stabilization of  $\alpha$  subunits by  $\beta$  subunits (5, 41, 42). The role of the ectodomain in the inability of  $\beta$ m to assemble with X,K-ATPase  $\alpha$  subunits is supported by the observation that, in contrast to  $\beta$ m, a  $\beta$ m $\beta$ 1 chimera containing the cytoplasmic domain of  $\beta$ m and the transmembrane and extracytoplasmic domain of  $\beta$ 1 NK can stabilize, in its full-length and short form, X,K-ATPase  $\alpha$  subunits. Since the sequence identity in the putative association domain between  $\beta$ m and X,K-ATPase  $\beta$  subunits is between 25% and 39% and therefore not significantly different from the overall sequence identity, and since only very few amino acids in  $\beta$ m that are clearly distinct from amino acids found in one or the other of the X,K-ATPase  $\beta$  isoforms, the presence or lack of a putative  $\alpha$ -association sequence is difficult to predict.

The results obtained in *Xenopus* oocytes on the lack of a chaperone function of  $\beta$ m for X,K-ATPase  $\alpha$  subunits are likely to be of physiological relevance since, in skeletal

muscle,  $\beta$ m is expressed as a similar protein species to that in oocytes and is located in the sarcoplasmic reticulum, indicating that, in situ,  $\beta$ m cannot be permanently associated with an X,K-ATPase  $\alpha$  subunit that is ultimately expressed at the cell surface. Since  $\beta$ m is an ER resident, we tested whether  $\beta$ m could associate with SERCA and potentially act as a regulatory subunit of this ER-located P2-ATPase. After coexpression in *Xenopus* oocytes, we could not demonstrate an association of  $\beta$ m with SERCA, but we cannot entirely exclude that transient interactions could occur under certain stringent physiological conditions. Such transient interactions, which are difficult to demonstrate, could potentially also occur with X,K-ATPase  $\alpha$  subunits, with other so far poorly characterized members of the P-type ATPases, such as the phospholipid translocases or heavy-metal ATPases, or hitherto unknown  $\alpha$  subunits. These possibilities remain to be explored.

$\beta$ m has three acidic clusters in the N-terminus, an Asp-rich (D<sup>22</sup>–E<sup>26</sup>) and two Glu-rich (D<sup>34</sup>–E<sup>42</sup>, D<sup>48</sup>–E<sup>70</sup>) regions, and it is tempting to speculate that they may be involved in a particular function of  $\beta$ m. Since our results do not support the hypothesis that the Glu-rich regions may be involved in Ca<sup>2+</sup>-sensing, future experiments should center on the question whether the acidic clusters are targets for specific protein–protein interactions as has been suggested for similar Asp-rich regions in the cytoplasmic tail of proprotein convertases (for review, see 43) or for similar Asp- and Glu-rich regions in the Ran GTPase activating protein (44).

In conclusion, despite the fact that, from a structural point of view,  $\beta$ m obviously belongs to the protein family, well-known as X,K-ATPase  $\beta$  subunits, our results suggest that  $\beta$ m does not have a chaperone function for X,K-ATPase  $\alpha$  subunits, which is the primary role of X,K-ATPase  $\beta$  subunits. This observation emphasizes the necessity to revise our understanding of the X,K-ATPase  $\beta$  subunit family as a structural and functional entity and suggests that the physiological roles of its members is more diverse than predicted.

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