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The βm Protein, a Member of the X,K-ATPase β-Subunits Family, Is Located Intracellularly in Pig Skeletal Muscle[1]

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The sequence of the pig cDNA encoding the muscle-specific βm-protein, a member of the X,K-ATPase β-subunits family, was determined. Two alternatively spliced transcripts encoding polypeptide chains of 355 and 351 residues were identified. The tissue specificity of expression of βm and other X,K-ATPase β-subunit genes was studied by RT-PCR performed on 24 tissues from newborn pigs. The βm expression was shown to be highly tissue-specific, being detected at the highest level in skeletal muscle, at a lower level in heart, and at much lower level in skin. The βm transcripts are more abundant in the tissues from the newborn than adult. Immunoblotting and deglycosylation shift assay indicated that skeletal muscle membranes of newborn pigs contain βm protein with an electrophoretic mobility and carbohydrate content very similar to that of human βm. Fractionation of membranes from both newborn and adult pig skeletal muscles by isopycnic centrifugation revealed that the majority of the βm protein is concentrated in the sarcoplasmic reticulum-containing fractions. This intracellular location is a unique property that distinguishes the βm protein from other members of the X,K-ATPase β-subunit family. © 2001 Elsevier Science

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The P-type ATPases comprise a large number of highly diverse transport proteins that are involved in active transport of cations across biological membranes and feature the formation of a phosphorylated Asp residue in the active site during the reaction cycle (1, 2). Members of the X,K-ATPase family, which consist of closely related potassium-dependent ATPases located in the plasma membrane that operate as cation exchangers pumping K^+ into the cell and Na^+ or H^+ out of the cell, include the Na,K-ATPases, the gastric H,K-ATPase, and the nongastric H,K-ATPase. Among the animal P-ATPases, only the X,K-ATPases require more than a single subunit for manifestation of the enzymatic and transport functions (2–6). All of the known X,K-ATPases are heterodimers composed of two types of subunits. The catalytic α-subunits (~110 kDa) contain most of the ATPase functional domains (2–4). The glycosylated β-subunits (core protein ~30–35 kDa) have a relatively short cytoplasmic N-terminal domain, a single transmembrane segment, and a large ectodomain that contains three conserved disulfide bridges and several carbohydrate chains (7–9). Other P-ATPases are not known to have analogs of the X,K-ATPase β-subunits (2, 3).

An absolute requirement for a β-subunit to participate in the formation of the active ATPase is a unique feature of the X,K-ATPase family members. The β-subunits play a crucial role in the structural and functional maturation of the active X,K-ATPase molecule. In this process, the β-subunit functions as a specific chaperone whose tight association with the catalytic α-subunit helps the latter in correct membrane insertion and folding and in translocation to the plasma membrane (9–12). In addition to this important structural role, β-subunits also influence enzymatic and transport functions of the X,K-ATPases through mod-

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ulation of the enzyme affinities for cations (3, 8–10, 13).

Both of the X,K-ATPase subunits are encoded by multigene families (3, 14, 15). Six different but closely related human genes for X,K-ATPase α-subunits have been identified; four are isoforms of Na,K-ATPase α-subunit, and the other two represent gastric and nongastric H,K-ATPases (3, 14–16). Three β-isoforms of the Na,K-ATPase and one β-subunit of the gastric H,K-ATPase are known, while a specific β-subunit of the nongastric H,K-ATPase has not been unambiguously identified. Known β-subunit proteins exhibit nearly a two times lower degree of sequence similarity than α-subunits and have no significant sequence homology with any other proteins (2, 3, 7–9).

All X,K-ATPase isoforms exhibit characteristic patterns of tissue-specific expression (for review see (3)). The combination $\alpha_1\beta_1$[3], a principal Na,K-ATPase isozyme, is present in all tissues. α2 is expressed largely in heart, brain, and skeletal muscle; α3 is mostly expressed in neuronal tissue; and α4 is a testis-specific isoform. β2 expression is known to be specific for heart, skeletal muscle, and neuronal tissue, while β3 is present in many tissues at varying levels. The expression of the gastric H,K-ATPase is restricted almost entirely to parietal cells of stomach, though low levels of mRNAs were found in kidney (4). The expression of the nongastric H,K-ATPase α subunit was detected in colon, kidney, skin, brain, and prostate (16–18).

Recently, we have identified a new member of the mammalian X,K-ATPase β-subunit gene family. It was determined that protein primary structures deduced from sequences of human and rat cDNAs are highly homologous (89% identity) and exhibit from 30 to 40% identity with known isoforms of the Na,K-ATPase and the H,K-ATPase β-subunits. Expression of the human and rat genes was shown to be strictly limited to skeletal and heart muscles; therefore, these hitherto unknown mammalian proteins were termed βm (β_{muscle}) (19).

Although the deduced primary structure of βm has all of the above mentioned structural features typical for known X,K-ATPase β-subunits, its structural organization differs significantly from that of other isoforms due to the presence of an additional N-terminal cytoplasmic domain of about 7 kDa containing two negatively-charged poly-Glu sequences (19, 20). None of the known members of the X,K-ATPase family contains this kind of sequence motif. Another specific feature of β_m proteins is the existence of two variants, products of alternative splicing, that differ in the presence or absence of four residues in the junction between cytoplasmic and transmembrane domains (19, 20).

In subsequent studies, we confirmed the existence of the βm protein in human skeletal muscle using specific antibodies generated against a recombinant polypeptide formed by the extramembrane βm domains (21). We also revealed that the status of βm glycosylation is unusual for the mature forms of X,K-ATPase β-subunits: in contrast to all other β-subunits, the carbohydrate moiety of βm is composed of short oligomannose or hybrid N-glycans. These observations argue in favor of the βm localization to the intracellular compartments of muscle cells (20, 21).

We herein report the characterization of pig βm subunit: its primary structure, tissue-specific expression in comparison with other X,K-ATPase β-subunit isoforms, and its localization in subcellular membrane fractions.

MATERIALS AND METHODS

Animals and tissues. Animal studies were approved by the Animal Care and Use Committee of the Medical College of Ohio. Female 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). Adult pig tissues were purchased in a local slaughterhouse, put on ice, and used as soon as possible. Unless otherwise stated, "heart" stands for left ventricle, "stomach," "duodenum," and "colon" for the corresponding mucosal scrapings; "airway" for the nasal cavity epithelium with underlying septa; and "skeletal muscle" for pooled samples of hindlimb muscles and diaphragm.

RT-PCR, DNA cloning, and sequencing. RNA isolation and RT-PCR were performed essentially as described before (18, 19). Primer pairs PU and TU, VEKO1 and BS1, and VEKO1 and TU (19) were used to independently amplify 3′-, 5′-fragments, and the full ORF, respectively. The PCR products were cloned using the pGEM-T PCR cloning kit (Promega, Madison, WI). The cloned PCR products were sequenced using the walking primer strategy and the *f*mol DNA cycle sequencing kit (Promega). Several clones resulting from PCR with primers VEKO1-BS1 (19) were sequenced to determine the structure of both alternative transcripts. To ensure the absence of PCR-introduced errors, every ORF region was covered with at least two reads of two clones from independent amplifications.

To analyze tissue-specific expression, primers DSBNF and DSBNB (19), which are complementary to the ectodomain region of βm, were used in a RT-PCR reaction, as described previously (18). For RT-PCR analysis of other β-subunit isoforms the following primer pairs complementary to nucleic acid sequences that are conserved between human, rat, and mouse were used: 1FBE (arggcagctggaagaaattcatc) and 1BBE (tcactgtacccaatgttctcacc) for β1; 2FBER (aragctgcgggcaggtggttgag) and 2BBE (cctgcatagaagttgatgacc) for β2; 3FBE (artaycgwgaccagattcctag) and 3BBE (ckgaacactrtgatctggaag) for β3. For pig βg, the primer pair P1HKBF (tacggggagaaaggcctggaca) and P1HKBB (gctatagtggggctgggctttc) was used and the annealing temperature was set at 63°C.

[3] Abbreviations used: α1-, α2-, α3-, α4-, β1-, β2-, β3-, subunits of Na,K-ATPase; βg, gastric H,K-ATPase β-subunit; βm, muscle-specific βm-protein, member of X,K-ATPase β-subunit family; PMSF, phenylmethylsulfonyl fluoride; RT-PCR, reverse transcription-polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PNGase F, peptide-*N*-glycosidase F; RT-PCR, reverse transcription and polymerase chain reaction; UTR, untranslated region; WGA, wheat germ agglutinin.

Agarose gels were stained with ethidium bromide or SYBR Gold (Molecular Probes, Eugene, OR), and imaged and analyzed using a Typhoon 8600 laser scanner (Amersham Pharmacia, Piscataway, NJ).

Membrane fractionation. Several published methods of rabbit or rat skeletal muscle membrane fractionation by sucrose gradient centrifugation (22–25) were used for pig skeletal muscle without significant changes except that all solutions were supplemented with protease inhibitors (PMSF 150 μM, 1 μM leupeptin, 1 μM pepstatin, 1 μM bacitracin). Briefly, the method of Chu *et al.* (22) allows the separation of fractions enriched in surface membranes (floating on 27% sucrose, F27) and in different reticular membranes referable as light or longitudinal (F32), intermediate (F34 and F38) and heavy reticulum, or junctional terminal cisternae (F45). The method of Muñoz *et al.* (23) produces a sarcoplasmic reticulum fraction (F35) as well as membranes enriched in GLUT4-containing vesicles (F26 and F29). In this method the fraction of surface membranes can be separated by wheat germ agglutinin (WGA) precipitation into fractions referable as sarcolemma and T-tubules. The method of Rosemblatt *et al.* (24) provides enrichment in T-tubules (F25), as well as in light (F35) and heavy (F50) reticulum. The method of Klip *et al.* (25) was designed to separate surface membranes (F25), GLUT4-enriched "intracellular stores" (F30), and sarcoplasmic reticulum (F35).

Immunoblotting and deglycosylation. Immunoblotting was performed as described with affinity purified polyclonal antibodies against recombinant human βm that lacks its transmembrane part (21). Densitometry was performed using a Bio-Rad imaging densitometer (Bio-Rad Laboratories, Hercules, CA). Na,K-ATPase β1-subunit was detected with polyclonal antibodies against the human recombinant ectodomain (26). Anti-53-kDa glycoprotein antibodies were mouse monoclonals against the protein from rabbit skeletal muscle (27). Deglycosylation was performed by treatment with PNGase F as described previously (21).

RESULTS

Primary Structure of Pig βm

The coding region of pig βm mRNA was reconstructed by sequencing RT-PCR products and was found to contain a 1068-nt open reading frame. The second, "short," form of cDNA lacking 12 bp within the open reading frame, has been identified in pig skeletal muscle, thus indicating that the alternative splicing of pig βm mRNA also takes place similarly to that previously demonstrated in human and rat tissues (19).

The primary product of the "long" pig cDNA translation consists of 355 amino acid residues and has a molecular mass of 41.3 kDa (p*I* 4.64) (Fig. 1). This new protein sequence exhibits all of the structural features that are common to the X,K-ATPase β-subunits and also shares specific peculiarities of the βm subgroup. These include the transmembrane topology of type II membrane proteins and a typical domain organization that contains a highly charged cytoplasmic N-terminal domain followed by a single hydrophobic transmembrane α-helical segment of 27 amino acids (Leu109–Leu134) and a large extracytoplasmic C-terminal domain (3, 7, 8). The tetrapeptide 105QSLS108, a part of the conserved sequence located in the junction between the cytoplasmic and the membrane domains, is deleted in the second, "short," βm variant as deduced from the alternatively spliced form of βm cDNA.

The pig βm primary structure contains structural elements that are common for the ectodomains of the X,K-ATPase β-subunits. These include: the highly conserved sequence motif Tyr 293-Tyr-Pro-Tyr-Tyr-Gly-Lys299, six Cys residues (positions 199, 218, 228, 246, 267, and 327) that are expected to form three sequential disulfide bridges, and four consensus sites for N-glycosylation (Asn residues in positions 166, 188, 227, 304).

The level of the overall sequence homology between pig βm protein and other known pig X,K-ATPase β-subunits comprises 29.5% for Na,K-ATPase β1 (28) and 31.5% for the β of gastric H,K-ATPase (29). In contrast to other pig β-subunits, the deduced amino acid sequence of βm includes about 60 additional residues located in the N-terminal cytoplasmic domain. This addition contains two negatively charged clusters formed predominantly by glutamic acid residues that make βm a very acidic protein (compare the calculated p*I* of βm (4.64) with that of β1 (8.54) or βg (8.19). As predicted from the secondary structure, these Glu-rich sequences can be organized in a "hairpin" structure formed by long α-helixes Asp22–Ala42 and Glu51–Glu70 connected by a flexible loop. None of the known members of the X,K-ATPase family contains this sequence motif that appears to be unique to βm proteins.

Tissue-Specific Expression of Pig βm by Comparison to Other X,K-ATPase β-Subunits

RT-PCR analysis of the tissue-specific gene expression of all of the known X,K-ATPase β-subunits in newborn pigs is presented in Fig. 2. These data illustrate that the expression of the βm (ATP1B4) in newborn pigs is restricted to skeletal muscle, heart, and skin. This pattern is the same as in adult human and rat where it is present in high amounts in skeletal muscle, lower in heart, even lower in skin, and absent in all other tissues tested (19). A high number of cycles used for βm in comparison with other isoforms in Fig. 2 strengthens the conclusion that βm expression is highly tissue-specific. No significant amount of its transcripts can be detected in any tissues other than skeletal muscle, heart, and skin. Very likely, the βm expression observed in skin is due to the presence of attached skeletal muscle; though we have no direct evidence to support this hypothesis. Only trace levels of expression can be observed in other tissues: faint bands can be seen in thymus, ovary, uterus, airway, and kidney (Fig. 2) and in most of the tissues when a very high number of PCR cycles is used (40 and more, results not shown).

It should be noted that in this set (Fig. 2) we included also tissues that were previously not tested in the rat and human (19). This was important because several ESTs matching 3′-UTR of human βm mRNA were se-

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Hum  M R R Q L R S R R A P s F P Y s Y r Y R L D D p D E a N Q N Y L A D E E E E A E  40
Pig  M R R Q L R S R R A P A F P Y G Y G Y R L D D Q D E V N Q N Y L A D E E E E A E  40
Rat  M R R Q L R S R R A P A F P Y G Y r Y R L D D Q D E m N H N Y L A D E E E E A E  40

Hum  E E A R V t V V P k s E E E E e E E E k E E e E E e E K E E E E g q g Q p T g n  80
Pig  E - A R V M V V P D L E E E E K E E E - E E K E E D E K E E E E S H H Q D T R S  78
Rat  E E A q V M m V P g L E E E E e E E E - g k e E E e E r E E E E g q g Q s T g n  79

Hum  A W W Q K L Q I m s E Y L W D P E r R M f L A R T G|Q S w S|L i L l I Y F F F Y 120
Pig  A W W Q K L Q I V N E Y L W D P E K R M S L A R T G|Q S L S|L L L V I Y F F F Y 118
Rat  A W W r K L Q I V N E Y L W D P E K R M S L A R T G|Q S r S|L i L V I Y F F F Y 119
                                                             =====transmembrane==
Hum  A S L A A V I T L C M Y T L F L T I S P Y i P T F T E R V K P P G V M I R P F A 160
Pig  A S L A A V I T L C M Y T L F L T I S P Y V P T F T E R V K P P G V M I R P F A 158
Rat  A S L A A V I T L f i Y m L F L a I S P Y m P T F T E q V K P P G V M I R P F A 159
     =segment===============
Hum  H S L N F N F N V S E P D T W Q H Y V I S L N G F L Q G Y N D S L Q E E M N V D 200
Pig  H S L N F N F N V S E P D T W Q H Y V I S L N G F L Q G Y N D S L Q E E M N V D 198
Rat  H S L N F N F N V S E P e T W Q r Y V I S L N G F L Q G Y N D S L Q E E M N i D 199

Hum  C P P G Q Y F I Q D G n E D E D K K A C Q F K R S F L K N C S G L E D P T F G Y 240
Pig  C P P G Q Y F I Q D G D E D E D K K A C Q F K R S F L K N C S G L E D P T F G Y 238
Rat  C P P G Q Y F I Q D G D E D E D K K A C Q F K R S F L K N C S G L E D P T F G Y 239

Hum  S T G Q P C I L L K M N R I V G F R P E L G D P V K V S C K V Q R G D E N D I R 280
Pig  S T G Q P C I L L K M N R I V G F R P E L G D P V K V S C K V Q R G D E N D I R 278
Rat  S T G Q P C I L L K M N R I V G F R P E f G D P V K V S C K V Q k G D E N D I R 279

Hum  S I S Y Y P E S A S F D L R Y Y P Y Y G K L T H V N Y T S P L V A M H F T D V V 320
Pig  S I S Y Y P E S A S F D L R Y Y P Y Y G K L T H V N Y T S P L V A M H F T D V V 318
Rat  S I n Y Y P E S A S F D L R Y Y P Y Y G K L T H V N Y T S P L V A M H F T D V V 319

Hum  K N Q A V P V Q C Q L K G K G v I N D V I N D R F V G R V I F T L N I E T       357
Pig  K N Q A V P V Q C Q L K G K G I I N D V I N D R F V G R V I F T L N I E T       355
Rat  K N Q e V P V Q C Q L K G K G I v N D V I N D R F V G R i I F T L N I E T       356
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FIG. 1. Comparison of the amino acid sequences of the pig, human, and rat βm proteins. "Long" alternative splice variants of proteins are shown. Tetrapeptides that are absent in "short" alternative splice variants are boxed. Nonconserved amino acid residues are depicted by lowercase letters in italic. Conserved cysteines are shown in bold. Consensus sequences of potential sites of N-glycosylation are underlined. The amino acids are numbered at the right of the sequences.

quenced in a cDNA library from fetal human retina (GenBank Accession Nos. AA490116, AA504702, and AA780676). Also, Wetzel *et al.* (30) have found that undifferentiated neuroblasts in postnatal mouse retina lacked detectable Na,K-ATPase β-subunits and this led to the hypothesis that an undiscovered β-subunit isoform must exist. As seen in Fig. 2, lane 1, newborn pig retina has no detectable amount of βm transcripts. However, the possibility cannot be excluded that the βm can be expressed in retina in a developmentally restricted manner.

The expression of other β-subunit isoforms in pig tissues had not been studied in detail. For this reason, and also for comparison with βm, we analyzed the expression of β1, β2, β3, and βg (Fig. 2). β1 was reliably detected in all tissues tested but the expression level was variable: the highest in kidney, choroid plexus, and colon, and somewhat less abundant in retina, duodenum, skeletal muscle, and thyroid. Its expression in uterus, ovary, thymus, and brown fat was relatively low. β2 content was the most abundant in retina, and also high in some brain regions and adrenal. In many tissues like colon or liver its expression level was very low. β3 could be detected in many tissues but at an apparently low level, the most prominent in colon. Adrenal, duodenum, mesenterium, salivary gland, and retina had a somewhat higher content of β3 transcripts than other tissues. Gastric β-subunit was abundant only in the stomach, though its transcripts were readily detectable also in heart and retina, and at trace levels (at high number of cycles)—in a majority of the tissues examined.

Relative abundance of βm mRNA in both newborn and adult skeletal muscle, as well as in heart and skin is illustrated in Fig. 3, where RT-PCR products were analyzed after a low number of cycles. Measuring their fluorescence intensities indicates that transcripts of pig βm are present in skeletal muscle at several fold higher levels than in heart. Moreover, the βm transcripts are more abundant in newborn skeletal muscle and in heart than in the corresponding adult tissues. Interestingly, β1 also has a significantly higher expres-

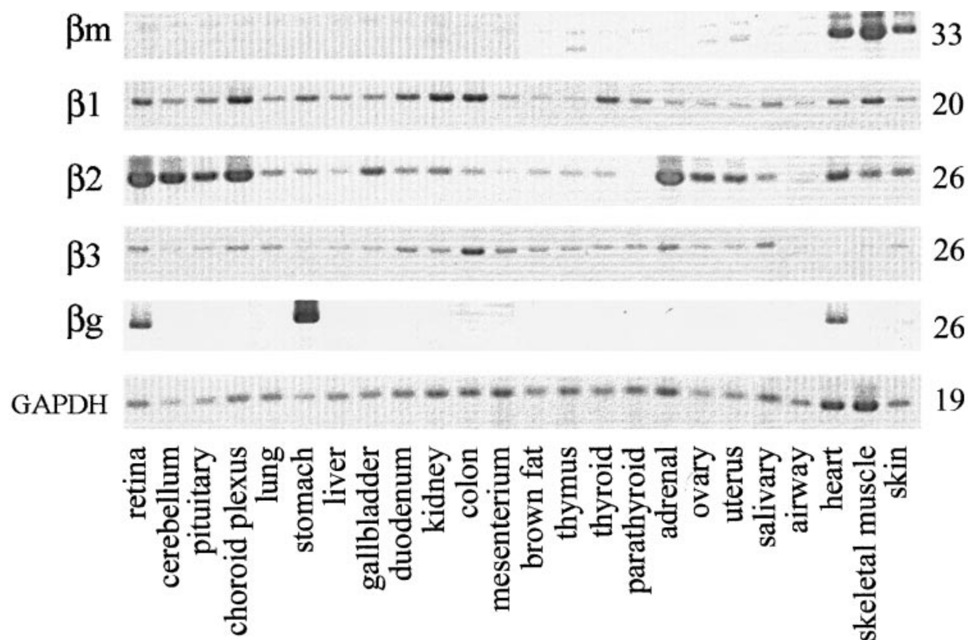

FIG. 2. RT-PCR analysis of tissue-specific expression of βm and known X,K-ATPase β-subunits in the newborn pig. RT-PCR products from 0.1 μg total RNA were electrophoresed and stained with ethidium bromide. The numbers of cycles shown at the right were chosen for each isoform to compare expression levels in different tissues. The band intensities may not necessarily reflect relative contents of different isoforms in same tissue.

sion level in newborn muscular tissues than in those of adult animals. However, in contrast to βm, β1 has little difference between the heart and muscle in the newborn and is more abundant in heart than in muscle in the adult pig. β2 and β3 are expressed at low levels in adult muscle and the heart which are comparable with those of newborn tissues (results not shown).

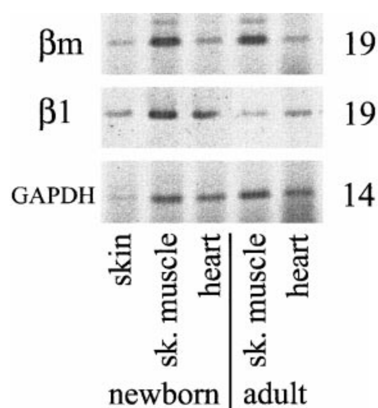

FIG. 3. RT-PCR analysis of the expression of porcine βm and β1 subunits of X,K-ATPase in newborn and adult skeletal muscle, heart, and skin. RT-PCR products from 0.1 μg total RNA were electrophoresed and stained with SYBR Gold. The numbers of cycles shown at the right were kept at a minimum so that to ensure proportionality of the fluorescence intensities of the RT-PCR products to the amount of the template. The results of one of three independent experiments which produced similar results are shown.

Detection of the βm Protein in Pig Skeletal Muscle by Western Blotting and Analysis of Its Glycosylation State

Affinity purified antibodies against human recombinant βm protein recognize a specific band in pig skeletal muscle microsomes with an electrophoretic mobility that corresponds to an apparent molecular weight of 57 kDa (Fig. 4). After deglycosylation with PNGase F the mobility is shifted to an apparent molecular weight of 52 kDa. The anomalously low electrophoretic mobility of pig βm protein is very similar to that of human βm protein (21). The electrophoretic mobility shift of βm after deglycosylation can be compared to that of

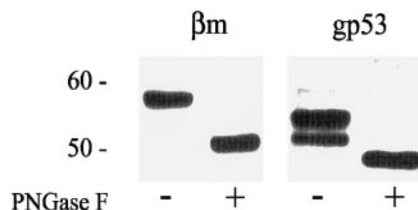

FIG. 4. Immunoblotting of microsome proteins from newborn pig skeletal muscle with antibodies against recombinant X,K-ATPase β_m-subunit and deglycosylation shift assay. Membrane proteins were treated or not treated with PNGase F, resolved on a 12% polyacrylamide–SDS gel and analyzed by Western blotting with antibodies against β_m or monoclonal antibodies against 53-kDa glycoprotein. Positions of molecular mass markers (10-kDa protein ladder, Life Technologies) are shown to the left.

one of the major sarcoplasmic reticulum proteins, the 53-kDa glycoprotein (Fig. 4; "gp53"). One can see that the shift of βm is somewhat larger than that of the 53-kDa glycoprotein, possibly reflecting the higher carbohydrate content of βm. Indeed, the 53-kDa glycoprotein has two N-glycosylation sites while βm has four and, most probably, all of these four sites are occupied with oligosaccharide chains. Interestingly, the 53-kDa glycoprotein in newborn pig skeletal muscle gives two closely migrating bands (Fig. 4; "gp53"), most likely due to the presence of two differentially glycosylated species.

Subcellular Localization of βm Protein Using Membrane Fractionation

Several published methods for enrichment (22–25) of various membranes were employed to investigate subcellular location of the βm protein in newborn and adult pig skeletal muscle. In general, these methods are variations of the isopycnic centrifugation of Ca-phosphate loaded membrane vesicles in sucrose density gradients. The necessity for the use of several procedures was dictated by the fact that each method was optimized for purification of particular membranes (sarcoplasmic reticulum, T-tubules etc.) and all of the methods were developed for skeletal muscles from animals other than pig. Fractions from the sucrose gradient centrifugations were analyzed by immunoblotting with antibodies against human recombinant βm (Fig. 5).

First, newborn pig skeletal muscle membranes were separated by isopycnic centrifugation according to the method of Chu *et al.* (22) (Fig. 5A). Though some βm protein was detected by chemiluminescence technique in all fractions, the highest content of the protein was found in fractions of 34% and 36% sucrose (F34 and F38; Fig. 5A) that were referred to as "intermediate" sarcoplasmic reticulum (22). On the contrary, fractions enriched in surface membrane (F27), longitudinal sarcoplasmic reticulum (F32), and junctional reticulum (F45) were relatively poor in βm content.

The separation method of Muñoz *et al.* (23) was also employed (Fig. 5B). This method was reported to achieve enrichment of glucose transporter GLUT4-containing vesicles and separation of plasmalemma and T-tubules by WGA precipitation of plasmalemmal vesicles. The highest concentration of the βm protein was found in fraction F35 (sarcoplasmic reticulum). Fraction F29 (GLUT4-containing vesicles) and F23 (surface membranes) are relatively poor in βm (Fig. 5B). βm was also present in WGA nonprecipitable membranes (F23w−). This can be interpreted as either βm is present in T-tubules or the protein is present in sarcoplasmic reticulum contamination of the fraction of "surface membrane." It should be noted that the WGA

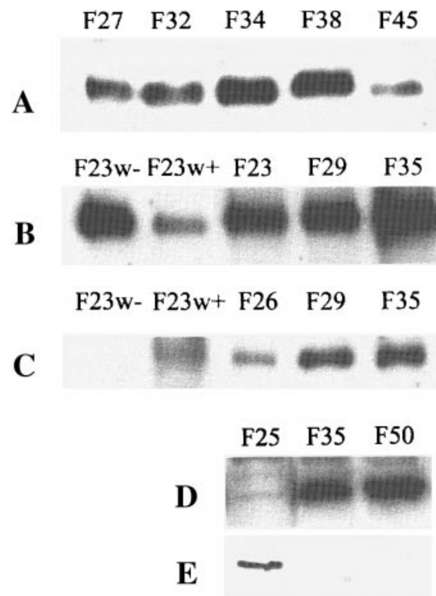

FIG. 5. Intracellular localization of βm protein in porcine skeletal muscle by fractionation of subcellular membranes. Equal amounts of protein from corresponding sucrose density gradient centrifugation fractions were analyzed by immunoblotting. The fractions are numbered according to their buoyant densities as described under Materials and Methods. (A) Separation according to Chu *et al.* (22), newborn pig; (B, C) Separation according to Muñoz *et al.* (23): (B) Newborn pig, (C) Adult pig, F23w+, and F23w− represent membranes from fraction F23 that are, respectively, precipitable and nonprecipitable by WGA; (D, E) Separation of adult pig membranes according to Rosemblatt *et al.* (24): (D) Anti-βm antibodies, (E) Anti-β1 antibodies. For the detection of β1 the proteins were deglycosylated with PNGase F.

precipitation method of Muñoz *et al.* (23) that was originally developed for adult rat skeletal muscle, gave different results for newborn pig membranes—almost all of the membranes were precipitated with WGA. Possibly this reflects the fact that in neonatal skeletal muscle the T-tubule system (WGA nonprecipitable membranes) is not yet completely developed (31). We propose that the anti-βm reactivity in the fraction F23w− most probably represents a contamination with sarcoplasmic reticulum. However, to exclude the possibility that the βm protein is present in T-tubules, we reproduced separation of adult pig skeletal muscle membranes by the method of Muñoz *et al.* (23) and also employed the method of T-tubule enrichment by Rosemblatt *et al.* (24). Results are shown in Figs. 5C and 5D. The strongest signals of βm protein were observed in fractions with buoyant density corresponding to 35% sucrose (F35) that represent sarcoplasmic reticulum. The protein was not detected in fractions that were enriched with T-tubules (F23w− in Fig. 5C, and F25 in Fig. 5D). On the contrary, Na,K-ATPase β1 protein was reliably detected only in the light fractions that represent the surface membranes (F25; Fig. 5E).

This also indicates that the method of Rosemblatt *et al.* (24) provides the effective separation of the sarcoplasmic reticulum from the surface membranes. However, the opposite is not true, since SERCA is readily detectable in all, including the lightest, fractions (results not shown). Thus the relatively weak band of βm detected in surface membrane fractions could reflect some contamination with reticular membranes.

It should be emphasized that the isopycnic ultracentrifugation techniques used here cannot completely and accurately separate different membrane fractions and some contamination is always present. One should keep in mind that most of these methods were optimized for white muscles of young rabbits (22, 24). Thus, it is impossible to distinguish whether weak signals observed in other fractions are due to the incomplete separation of the membranes or due to the presence of the protein in these membranes *in vivo.* Although at this time we cannot rule out that some βm protein exists in other membrane compartments, it is clear that the βm is enriched in sarcoplasmic reticulum. Also, considering the absolute amounts of the protein, it can be concluded that, as detected with specific antibodies, the majority of the βm in pig skeletal muscle is located intracellularly in the sarcoplasmic reticulum.

DISCUSSION

The availability of three closely related sequences from different species (Fig. 1) allows us to consider the specific structural features of this group of hitherto unknown proteins which (from the structural point of view) obviously belong to the family of the X,K-ATPase β-subunits (19, 20). The overall sequence identity between pig and human βm proteins is 92% and between pig and rat βm is 89%. These data clearly indicate that the pig, human and rat βm proteins are conservative homologues. In this respect βm proteins occupy an intermediate position between very conserved β1 and β2 (~94% identity) and more divergent β3 and βg (~81%).

It should be noted that variable amino acid residues are not evenly distributed along the βm polypeptide chains (Fig. 1). In the most conserved ectodomains, pig and human sequences differ only by two isofunctional residues (98.6% identity); pig and rat sequences share 95% identical amino acids, and human and rat 93.7%. The level of identity is slightly lower within intramembrane segments and comprises 92.3% for pig–human pair and 84.6% for both pig–rat and human–rat pairs. Cytoplasmic domains are the most variable parts of βm polypeptides. In contrast to ectodomains and membrane segments, pig and human sequences of cytoplasmic domains exhibit higher diversity (76.8% identity) than pig and rat (80.9%) or human and rat (80.2%). Variable residues are predominantly concentrated in junctions between strictly conserved N-terminal undecapeptides containing 5 Arg residues and Glu-rich stretches presumably organized into α-helixes. This may reflect that sequence requirements for the particular parts of βm cytoplasmic domains were not as strict during the evolution as in the case of the ectodomains that are highly conserved. As an alternative explanation, one can think of sequence variability of the N-terminal part as a requirement for the functional fitness for the molecular interactions in which the cytoplasmic domain may be involved.

The presence of clusters of acidic, predominantly Glu, residues within the N-terminal cytoplasmic domain is a striking feature unique to the βm proteins within the X,K-ATPase family. The region Asp22–Glu70 of pig βm sequence contains 23 Glu and 6 Asp and only 1 Arg and 3 Lys. Similar highly acidic domains have been found in a variety of proteins unrelated to the X,K-ATPase family, such as bone sialoprotein, N-arginine dibasic convertase, PP2Cg phosphatase, etc; but their functions are largely undefined. Here, it should be mentioned that acidic amino acids stretches were found in a number of Ca-binding and Ca-transporting proteins in muscle cells, such as ryanodine receptor, voltage-gated calcium channel, calsequestrin, calreticulin, etc. (See for review (32)). This raises the possibility that the cytoplasmic domain of βm may interact with calcium.

The clusters of Glu-rich sequences are encoded by an additional exon 2 in the human βm gene (19) that was not found in other related X,K-ATPase genes. It is possible to speculate that this particular exon was acquired during evolution, as a component needed for performance of a yet unknown βm function. We suggest that the cytoplasmic acidic region of the βm may be important for intracellular targeting or for interactions with specific ligands or regulators. This may also indicate that actual functional role of the βm proteins is different from that of other known members of the X,K-ATPase β-subunit family.

The results presented here imply that the βm protein is not translocated from the ER (SR) to the plasma membrane (at least, most of the protein). Indirect support for βm being an ER (SR) resident derives from certain peculiarities of the βm structure. All of the known orthologous βm proteins (Fig. 1) have identical sequence of N-terminal undecapeptides MRRQLRSR-RAP. Their characteristic feature is two consecutive double-arginine or XXRR-motifs whose essential elements are two Arg residues close to the N-terminus. This kind of sequence motif has been identified as being responsible for localizing some type II membrane proteins to the endoplasmic reticulum (33, 34).

Previously, we hypothesized that βm may be involved in recycling of the “intracellular stores” that

contain Na,K-ATPase (21). These membrane compartments have been postulated from fractionation experiments of rat skeletal muscle membranes before and after insulin treatment (35, 36). It was proposed that these vesicles are analogous to the glucose transporter GLUT4-containing endosomes, but are distinct species (37). However, our attempts to reproduce the method of Klip *et al.* (25) that was developed for fractionation of membranes of adult rat skeletal muscle, resulted in a relatively poor separation of specific membrane compartments and we did not observe enrichment of βm in the corresponding fractions (results not shown). Using the method of Muñoz *et al.* (23), we detected βm in GLUT4-containing fractions but at a much lower abundance than in reticular fractions (Figs. 5B and 5C). Thus, the possibility of βm involvement in the insulin-responsive membrane vesicle recycling in skeletal muscle cannot be excluded at this point. However, the predominant abundance of βm in the light sarcoplasmic reticulum fractions and the absence of complex type glycosylation suggest that the presence of βm in the recycled vesicles is not likely.

Our data on the tissue-specific expression of X,K-ATPase β-subunit genes in newborn piglets are in agreement with studies in other species (see for review (3)). This demonstrates that among the five known members of the X,K-ATPase β-subunit family, two of them (βm and βg) are highly tissue-specific, while β1 and β3 are almost ubiquitous, though β3 is a rare species, and their expression levels vary between tissues. We observed that β3 has the highest level of expression in colon (among the tissues tested). This does not contradict the previous observation that β3 is the most abundant in rat testes (38, 39) because here we studied only female piglets. But, curiously, this supports the extravagant classification of β3 as the “colonic” isoform (40). However, from these and other studies (30, 38, 39, 41–43) it is clear that β3 expression lacks the high specificity of βm and βg isoforms. From the initial studies on cDNA cloning, the gastric H,K-ATPase β-subunit, βg, was known as an isoform expressed exclusively in stomach (44). However, later studies demonstrated that lower levels of this isoform can also exist in other tissues. For example, βg transcripts were detected in inner ear (45). Here, we presented novel results that indicate the possible existence of appreciable amounts of βg also in retina and heart.

The major physiological function of βg is well known—it assembles with the catalytic H,K-ATPase α-subunit to form an active proton pump of the stomach. βm, the most recently discovered isoform, should similarly have a specialized and important function. Determination of primary structure of βm and optimization of membrane preparations from the pig—a convenient animal for large-scale protein experiments—provide the basis for isolation and further characterization of βm-protein.

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REFERENCES

1. Pedersen, P. L., and Carafoli, E. (1987) *Trends Biochem. Sci.* **12,** 146–150; 186–189.
2. Møller, J. V., Juul, B., and Le Maire, M. (1996) *Biochim. Biophys. Acta* **1286,** 1–51.
3. Blanco, G., and Mercer, R. W. (1998) *Am. J. Physiol.* **275,** F633–F650.
4. Hersey, S. J., and Sachs, G. (1995) *Physiol. Rev.* **75,** 155–190.
5. Modyanov, N. N., Mathews, P. M., Grishin, A. V., Beguin, P., Beggah, A. T., Rossier, B. C., Horisberger, J.-D., and Geering, K. (1995) *Am. J. Physiol.* **269,** C992–C997.
6. Adams, G., Tillekeratne, M., Yu, C., Pestov, N. B., and Modyanov, N. N. (2001) *Biochemistry* **40,** 5765–5776.
7. McDonough, A. A., Geering, K., and Farley, R. A. (1990) *FASEB J.* **4,** 1598–1605.
8. Chow, D.-C., and Forte, J. C. (1995) *J. Exp. Biol.* **198,** 1–17.
9. Geering, K. (2000) *J. Membr. Biol.* **174,** 181–190.
10. Béguin, P., Hasler, U., Beggah, A., Horisberger, J. D., and Geering, K. (1998) *J. Biol. Chem.* **273,** 24921–24931.
11. Beggah, A. T., Béguin, P., Bamberg, K., Sachs, G., and Geering, K. (1998) *J. Biol. Chem.* **274,** 8217–8223.
12. Geering, K., Crambert, G., Yu, C., Korneenko, T. V., Pestov, N. B., and Modyanov, N. N. (2000) *Biochemistry* **39,** 12688–12698.
13. Crambert, G., Hasler, U., Beggah, A. T., Yu, C., Modyanov, N. N., Horisberger, J. D., Lelievre, L., and Geering, K. (2000) *J. Biol. Chem.* **275,** 1976–1986.
14. Sverdlov, E. D., Monastyrskaya, G. S., Broude, N. E., Ushkaryov, Yu. A., Allikmets, R. L., Melkov, A. M., Smirnov, Yu. V, Malyshev, I. V., Dulobova, I. E., Petrukhin, K. E., Grishin, A. V., Kijatkin, N. I., Kostina, M. B., Sverdlov, V. E., Modyanov, N. N., and Ovchinnikov, Yu. A. (1987) *FEBS Lett.* **217,** 275–278.
15. Shull, M. M., and Lingrel, J. B. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 4039–4043.
16. Sverdlov, V. E., Kostina, M. B., and Modyanov, N. N. (1996) *Genomics* **32,** 317–327.
17. Grishin, A. V., Sverdlov, V. E., Kostina, M. B., and Modyanov, N. (1994) *FEBS Lett.* **349,** 144–150.
18. Pestov, N. B., Romanova, L. G., Korneenko, T. V., Egorov, M. V., Kostina, M. B., Sverdlov, V. E., Askari, A., Shakhparonov, M. I., and Modyanov, N. N. (1998) *FEBS Lett.* **440,** 320–324.
19. Pestov, N. B., Adams, G., Shakhparonov, M. I., and Modyanov, N. N. (1999) *FEBS Lett.* **456,** 243–248.
20. Pestov, N. B., Adams, G., Kostina, M. B., Shakhparonov, M. I., and Modyanov, N. N. (2000) *in* The Na,K-ATPase and Related ATPases (Taniguchi, K., and Kaya, S., Eds.), pp. 257–260. Elsevier.
21. Pestov, N. B., Korneenko, T. V., Zhao, H., Adams, G., Shakhparonov, M. I., and Modyanov, N. N. (2000) *Biochem. Biophys. Res. Commun.* **277,** 430–435.
22. Chu, A., Dixon, M. C., Saito, A., Seiler, S., and Fleischer, S. (1988) *Methods Enzymol.* **157,** 36–46.

23. Muñoz, P., Rosemblatt, M., Testar, X., Palacín, M., and Zorzano, A. (1995) *Biochem. J.* **307,** 271–280.
24. Rosemblatt, M., Hidalgo, C., Vergara, C., and Ikemoto, N. (1981) *J. Biol. Chem.* **256,** 8140–8148.
25. Klip, A., Ramlal, T., Young, D. A., and Holloszy, J. O. (1987) *FEBS Lett.* **224,** 224–230.
26. Wang, J., Schwinger, R. H., Frank, K., Muller-Ehmsen, J., Martín-Vasallo, P., Pressley, T. A., Xiang, A., Erdmann, E., and McDonough, A. A. (1996) *J. Clin. Invest.* **98,** 1650–1658.
27. Leberer, E., Charuk, J. H., Clarke, D. M., Green, N. M., Zubrzycka-Gaarn, E., and MacLennan, D. H. (1989) *J. Biol. Chem.* **264,** 3484–3493.
28. Ovchinnikov, Y. A., Modyanov, N. N., Broude, N. E., Petrukhin, K. E., Grishin, A. V., Arzamazova, N. M., Aldanova, N. A., Monastyrskaya, G. S., and Sverdlov, E. D. (1986) *FEBS Lett.* **201,** 237–245.
29. Toh, B.-H., Gleeson, P. A., Simpson, R. J., Moritz, R. L., Callaghan, J. M., Goldkorn, I., Jones, C. M., Martinelli, T. M., Mu, F.-T., Humphris, D. C., Pettitt, J. M., Mori, Y., Masuda, T., Sobieszczuk, P., Weinstock, J., Mantamadiotis, T., and Baldwin, G. S. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 6418–6422.
30. Wetzel, R. K., Arystarkhova, E., and Sweadner, K. J. (1999) *J. Neurosci.* **19,** 9878–9889.
31. Yuan, S., Arnold, W., and Jorgensen, A. O. (1990) *J. Cell Biol.* **110,** 1187–1198.
32. Mackrill, J. J. (1999) *Biochem. J.* **337,** 345–361.
33. Schutze, M. P., Peterson, P. A., and Jackson, M. R. (1994) *EMBO J.* **13,** 1696–1705.
34. Teasdale, R. D., and Jackson, M. R. (1996) *Annu. Rev. Cell Dev. Biol.* **12,** 27–54.
35. Hundal, H. S., Marette, A., Mitsumoto, Y., Ramlal, T., Blostein, R., and Klip, A. (1992) *J. Biol. Chem.* **267,** 5040–5043.
36. Lavoie, L., Roy, D., Ramlal, T., Dombrowski, L., Martín-Vasallo, P., Marette, A., Carpentier, J. L., and Klip, A. (1996) *Am. J. Physiol.* **270,** C1421–C1429.
37. Lavoie, L., He, L., Ramlal, T., Ackerley, C., Marette, A., and Klip, A. (1995) *FEBS Lett.* **366,** 109–114.
38. Malik, N., Canfield, V. A., Beckers, M.-A., Gros, P., and Levenson R. (1996) *J. Biol. Chem.* **271,** 22754–22758.
39. Malik, N., Canfield, V., Sanchez-Watts, G., Watts, A. G., Scherer, S., Beatty, B. G., Gros, P., and Levenson, R. (1992) *Mamm. Genome* **9,** 136–143.
40. Sangan, P., Kolla, S. S., Rajendran, V. M., Kashgarian, M., and Binder, H. J. (1999) *Am. J. Physiol.* **276,** C350–C360.
41. Arystarkhova, E., and Sweadner, K. J. (1997) *J. Biol. Chem.* **272,** 22405–22408.
42. Wetzel, R. K., and Sweadner, K. J. (2001) *Invest. Ophthalmol. Vis. Sci.* **42,** 763–769.
43. Martín-Vasallo, P., Wetzel, R. K., Garcia-Segura, L. M., Molina-Holgado, E., Arystarkhova, E., and Sweadner, K. J. (2000) *Glia* **31,** 206–218.
44. Canfield, V. A., Okamoto, C. T., Chow, D., Dorfman, J., Gros, P., Forte, J. G., and Levenson, R. (1990) *J. Biol. Chem.* **265,** 19878–19884.
45. Lecain, E., Robert, J. C., Thomas, A., and Tran Ba Huy, P. (2000) *Hear. Res.* **149,** 147–154.