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Accumulation of β_m, a structural member of X,K-ATPase β-subunit family, in nuclear envelopes of perinatal myocytes

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Zhao, Hao, Nikolay B. Pestov, Tatyana V. Korneenko, Mikhail I. Shakhparonov, and Nikolai N. Modyanov. Accumulation of β_m, a structural member of X,K-ATPase β-subunit family, in nuclear envelopes of perinatal myocytes. *Am J Physiol Cell Physiol* 286: C757–C767, 2004. First published December 3, 2003; 10.1152/ajpcell.00358.2003.—Recently discovered muscle-specific β_m protein is structurally closely related to the X,K-ATPase β-subunits. However, it has a number of unique properties such as predominant localization in intracellular stores and lack of association with known X,K-ATPase α-subunits on heterologous coexpression. In this study, the primary structure of mouse β_m was determined and developmental regulation of the gene (ATP1B4) was analyzed. The expression is first detected at *day 14* of gestation, is sharply increased at *day 16*, and reaches its maximum at *day 18*. After birth, the expression quickly decreases and is hardly detectable in adult mice. A more detailed subcellular localization study was undertaken, and its results indicate that β_m not only is located in sarcoplasmic reticulum but is concentrated in nuclear envelopes of both prenatal and postnatal skeletal muscles. Immunohistochemical studies show that β_m is specific to myocytes and, at the subcellular level, many nuclear envelopes are intensively labeled in both fetal and newborn skeletal muscles. Accordingly, β_m is detected by immunoblotting in purified nuclei and nuclear membranes from neonatal skeletal muscles. On transfection of human rhabdomyosarcoma cell line RD, green fluorescent protein-tagged β_m resides intracellularly with significant enrichment in nuclear envelopes, whereas β_m with transmembrane domain deleted localizes in both cytoplasm and nucleoplasm. Nuclear β_m apparently is not in association with Na,K-ATPase because we never detected its α-subunit in myonuclear membranes. These results indicate that β_m has a specialized function in mammalian perinatal myocytes, different from functions of other X,K-ATPase β-subunits. The unique temporospatial distribution of β_m protein expression suggests its important role in development of growing skeletal muscle.

ATP1B4; sodium, potassium-adenosine 5′-triphosphatase; nuclear membrane; skeletal muscle development

AMONG MORE THAN 200 P-ATPases identified in prokaryotes and eukaryotes (1), most enzymes have just a single catalytic subunit. However, the animal X,K-ATPases (Na,K-ATPase, gastric H,K-ATPase, and nongastric H,K-ATPase) contain, in addition to the catalytic α-subunit, an accessory β-subunit. Although major functions such as ATP binding, phosphorylation, and ion transport are performed by the α-subunit, the α-β interaction is required for maturation, stability, and translocation of the ATPases to plasma membrane (4, 44). Moreover, the β-subunit seems to be related to K transport, the unique feature of the X,K-ATPase family members (16, 45). The β-subunit is involved in K affinity and cardiac glycoside sensitivity (25, 45). Different α- and β-isoform combinations exhibit functional properties that may be necessary for maintaining ion homeostasis in vivo (11).

The last identified member of the X,K-ATPase β-subunit family is β_m, so named because its expression is strictly confined to skeletal muscle and heart (36). This protein shares typical X,K-ATPase β-subunit features including the overall domain disposition and possesses all conserved sequence motifs. However, only β_m possesses a very acidic NH_2 terminus and short high-mannose or hybrid *N*-glycans (40), whereas mature forms of other βs always have complex-type glycans (4, 20, 44). This glycosylation pattern suggested the subcellular location of β_m in an intracellular compartment, and not the plasma membrane as is normally the case for various X,K-ATPases (4, 20). Fractionation of porcine skeletal muscle membranes showed that β_m is concentrated in fractions corresponding to sarcoplasmic reticulum (SR) (39). Also, Crambert et al. (10) recently expressed β_m in *Xenopus laevis* oocytes and found that, as in muscles, β_m protein does not acquire complex glycans and is retained in endoplasmic reticulum (ER).

The presence of Na,K-ATPase subunits in intracellular stores of skeletal muscles has been described in several papers (14, 23, 24). However, Na,K-ATPase β_1- and β_2-subunit isoforms detected in these studies have complex-type glycosylation and appear to be in the process of recycling (stimulated by hormones such as insulin or aldosterone) (14, 24); β_m is different from these isoforms in that a major pool of the protein is located intracellularly and there is no information whether an exogenous stimulus can promote its translocation to the cell surface.

Here we demonstrate that the subcellular localization of β_m protein is unique among members of the X,K-ATPase β-subunit family. The β_m protein is especially abundant in nuclear envelopes. Also, we show that mouse β_m is induced during fetal development and decreases after birth. Thus the highest level of the β_m protein occurs in perinatal nuclear envelopes. This fact may provide a clue for future studies on the function of the β_m protein. A preliminary account of this work has been presented (37, 53).

MATERIALS AND METHODS

Animals and tissues. Female piglets were supplied within 24 h after parturition and were euthanized by intramuscular injection of 4–6 mg/kg Telazol followed by intraperitoneal pentobarbital sodium (100

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mg/kg). Adult pig tissues were purchased from a nearby abattoir, placed on ice, and used as soon as possible. Paraffin-embedded mouse embryo sections were purchased from Paragon Bioservices (Baltimore, MD). Frozen IRC mouse embryos and tissues were kindly provided by Dr. Yu Sviridov (Institute of Medicinal Chemistry, Moscow, Russia). Newborn rat tissues were kindly provided by Dr. Zi-Jian Xie (Medical College of Ohio, Toledo, OH). All procedures were conducted in conformance with the "Guiding Principles for Research Involving Animals and Human Beings" of the American Physiological Society.

RT-PCR, cDNA cloning, and sequencing. Conditions of routine RT-PCR and primer sequences for β_m expression studies were essentially as described previously (36). Agarose gels were stained with ethidium bromide and imaged with a Typhoon 8600 laser scanner (Amersham Pharmacia, Piscataway, NJ). The mouse β_m open reading frame (ORF) plus most of its 3′-untranslated region (UTR) was amplified from a 17.5-day whole fetus cDNA with primers VEKO (39) and 3MK (CCTTTTTATTTCGTTTATTGTTGGCACC) and Expand Long DNA polymerase (Roche Molecular Biochemicals, Indianapolis, IN). The following cycling parameters were used: 94°C for 30 s, 60°C for 30 s, 68°C for 10 min, 33 cycles, to give a 4-kb product. The primer VEKO is complementary to human and rat β_m, whereas the primer 3MK was designed from mouse expressed sequence tag (EST) sequences homologous to rat β_m. The PCR product was codigested with *Alu*I and *Sma*I, and the fragments were cloned in pGEM7xf vector (Promega, Madison, WI). Eight clones with inserts from 140 bp to 1 kb were sequenced, and the complete assembly was obtained by independent amplification of gaps and direct sequencing of these shorter PCR products.

Bacterial protein expression and purification. To produce the recombinant ectodomain of mouse β_m, the corresponding region (Phe^{139}–Thr^{356}) was amplified with primers MECTO (GGATAGATCTCCGACCTTCACCGAGCAGGTG) and BCHINM (ATTAAGCTTATGGTGGAGTGGCA) from a 17.5-day mouse fetus cDNA. The PCR product was digested with *Bgl*II and *Hin*dIII and cloned at *Bam*HI/*Hin*dIII sites of pQE30 expression vector (Qiagen, Valencia, CA). Conditions of purification of the His-tagged protein by immobilized metal chelate affinity chromatography under denaturing conditions were essentially as described previously (38).

Antibodies. Immunization and affinity purification of rabbit polyclonal antibodies against human β_m lacking transmembrane region were described previously (40). Similarly, this rabbit serum was subsequently used to isolate antibodies against mouse COOH-terminal ectodomain by affinity purification. Mouse monoclonal antibody α6F against Na,K-ATPase α_1-subunit (47) was obtained from the Developmental Studies Hybridoma Bank (Iowa City, IA). Goat anti-lamin A/C antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) monoclonal antibody A52 (54) was a generous gift from Dr. David MacLennan (University of Toronto, Toronto, Canada). Affinity-purified rabbit polyclonal antibodies against COOH-terminal peptide KETYY (which is specific for all Na,K-ATPase isoforms; Ref. 3) were kindly provided by Dr. Jack Kyte (University of California, San Diego, CA).

Preparation of crude microsomes. Approximately 100 g of tissue was homogenized with a polytron in 400 ml of *buffer A* [in mM: 250 sucrose, 500 KCl, 20 HEPES, 5 EDTA, 5 benzamidine, pH 7.2, and 0.2 PMSF with protease inhibitor cocktail (P2714, Sigma)]. The homogenate was then centrifuged at 6,000 *g* for 20 min at 4°C. The supernatant was collected, and the microsomal fraction was pelleted at 100,000 *g* for 1 h. The microsomes were washed twice with *buffer A* without KCl, pelleted, and stored at −80°C. Protein concentration was determined by the Lowry assay.

Fractionation of nuclei and microsomes. Simultaneous preparation of microsomes and nuclear membranes from newborn pig skeletal muscle was done according to a slightly modified method of Mislow et al. (28). Briefly, skeletal muscle tissue was homogenized in PBS buffer supplemented with protease inhibitor cocktail (P2714, Sigma), pelleted, and washed twice in PBS at 4°C. Cells were treated with a hypotonic buffer (in mM: 10 HEPES, pH 7.9, 1.5 $MgCl_2$, 10 KCl, and 0.5 DTT with protease inhibitor cocktail) on ice for 30 min with occasional vortexing. Nuclei were pelleted at 14,000 *g* for 10 s at 4°C. The supernatant was centrifuged at 100,000 *g* for 1 h to sediment the microsomal fraction.

Purification of skeletal muscle nuclei by Percoll density gradient centrifugation. Skeletal muscles from newborn piglets or rats were collected on ice and processed immediately for isolation of nuclei according to a modified procedure of Hahn and Covault (18): 10 g of muscle was homogenized with a polytron generator in 250 ml of *buffer B* (0.3 M sucrose, 60 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 0.5 mM EGTA, 2 mM EDTA, 14 mM β-mercaptoethanol, 10 mg/ml BSA, and 25 mM HEPES, pH 7.5). The homogenate was centrifuged at 2,500 *g* for 5 min. The pellet was rehomogenized in 200 ml of *buffer C* (same as *buffer B*, except with 0.1 mM EGTA and EDTA) with a Potter homogenizer and filtered through a 100-μm-pore nylon mesh to remove poorly disrupted tissue pieces. Percoll was then added to the filtrate to a final concentration of 27% (vol/vol), and the mixture was centrifuged at 27,000 *g* for 30 min. The nuclear layer was collected, diluted 10 times with *buffer C*, and layered on a 2-ml pad of *buffer N* (50% glycerol, 75 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, and 20 mM Tris·HCl, pH 7.9). After 10-min centrifugation at 1,000 *g*, the pelleted nuclei were resuspended in *buffer D* (0.25 M sucrose, 5 mM $MgCl_2$, 1 mM DTT, and 50 mM Tris·HCl, pH 7.4) and stored at −80°C. Protease inhibitor cocktail (P2714, Sigma) was present in all buffers.

Purification of skeletal muscle nuclei by high-density sucrose centrifugation. Nuclear preparation was done by centrifugation through a dense sucrose solution according to a modified method of Humbert et al. (22). Skeletal muscles (50 g) were sheared with a Waring blender and homogenized in 200 ml of *buffer E* (1.3 M sucrose, 1 mM $MgCl_2$, 10 mM potassium phosphate, pH 6.8). The homogenate, after filtration through four layers of cheesecloth, was centrifuged at 1,000 *g* for 15 min. The pellet was resuspended in a small volume of *buffer E*, and the suspension was mixed with *buffer F* (2.4 M sucrose, 1 mM $MgCl_2$, and 10 mM potassium phosphate, pH 6.8) to give a final 2.2 M sucrose concentration. The mixture was centrifuged at 100,000 *g* for 60 min. The resulting nuclear pellet was resuspended in *buffer D* and centrifuged at 1,000 *g* for 15 min. Pelleted nuclei were stored at −80°C. Protease inhibitor cocktail was present in all buffers.

Nuclear envelope preparation. Nuclei were diluted in *buffer D* to 5 mg/ml protein and digested on ice for 1 h with 250 μg/ml each of DNase I and RNase A (26). The digested nuclei were pelleted at 16,000 *g* for 10 min and resuspended in *buffer D*. An equal volume of 2 M NaCl was added, and the mixture was incubated on ice for 60 min with occasional swirling. Nuclear envelopes were pelleted, washed, and resuspended in *buffer D*.

Extraction of nuclear envelopes. Nuclear envelopes were extracted according to a modified method of Gilchrist and Pierce (17). Membranes (2.5 mg/ml protein) were incubated in *buffer D* plus either 1% Triton X-100 or 6 M urea. After incubation at room temperature for 60 min, insoluble material was pelleted at 14,000 *g* and proteins in the supernatant were precipitated with addition of 0.02% deoxycholate-10% trichloroacetic acid and washed with acetone.

SDS-PAGE and Western blotting. Protein samples were resuspended in SDS-PAGE sample buffer [4% SDS, 12% glycerol (wt/vol), 50 mM Tris·HCl, pH 7.0, 2% β-mercaptoethanol, and 0.01% brilliant blue G-250] and resolved by 12% SDS-PAGE with a Laemmli buffer system. After electrophoresis, the proteins were blotted onto Nucleobond P polyvinylidene difluoride (PVDF) membrane (Amersham-Pharmacia) and nonspecific binding sites were blocked by 5% nonfat milk in TTBS (20 mM Tris·HCl, pH 7.6, 137 mM NaCl, and 0.1% Tween 20). The membranes were incubated for 60 min at room temperature with primary antibodies, thoroughly washed in

TTBS, incubated with peroxidase-conjugated secondary antibodies, washed in TTBS, and visualized with a chemiluminescent peroxidase substrate (ECL, Amersham-Pharmacia). Densitometry analysis was performed on a Bio-Rad GS-670 imaging densitometer (Bio-Rad Laboratories, Hercules, CA).

Immunohistochemistry. For cryosectioning, tissues were frozen in liquid nitrogen, cut at 10-μm thickness, fixed in acetone-methanol (3:5) at −15°C for 30 min, dried in air, and stored at −20°C. Formaldehyde-fixed, paraffin-embedded sections were dewaxed in xylenes followed by graded alcohols and water and then treated with PBS containing 0.3% Triton X-100 for 30 min. For peroxidase labeling, sections were also incubated for 1 h in PBS with 3% hydrogen peroxide and thoroughly washed in PBS. From this point, both frozen and paraffin sections were then processed identically: blocked in PBS with 5% porcine serum, incubated with primary antibodies diluted in PBS with 1% porcine serum, followed by washes in PBS and secondary antibodies and more washes in PBS. For peroxidase labeling, a tyramide-488 peroxidase fluorescent substrate (Molecular Probes, Eugene, OR) was used. Labeled sections were mounted in VectaShield (Vector Laboratories, Burlingame, CA) containing either 0.5 μg/ml ethidium bromide or SybrGold 1:100,000 (Molecular Probes) to visualize nuclei. Images were collected with a Nikon Optiphot 2 fluorescent microscope equipped with a SPOT digital camera (Diagnostic Instruments, Sterling Heights, MI) with automatic exposure control.

Cell culture and transfection by β_m-green fluorescent protein. To produce green fluorescent protein (GFP)-fused β_m, three splice variants of human β_m (A, B, C; Ref. 39) were amplified with primers UHMUF (CATGAGATCTTGAACAGCCATGAGAAGG) and UHMUB (CTTAAGATCTAGTTTCTATGTTCAGGGT) and cloned at the *Bgl*II site of vector pEGFP-N3 (BD Biosciences). In addition, a human β_3 construct was prepared in the same way from plasmid carrying full-length ORF (51). Human rhabdomyosarcoma line RD, C_2C_{12} mouse myoblasts, HT-29 human colon carcinoma cells, and 3T3 mouse fibroblasts (all from American Type Culture Collection) were grown in glass-bottomed chambers in DMEM supplemented with 10% FCS, 2 mM glutamine, 50 μg/l carbenicillin, and 10 μg/l tetracyclin. Cells were transfected by addition of the plasmids complexed with Superfect (Qiagen, Valencia, CA) and incubated from 8 to 24 h. Images were collected with an Olympus fluorescent microscope equipped with a Radiance laser scanning head (Bio-Rad Laboratories).

RESULTS

Primary structure of mouse β_m cDNA, deduced protein, and ATP1B4 gene. Because the mouse is a widely used model organism, we determined the primary structure of murine β_m ORF by sequencing RT-PCR products from a 17-day embryo. This allowed identification of two alternatively spliced forms of the mouse β_m transcript (GenBank accession nos. AF348324 and AF348325), one of them lacking 12 bp within the ORF, similar to that previously demonstrated in human, rat, and pig muscles (36, 39, 40). The first translation product (variant A) consists of 356 amino acid residues and has a molecular mass of 41.4 kDa (Fig. 1), whereas the second β_m variant, termed B, does not contain tetrapeptide Gln^{106}-Ser-Arg-Ser^{109} (boxed in Fig. 1; all amino acid numbering in text corresponds to mouse β_m).

Mouse β_m shares common structural features with other X,K-ATPase β-subunits (9), including the domain organization of type II membrane glycoproteins: NH_2 terminus exposed to cytoplasm, a single transmembrane α-helical segment (Leu^{110}–Leu^{134}), and a large COOH-terminal extracytoplasmic domain. The COOH-terminal domain contains a strictly conserved motif 294-YYPYYGK-300, six conserved Cys residues (200, 219, 229, 245, 268, and 328), presumably forming three consecutive disulfide bridges, and four consensus *N*-glycosylation sites (NXS/T) at Asn residues 167, 189, 228, and 305. Mouse β_m protein exhibits 39.4% and 37.1% overall sequence identity with Na,K-ATPase β_2 and β_3, respectively, and is slightly more distant from both Na,K-ATPase β_1- and gastric H,K-ATPase β-subunit (28.7% and 32.4% identity, respectively). Hence, from a structural point of view, mouse β_m obviously belongs to the family of X,K-ATPase β-subunits.

Characterization of β_m proteins from four different mammalian species allows us to outline the specific structural properties that distinguish them from other members of the family of X,K-ATPase β-subunits. The most remarkable and unique structural feature of β_m proteins is the presence of two extended Glu-rich regions (Asp^{22}–Glu^{42} and Glu^{42}–Glu^{71}) in their NH_2-terminal domains. There are 25 Glu and 4 Asp residues in this portion of mouse β_m. Because of these negatively charged clusters, β_m is much more acidic (pI ≈ 4.6) than related X,K-ATPase β-subunits, whose pI values are in the range of 8.1–8.7. The presence of these acidic domains explains the abnormal electrophoretic mobility of β_m proteins (40). According to a secondary structure prediction, these poly-Glu stretches may exist as long α-helixes connected by a flexible loop (36). None of the structurally related ATPase subunits contains this kind of sequence motif.

Data presented in Fig. 1 demonstrate that primary structures of mammalian β_m proteins are highly conserved. Rodent β_m proteins are the most closely related, exhibiting 98.9% sequence identity. Identity of mouse β_m with human and pig proteins is only slightly lower, being 89% and 90%, respectively. Most of the variable amino acid residues are located in the cytoplasmic domains, being especially abundant in a loop that connects the strictly conserved NH_2-terminal Arg-rich undecapeptide with the first Glu-rich cluster and the nearby COOH-terminal end of the second Glu-rich cluster.

It should be noted that the nucleotide sequences of β_m mRNAs also demonstrate a significant degree of conservation between species and a high level of structural similarity. All of them are ~4 kb long with short 5′-UTRs (~80 nt) and unusually long 3′-UTRs of ~3 kb, which are the longest among the mRNAs of mammalian X,K-ATPase β-subunit family members. The most closely related mouse and rat β_m mRNAs exhibit 97% sequence identity in coding regions and 85% in 3′-UTRs. Sequence identity within coding regions comprises 89.5% even for the most divergent human-mouse pair of β_m mRNAs. Alignment of their long 3′-UTRs reveals four conserved sequences (400–1,200 bp) with similarity exceeding 62%.

Also, a remarkable conservation in sequences of human and mouse β_m genes, termed ATP1B4 according to standard nomenclature, has been revealed on comparison of corresponding regions of human Xq24 and mouse XA2 chromosome fragments (GenBank accession nos. NT004668 and NT039702). These genomic intervals represent conserved linkage in which the order of multiple orthologous genes is the same in both species (19, 41). Both genes span ~21 kb of genomic DNAs and are divided into eight exons. The exon-intron organization of β_m genes is quite similar to that of related X,K-ATPase β-subunit genes that contain six or seven exons (44). The unique NH_2-terminal Glu-rich domain is encoded by the additional exon 2. Presumably, this

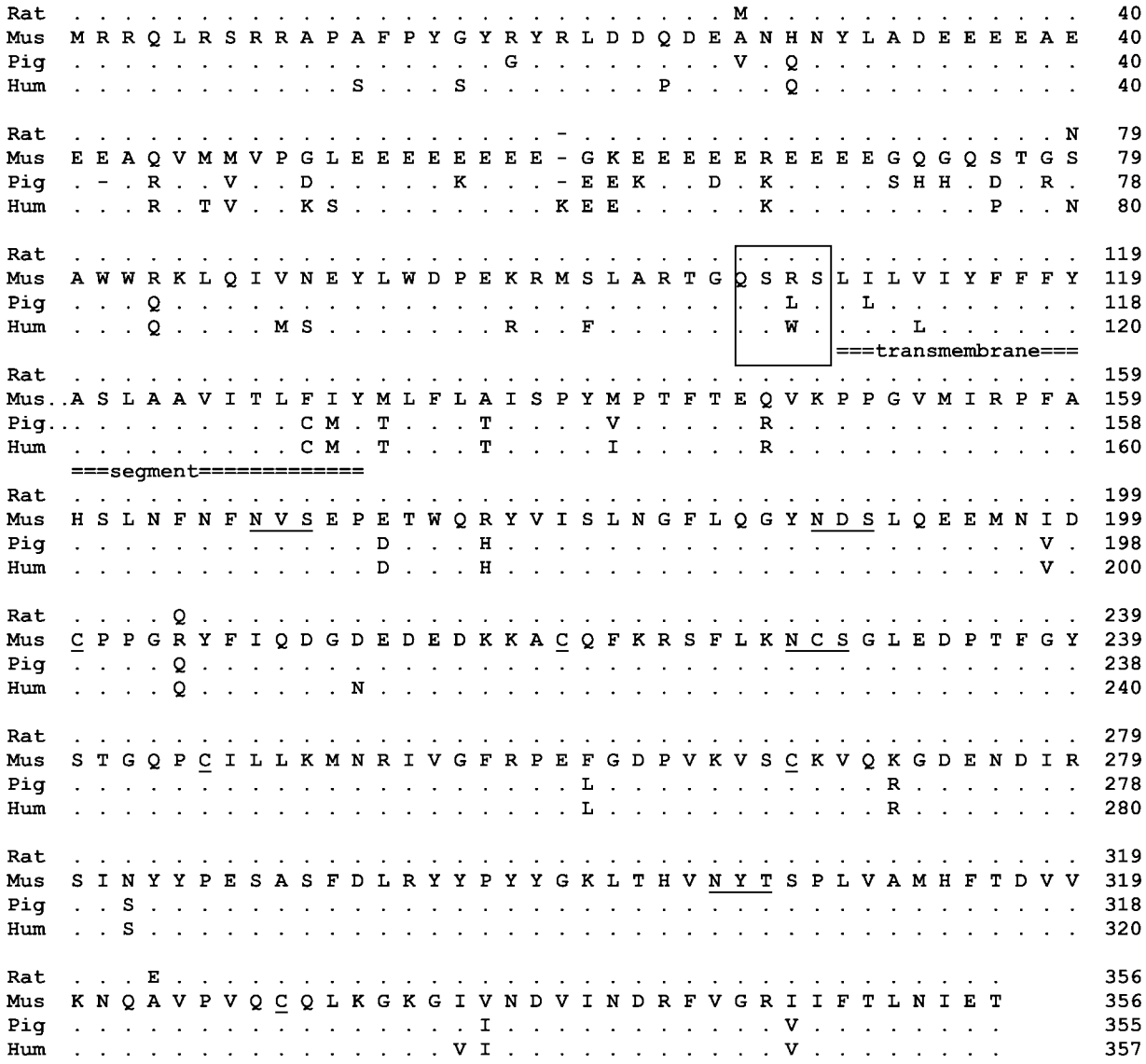

Fig. 1. Comparison of amino acid sequences of mammalian β_m proteins. Alternative splice variants A of the proteins are shown. Tetrapeptides absent in variants B are boxed. Gaps (represented by dashes) were introduced to maintain alignment. Dots indicate identity to the corresponding residues of the mouse protein. Conserved cysteines and consensus sequences of *N*-glycosylation sites are underlined. The amino acids are numbered at *right* of the sequences.

exon was acquired during evolution for an yet-unknown function of β_m. It is important to note that, in contrast to genes encoding other members of the X,K-ATPase β-subunit family, mouse and human β_m ATP1B4s have extended conserved sequences not only in coding regions of exons but also in all introns whose lengths are also similar. Typical examples are introns 3 (262 bp human and 237 bp mouse, 78.8% identity), introns 7 (699 bp human and 711 mouse, 60% identity), and introns 4 (4161 bp human and 4148 bp mouse). These contain several long conserved sequences of ~500–700 bp where similarity index exceeds 60%. Most importantly, alignment of 5′-UTRs of mouse and human mRNAs and 5′-flanking gene regions also revealed a high level of homology, exceeding 81% between ~900-bp sequences immediately preceding the ATG start codons. The strong conservation of noncoding sequences between human and mouse ATP1B4 genes, which putatively diverged about 75 million years ago, indicates a functional importance of these genomic segments. Most probably, the conserved noncoding sequences of the ATP1B4 genes, especially those located upstream of protein-coding regions, contain transcriptional regulatory elements that predetermine unique temporospatial patterns of β_m expression.

Developmental regulation of β_m. The expression of various X,K-ATPase β-subunits in mouse fetuses was analyzed by RT-PCR (Fig. 2*A*). Results of this analysis indicate that both β_1- and β_2-subunit expression tend to increase with progression of fetal development, whereas β_3-subunit expression stays at approximately the same level. The results on the β_1-subunit are in accord with an early Northern blotting study of Orlowski and Lingrel (Ref. 33; β_2 and β_3 were not known at that time). In a sharp contrast with these isoforms, β_m has a very strict regulation, as its transcripts can be hardly detected at *days 10.5–13.5* even with high detection sensitivity. The β_m transcripts appear at *days 14.5–15.5*. Their levels become high at *day 16.5* and reach a maximum at *day 18.5*. Considering the

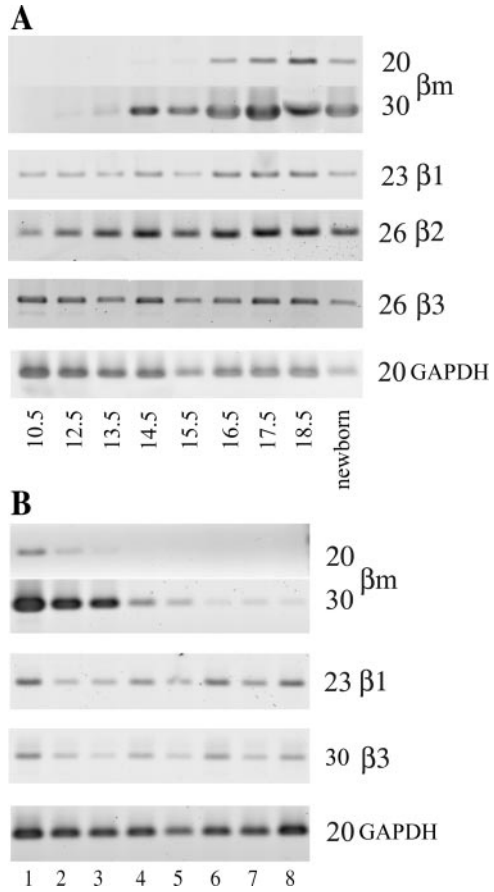

Fig. 2. RT-PCR detection of β_m transcripts in mouse fetuses and in postnatal muscles compared with other X,K-ATPase β-subunits. One-half of RT-PCR reaction from 50 ng of total RNA was electrophoresed and imaged. Number of cycles is indicated on *right*. *A*: analysis of RNA from whole mouse fetuses. *B*: analysis of postnatal development in tongue skeletal muscle (*lanes 1–5*) and heart (*lanes 6–8*): *lanes 1* and *6*, newborn; *lanes 2* and *7*, 7 days postpartum; *lane 3*, 14 days postpartum; *lanes 4* and *8*, 28 days; *lane 5*, 70 days.

number of PCR cycles, the apparent level of β_m transcripts at late fetal stages is higher than levels of other β-subunits.

Another experiment was performed with skeletal muscles and heart of growing mice to study the regulation of β-subunit expression in postnatal development. Figure 2*B* illustrates that the level of β_m transcripts in skeletal muscle (tongue) decreases rapidly after birth. This decrease is especially pronounced after 2 wk postpartum, and in adult mouse the level of β_m is very low. Other β-subunits studied, β_1 and β_3, also demonstrate some decrease immediately after birth, but then their levels seem to be stabilized, without such dramatic changes as in the case of β_m. In postnatal mouse heart, β_m has a very low expression level immediately after birth without significant changes later (Fig. 2*B*).

The difference between newborn and adult pig skeletal muscles in β_m level has been reported before (39), but it does not appear so dramatic as in the mouse. Because there may exist species-specific differences, we also compared newborn to adult changes of β_m protein level in pig and rat membranes (Fig. 3). In both species, the signals of β_m are much stronger in samples from neonatal animal skeletal muscles than in adult samples. In both adult rat and adult pig skeletal muscle, the β_m level is rather low, and in adult hearts the protein cannot be detected at all.

Thus β_m is highly expressed in newborn skeletal muscles of all mammalian species studied, and this level decreases after birth. The rate of this decrease, however, may be rather different between species.

Immunohistochemical detection of β_m in perinatal myocytes. Rabbit polyclonal antibodies were affinity purified with solid support-absorbed ectodomain of mouse β_m and were used for immunohistochemical experiments with fluorophore-conjugated secondary antibodies or, to achieve high sensitivity, peroxidase-conjugated secondary antibodies and fluorogenic tyramide substrate. Figure 4, *A–C*, illustrates such an experiment on formalin-fixed, paraffin-embedded newborn rat tongue. Low-magnification images (Fig. 4, *A* and *B*) demonstrate that all myocytes are brightly labeled, to a comparable extent among each other, whereas connective tissue, blood vessels, and epidermis are negative. This shows that, as expected, β_m is specific to the principal cells of skeletal muscle—the myocytes.

Quite unexpectedly, bright labeling of many nuclei was observed at higher magnification (Fig. 4*C*). The whole of the myocyte is also labeled, but to a much lesser extent, and this labeling usually has a cross-striated pattern on longitudinally positioned cells (Fig. 4*D*). Prenatal mouse skeletal muscle is labeled similarly (Fig. 4, *E* and *F*). Comparison of DNA labeling (Fig. 4*E*) and β_m (Fig. 4*F*) illustrates that only some of the nuclei are β_m positive, although they appear to accumulate the protein to a much higher extent than the rest of the cell (from this image, however, we cannot judge whether all or only some of myonuclei contain β_m because many of the nuclei visualized belong to nonmuscle cells).

Figure 4*G* demonstrates labeling of acetone-methanol-fixed cryosections of newborn pig skeletal muscle with anti-β_m antibodies. These results are similar to those described above:

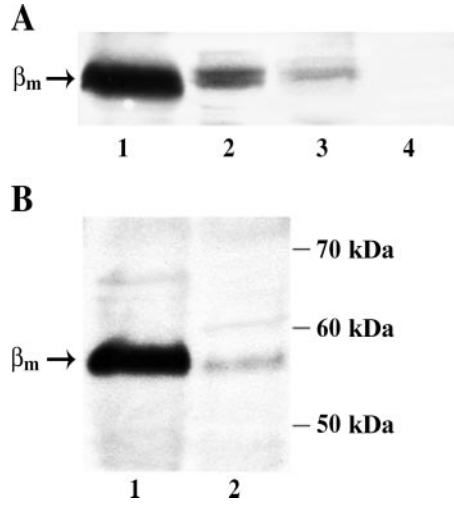

Fig. 3. Immunoblotting of membrane proteins from newborn and adult skeletal muscle with anti-β_m antibodies. Microsome proteins were prepared from the skeletal muscle and heart of neonatal and adult rat (*A*) and pig (*B*). Protein (200 μg) was resolved on 12% SDS-PAGE and immunoblotted with β_m-specific antibodies. *A*: *lane 1*, neonatal rat skeletal muscle; *lane 2*, neonatal rat heart; *lane 3*, adult (2 month old) rat skeletal muscle; *lane 4*, adult (2 month old) rat heart. *B*: *lane 1*, newborn pig skeletal muscle; *lane 2*, adult (6 month old) pig skeletal muscle. Molecular mass markers are shown on *right*.

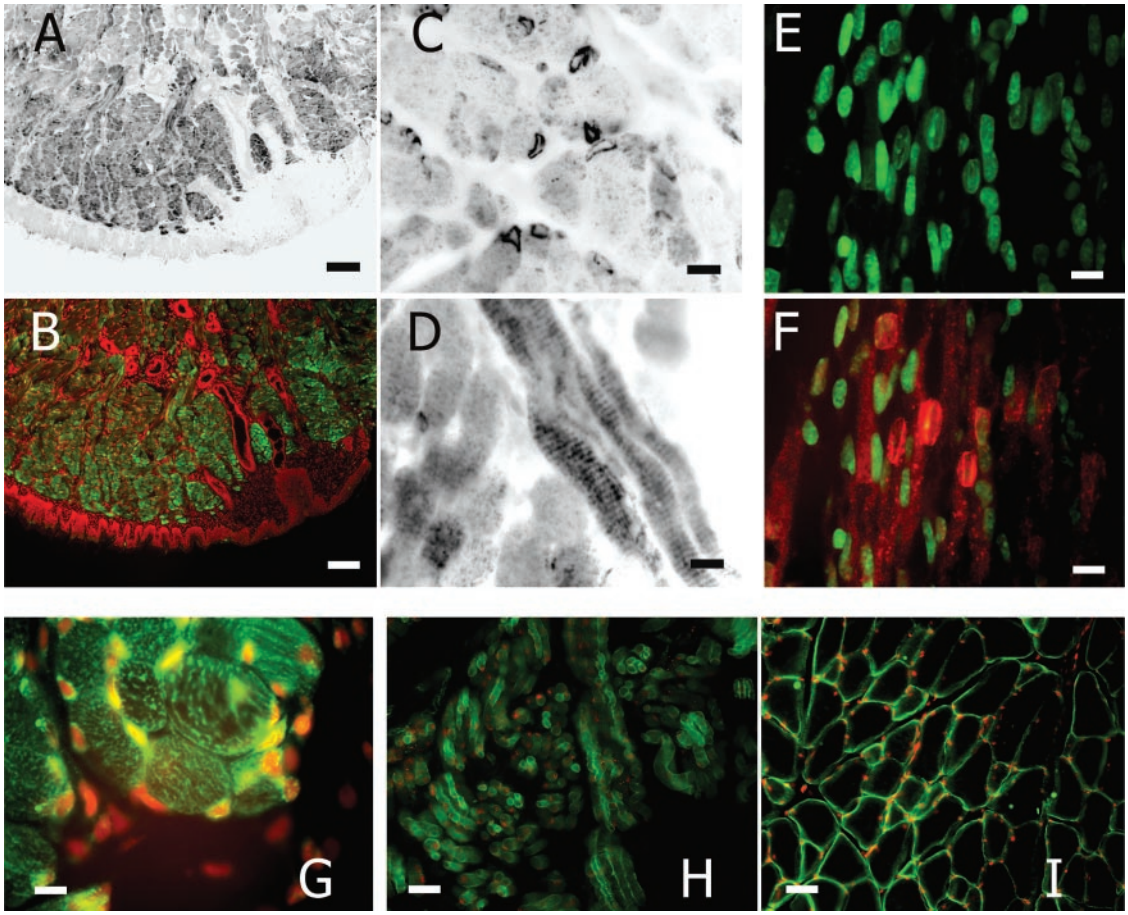

Fig. 4. Immunohistochemical detection of β_m protein in perinatal skeletal muscle. *A–G*: labeling with affinity-purified rabbit antibodies against recombinant ectodomain of β_m of perinatal skeletal muscles. *H* and *I*: labeling with anti Na,K-ATPase α_1-monoclonal antibody (green). *A–D*: anti-β_m labeling visualized with tyramide-488 (green fluorescence). *A*, *C*, *D*: inverted black and white images of green anti-β_m labeling. *F*: anti-β_m labeling visualized with Alexa Fluor 595-conjugated secondary antibodies (red fluorescence). *G*: anti-β_m labeling visualized with fluorescein-conjugated secondary antibodies (green fluorescence). *B* and *G–I*: nuclei stained in red with ethidium bromide. *E* and *F*: nuclei stained in green with SybrGold. *A–D*: rat tongue, 4 days postpartum. *E* and *F*: fetal mouse dorsal muscles, 18 days postcoitum. *G*: newborn piglet limbic muscle. *H*: newborn piglet periocular muscles. *I*: adult rat diaphragm. *A–F*: formalin-fixed, paraffin-embedded sections. *G–I*: methanol-acetone-fixed cryosections. Bars: 100 (*A* and *B*), 10 (*C–G*), and 50 (*H* and *I*) μm.

many nuclei are labeled very brightly, whereas a weaker labeling of the whole body of the myocytes can be observed. The lack of a significant labeling of the surface of the cells indicates that β_m is not accumulated in plasma membranes, in accordance with our previous results (39). The punctate labeling on transverse sections and cross-striated labeling on longitudinal sections may be SR or T tubules.

We were not able to reliably detect β_m on sections of adult muscles, both porcine and rodent, even with the sensitive tyramide substrate (results not shown). The distribution of β_m in postnatal mouse skeletal muscle appears not to change from that in fetal/newborn muscles, but the labeling intensity drops dramatically during growth, in accordance with RT-PCR detection of β_m transcripts (results not shown). Satellite cells [identified as small neural cell adhesion molecule (NCAM)-positive cells] were found negative for β_m (results not shown). No significant labeling of neuromuscular junction sites (identified with fluorescein-conjugated α-bungarotoxin) was observed in newborn or adult skeletal muscles (results not shown).

The Na,K-ATPase α-subunit is detected on the surface of newborn and adult myocyte membranes (Fig. 4, *H* and *I*). This suggests that the α-subunit is located in the surface plasma membrane compartment or in intracellular stores adjacent to the surface as suggested by Klip and coworkers (23, 24). Nuclear labeling with anti-Na,K-ATPase α-subunit antibodies was absent in both adult and newborn skeletal myocytes.

Detection of β_m in isolated nuclei and nuclear envelope. We separated membrane fractions of neonatal pig skeletal muscle into microsomes and nuclear membranes according to a modified method of Mislow et al. (28). Lamins, which are proteins present exclusively in the nucleus, were enriched in the nuclear fractions obtained as demonstrated by Western blotting with anti-lamin antibody (Fig. 5*A*). A quantitative analysis of lamin distribution indicates its presence predominantly in the nuclear fraction, as expected (Fig. 5*A*, *bottom*). Analysis of β_m distribution by Western blotting reveals its presence in both nuclei and microsomes with a stronger signal in nuclei (Fig. 5*A*, *right*). The presence of β_m in microsomes is consistent with our previous finding that β_m is present in SR (39). The nuclear

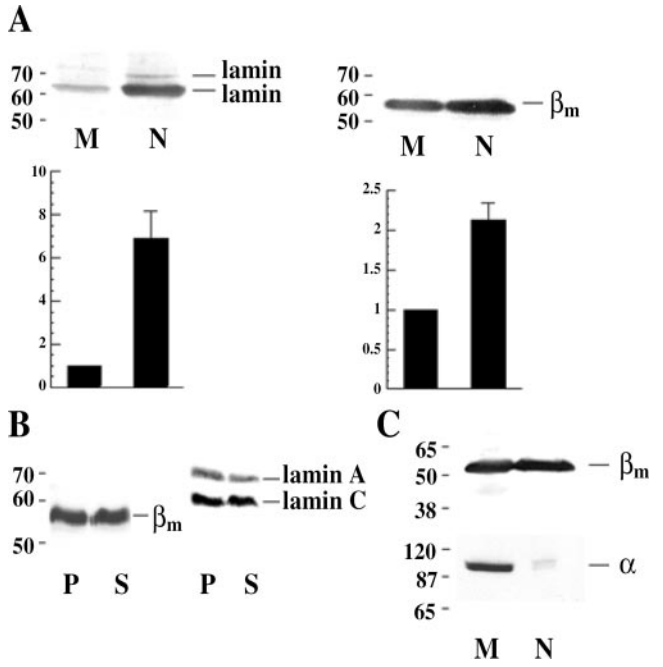

Fig. 5. Detection of β_m in isolated nuclei. *A*: newborn pig skeletal muscle was homogenized and treated with a hypotonic buffer, and nuclei were separated from microsomes. Equal amounts of proteins from both fractions were resolved on SDS-PAGE, and distribution of lamins (*left*) and β_m (*right*) was determined by Western blotting. Densitometric data represent results of 5 experiments (means ± SE). M, microsomal fraction; N, nuclei. Molecular mass markers are shown on *left*. *B*: nuclei from neonatal pig skeletal muscle cells were purified by either Percoll density-gradient centrifugation (P) or high-density sucrose centrifugation (S) and analyzed with immunoblotting. *C*: equal amounts of proteins from microsomal fraction (M) and purified nuclei (N) were resolved on SDS-PAGE and analyzed by Western blot with β_m or specific antibody against COOH-terminal KETYY peptide of Na,K-ATPase α-subunits (α).

fraction is not significantly contaminated with SR membranes because SERCA is detected predominantly in the microsomal fraction (results not shown). Densitometric quantification of the data suggests that the concentration of β_m protein in the nuclear fraction is twice that in microsomes (Fig. 5*A*, *bottom*).

To confirm the nuclear localization of β_m, two published methods for purification of nuclei were used. Percoll density-gradient centrifugation is a well-established procedure for the preparation of skeletal muscle nuclei free of myofibril contaminations (18). Isolation by centrifugation through a high concentration of sucrose was also used for comparison of yield and purity. An equal amount of nuclear proteins prepared by each approach was resolved on SDS-PAGE and probed with antibodies against β_m. In both samples, β_m was found in purified nuclei (Fig. 5*B*, *left*). Lamins were used as nuclear markers (Fig. 5*B*, *right*). Percoll density-gradient centrifugation gives a somewhat higher yield of nuclear proteins (results not shown), and this method was used in subsequent studies.

Comparison of microsomes and nuclei by Western blot with antibody against COOH-terminal KETYY peptide of Na,K-ATPase α-subunits shows that the α-subunits are present only in microsomes (Fig. 5*C*). This result not only attests to little cross-contamination of the purified nuclei from microsomes (either plasma membranes or Na,K-ATPase-containing intracellular stores) but also argues that Na,K-ATPase is not present in nuclear membranes, in accordance with our immunohistochemical results. Results of the subcellular fractionation suggest that the nuclear preparations obtained are devoid of significant microsomal contamination and the β_m protein detected really represents that located in nuclei.

Detection of β_m in nuclear envelopes and extraction from nuclear membranes. Neonatal pig skeletal muscle nuclei were separated into nuclear membranes and nucleoplasm by high-salt extraction (26). The electrophoretic profile of nuclear envelope and nucleoplasm exhibited different protein patterns (data not shown). In particular, pelleted nuclear envelopes were enriched in lamins (Fig. 6*A*, *bottom*). β_m was only detected in the nuclear envelopes and not in nucleoplasm (Fig. 6*A*, *bottom*).

In agreement with the reports of others (26), our result shows that lamins remain insoluble after extraction with 1% Triton X-100 (Fig. 6*B*, *top*). However, when treated with 6 M urea, these peripheral proteins can be washed off the nuclear envelope (Fig. 6*B*, *top*). On the other hand, β_m is readily solubilized by the nonionic detergent 1% Triton X-100 in the absence of salt (Fig. 6*B*, *bottom*), suggesting that this protein is not in a tight association with the nuclear lamina or anchored to nuclear pore complex. Also, β_m is resistant to 6 M urea treatment, indicating that β_m is an intrinsic nuclear membrane protein (Fig. 6*B*, *bottom*).

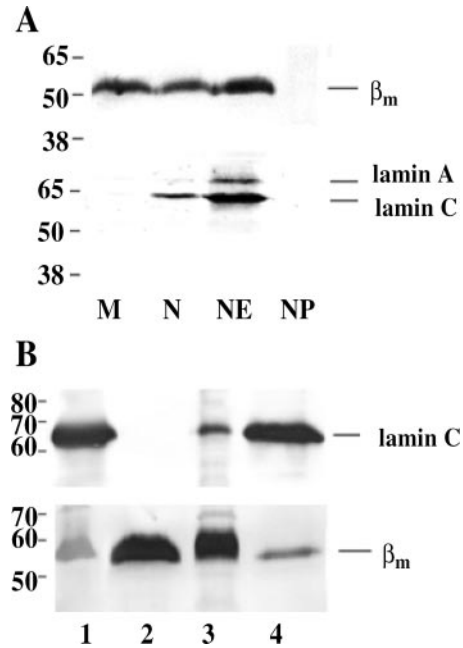

Fig. 6. Extraction of β_m from newborn pig nuclear membranes. *A*: detection of β_m in nuclear envelopes. Nuclei were treated with DNase and RNase and extracted with 1 M NaCl to separate nuclear envelopes and nucleoplasm. Equal amounts of protein were resolved on SDS-PAGE and analyzed by Western blot with β or lamin A/C antibodies. M, microsomes; N, DNase- and RNase-treated nuclei; NE, nuclear envelopes; NP, nucleoplasm. Molecular mass markers are shown on *left*. *B*: extraction of β_m and lamins from purified nuclear envelopes. The nuclear envelopes (2 mg/ml) were incubated with either 1% Triton X-100 or 6 M urea for 60 min. Insoluble material was pelleted, and soluble material was precipitated by deoxycholate-trichloroacetic acid. Proteins were resolved on 12% SDS-PAGE and analyzed by Western blotting with lamin A/C or β_m antibodies. *Lane 1*, insoluble fraction after 1% Triton X-100 treatment; *lane 2*, soluble fraction after 1% Triton X-100 treatment; *lane 3*, pellet resulting from 6 M urea extraction; *lane 4*, supernatant resulting from 6 M urea extraction.

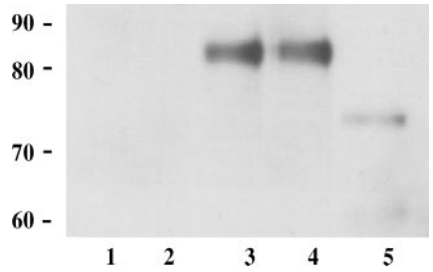

Fig. 7. Western blotting of RD cells transfected with green fluorescent protein (GFP)-tagged proteins. Cultured rhabdomyosarcoma RD cells were transfected for 24 h and lysed in sample loading buffer, and 10 μg of protein each were analyzed with separation in 8% SDS-PAGE and Western blotting with affinity-purified rabbit anti-β_m antibodies. *Lane 1*, control pEGFP-N3 vector; *lane 2*, β_3-GFP; *lane 3*, β_m-GFP, variant A; *lane 4*, β_m-GFP, variant B; *lane 5*, β_m-GFP, variant C.

Localization of GFP-tagged β_m in transfected cultured cells. An important question to answer is whether the observed accumulation of β_m in the nuclear envelope is unique for muscle cells in vivo. For this reason, we studied several available cell lines for the presence of β_m (mouse C_2C_{12} and rat L6 myoblasts, human rhabdomyosarcoma RD, primary rat skeletal muscle myoblasts, primary rat cardiomyocytes). However, all of them were found to contain only trace levels of β_m transcripts, and, moreover, in vitro fusion of either C_2C_{12} or L6 rodent myoblasts did not result in any significant upregulation of the β_m gene (results not shown).

Thus we decided to expand our studies using transient heterologous expression of β_m tagged with GFP. Human β_m was used because cloned full-length alternative splice variants were available (40). Their ORFs were cloned into a mammalian expression vector in frame COOH-terminally with GFP. For comparison, β_3 was also included in the study. Human rhabdomyosarcoma RD cells transfected with the plasmids show the appearance of proteins with electrophoretic mobilities corresponding to apparent molecular mass of ~85 kDa in the case of variants A and B and 74 kDa in the case of variant C (Fig. 7). These values correspond to theoretical molecular masses amended for abnormal mobility because of the presence of the acid NH_2 terminus (40).

Confocal images of intracellular distribution of GFP-tagged proteins in live cells are presented in Fig. 8. Unfused GFP is present in both cytoplasm and nucleoplasm (Fig. 8, *A* and *F*). Both variants A and B of β_m show reticular intracellular fluorescence with a clear and bright, but not exclusive, signal in the nuclear envelope (Fig. 8, *B*, *C*, and *G*). Variant C, which lacks a transmembrane domain, is localized similarly to GFP itself, in both nucleus and cytoplasm (Fig. 8*D*). The β_3-GFP chimera has an intracellular localization (Fig. 8*E*) with a significant difference that can be more easily observed on false

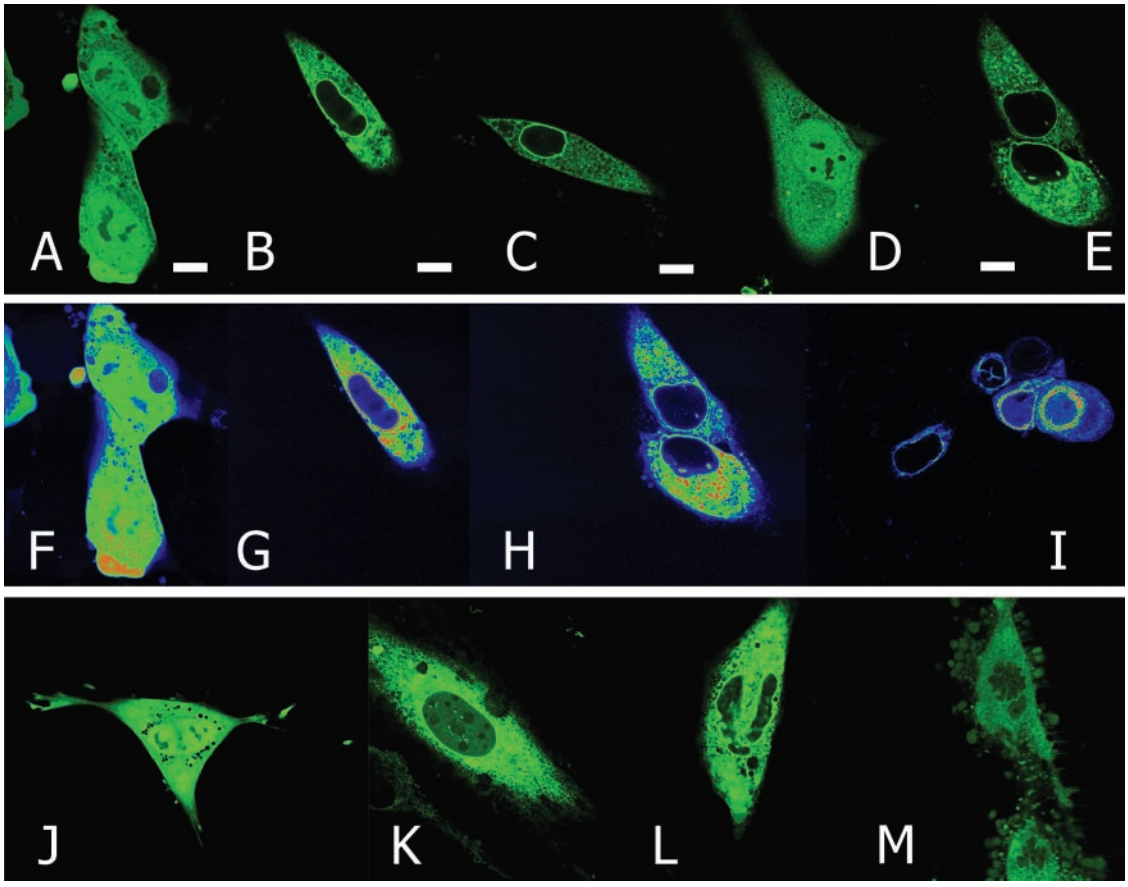

Fig. 8. Localization of GFP-tagged β_m in cultured cells. Live cells were imaged with the help of a confocal microscope. *A*, *F*, *J*: unfused GFP. *B–D*, *G*, *I*, *K–M*: β_m-GFP chimeras. *B*, *G*, *K–M*: β_m splice variant A. *C*, *I*: β_m splice variant B. *D*: β_m splice variant C (lacking transmembrane domain). *E*, *H*: β_3-GFP chimera. *A–H*: human rhabdomyosarcoma RD. *I*: human intestinal carcinoma HT-29. *J–M*: mouse 3T3 fibroblasts. *F–I*: false color images. Transfection times: 24 h for *A–C*, *E–I*; 8 h for *D*, *J–M*. Bars: 10 μm.

color images (Fig. 8, *G* and *H*): β_m-GFP is always present in the nuclear envelope (Fig. 8*G*), whereas β_3-GFP is accumulated in unidentified intracellular granules instead of nuclear membranes (Fig. 8*H*).

Transfection of cells of nonmyogenic origin with β_m-GFP produced results similar to but not as evident as in the case of RD. Human intestinal carcinoma HT-29 cells show relatively weak perinuclear labeling (Fig. 8*I*). The use of cells unrelated to muscle may be compromised by high toxicity of β_m-GFP in these cells. For example, mouse 3T3 fibroblasts withstand unfused GFP (Fig. 8*J*) but, in the case of β_m-GFP, no labeled cells can be found after 24 h of transfection. This is due to death of the transfected cells, as illustrated by acquisition of images of the cells after just 8 h of transfection (Fig. 8, *K–M*). There, three types of cells can be found: apparently normal cells with β_m-GFP with a distribution similar to that in RD cells (Fig. 8*K*), cells with fragmented nuclei (Fig. 8*L*), and dead cells (Fig. 8*M*). Variant C is especially toxic, because we were not able to observe any transfected HT-29 or 3T3 cells and only a few RD cells can be found, predominantly in the dying state (results not shown). Also, both RD and C_2C_{12} cells transfected with either β_m-GFP or untagged β_m were unable to grow (results not shown). Hence, β_m shows significant toxicity when expressed at high levels in mammalian cells.

DISCUSSION

Developmental regulation of β_m. Differentiation of cells during organogenesis imposes fluctuating demands on ion homeostasis. For this reason, the expression of cation pumps including X,K-ATPases is subject to strict developmental regulation. The fundamental work of Orlowski and Lingrel (33) compared mRNA levels of three Na,K-ATPase α-subunit isoforms and β_1-subunit in several rat tissues at fetal, neonate, juvenile, and adult stages. It was observed that the β_1 level in skeletal muscle increases slowly from fetus to neonate, followed by a sevenfold increase from neonate to juvenile and a twofold decrease in the adult rat. Comparing the regulation patterns of α-subunit isoforms and β_1, Orlowski and Lingrel (33) predicted: "It is possible that an unidentified β-subunit isoform exists, perhaps muscle-specific, which is not detected using experimental conditions."

The present study demonstrates that β_m has a pattern of developmental regulation different from other β-subunits. In the mouse, the β_m transcripts peak a few days before birth and remain very high in newborn skeletal muscle. The induction of expression occurs significantly later than formation of primary myotubes by myoblast fusion. This fact accounts for the absence of β_m in differentiating myoblasts in vitro. Obviously, β_m expression is induced by signals that occur after primary myogenesis. Regulation of some other X,K-ATPase subunits is also not identical in vitro and in vivo (46).

During postnatal maturation of myocytes β_m gradually disappears, and in adult murine skeletal muscle β_m transcripts are difficult to detect even with RT-PCR. In other, less studied species, this regulation may be similar but not so sharp; for example, β_m protein can be detected in adult pig or human skeletal muscle membranes (39, 40) and in the pig the transcript levels differ between newborn and adult stages just about two times (39). It is clear, however, that the highest levels of β_m in all mammalian species occur in perinatal skeletal muscles.

Accumulation of β_m protein in nuclear envelope membrane. Here we demonstrated that β_m protein is accumulated in nuclear envelopes of mammalian perinatal myocytes. It is impossible to quantitate accurately the relative portions of nuclear and extranuclear protein, but it is clear that, at least in some myonuclei, the concentration of β_m is very high. When expressed in cultured cells of both myogenic and nonmyogenic origin, β_m also accumulates in nuclear envelopes, although apparently not to such a great extent as in myocytes in vivo.

The nuclear envelope is composed of a "double" membrane structure: inner and outer nuclear membranes. Recent studies suggest that distribution of ions across the nuclear membranes is not uniform (13) and may be controlled by ion transporters such as SERCA-type Ca-ATPase (2), K/H exchanger, Cl channel, and Ca-dependent K channel in the nuclear envelope (35). It was reported that a significant portion of Na,K-ATPase α-subunit is localized to the inner nuclear membrane of hepatocytes (Ref. 15; no data about β-subunit were presented). Also, other immunohistochemical experiments produced nuclear or juxtanuclear staining for Na,K-ATPase (27, 29, 31). Moreover, Na,K-ATPase can be detected in the nuclear fraction isolated from HeLa cells treated with ouabain (50). Recently, peptides of Na,K-ATPase α-subunit have been detected in a large proteomic study of rodent liver nuclear envelope (43), thus confirming results of Garner (15). Curiously, one peptide was attributed to β_m (15), but this is certainly an error because that sequence is present only in pig and human β_m and the animal used in that study was the mouse.

The detection of the α-subunit in the nucleus prompted Garner to hypothesize that Na,K-ATPase is a driving force of Na and K gradient generation across the nuclear envelope (15). In our work, however, no myonuclear Na,K-ATPase α-subunit has been detected. Thus nuclear β_m may exist as a free subunit or, alternatively, in association with a different protein(s).

The identification of β_m in both ER and nuclear membranes raises an interesting question: How is it targeted and retained in the nuclear envelope? Many other proteins do have similar patterns of subcellular distribution, for example, calnexin (17), inositol 1,4,5-trisphosphate receptor (22), and nesprin (52). Most likely, the generally accepted mechanism (6, 21) for these proteins applies for β_m as well: it initially integrates into the ER membrane and then diffuses laterally in the lipid bilayers until it is retained in the inner nuclear membrane.

Interestingly, β_m is easy to solubilize with a nonionic detergent, in contrast to components of two major structures of the nuclear envelope, nuclear pore complex (NPC) and nuclear lamina. NPC is a large macromolecular assembly embedded in the double membrane of the nuclear envelope. The nuclear lamina, the major framework of the nucleus, is formed by lamins and by integral and peripheral proteins of the inner membrane. Lamina-associated proteins and NPC components are distinguished by their resistance to extraction with high-salt or nonionic detergent, respectively (26). Complete solubilization of β_m in 1% Triton X-100 provides evidence that β_m is not tightly associated with nuclear lamina or NPC. This apparently weak association of β_m with the nuclear envelope appears to be an uncommon feature among known nuclear envelope residents. For example, nurim, a protein also not associated with lamins or NPC, resists extraction even by detergent plus high

salt (42). Indeed, the diversity of properties of the inner membrane proteins may be enormous (42, 43).

What structural determinants are responsible for the subcellular distribution of β_m that is unique among the structural members of the X,K-ATPase β-subunit family? β_m has no known nuclear localization signals (NLSs); however, β_m is very acidic (pI 4.6) and many nuclear proteins possess acidic clusters (7). Strong negative charge is conferred on β_m by its NH_2-terminal domain, a structural feature that distinguishes β_m from other β-subunits. Thus it is easy to hypothesize that the acidic NH_2-terminal portion contains a nuclear targeting and retention signal of β_m. However, this region appears not to be important for intracellular retention during heterologous expression in *Xenopus* oocytes, as neither its truncation in β_m nor its fusion to the β_1-isoform changes the distribution between intracellular stores and plasma membrane (10). Also, the sequence of the NH_2-terminal portion is relatively variable between species (Fig. 1). All these facts argue against the possibility that the NH_2-terminal domain of β_m is a strong NLS, although the possibility still exists that this domain can serve as an enhancer of the accumulation in the nuclear envelope.

Putative physiological function of β_m. A nuclear function of β_m is intriguing but difficult to understand in physiological terms. It is well known that skeletal muscle is highly sensitive to abnormalities in nuclear envelope proteins, such as mutations of lamin or emerin in muscular dystrophies (8, 34). Thus the existence of muscle-specific nuclear membrane proteins is not surprising. However, specific ontogenic regulation, especially the transient pattern of β_m expression in the mouse, indicates that function of β_m may be highly specialized and related exclusively to concurrent developmental events.

Skeletal muscle cells form in two or more successive waves, termed primary and secondary myogenesis, both requiring distinct myoblast populations (12). The first phase of myogenesis in vivo, formation of primary myotubes, appears to be completed in the absence of β_m protein. The induction of high-level β_m expression occurs (embyronic *day 16.5*) just after the onset of downstream developmental processes such as innervation and secondary myogenesis, the formation of new myotubes by fusion of myoblasts along the primary myotubes (32). The absence of β_m in neuromuscular junctions indicates that β_m is unlikely to be involved in innervation. On the other hand, β_m may be important for secondary myogenesis and terminal differentiation of the young myocytes.

During the formation of myotubes, the nuclei must accommodate to their transfer from mononucleated to polynucleated cells. The terminal differentiation into skeletal muscle fibers requires exit from the cell cycle (8, 48), although some myonuclei continue their division even after cell fusion (49). In vitro fusion of C_2C_{12} myoblasts evokes certain rearrangements of nuclear lamina that appear to be unique for myogenesis (30). Unfortunately, nothing is known about the fine processes specific to nuclei of perinatal myocytes (48).

It is becoming evident that many proteins previously considered to be associated with plasma membrane, and lacking classic NLS, also exist in nuclear membranes, where they perform additional, regulatory functions (5). Their nucleocytoplasmic trafficking may be important for signal transduction from the plasma membrane, and a similar function may be theoretically reserved for β_m.

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