Evolution of Na,K-ATPase βm-subunit into a coregulator of transcription in placental mammals

Nikolay B. Pestov*†, Nisar Ahmad*, Tatiana V. Korneenko*†, Hao Zhao*, Rossen Radkov*, Danièle Schaer‡, Sophie Roy‡, Stéphanie Bibert‡, Käthi Geering‡, and Nikolai N. Modyanov*§

*Department of Physiology and Pharmacology, University of Toledo College of Medicine, 3000 Arlington Avenue, Toledo, OH 43614; †Shemyakin–Ovchinnikov Institute of Bioorganic Chemistry, 16/10 Miklukho-Maklaya, Moscow 117997, Russia; and ‡Department of Pharmacology and Toxicology, University of Lausanne, Rue du Bugnon 27, CH-1005 Lausanne, Switzerland

Communicated by Edward A. Adelberg, Yale University, New Haven, CT, May 22, 2007 (received for review February 20, 2007)

Change in gene functions (gene cooption) is one of the key mechanisms of molecular evolution. Genes can acquire new functions via alteration in properties of encoded proteins and/or via changes in temporal or spatial regulation of expression. Here we demonstrate radical changes in the functions of orthologous ATP1B4 genes during evolution of vertebrates. Expression of ATP1B4 genes is brain-specific in teleost fishes, whereas it is predominantly muscle-specific in tetrapods. The encoded βm-proteins in fish, amphibian, and avian species are β-subunits of Na,K-ATPase located in the plasma membrane. In placental mammals βm-proteins lost their ancestral functions, accumulate in nuclear membrane of perinatal myocytes, and associate with transcriptional coregulator Ski-interacting protein (SKIP). Through interaction with SKIP, eutherian βm acquired new functions as exemplified by regulation of TGF-β-responsive reporters and by augmentation of mRNA levels of Smad7, an inhibitor of TGF-β signaling. Thus, orthologous vertebrate ATP1B4 genes represent an instance of gene cooption that created fundamental changes in the functional properties of the encoded proteins.

ATP1B4 | gene cooption | skeletal muscle development | TGF-β | Smad7

Isozymes of Na,K-ATPase and closely related gastric and nongastric H,K-ATPases (X,K-ATPases) are the only animal ion pumps among P-type ATPases (1–2), which, in addition to the catalytic α-subunit, contain a second indispensable component, the β-subunit that plays a crucial role in the formation of functionally active X,K-ATPases (3).

We identified a protein encoded by the ATP1B4 gene in placental mammals as a fifth member of the X,K-ATPase β-subunit family and named it "βm" to emphasize its predominant expression in skeletal muscle (4, 5). Eutherian βm has all of the structural features and signature motifs specific to X,K-ATPase β-subunits, which are type II integral membrane proteins (4, 6, 7), and exhibits 30–40% sequence homology with known X,K-ATPase β-subunits (4). However, βm possesses a unique N-terminal domain containing two long Glu-rich clusters (4, 6, 7). Moreover, in contrast to other family members, which are located at the plasma membrane associated with X,K-ATPase α-subunits, βm resides in intracellular stores, being accumulated in nuclear membranes of perinatal myocytes (6–8). Finally, βm does not assemble with any of the X,K-ATPase α-subunits, thus indicating that it does not function as a subunit of X,K-ATPase in placental mammals (8, 9).

The unique characteristics of the eutherian βm raise questions about its functional role and whether sharp distinctions of ATP1B4 gene products from other family members are consequences of gene cooption during vertebrate evolution (10–12). To gain insight, we performed comparative structure-function analysis of fish, amphibian, avian, and eutherian βm proteins.

Results and Discussion

The ATP1B4 genes have been found in all vertebrate genomes sequenced to date, from teleost fishes to mammals, in a highly conserved chromosomal segment located in Xq24 in case of the human genome. The order of multiple genes in this segment has been remarkably preserved during evolution (13, 14) indicating that they are true orthologs [supporting information (SI) Fig. 5]. Comparison of eutherian βm structures (4, 6, 7) with sequences derived from cloned full-length ORFs from teleost fish (*Tetraodon nigroviridis*), amphibian (*Xenopus laevis*), and avian (*Gallus gallus*) species revealed that their polypeptide chains can be divided into two parts, which exhibit sharply different sequence homology (Fig. 1*a*). Transmembrane and C-terminal domains, which contain all structural features of X,K-ATPase β-subunits (e.g., a strictly conserved YYPYYGK signature motif), exhibit 48–64% sequence identity that is similar to overall homology of other vertebrate X,K-ATPase β-subunit isoforms (4, 7). In contrast, sequences of βm N-terminal domains are very divergent and specific for each vertebrate class, and only nonadecapeptides adjacent to the transmembrane segments exhibit detectable homology. Thus, N-terminal βm-domains (encoded by two exons, three in the case of chicken) evolved at a much higher rate than other protein domains. The structure of N-terminal domains radically changed in placental mammals, acquiring an N-terminal Arg-rich nonapeptide and two extended Glu-rich clusters (Fig. 1*a*). Eutherian βm-proteins (e.g., mouse and human shown in Fig. 1*a*) are highly conserved and exhibit ≥90% overall identity (4, 7). Importantly, corresponding ATP1B4 genes show strong homology in promoter regions, which exceeds 80% within ≈900 nt preceding protein-coding sequences (7), indicating close similarity in regulation and function of ATP1B4 genes in placental mammals.

Expression of eutherian βm-proteins is confined to skeletal muscle and, at a lower level, to heart and skin (4, 6, 7) and is strictly developmentally regulated, being the highest in perinatal myocytes (7). For instance, expression of the mouse ATP1B4 gene is induced in the last quarter of pregnancy, but transcripts almost completely disappear within 14 days postpartum (7). RT-PCR data of Fig. 1*b* show that ATP1B4 genes in nonmammalian tetrapods are expressed at high levels in skeletal muscles. However, βm-transcripts are also present at comparable levels in

Author contributions: N.B.P. and N.A. contributed equally to this work; N.B.P., N.A., K.G., and N.N.M. designed research; N.B.P., N.A., T.V.K., H.Z., R.R., D.S., S.R., and S.B. performed research; N.B.P., N.A., K.G., and N.N.M. analyzed data; and N.B.P., N.A., K.G., and N.N.M. wrote the paper.

The authors declare no conflict of interest.

Abbreviations: hα1, human α1-subunit; hβm, human βm; SBE, Smad-binding element; SKIP, nuclear transcriptional coregulator Ski-interacting protein; Xα1, *Xenopus* α1-subunit; Xβm, *Xenopus* βm; X,K-ATPase, Na,K-ATPase and gastric and nongastric H,K-ATPases.

Data deposition: The sequences described in this paper have been deposited in the GenBank database (accession nos. DQ358914, DQ413025, and DQ417195).

§To whom correspondence should be addressed at: Department of Physiology and Pharmacology, University of Toledo College of Medicine, Health Science Campus, 3000 Arlington Avenue, Mail Stop 1008, Toledo, OH 43614-2598. E-mail:nikolai.modyanov@utoledo.edu.

This article contains supporting information online at www.pnas.org/cgi/content/full/0704809104/DC1.

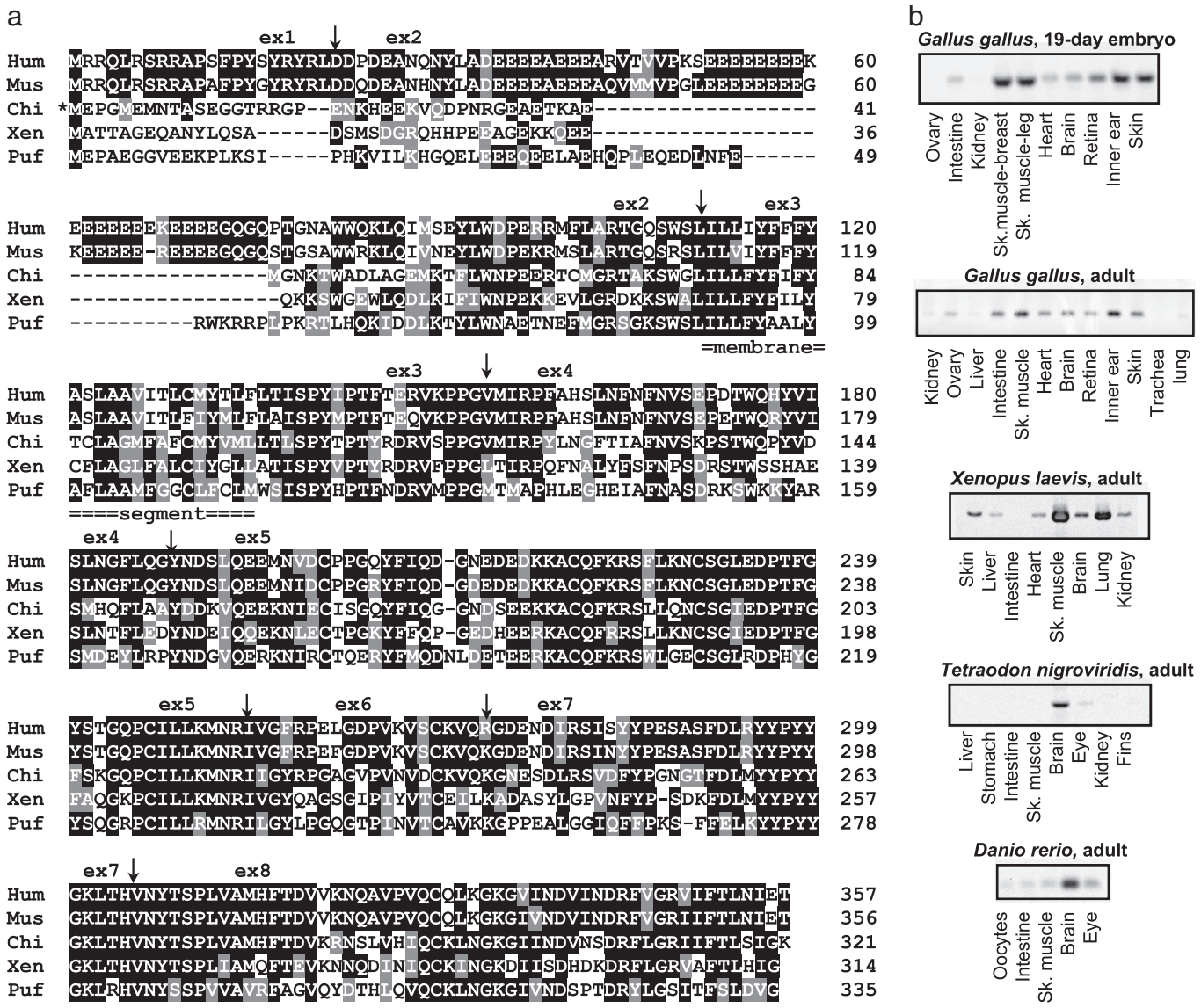

Fig. 1. Evolutionary changes in the structure of βm N-terminal domains and tissue specificity of expression. (*a*) Sequence alignment of βm-proteins: human (Hum); mouse (Mus); chicken (Chi); clawed toad, *Xenopus laevis* (Xen); and pufferfish, *Tetraodon nigroviridis* (Puf). Identical residues in two or more sequences are highlighted in black, and conserved substitutions are highlighted in gray. Gaps (represented by dashed lines) were introduced to maintain alignment. Arrows mark positions of the exon boundaries, except for the exon encoding the first Met in the chicken βm (represented by *). (*b*) Tissue-specific expression of ATP1B4 genes in chicken, *Xenopus*, pufferfish, and zebrafish (*Danio rerio*). RT-PCR analysis of βm-transcripts was done with total RNA extracted from tissues by using specific primers (*SI Materials and Methods*). The band intensities were normalized using GAPDH RT-PCR products.

some other tissues such as chicken inner ear, chicken skin, and frog lungs. Unlike eutherian species, mRNA of chicken βm is present in both embryonic and adult tissues. In contrast to tetrapods, βm-mRNA in pufferfish has been detected exclusively in brain. The same pattern of expression has been found in zebrafish (*Danio rerio*), thus demonstrating that brain-specific expression of ATP1B4 may be a common feature of teleost fishes. These differences in βm-structure and patterns of expression among animal classes provide initial evidence that functions of ATP1B4 genes may have changed during vertebrate evolution.

To test the ability of βm-proteins to interact with ubiquitous Na,K-ATPase α1-subunits, they were coexpressed in *Xenopus* oocytes, metabolically labeled, and immunoprecipitated with an α-antibody under nondenaturing conditions. Human βm (hβm) coexpressed with human α1-subunit (hα1) remains in an endo H-sensitive core-glycosylated form throughout the chase period (Fig. 2*a*, lanes 1–6) indicating its retention in the endoplasmic reticulum (9). In contrast to human Na,K-ATPase β1(hβ1) used as a control (lanes 13–18), hβm was not coimmunoprecipitated with an α-antibody (lanes 7–12). Due to lack of association with hβm, hα1 was degraded (lanes 7–12), whereas the control hα1/hβ1 complex was stable (lanes 13–18) as expected (3).

Xenopus βm (Xβm) coexpressed with *Xenopus* α1-subunit (Xα1) was coimmunoprecipitated with an α-antibody (Fig. 2*b*, lanes 1–6), indicating formation of stable Xα1/Xβm complex. Similar to control Xβ1 coexpressed with Xα1 (lanes 7–12), Xβm became partially endo H-resistant during the chase period, reflecting the acquisition of complex and hybrid-type sugars during its passage through the Golgi to the plasma membrane. Likewise, chicken βm (chβm) (Fig. 2*c*, lanes 1–6) and pufferfish βm (pβm) (lanes 7–12) were associated with chicken α1-subunit (chα1) and Xα1, respectively, stabilized the α-subunit, and became partially endo H-resistant. In contrast, hβm was not coimmunoprecipitated with chα1 or Xα1, and did not stabilize

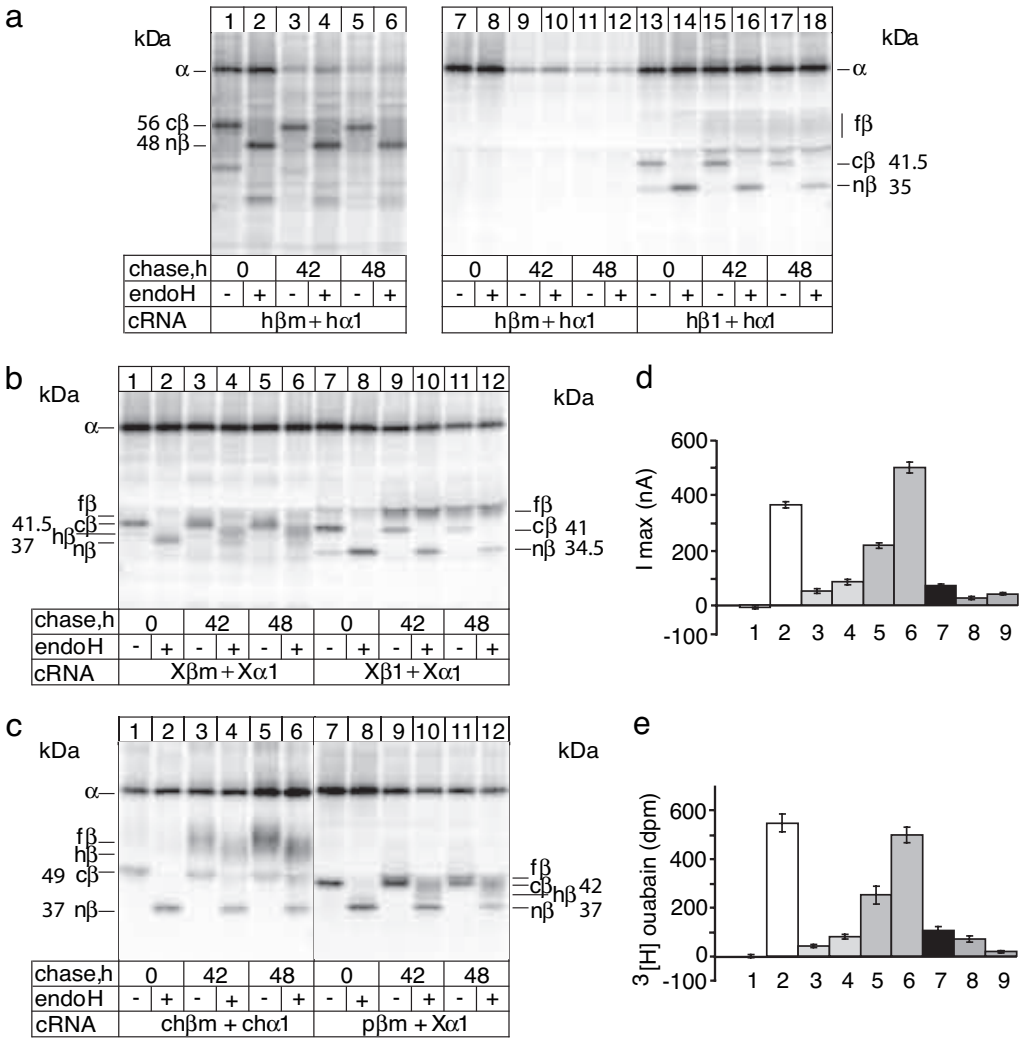

Fig. 2. Xβm, chicken βm (chβm), and pufferfish βm (pβm), but not hβm, associate with Na,K-ATPase α-subunits and form functional Na,K-ATPase at the cell surface. (*a–c*) Expression in *Xenopus* oocytes and coimmunoprecipitation of ^{35}S-metabolically labeled βm of different species with Na,K-ATPase α-subunits. Shown are SDS/PAGE (*a*, lanes 1–6) and immunoprecipitations with Na,K-ATPase α-subunit antibody (*a*, lanes 7–18, and *b* and *c*). Indicated are the positions of the α-subunit (α), nonglycosylated β (ng), coreglycosylated β (cg), hybrid glycosylated β (hβ), and fully glycosylated β (fg) subunits. endo H, endoglycosidase H. (*d*) Na,K-pump currents of α1 plus human βm (bar 1) or human β1 (bar 2); chicken α1 plus chicken βm (bar 3) or chicken β1 (bar 4); *Xenopus* α1 plus *Xenopus* βm (bar 5) or *Xenopus* β1 (bar 6); *Xenopus* α1 plus puffer fish βm (bar 7); *Xenopus* βm alone (bar 8); *Xenopus* β1 alone (bar 9). Shown are means ± SE of 21 oocytes from four different batches. All βm vs. β1, $P < 0.01$. (*e*) [^{3}H]Ouabain binding. Injection of cRNAs are the same as in *d*. Shown are means ± SE of 21 oocytes from four different batches.

the α-subunits (SI Fig. 6*a*). However, chβm, Xβm, and pβm did form a stable complex with hα1 and the nonmammalian βm-proteins became fully glycosylated (SI Fig. 6*b*).

To test whether nonmammalian βm can produce functional Na,K-ATPase at the cell surface, we measured the maximal Na,K-pump current by electrophysiological means in oocytes expressing the different βm-proteins together with α1-subunits. As expected, hβm expressed with hα1 did not increase the Na,K-pump current compared with noninjected oocytes (Fig. 2*d*, bar 1) in contrast to control human Na,K-ATPase β1 (hβ1) expressed with hα1 (bar 2). However, chicken βm (chβm) expressed with chicken α1-subunit (chα1) (bar 3), Xβm expressed with Xα1 (bar 5) and pufferfish βm (pβm) expressed with Xα1 (bar 7) produced a significant increase in Na,K-pump currents. Both Xβm (bar 8) and Xβ1(bar 9) expressed alone in oocytes slightly increased the Na,K-pump current through their association to the endogenous α-subunit expressed over β-subunits (3). The somewhat lower Na,K-pump activity in βm-expressing oocytes compared with β1-expressing oocytes is not due to a decrease in the intrinsic activity of Na,K-ATPase but is due to reduced cell surface Na,K-ATPase expression as indicated by the parallel decrease in ouabain binding in intact oocytes (Fig. 2*e*, bars 2–6), except for Xβm (bar 8) and Xβ1 (bar 9) expressed alone.

It is well established that β-subunits differentially modulate the apparent affinity of Na,K-ATPase for extracellular K^+ (3). This property was examined in oocytes expressing Xα1 together with control Xβ1 or Xβm. Xα1/Xβ1 complexes exhibit a voltage-dependent K^+ activation. Xα1/Xβm complexes have a lower K^+ affinity than Xα1/Xβ1 complexes over the whole range of membrane potentials and K^+ activation is only slightly voltage dependent between −140 mV and 0 mV (SI Fig. 7).

These results demonstrate that nonmammalian βm-proteins do associate with Na,K-ATPase α-subunits of the same or other species, prevent α-subunit degradation, and produce functional ion pumps at the cell surface indicating that they function as genuine Na,K-ATPase subunits.

BIOCHEMISTRY

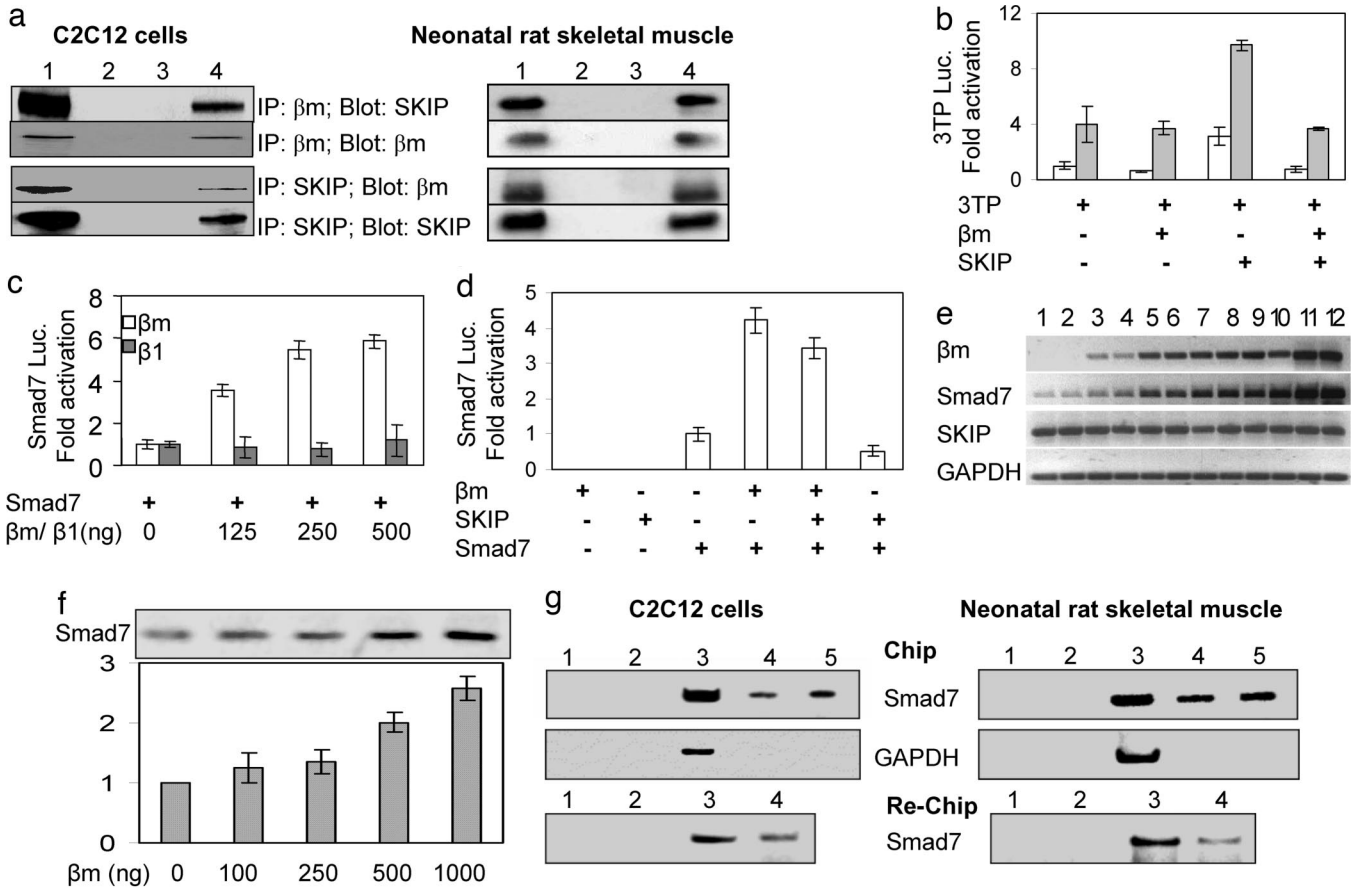

Fig. 3. βm functions as a transcriptional coregulator in mammalian cells. (*a*) Interaction of βm and SKIP coexpressed in C2C12 cells and endogenous βm and SKIP in rat neonatal skeletal muscle. Controls were as follows: input, C2C12 (10%), muscle (5%) (lane 1), no cell extract (lane 2), no antibodies (lane 3). Immunoprecipitated (IP) sample is shown in lane 4. (*b*) Effect of βm–SKIP interaction on the activity of TGF-β-responsive 3TP-luciferase construct in the absence (open bars) and presence of TGF-β, 2 ng/ml (filled bars). (*c*) βm induces the Smad7 luciferase reporter gene in a dose-dependent manner. Na,K-ATPase β1 subunit was used as a control. (*d*) Effect of βm-SKIP interaction on Smad7 luciferase reporter gene expression. (*b–d*) The basal activities of reporter plasmids were set to 1, and all results are shown as means ± SD; $n = 3$. Transfection efficiencies were normalized by measuring the activities of β-galactosidase (*c* and *d*) or *Renilla* luciferase (*b*). (*e*) Effect of βm on endogenous mRNA levels of Smad7, SKIP, and GAPDH. C2C12 cells were transfected with increasing amounts of βm in ng/ml (lane 1, 0; lane 2, 10; lane 3, 20; lane 4, 40; lane 5, 60; lane 6, 100; lane 7, 150; lane 8, 200; lane 9, 250; lane 10, 500; lane 11, 1,000; lane 12, 1,500) and analyzed by RT-PCR using specific primers in ng/ml (*SI Materials and Methods*). (*f*) Up-regulation of endogenous Smad7 protein by increasing amounts of transfected βm in C2C12 cells. Shown are the Western blot analyses of equal amounts of proteins and densitometry results of three experiments (means ± SE). (*g*) ChIP and re-ChIP assays in neonatal rat skeletal muscle and C2C12 cells cotransfected with βm and SKIP. Controls were as follows: chromatin alone (lane 1); IgG (lane 2); and input, 0.05% of total (lane 3). ChIP with antibodies against βm (lane 4) and SKIP (lane 5). For re-ChIP, βm antibody was used first followed by SKIP antibody (lane 4).

As a first step to understand the function(s) of eutherian βm in the myonuclear membrane, we sought to identify βm-interacting proteins by using the yeast two-hybrid system. Although membrane proteins are known to be less amenable to classical yeast two-hybrid assay (15), in case of βm, a natural resident of inner nuclear membranes (ref. 7 and SI Figs. 8 and 9), this approach turned out to be productive. Among potential βm-interacting proteins, which met criteria for specificity in yeast two-hybrid assay, two known residents of the inner nuclear membrane were identified: Syne1/Myne1, spectrin-repeat-containing protein (16), and LAP1, Lamina-associated polypeptide1 (17). The set of identified βm-interacting proteins also included βm itself, indicating that it may exist as a dimer *in vivo*. Direct interaction of βm with LAP1 in rat neonatal skeletal muscle was confirmed by coimmunoprecipitation (SI Fig. 9). These observations are congruent with subcellular βm location in inner nuclear membranes.

More importantly, yeast two-hybrid screen identified nuclear transcriptional coregulator SKIP, Ski-interacting protein (18), as a βm-interactor. To further confirm specificity of this interaction, βm and SKIP were coexpressed in a mammalian C2C12 myoblast cell line, which has endogenous SKIP but lacks βm (SI Fig. 10). Both SKIP and βm were detected in samples immunoprecipitated with either βm- or SKIP-antibodies, demonstrating the ability of βm to interact with SKIP in mammalian cells (Fig. 3*a*). The same results were obtained with rat neonatal muscle samples (Fig. 3*a*), thus demonstrating that βm and SKIP interaction is biologically relevant and not an artifact of overexpression.

SKIP has been shown to affect transcriptional programming of a variety of signaling networks including TGF-β/Smad pathway, in which SKIP augments the TGF-β-dependent transcription via interaction with Smad proteins (18, 19). Therefore, we sought to investigate whether βm interaction with SKIP would affect transcriptional activity by using TGF-β responsive luciferase reporter gene constructs.

The first set of experiments was done with 3TP-Lux reporter (20), which contains multiple TGF-β response elements in the promoter, including Smad-binding element (SBE). In agreement with published data (19), overexpression of SKIP in C2C12 cells increased the basal and the TGF-β-induced 3TP-Lux activity (Fig. 3*b*). Expression of βm alone did not affect the transcrip-

tional activity, whereas coexpression of βm and SKIP almost completely repressed the SKIP-dependent activation of the basal and the TGF-β-induced 3TP-Lux activity, demonstrating that the interaction of βm with SKIP antagonizes the effect of TGF-β on 3TP-Lux. Experiments with the SBE reporter containing only SBEs in the promoter (21) also revealed that βm–SKIP interaction results in elimination of the SKIP-dependent reporter activation (SI Fig. 11). These findings suggest that βm–SKIP interaction represses the activity of these TGF-β responsive reporters either by competing with Smad proteins for binding to SKIP or by preventing the interaction of Smad–SKIP complexes with SBEs.

Because the TGF-β/Smad signaling network is tightly regulated by an autoinhibitory feedback mechanism via inhibitory Smad7 expression induced by TGF-β (22–25), we next investigated whether βm and SKIP could affect the Smad7 gene expression by using the luciferase reporter (ps7–5) (26). In contrast to results obtained with SBE and 3TP-Lux reporters, the basal level of the Smad7 reporter expression in C2C12 cells transfected with SKIP was reduced by 50% (Fig. 3*d*). This negative effect of SKIP was reversed to stimulation when βm was coexpressed with SKIP. The expression of βm alone suppressed endogenous SKIP function leading to the up-regulation of Smad7 reporter activity up to 5-fold in a dose-dependent manner (Fig. 3*c*). Transfection with Na,K-ATPase β1 did not affect the Smad7 luciferase reporter activity, indicating that up-regulation of Smad7 by βm is specific (Fig. 3*c*).

These results demonstrate that SKIP can regulate the rearrangement between repressing and activating transcriptional complexes and that βm–SKIP interaction does play a role in the transactivation of the Smad7 luciferase reporter.

To examine the effect of βm on Smad7 gene expression, C2C12 cells were transfected with varying amounts of βm, and levels of endogenous Smad7, SKIP, and GAPDH mRNAs were detected by RT-PCR (Fig. 3*e*). Smad7 basal mRNA levels were increased even at low concentrations of transfected βm, whereas no effect on the level of SKIP mRNA was observed. Endogenous Smad7 protein expression was also increased in βm-transfected C2C12 cells (Fig. 3*f*). These results demonstrate the ability of βm to up-regulate Smad7 gene expression, which is consistent with the results obtained in luciferase assays (Fig. 3 *c* and *d*).

To demonstrate directly the presence of the authentic βm and SKIP in the Smad7 transcriptional complex *in vivo*, we performed a ChIP analysis. The conserved 141-bp fragment of Smad7 promoter containing SBE, activator protein 1 (AP1), E-box, and CCAAT/enhancer binding protein (C/EBP) response elements (26–28) was amplified using DNA fragments obtained from SKIP or βm-antibody immunoprecipitated rat neonatal skeletal muscle chromatin. As shown in Fig. 3*g*, the amplified bands were obtained with Smad7 primers, but not with control GAPDH primers. Further re-ChIP experiments were performed to demonstrate the presence of SKIP and βm on the same Smad7 promoter (Fig. 3*g*). In this case, the cross-linked chromatin was precipitated first with βm-antibody and then with SKIP antibody. Amplification of the immunoprecipitated DNA from re-ChIP assay produced a positive result for the Smad7 promoter. These observations clearly demonstrate that βm and SKIP are the components of the Smad7 transcriptional complex *in vivo*. Similar results were obtained with C2C12 cells transfected with βm and SKIP (Fig. 3*g*).

Thus, we show that eutherian βm, which is accumulated in nuclear membranes of perinatal myocytes (7), lost its ancestral function as a Na,K-ATPase β-subunit and associates with transcriptional coregulator SKIP (Fig. 4). Through this interaction, βm is involved in the regulation of the activity of TGF-β responsive reporters and is able to up-regulate mRNA levels of inhibitory Smad7 in C2C12 myoblast cells, thereby suggesting that βm may play a role in TGF-β signaling. These properties of

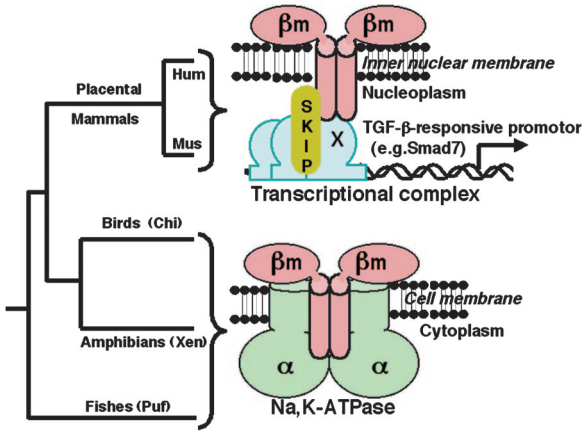

Fig. 4. Changes in functional properties of βm-proteins encoded by orthologous vertebrate ATP1B4 genes during evolution. Direct β,β association in Na,K-ATPase (37) and results of present yeast two-hybrid assays indicate that βm may exist as a dimer *in vivo*. DNA binding components of transcriptional complex are denoted by X.

βm together with its previously described spatiotemporal pattern of expression (4, 6, 7) allow us to suggest that βm might function *in vivo* as a coregulator of gene expression during muscle development (29–31). Eutherian βm may also affect other cellular processes through interaction with SKIP, which in turn can interact with different nuclear proteins involved in regulation of various signaling pathways and in RNA processing (18).

This study demonstrates evolutionary shifts in functions of orthologous vertebrate ATP1B4 genes, such as changes in tissue-specific expression, changes in subcellular location, alterations in protein structures and loss of ancestral function that created fundamental changes in functional properties of encoded proteins. Further investigations are needed to reveal the nature of evolutionary forces that underlie the necessity and physiological importance of ATP1B4 gene cooption in placental mammals.

Materials and Methods

Cloning of βm-cDNA. Chicken βm-cDNA(DQ358914) has been cloned using RACE from 19-day-old chicken embryo skeletal muscle essentially as described in ref. 4 by using primers that are based on EST sequences from GeneBank. Full-length ORF was amplified with primers complementary to 5′- and 3′-termini. *Tetraodon nigroviridis* βm-ORF (DQ417195) was amplified from brain cDNA by using primers that are based on the sequence of genomic DNA from GeneBank. The sequence of *Xenopus laevis* βm (DQ413025) was derived from the full-length insert of an XL095c22 EST clone.

Protein Expression in *Xenopus* Oocytes. *Xenopus* oocytes were injected with cRNAs coding for human, chicken, *Xenopus*, or pufferfish β1 or βm (4 ng per oocyte) and Na,K-ATPase α1 (14 ng per oocyte) subunits. Oocytes were ^{35}S-metabolically labeled (6 h) and subjected to 24-h and 48-h chase periods. Microsomes were prepared, and immunoprecipitations were performed with Na,K-ATPase α-antibodies under nondenaturing conditions as described in refs. 9 and 32. Immunoprecipitated proteins were detected by SDS/PAGE and fluorography.

Electrophysiological Measurements. Maximal Na,K-pump currents and $K_{1/2}K^+$ values were measured 3 days after cRNA injection of *Xenopus* oocytes by using the two-electrode voltage-clamp technique as described in refs. 9 and 32. Na,K-pump currents measured in noninjected oocytes were deduced from those

BIOCHEMISTRY

measured in cRNA-injected oocytes. Statistical analysis was performed by unpaired Student's *t* test.

[^{3}H]Ouabain-Binding on Intact Oocytes. Three days after cRNA injection, the total number of Na,K pumps expressed at the cell surface was determined by [^{3}H]ouabain binding as described in ref. 32. Nonspecific binding was determined on noninjected oocytes.

Yeast Two-Hybrid Screening. The bait plasmid constructed by cloning the full-length βm-coding region in frame with the GAL-binding domain of pGBKT7 vector was used to screen BD Matchmaker 17-day-old mouse embryo cDNA library (BD Biosciences, San Jose, CA) according to the manufacturer's protocols. Plasmids from positive yeast transformants were rescued and sequenced. The interaction of the hybrid proteins was examined by cotransformation of the prey and bait plasmids by using empty pGBKT7 vector as a negative control.

Luciferase Reporter Gene Assay. The C2C12 cell line (1×10^5 cells) was transiently transfected with Smad7 luciferase reporter plasmid (ps7–5) (26) with or without plasmids containing SKIP and βm in different combinations. The luciferase activity was determined as described in ref. 33 by using the Luciferase Assay System (Promega, Madison, WI). Luciferase assays with 3Tplux (20) and SBE (21) reporter plasmids were done in the same way. Cells were treated with TGF-β (2 ng/ml) for 60 min to examine the TGF-β effect.

ChIP Assays. ChIP assays were performed according to the standard procedure (33–35). Samples of cross-linked chromatin were precipitated with βm-antibody (5) or SKIP antibody (36). The immunoprecipitated Smad7 DNA fragments obtained from ChIP and re-ChIP assays were detected by PCR amplification by using [^{32}P]αdCTP.

More detailed descriptions of the methods are available in *SI Materials and Methods*.

We thank T. Shin-I (National Institute of Genetics, Mishima, Japan) for the XL095c22 EST clone; E. P. Bottinger (Mount Sinai School of Medicine, New York, NY), J. Massagué (Memorial Sloan–Kettering Cancer Center, New York, NY), and Y. Chen (Indiana University School of Medicine, Indianapolis, IN) for luciferase constructs ps7–5, 3TPlux, and SBE; O. Vagin (University of California, School of Medicine, Los Angeles, CA) for NK β1 vector; P. N. MacDonald (Case Western Reserve University, Cleveland, OH) for NCoA62/SKIP antibodies; and A. D. Beavis for a critical reading of the manuscript. This work was supported by National Institutes of Health Grant HL-36573 (to N.N.M.), Swiss National Science Foundation Grant 3100A0-107513 (to K.G.), and Russian Foundation for Basic Research Grant 00-04-48153 (to N.B.P.).

1. Moller JV, Juul B, le Maire M (1996) *Biochim Biophys Acta* 1286:1–51.
2. Jorgensen PL, Hakansson KO, Karlish SJ (2003) *Annu Rev Physiol* 65:817–849.
3. Geering K (2001) *J Bioenerg Biomembr* 33:425–438.
4. Pestov NB, Adams G, Shakhparonov MI, Modyanov NN (1999) *FEBS Lett* 456:243–248.
5. Pestov NB, Korneenko TV, Zhao H, Adams G, Shakhparonov MI, Modyanov NN (2000) *Biochem Biophys Res Commun* 277:430–435.
6. Pestov NB, Korneenko TV, Zhao H, Adams G, Kostina MB, Shakhparonov MI, Modyanov NN (2001) *Arch Biochem Biophys* 396:80–88.
7. Zhao H, Pestov NB, Korneenko TV, Shakhparonov MI, Modyanov NN (2004) *Am J Physiol Cell Physiol* 286:C757–C767.
8. Pestov NB, Crambert G, Zhao H, Korneenko TV, Shakhparonov MI, Geering K, Modyanov NN (2003) *Ann N Y Acad Sci* 986:304–305.
9. Crambert G, Beguin P, Pestov NB, Modyanov NN, Geering K (2002) *Biochemistry* 41:6723–6733.
10. True JR, Carroll SB (2002) *Annu Rev Cell Dev Biol* 18:53–80.
11. Ganfornina MD, Sanchez D (1999) *Bioessays* 21:432–439.
12. Eizinger A, Jungblut B, Sommer RJ (1999) *Trends Genet* 15:197–202.
13. Jaillon O, Aury JM, Brunet F, Petit JL, Stange-Thomann N, Mauceli E, Bouneau L, Fischer C, Ozouf-Costaz C, Bernot A, *et al.* (2004) *Nature* 431:946–957.
14. Hillier LW, Miller W, Birney E, Warren W, Hardison RC, Ponting CP, Bork P, Burt DW, Groenen MAM, Mary E, *et al.* (2004) *Nature* 432:695–717.
15. Fields S (2005) *FEBS J* 272:5391–5399.
16. Mislow JM, Kim MS, Davis DB, McNally EM (2002) *J Cell Sci* 115:61–70.
17. Martin L, Crimaudo C, Gerace L (1995) *J Biol Chem* 270:8822–8828.
18. Folk P, Puta F, Skruzny M (2004) *Cell Mol Life Sci* 61:629–640.
19. Leong GM, Subramaniam N, Figueroa J, Flanagan JL, Hayman MJ, Eisman JA, Kouzmenko AP (2001) *J Biol Chem* 276:18243–18248.
20. Wrana JL, Attisano L, Cardamom J, Zentella A, Doody J, Laiho M, Wang XF, Massague J (1992) *Cell* 71:1003–1014.
21. Nagarajan RP, Chen F, Li W, Vig E, Harrington MA, Nakshatri H, Chen Y (2000) *Biochem J* 348:591–596.
22. Nakao A, Afrakhte M, Moren A, Nakayama T, Christian JL, Heuchel R, Itoh S, Kawabata M, Heldin NE, Heldin CH, *et al.* (1997) *Nature* 389:631–635.
23. Hayashi H, Abdollah S, Qiu Y, Cai J, Xu YY, Grinnell BW, Richardson MA, Topper JN, Gimbrone MA, Jr., Wrana JL, *et al.* (1997) *Cell* 89:1165–1173.
24. Massague J, Seoane J, Wotton D (2005) *Genes Dev* 19:2783–2810.
25. Feng XH, Derynck R (2005) *Annu Rev Cell Dev Biol* 21:659–693.
26. von Gersdorff G, Susztak K, Rezvani F, Bitzer M, Liang D, Bottinger EP (2000) *J Biol Chem* 275:11320–11326.
27. Brodin G, Ahgren A, ten Dijke P, Heldin CH, Heuchel R (2000) *J Biol Chem* 275:29023–29030.
28. Hua X, Miller ZA, Benchabane H, Wrana JL, Lodish HF (2000) *J Biol Chem* 275:33205–33208.
29. Liu D, Black BL, Derynck R (2001) *Genes Dev* 15:2950–2966.
30. Molkentin JD, Olson EN (1996) *Curr Opin Genet Dev* 6:445–453.
31. Charge SB, Rudnicki MA (2004) *Physiol Rev* 84:209–238.
32. Jaunin P, Horisberger JD, Richter K, Good PJ, Rossier BC, Geering K (1992) *J Biol Chem* 267:577–585.
33. Ahmad N, Lingrel JB (2005) *Biochemistry* 44:6276–6285.
34. Huddleson JP, Ahmad N, Srinivasan S, Lingrel JB (2005) *J Biol Chem* 280:23371–23379.
35. Wells J, Farnham PJ (2002) *Methods* 26:48–56.
36. Zhang C, Dowd DR, Staal A, Gu C, Lian JB, van Wijnen AJ, Stein GS, MacDonald PN (2003) *J Biol Chem* 278:35325–35336.
37. Ivanov AV, Modyanov NN, Askari A (2002) *Biochem J* 364:293–299.