Identification of a novel gene of the X,K-ATPase β-subunit family that is predominantly expressed in skeletal and heart muscles[1]

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Abstract We have identified the fifth member of the mammalian X,K-ATPase β-subunit gene family. The human and rat genes are largely expressed in skeletal muscle and at a lower level in heart. The deduced human and rat proteins designated as β_{muscle} (β_m) consist of 357 and 356 amino acid residues, respectively, and exhibit 89% identity. The sequence homology of β_m proteins with known Na,K- and H,K-ATPase β-subunits are 30.5–39.4%. Unlike other β-subunits, putative β_m proteins have large N-terminal cytoplasmic domains containing long Glu-rich sequences. The data obtained indicate the existence of hitherto unknown X,K-ATPase (most probably Na,K-ATPase) isozymes in muscle cells.

Key words: P-ATPase; Na,K-ATPase; H,K-ATPase; β-Subunit; Tissue-specific expression; Muscle-specific isoform

1. Introduction

The X,K-ATPases that pump K^+ into and Na^+ or H^+ out of the cell are the most closely related P-ATPases [1,2]. They are composed of catalytic α-subunits (~110–115 kDa) and glycosylated β-subunits (core protein ~30–35 kDa) [2–6]. The β-subunits, which are absent in other P-ATPases, are indispensable for structural and functional maturation of X,K-ATPases [7,8]. The X,K-ATPase family consists of three distinct groups of ion pumps: the Na,K-ATPases, cardiac glycoside receptors [3,4], the gastric H,K-ATPases [5] and ouabain-sensitive, non-gastric H,K-ATPases [6,9,10]. The α-subunits of Na,K-ATPase (four isoforms), gastric and non-gastric H,K-ATPases exhibit ~63% sequence identity [2,11–16]. Four known X,K-ATPase β-subunits (β1, β2, β3 and β_g) share a common domain structure, but exhibit a twice lower similarity than α-subunits [5,7,8,13].

Each of these proteins has a characteristic pattern of expression (for review, see [13]). The principle Na,K-ATPase isozyme (α1β1) is expressed in all tissues. The α2 is largely expressed in heart, brain and skeletal muscle, the α3 is mostly expressed in neuronal tissue and the α4 is a testis-specific isoform [14]. The β2 expression is limited to heart, skeletal muscle and neuronal tissue, while β3 is present in many tissues. In addition to stomach, the gastric H,K-ATPase αmRNA was traced in kidney [5]. The expression of non-gastric H,K-ATPase α-subunits encoded by mammalian ATP1AL1 genes was detected in colon, kidney, skin and brain [9,10,14,16,17]. The real β-subunit of non-gastric H,K-ATPase is unknown. Our studies indicate that a yet unidentified β-subunit rather than any of the known ones serves as a real counterpart of ATP1AL1 in vivo [6,10,17].

In search of a candidate for a β-subunit of non-gastric H,K-ATPase, we detected in rat neonatal skin a transcript capable to encode a 'β-like' protein. The rat and homologous human cDNAs have been cloned and sequenced. These new members of the X,K-ATPase β-subunit gene family are predominantly expressed in skeletal muscles and at a lower level in hearts. Since only traces of mRNAs were detected in skin and other tissues, it is unlikely that these novel proteins are subunits of the non-gastric H,K-ATPases. We term these novel β-subunits β_{muscle} (β_m). The data obtained suggest the existence of novel distinct X,K-ATPase isozymes.

2. Materials and methods

2.1. RNA isolation

6 Months (adult) and 3 days (neonatal) old Sprague-Dawley rats were killed by decapitation. Samples of human tissues were obtained from the Cooperative Human Tissue Network (Columbus, OH, USA) and from the Medical College of Ohio Hospital. Small scale total RNA preparations were made from about 30 mg tissue using the SV Total RNA Isolation system (Promega). For mRNA isolation, total RNA was extracted using Trizol reagent (Life Technologies), followed by chromatography on an oligo-(dT)-cellulose using a PolyA Spin mRNA isolation kit (New England Biolabs).

2.2. Reverse transcription-polymerase chain reaction (RT-PCR)

Routine first strand cDNA synthesis and PCR with Taq DNA polymerase were performed as described before [17]. The quality of cDNA was monitored using amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA [17]. For tissue-specific expression studies, primer pairs DSBNF (ATCAGACCCTTCGCCCATAGC, coordinates 466–486 from the initiation codon)/DSBNB (ACGCCTTTGCCCTTCAGTTG, 988–1007) and DSRF (GATCAGACCCTTCGCTCATAGC, 462–483)/DSRB (GATGCCTTTGCCCTTCAGCTG, 985–1005) were used for human and rat tissues, respectively. Most of the translated regions of both cDNAs were amplified with primers PU (CAATGCCTGGTGGCAGAAATTGCAGATC, 237–264) and TU (AGATGATCGGCTGTCTTAGATG-

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[1] The nucleotide sequences reported here have been submitted to the GenBank/EBI DATA Bank with accession numbers AF15383, AF15384, AF15385 and AF15386.

Abbreviations: α1, α2, α3, α4, α1-, α2-, α3- and α4-subunits of Na,K-ATPase; β1, β2, β3, β1-, β2- and β3-subunits of Na,K-ATPase; α_g and β_g, α- and β-subunits of the gastric H,K-ATPase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT-PCR, reverse transcription-polymerase chain reaction; RACE, rapid amplification of cDNA ends; UTR, untranslated region

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TCC, 1176–1200) using *Pfu* DNA polymerase (Promega). The PCR products were analyzed by gel electrophoresis.

2.3. 5′- and 3′-end regions determination by rapid amplification of cDNA ends (RACE)-PCR

The SMART RACE cDNA Amplification kit and Advantage 2 PCR kit (Clontech) were applied according to the manufacturer's instructions with some modifications. cDNA was synthesized with 2 μg poly-A+ RNA and 'Superscript I' reverse transcriptase (Life Technologies) for 1 h at 42°C and 1 h at 50°C. PCR was performed with 1/50 of the cDNA using Advantage 2 DNA polymerase (Clontech) with the supplied buffer, 0.2 μm of each primer and 200 μM dNTP. 3′-RACE was performed with primers FBNR (GATGAGGACAAGAAGGCCTGCCAATTTAAACGC, 637–669) for rat and FMHR (GATGAGGACAAGAAGGCCTGCCAATTTAAGCG, 640–671) for human cDNAs and universal primer mix (Clontech) for 22 cycles at 94°C (25 s), 70°C (4 min and 25 s), followed by a second amplification using 1/50 of the first reaction with nested primers MODNES (GGCGATCAGTGGTAACAACCCAGAGTAC) and primers (FKR (GCTACTACCCTTACTATGGCAAACTGAC, 878–905) for rat or FK (TACTACCCTTACTACGGCAAACTGAC, 883–908) for human cDNAs) for 17 cycles at 94°C (25 s), 60°C (25 s), 72°C (4 min and 25 s). 5′-RACE of both human and rat cDNA was achieved with the human primer BMHR (CGCTTAAATTGGCAGGCCTTCTTGTCCTCATC, 640–671) and universal primer mix for 28 cycles at 94°C (25 s) 70°C (2 min and 25 s) (for rat cDNA, annealing at 68°C for 25 min was included) followed by PCR with nested primers BNNB (AGRCCTGTTCGGGCCAGARACATCC, 296–317) and MODNES for 28 cycles at 94°C (25 s), 60°C (25 s), 72°C (2 min and 25 s).

3. Results

3.1. Identification and characterization of rat and human β_m cDNAs

In a search of the GenBank database with sequences of known X,K-ATPase β-subunits using the TBLASTN program, we identified a rat cDNA EST clone (Accession No AI071721) encoding sequence of 50 amino acids with 42% similarity to the rat β2 fragment, D^{75}-Q^{124} [18], and 32% similarity to rat β_g [19]. We used this sequence to design primers for PCR analysis and for the first time, the existence of a transcript was detected in rat neonatal skin cDNA. In the majority of subsequent experiments, skeletal muscle cDNAs were used as templates.

When the present study was in progress, a draft of three unordered pieces of human genomic sequence from clone DA0052J04 was deposited to the high throughput genomic sequences division of the GenBank database (Waterston, R.H., accession number AC006962). One of these fragments represents a contig of 62 520 bp and was found to contain several clusters of sequences exhibiting a high level of identity with the rat cDNA under study. We suggested that the rat and human sequences represent either homologous or closely related genes. Genomic data were used to design specific primers to amplify most of the coding region and to determine 5′- and 3′-ends of the cDNAs by RACE-PCR. Both human and rat mRNAs were reconstructed by direct sequencing of PCR products from coding regions and cloned fragments of their 3′-untranslated regions (UTRs).

As judged from these results, both the human and rat mRNAs are about 4.0 kbp long with short 5′-UTRs (~80 nucleotides (nt)). Their 3′-UTRs are unusually long (3.2 kbp), being the longest among known β-subunit mRNAs.

The composite human and rat cDNA sequences generated from these experiments contain 1074 nt and 1071 nt open reading frames, respectively. The nucleotide and amino acid sequence identities between human and rat isoforms are 86.5 and 89%, respectively. Thus, the rat and the human genes and the encoded proteins are highly conserved homologues.

3.2. Structural features of β_m proteins

The primary products of human and rat gene translations consist of 357 and 356 amino acid residues and have molecular masses of 41.6 and 41.55 kDa, respectively. Coding human cDNA sequences and the deduced structures of the human and rat proteins are shown in Fig. 1.

Hydropathy analysis predicts that the transmembrane topology of the putative proteins is similar to that of previously identified X,K-ATPase β-subunits, containing a highly charged cytoplasmic N-terminal domain followed by a transmembrane segment (L^{111}-L^{136}) and a large ectodomain [7,8,13,20]. Other structural features specific to X,K-ATPase β-subunits include the conserved motif[2] ^{295}Y(Y/F)PYYGK^{301}, six conserved Cys residues (201, 220, 230, 246, 269 and 329) and four potential sites for *N*-glycosylation (Fig. 1). The new proteins exhibit 30–39% of overall sequence identity with known X,K-ATPase β-subunits [8,13,18,19,21,22].

The above indicates that the human and rat cDNAs described here encode a new type of X,K-ATPase β-subunits designated as β_m. The novel β-subunits exhibit a unique structural feature. Their N-terminal cytoplasmic domains are significantly larger than those of known β-subunits and contain long Glu-rich sequences which, according to a secondary structure prediction [23], form two long α-helices, E^{26}-V^{45} and E^{54}-E^{72}. None of the other P-type ATPase subunits contains this kind of sequence motif.

Another specific feature of β_m proteins is the existence of the variants lacking tetrapeptide ^{107}QSWS^{110} (human) or ^{106}QSRS^{109} (rat). This was predicted when two alternative splice forms of cDNA, one of them lacking 12 bp, were identified in human and rat PCR products. This protein fragment is a part of a conserved sequence (see below) which includes the junction between cytoplasmic and membrane domains (Fig. 1).

3.3. Organization of the human β_m gene and tissue-specificity of β_m expression

Detailed sequence comparison of the human β_m cDNA with genomic clone DA0052J04 allowed for the reconstruction of the exon-intron structure of the β_m human gene which is divided into eight exons (Fig. 1). All splice donor and acceptor sequences conform to the standard consensus (data not shown) [24]. Two different donor sites at the exon 2-intron 2 boundary (Fig. 1) have been used in the alternative splicing event described above. The exon organization of the β_m gene and locations of the exon/intron boundaries relative to the protein sequence are quite similar to that of β-subunit genes [25]. The β1 gene contains six exons, whereas those of β2, β3 and β_g are divided into seven exons [25]. The additional exon in the β_m gene encodes the extreme N-terminal domain which has no homology in other β-subunits. This similarity is consistent with the view that all these genes originated from a common evolutionary ancestor.

Screening of the STS division of the GenBank database

[2] Here and throughout, all amino acid numbering in the text is indicated according to the human β_m sequence.

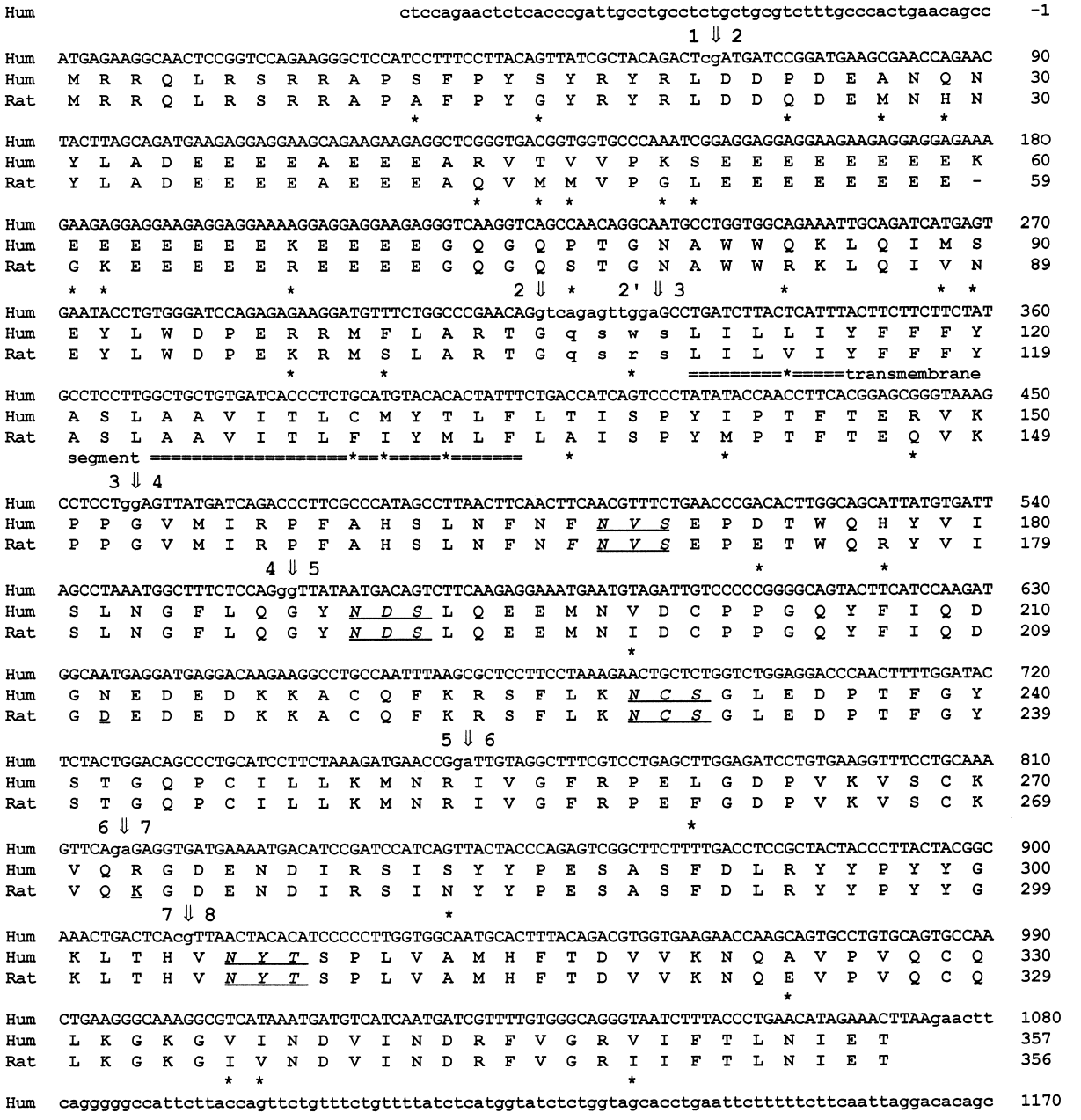

Fig. 1. Nucleotide sequence of the human X,K-ATPase β_m subunit and deduced amino acid sequences of human and rat β_m proteins. The human nucleotide sequence is shown above the deduced amino acid sequences of human and rat proteins. Nucleotides and amino acids are numbered to the right of the sequences beginning with the initiating ATG and Met, respectively. Consensus sequences of potential sites of *N*-glycosylation are underlined. Non-conserved amino acid residues are marked by asterisks. Positions of the introns in the human gene are indicated by arrows, lowercase letters and flanked by exon numbers. The sequence of the alternative splicing site and corresponding amino acid residues are depicted by lowercase letters.

revealed sequence identity of two regions of human β_m cDNA corresponding to exons 4 and 8 of the gene, with the X chromosome sequence-tagged sites stSG34617 and stSG45811. These data indicate that the human β_m gene is located on the X chromosome.

A qualitative analysis of the β_m gene expression in various rat and human tissues was done using RT-PCR. A prominent signal was observed in skeletal muscle and a weaker one in heart tissue (Fig. 2). In other tissues tested, only a trace or no expression was detected. The list of rat tissues with trace expression is: skin, uterus, lung, pancreas, prostate, esophagus, vena cava, adrenal, adipose tissue, uterus, ovary, stomach, spleen, vas deferens and thymus. The transcript seems to be absent in testis, preputial, vesicular and coagulating glands, kidney, liver, bone marrow, colon mucosa, brain and duodenum. Human tissues tested and found to almost or completely lack the transcript are skin, jejunum, uterus, brain, prostate, liver, skin, kidney and placenta.

Different rat skeletal muscles (diaphragm, tongue, soleus and extensor digitorum longum) showed the presence of the transcript without a dramatic difference in the expression level (results not shown).

These data allowed us to conclude that the gene exhibits a muscle-specific expression pattern being especially abundant

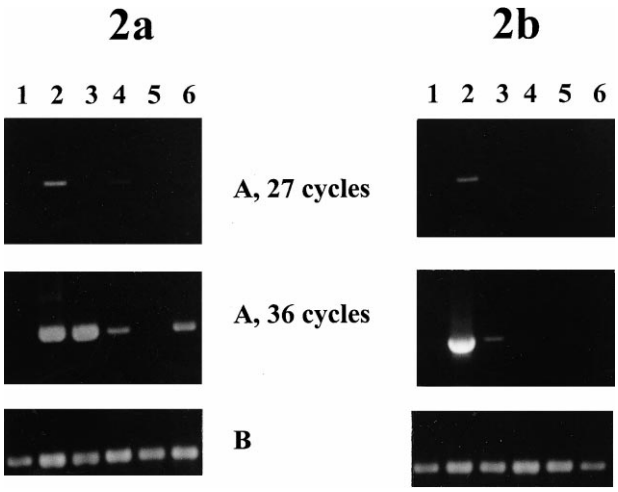

Fig. 2. RT-PCR analysis of tissue-specific expression of the X,K-ATPase β_m-subunit. (A) Amplification products of the β_m mRNA. (B) Control amplification of GAPDH. 2a: Rat: 1, brain; 2, skeletal muscle; 3, heart; 4, skin; 5, duodenum; 6, uterus. 2b: Human: 1, brain (cortex, white matter and meninges); 2, skeletal muscle; 3, heart; 4, skin; 5, jejunum; 6, uterus (myometrium).
←

in skeletal muscle and at a lower level in heart. In other adult tissues, the expression seems to be absent or at a trace level that may be attributed to smooth muscle cells. The initial detection of the transcript in rat skin may be due to the presence of skeletal muscles in the samples used. To underline muscle-specific patterns of expression, we termed these novel β-subunits β_m.

4. Discussion

We have identified the human and rat cDNAs encoding a novel muscle-specific X,K-ATPase β_m subunit whose existence

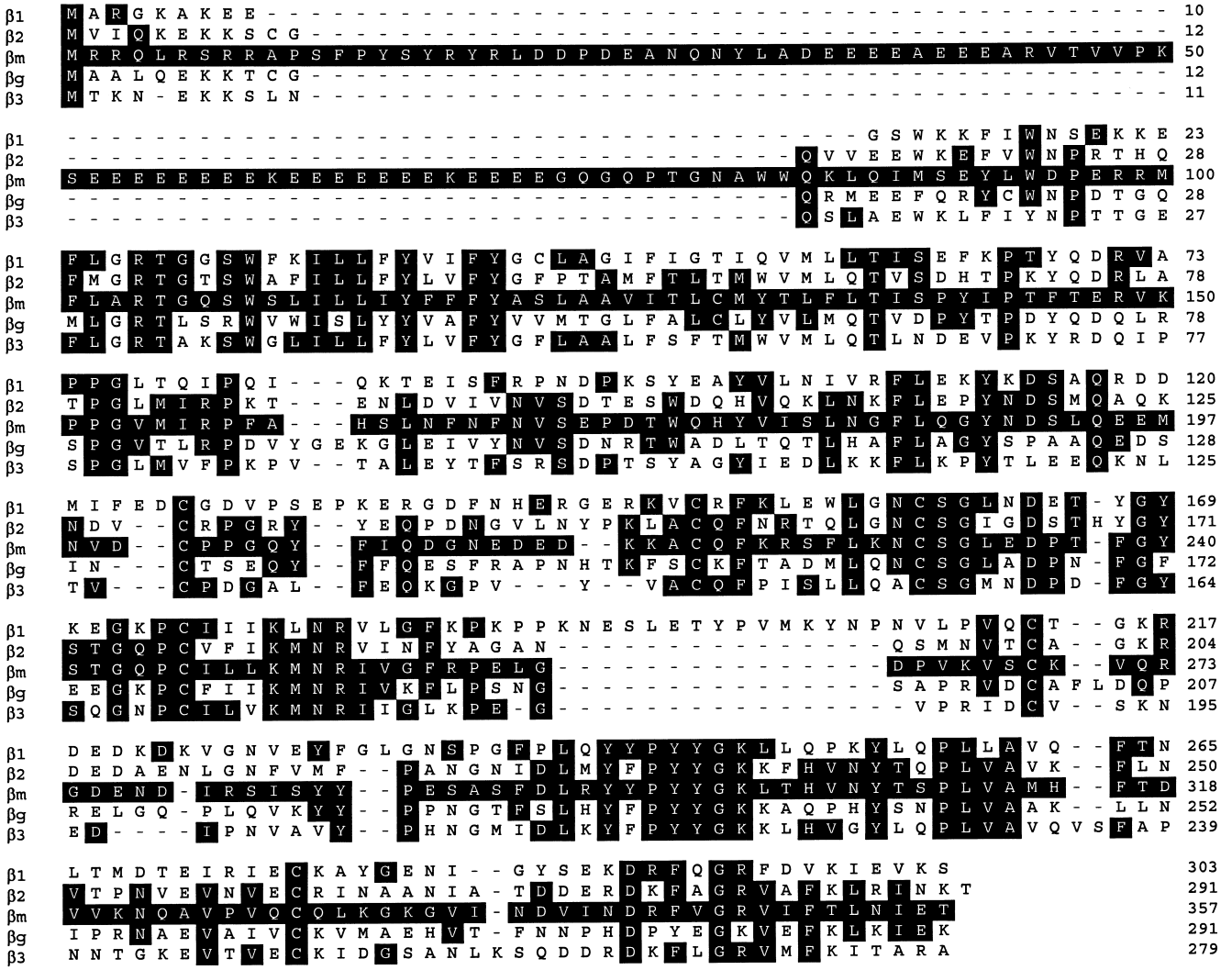

Fig. 3. Comparison of the amino acid sequences of the human X,K-ATPase β_m subunit, β_1-, β_2- and β_3-isoforms of human Na,K-ATPase (β_1, β_2 and β_3) and human gastric H,K-ATPase β-subunit (β_g). The following sequences were obtained from the GenBank database: β_1 (NM_001677), β_2 (NM_001678), β_3 (NM_001679) and β_g (NM_000705). The amino acid sequence of β_m and identical residues in other proteins are shaded. Gaps (represented by dashes) were introduced to maintain the alignment. Transmembrane domains are underlined. Conserved cysteines are indicated by asterisks. The amino acids are numbered at the right of the sequences.

was not predicted in previous biochemical and physiological studies on P-type ATPases in heart and skeletal muscles. Discovery of the β_m proteins has substantially expanded the possibility for a more detailed analysis of structural relatedness between different members of the X,K-ATPase β-subunit gene family (Fig. 3).

In respect to sequence homology, rat and human β_m proteins (89.1% identity) occupy an intermediate position between extremely conserved β1 (94.4%) and β2 (96.2%) and more divergent β_g (81.4%) and β3 (74.9%) [8,13,25]. The human β_m protein exhibits 39.4% identity with the β2 sequence [18], 32.6% with β1 [21], 35.1% with β3 [22] and 33.4% with β_g [19]. The similarities of corresponding cDNA sequences are 45.4, 40.8, 30.7 and 45.6%, respectively. Thus, β_m is slightly more related to β2 and almost equally distant from all other β-subunits.

All X,K-ATPase β-subunits exhibit characteristic features, such as the presence of three disulfide bridges within all β-ectodomains. There are 2–8 (β3 and β2, respectively) sites of *N*-glycosylation in human β-isoforms [8,18,22,25]. Locations of these sites in the polypeptides are not conserved and only one motif, ^{227}LXNCS^{231} (X = any residue), is present in all human proteins except β3.

Data obtained allowed us to trace several other conserved stretches of amino acids. The most prominent examples of these sequences are ^{101}(F/M)-(L/M)-(A/G)-R-T-X-X-(S/R)-W-X-X-I-(L/S)-L-(X)^3-F-Y^{120}, ^{220}C-X-F-(X)^5-L-X-(N/A)-C-S-G-X-X-D-(X)$_3$-(F/Y)-G-(Y/F)-X-X-G-X-P-C-(X)$_3$-K-M-N-R-(I/V)-(I/V)^{255} and ^{291}(F/I)-X-L-X-Y-(Y/F)-P-Y-Y-G-K-(K/L)-X-(H/Q)-(V/P)-X-Y-X-X-P-L-(V/L)-A^{313}. We consider these conserved motifs as signature sequences that may simplify the search of hitherto unknown β-subunits.

As it was mentioned above, the β_m is the only known P-ATPase subunit with the long Glu-rich N-terminus. Acidic domains including oligo-Glu or oligo-Asp stretches are suspiciously frequent in Ca-binding and Ca-transporting proteins in muscle cells, such as ryanodine receptor, voltage-gated calcium channel, calsequestrin, calreticulin, etc. (see for review [26]). From this, one can speculate that the acidic N-terminal domain of β_m may be a Ca-binding site with a potential of Ca-dependent regulation of X,K-ATPase(s).

The highly specific expression pattern of the β_m gene and the structural features of the β_m protein suggest its specialized function in the muscle cells. It is known that skeletal muscles contain several Na,K-ATPase isoforms but gastric and non-gastric H,K-ATPases are absent. The α1 and α2 are expressed in both muscle fiber types while expression of β-subunits is muscle type-specific: β1 is abundant in slow oxidative type muscles while β2 is restricted to fast glycolytic fibers [27]. The skeletal muscle Na,K-ATPase constitutes the major pool of the organism and its regulation is important for plasma K^+-homeostasis under some conditions. Significant augmentation of skeletal muscle Na,K-ATPase during training is thought to increase the plasma K^+-clearance capacity [28–30]. Downregulation of the skeletal muscle ATPase in hypokalemia [31–33] is controlled by the central nervous system [34] and is isoform- and muscle type-specific: it is substantial only for α2 and β2 genes and is more pronounced in glycolytic muscles [35].

Interestingly, the total pool of β1 and β2 proteins in skeletal muscle was reported to be several fold higher than the total pool of α-subunits (unlike kidney and brain where the ratio is about 1:1) [36]. It was suggested that the ratio of β- to α-subunits may serve to regulate the catalytic activity of Na,K-ATPase in skeletal muscle [37]. The identification of yet another β-subunit adds a new aspect to this problem and makes the situation even more complex.

The novel β_m-subunit cannot a priori be classified as a subunit of the Na,K-ATPase. The possibility cannot be excluded that β_m is a component of an unknown X,K-ATPase whose catalytic subunit is not identified. Also, it cannot be excluded that β_m may have a function apart from its role of X,K-ATPase subunit, similar to β2 which acts as an adhesion molecule [7,8].

Identification of this novel β-isoform shows that the diversity of X,K-ATPases is still underestimated and also suggests a possibility that yet some other unknown β-isoforms are to be discovered. The physiological significance of the human β-isoform diversity and nature of potential tissue-specific and developmental regulation of α-β-subunits pairing remains to be a goal of future studies.

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