The major myosin phosphatase in skeletal muscle is a complex between the β-isoform of protein phosphatase 1 and the MYPT2 gene product

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Abstract Myosin is dephosphorylated by distinct forms of protein phosphatase 1 (PP1) in smooth muscle and skeletal muscle that are composed of PP1 complexed to different regulatory subunits. The smooth muscle myosin phosphatase (smPP1M) has been characterised previously and is composed of PP1β complexed to M_{110} and M_{21} subunits that enhance the dephosphorylation of smooth muscle myosin, but not skeletal muscle myosin. In contrast, the regulatory subunit(s) of skeletal muscle myosin phosphatase (skPP1M) greatly enhance(s) the dephosphorylation of skeletal muscle myosin. Here we identify a regulatory subunit of skPP1M as the product of the MYPT2 gene, a protein whose sequence is 61% identical to the M_{110} subunit of smPP1M. Surprisingly, the M_{21} subunit of smPP1M appears to be produced from the same gene that encodes MYPT2.

Key words: Protein phosphatase 1; Myosin; Muscle contraction

1. Introduction

Protein phosphatase 1 (PP1), one of the major protein serine/threonine phosphatases of eukaryotic cells, participates in the control of a variety of cellular functions that include glycogen metabolism, muscle concentration, protein synthesis and pre-mRNA splicing (reviewed in [1–3]). It is now established that each of these physiological processes is regulated by a distinct form of PP1 in which the phosphatase catalytic subunit is complexed to specific targeting subunits containing an Arg/Lys-Val/Ile-Xaa-Phe/Trp motif that binds to a hydrophobic groove on the surface of PP1 [2,4,5]. These proteins not only direct PP1 to particular subcellular locations, but also modify its specificity in unique ways and confer the ability to be regulated by extracellular signals.

A form of PP1 that is associated with myofibrils [6] and has enhanced myosin phosphatase activity compared to the free catalytic subunit [7] was identified in skeletal muscle many years ago and termed PP1M. An enzyme with these properties was highly purified from the myofibrillar fraction of skeletal muscle and shown to be a complex between the catalytic subunit of PP1 and another protein(s) that greatly enhanced the rate of dephosphorylation of skeletal muscle myosin [8]. Subsequently, two such forms of skeletal muscle PP1M were resolved by chromatography on MonoQ termed PP1Mα and PP1Mβ. Both enzymes were heterodimers in which the PP1 catalytic subunit was complexed to proteins with apparent molecular masses of 33 kDa (Mα) and 50 kDa (Mβ) [9].

A form of PP1M was purified to homogeneity from chicken gizzard smooth muscle and shown to be composed of the β-isoform of PP1 bound to an 'M complex' consisting of two other proteins with apparent molecular masses of 130 kDa and 20 kDa [10]. Subsequent cDNA cloning revealed that the true molecular masses of the latter proteins were 110 kDa [11] and 21 kDa [12] and they were therefore termed the M_{110} and M_{21} subunits. cDNAs encoding proteins that are highly homologous to the M_{110} subunit from avian gizzard were also cloned from mammalian libraries [12–14]. The M_{110} subunit from chicken gizzard is not only complexed to the M_{21} subunit, but also to PP1 [10] and the Arg-Val-Lys-Phe sequence near its N-terminus appears to be critical for interaction with the phosphatase catalytic subunit [15]. In contrast the binding site for the M_{21} subunit is near the C-terminus of the M_{110} subunit [15]. Both the M_{110} and M_{21} subunits are myosin-binding proteins [16].

The binding of the chicken gizzard M_{110} subunit to PP1 was found to cause a several-fold enhancement of the rate of dephosphorylation of smooth muscle myosin, but did not increase the rate of dephosphorylation of skeletal muscle myosin [10]. In contrast, the equivalent proteins in skeletal muscle enormously enhanced (25-fold) the rate at which PP1 dephosphorylated skeletal muscle myosin, with a more modest (4-fold) effect on the dephosphorylation of smooth muscle myosin [9,10]. These observations indicated that Mα and Mβ from skeletal muscle were distinct gene products from their counterparts in smooth muscle.

Recently, a cDNA was cloned from a human brain library encoding MYPT2 (myosin phosphatase targeting subunit 2), a 110 kDa protein whose sequence is 61% identical to that of the M_{110} subunit from smooth muscle [17]. However, the mRNA encoding MYPT2 was most abundant in cardiac muscle and skeletal muscle [17]. Here we show by peptide sequencing that the 33 kDa Mα and 50 kDa Mβ subunits of skeletal muscle PP1Mα and PP1Mβ correspond to N-terminal fragments of MYPT2, indicating that MYPT2 encodes the myosin-targeting subunit of skeletal muscle PP1M.

The C-terminal 72 residues of the M_{110} subunit from chicken gizzard are 43% identical to residues 87–161 of the M_{21} subunit [12]. However, a second form of the M_{110} subunit from avian or mammalian smooth muscle can be produced by alternative splicing, whose C-terminal sequence is extremely similar (but not identical) to the C-terminal 50 residues of the M_{21} subunit from chicken gizzard [12]. These observations led us to speculate that the M_{21} subunit from chicken gizzard might be transcribed from a second M_{110} gene [12]. This hypothesis appears to be correct because the amino acid sequence of the M_{21} subunit from chicken gizzard is virtually identical to the C-terminus of the protein encoded by the human MYPT2 gene.

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2. Materials and methods

2.1. Isolation of tryptic peptides from rabbit skeletal muscle PP1M

PP1Mα and PP1Mβ were purified to homogeneity from the myofibrillar fraction of rabbit skeletal muscle by affinity chromatography on microcystin-Sepharose and chromatography on MonoQ [9]. The preparations were subjected to SDS-polyacrylamide gel electrophoresis and the bands corresponding to PP1, Mα and Mβ, excised and digested with trypsin [18]. The resulting peptides were chromatographed on a C18 column and analysed on an Applied Biosystems 476A Amino Acid Sequencer [18].

2.2. Preparation of tissue extracts

Tissues were rapidly removed post mortem, frozen in liquid nitrogen, ground in a pestle and mortar and homogenised with 6 vols of 20 mM Tris-HCl pH 7.5, 500 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.1% (by vol) 2-mercaptoethanol, 0.2 mM phenylmethylsulphonyl fluoride, 1 mM benzamidine and 10 μg/ml of the proteinase inhibitors leupeptin, chymostatin, pepstatin and lima bean trypsin inhibitor. The homogenates were then centrifuged at $13\,000 \times g$ for 5 min at 4°C, the supernatants removed and denatured in SDS by heating for 5 min at 95°C.

2.3. Antibodies and immunoblotting

Polyclonal antibodies were raised in sheep against the PP1M holoenzyme from chicken gizzard. Affinity-purified antibodies that recognise the M_{110} subunit (but not the M_{21} subunit) were obtained by passing the antiserum through Sepharose to which the C-terminal 290 residues of the chicken gizzard M_{110} subunit had been coupled [15]. Antibodies that recognise the M_{21} (but not the M_{110} subunit) of chicken gizzard PP1M were obtained in an identical manner, except that the antiserum was passed through a Sepharose column to which the chicken gizzard M_{21} subunit had been coupled covalently [15]. The specificity of both antibodies has been demonstrated previously [15].

3. Results and discussion

Previous peptide sequencing studies indicated that the major isoform of the PP1 catalytic subunit associated with the 'M subunit' from skeletal muscle was the β-isoform [8] (also termed PP1δ [19]) and this finding was confirmed in the present work by sequencing additional peptides. Sequence analysis of the catalytic subunit of PP1 that is complexed to Mα and Mβ revealed six tryptic peptides whose sequences were common to all three human isoforms of PP1, PP1α, PP1β and PP1γ, and two that were specific to PP1β (Fig. 1). Since each peptide was recovered from the tryptic digest in similar amounts, these results indicate that PP1β is the major PP1 isoform that is complexed to Mα and Mβ in skeletal muscle; i.e. the same isoform that is complexed to the glycogen-targeting (G_M) subunit of PP1 in skeletal muscle [20] and to the M_{110} subunit in chicken gizzard [10]).

A number of tryptic peptides were also isolated after tryptic digestion of Mα and Mβ from rabbit skeletal muscle and their sequences are shown in Fig. 1. All were virtually identical to particular regions of the protein encoded by the human MYPT2 gene [17] with 94 identities out of 100 residues sequenced. The peptide sequences were much less similar to MYPT1 (65% identity), the human homologue of the smooth muscle M_{110} subunit [12,14]. Since the predicted molecular mass of MYPT2 is 110 kDa, these results indicate that Mα and Mβ are both derived from MYPT2 by limited proteolysis during extraction and/or purification from skeletal muscle. All the peptides sequenced are located within the residues 81–412 of MYPT2, indicating that Mα and Mβ are N-terminal fragments of this protein. Since the complexes formed between Mα and PP1 or Mβ and PP1 both have greatly enhanced

Peptide Sequences from the M-subunits of Rabbit Skeletal Muscle PP1M	Found in	Residue number in Human MYPT2
1. GADINTVNVDGLTALHQACI	Mα, Mβ	81-100
2. FLVENR	Mβ	109-114
3. ANVLQQDNEGXXPLHAAAS	Mβ	115-133
4. EACSILAEALCDMDIR	Mα, Mβ	264-279
5. LGQTPFDVADEGLVE	Mβ	282-296
6. LQSGLFK	Mβ	327-333
7. FSIMEQIPAXAQNAFR	Mβ	397-412

Peptide Sequences from Rabbit Skeletal Muscle PP1	Found in	Residue number in PP1β
1. EIFLSQ	PP1Mβ	43-48
2. IYGFYDECK	PP1Mβ	132-140
3. TFTDCFNCLPIAAIVD	PP1Mβ	150-165
4. IMRPTDVPDTGLLCDLL	PP1Mβ	188-204
5. AHQVVEDGYE	PP1Mβ	246-255
6. YPENFFLLR	PP1Mα	113-121
7. IFCCHGGLSPDLQSMEQIR	PP1Mα	168-186
8. SAPNYCGEFDNAGGMMSVDE	PP1Mα	267-286

Fig. 1. Amino acid sequences of tryptic peptides from PP1Mα and PP1Mβ. The catalytic and regulatory subunits of rabbit skeletal muscle PP1Mα and PP1Mβ were resolved by SDS-polyacrylamide gel electrophoresis, digested with trypsin and the peptides separated and sequenced as in Section 2. The positions of these peptides in the sequence of the human MYPT2 are indicated. Underlined residues are unique to the β-isoform of PP1. An X indicates residues that could not be identified unambiguously.

activity towards skeletal muscle myosin compared to the free PP1 catalytic subunit, the information required to interact with PP1 and enhance its myosin phosphatase activity are both contained in the N-terminal portion of MYPT2. This finding is as anticipated, since the first 309 residues of M_{110} subunit from smooth muscle interact with PP1 and enhance the dephosphorylation of smooth muscle myosin in a similar manner to the full length protein [15]. The first 300 residues of the skeletal muscle and smooth muscle proteins both contain seven ankyrin repeats, the first of which is preceded by a canonical PP1-binding motif (see Section 1). In MYPT2, this Arg-Val-Arg-Phe sequence is located between residues 53 and 56 [17]. It will clearly be of great interest to understand why the N-terminal domain of the M_{110} subunit from smooth muscle stimulates the dephosphorylation of smooth muscle myosin, but not skeletal muscle myosin, and why MYPT2 enhances the dephosphorylation of skeletal muscle myosin far more than it enhances the dephosphorylation of smooth muscle myosin.

The C-terminus of the M_{110} subunit from smooth muscle is homologous, but not identical, to the M_{21} subunit from smooth muscle (Fig. 2). Remarkably, however, the last 120 residues of chicken gizzard M_{21} (residues 67–186) show 91% identity and nearly 100% similarity (if highly conservative replacements are included) to the last 120 residues of human MYPT2 (Fig. 2). This suggests that the M_{21} subunit from

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M21     31   KTLRHERLSRLE.AATTPATSDSYSDRASSRSSAYSRRENRLAALSSRAE
MYPT2  827   KETWHERLSRLESGGSNPTTSDSYGDRASARARR.EARREARLATLTSRVE
MYPT1  880   KETQTDSISRYETSSTSA..GDRY.DSLLGRSGSYSYLEER.KPYSSRLE
M110   826   RDTQTDSVSRYDSSSTSS..SDRY.DSLLGRSASYSYLEER.KPYGSRLE

M21     80   EESNRDYKKLYESALSENQKLKSKLQEAQLELADIKSKLEKAAQQKHEKT
MYPT2  876   EDSNRDYKKLYESALTENQKLKTKLQEAQLELADIKSKLEKVAQQKQEKT
MYPT1  926   KDDSTDFKKLYEQILAENEKLKAQLHDTNMELTDLKLQLEK.ATQRQERF
M110   872   KDDSTDFKKLYEQILAENEKLKAQLHDTNMELTDLKLQLEK.ATQRQERF

M21    130   SDRSSMLEMEKRERKRALERKLSEMEEEMKILTELKSDNQRLKDENGALIR
MYPT2  926   SDRSSVLEMEKRERRRALERKMSEMEEEMKVLTELKSDNQRLKDENGALIR
MYPT1  975   ADR.SLLEMEKRERRRALERRISEMEEELKMLPDLKADNQRLKDENGALIR
M110   921   ADR.SLLEMEKRERRRALERRISEMEEELKMLPDLKADNQRLKDENGALIR

M21    180   VISKLSK
MYPT2 1076   VISKLSK
MYPT1 1024   VISKLSK
M110   970   VISKLSK
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Fig. 2. Alignment of the M_{21} subunit from turkey gizzard with the C-termini of human MYPT2, human MYPT1 and the rat aorta M_{110} subunit. Identities with the M_{21} subunit are highlighted in black and the most conservative replacements are shaded grey. The sequences are taken from [11,12,14,17].

chicken gizzard is likely to be produced from the gene encoding an avian homologue of MYPT2. Similarity with MYPT2 disappears N-terminal to residue 30 of the M_{21} subunit (Fig. 2), suggesting that the N-terminal 30 residues may be encoded within an intron of the MYPT2 gene. If the M_{21} subunit is indeed produced from the MYPT2 gene, then anti-M_{21} antibodies should recognise a 21 kDa protein in smooth muscle and a 110 kDa protein in tissues where the full length MYPT2 protein is expressed. The results presented in Fig. 3 are consistent with this scenario. The antibody raised against M_{21} from chicken gizzard recognised a 22 kDa protein in five rat smooth muscles (small intestine, aorta, stomach, bladder and uterus) whose electrophoretic mobility was slightly slower than that of the M_{21} subunit from chicken gizzard, but the same antibody detected a 110 kDa protein in rat brain and rat cardiac muscle (Fig. 3). Similar results were obtained in chicken tissues, where the M_{21} antibody recognised 20 kDa and/or 21 kDa proteins in three smooth muscles (gizzard, aorta and small intestine), but recognised 110 kDa proteins in brain and cardiac muscle that were also expressed (albeit more weakly) in small intestine and aorta (data not shown). Brain and cardiac muscle are the two human tissues where the MYPT2 protein was shown to be expressed most prominently [17]. The myosin P-light chain present in cardiac muscle is not the same gene product as the P-light chain found in skeletal muscle [21]. The expression pattern of MYPT2 therefore suggests that this protein may not only enhance the dephosphorylation of skeletal muscle myosin, but also the dephosphorylation of cardiac myosin and brain myosin.

Although the mRNA encoding MYPT2 is highly expressed in skeletal muscle, Fujioka et al. [17] failed to detect any MYPT2 protein in this tissue by immunoblotting. Our antibody raised against the chicken gizzard M_{21} subunit also failed to detect an immunoreactive 110 kDa protein in skeletal muscle extracts, although it weakly recognised a 90 kDa protein that might correspond to a proteolytic fragment of MYPT2 (Fig. 3). However, MYPT2 is clearly expressed in skeletal muscle because we isolated it as fragments of a subunit of the major myosin phosphatase in this tissue, and a 55 kDa fragment of MYPT2 was also detected recently by others [22] as a myofibrillar protein from skeletal muscle that binds to microcystin-Sepharose. The failure to detect MYPT2 by immunoblotting could be explained by its low abundance, since the isolation of PP1M requires over 100 000-fold purification from the myofibrillar fraction of skeletal muscle [8,9], in contrast to the much smaller (1000-fold) purification needed to isolated PP1M from the myofibrils of avian smooth muscle [10]. This low level is consistent with the slow rate of dephosphorylation of the skeletal muscle myosin P-light chain in vivo [23]. Alternatively, the extreme susceptibility of the C-terminus of MYPT2 to proteolysis in skeletal muscle extracts

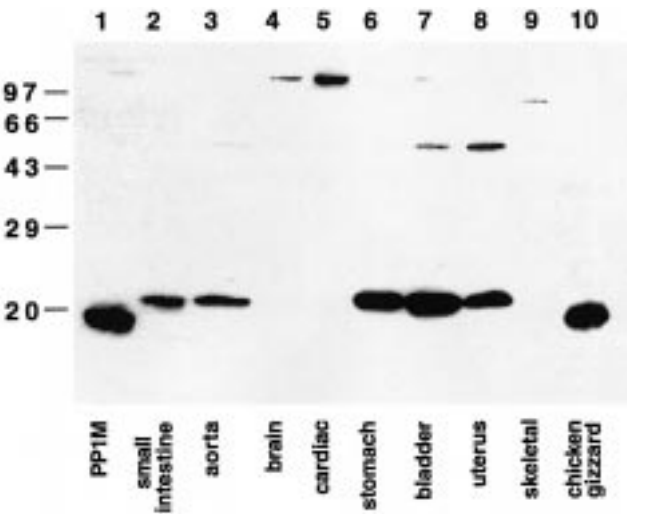

Fig. 3. Immunoblotting of rat tissues with antibody raised against the M_{21} subunit from chicken gizzard. Tissue extracts (15 μg extract protein, except for chicken gizzard where 10 μg protein was used) were electrophoresed on 15% SDS-polyacrylamide gels, transferred to nitrocellulose and immunoblotted with affinity-purified anti-M_{21} antibody using the enhanced chemiluminescence detection system (Amersham). Lane 1, PP1M (0.1 μg) purified from chicken gizzard [10]; lane 2, small intestine; lane 3, aorta; lane 4, brain; lane 5, cardiac muscle; lane 6, stomach; lane 7, bladder; lane 8, uterus; lane 9, skeletal muscle; lane 10, chicken gizzard. The positions of the marker proteins glycogen phosphorylase (97 kDa), bovine serum albumin (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa) and soybean trypsin inhibitor (20 kDa) are indicated.

may have prevented detection by our antibody which recognises the C-terminus of the protein.

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