Expression of the AMP-activated protein kinase β1 and β2 subunits in skeletal muscle

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Abstract A heterotrimeric member of the AMP-activated protein kinase (AMPK) isoenzyme family was purified from rat skeletal muscle by immunoaffinity chromatography, consisting of an α2 catalytic and two non-catalytic subunits, β2 and γ1. The AMPK β2 cDNA (271 amino acids (aa), molecular weight (MW) = 30 307, pI 6.3) was cloned from skeletal muscle and found to share an overall identity of 70% with β1 (270 aa, MW = 30 475, pI 6.0). In the liver AMPK β1 subunit, Ser-182 is constitutively phosphorylated whereas in skeletal muscle β2 isoform, we find that Ser-182 is only partially phosphorylated. In addition, the autophosphorylation sites Ser-24, Ser-25 found in the β1 are replaced by Ala-Glu in the β2 isoform. β2 contains seven more Ser and one less Thr residues than β1, raising the possibility of differential post-translational regulation. Immunoblot analysis further revealed that soleus muscle (slow twitch) contains exclusively β1 associated with α2, whereas extensor digitorum longus muscle α2 (EDL, fast twitch) associates with β2 as well as β1. Sequence analysis revealed that glycogen synthase, a known AMPK substrate, co-immunoprecipitated with the AMPK α2β2γ1 complex.

Key words: AMP-activated protein kinase; Skeletal muscle isoform

1. Introduction

The AMP-activated protein kinase (AMPK) consists of a catalytic α subunit (550 amino acids (aa)) and non-catalytic β (270 aa) and γ (323 aa) subunits and multiple isoforms exist for each subunit [1–3]. Both non-catalytic β and γ subunits are required for optimal activity of the α catalytic subunit [4]. Several isomers of the AMPK have been identified whereby the various subunit isoforms can be found in different combinations. We will use the nomenclature AMPK α1β1γ1 to describe an AMPK containing the α1 catalytic subunit together with β1 and γ1 non-catalytic subunits [5]. AMPK was initially recognised as a kinase that phosphorylated and inactivated acetyl-CoA carboxylase and HMG-CoA reductase, key regulatory enzymes in the control of fatty acid and cholesterol synthesis, respectively [6]. Recently, it has become clear that AMPK plays a more extensive and dynamic role in modulating metabolism in response to energy demand and nutrient availability, mediating exercise-induced glucose uptake in muscle and activation of endothelial NO synthase [5,7]. AMPK is activated by elevation of intracellular AMP caused by metabolic stress that can be induced by high-energy demand, heat shock or toxins [5]. The activation of AMPK by AMP results from three contributing mechanisms. These are direct allosteric activation of the enzyme, activation of an upstream kinase kinase [8] and inhibition of AMPK dephosphorylation [9] that is thought to provide an exquisitely sensitive mechanism for monitoring the cellular energy status [10].

Previously, we reported the identification of two catalytic isoforms (α1 and α2) of the AMPK in rat liver [11]. Both isoforms could be stimulated by AMP and contained non-catalytic β and γ subunits. The α1 and α2 catalytic isoforms of the AMPK are 90% identical in the catalytic core region, but only 60% identical in their COOH-terminal tails. In addition, sequence analysis of β and γ non-catalytic subunits has revealed the presence of additional genes for each subunit within the human genome [12]. Since the AMPK consists of a family of isoenzymes, it is important to establish which β and γ subunit isoforms are bound to each catalytic isoform and whether the isoenzymes are differentially expressed in different tissues and cell types. Rat and porcine liver contain the AMPK isoenzymes α1β1γ1 and α2β1γ1 [3,13,14]. It was initially reported that skeletal muscle AMPK was not associated with β or γ non-catalytic subunits [15] but we noted the presence of a β2 isoform form in the EST database [14]. Thornton et al. [16] have now reported that the human β2 isoform interacts with human AMPK α2 subunit using the yeast two hybrid technique. They claimed that β2 was the dominant isoform expressed in skeletal muscle. In the present study, we show that skeletal muscle AMPK α2 is associated with γ1 and either β2 or β1 depending on the muscle type.

2. Materials and methods

2.1. AMPK α2 purification

Rattus norvegicus skeletal muscle (hind leg, approximately 500 g) was homogenised in 2 l buffer (50 mM Tris-HCl pH 8.5, 250 mM sucrose, 50 mM NaF, 5 mM Na-pyrophosphate, 1 mM EGTA, 1 mM EDTA, 1 mM DTT, 1 mM PMSF and 1 mM benzamidine) using an Ultra-Turrax (IKA-Labortechnik, Bremen, Germany). The homogenate was centrifuged at 5000×*g* for 30 min and the supernatant was fractionated by 1–7% polyethylene glycol (PEG 6000) fractionation. The final pellet was resuspended in 1 l of 50 mM Tris-HCl pH 7.5, 50 mM NaF, 5 mM Na-pyrophosphate, 1 mM EGTA, 1 mM EDTA, 1 mM DTT and 5% glycerol (buffer A) before batch application to 1 l of DE52 Sepharose (Whatman, Maidstone, UK). The resin was eluted with buffer A containing 0.5 M NaCl and the volume reduced by 10% PEG 6000 precipitation. The pellet was suspended in 30 ml buffer A and passed through an ADR1 (222–234)P^{229}-peptide affinity column [17] to deplete contaminating AMPK α1. The AMPK α2 present in the flow-through was affinity-purified by immunoprecipitation with

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equal amounts (250 μg) of anti-AMPK α2 (352–366) and α2 (490–516) polyclonal antibodies [11] coupled to 250 μl (slurry) of Tosyl-activated Dynabeads (Dynal, Oslo, Norway) according to the manufacturer's instructions. The magnetic beads were collected, washed twice with 1 ml phosphate-buffered saline, 3×1 ml 50 mM Tris pH 7.4, 1 mM EDTA, 0.5 M NaCl and 1% Triton X-100, and 2×1 ml with the same buffer containing 2% Triton X-100. Proteins were eluted by boiling in sodium dodecyl sulfate (SDS) sample buffer containing 10 mM DTT and subjected to 12% SDS-polyacrylamide gel electrophoresis (PAGE).

2.2. Antibody production

Polyclonal antibodies against AMPK peptides were prepared as described [11]. AMPK α1 antibodies were raised against the peptide CARHTLDELNPQKSKHQG-COOH (373–390)C^{373}. AMPK β1 antibodies were raised against the peptide CSSPPGPYHQEPYISKPE-NH_2 (181–197)C^{181} and AMPK β2 antibodies against the peptide CGPGKEHKIMVGSTDDPSV-NH_2 (26–44)C^{26} while AMPK γ1 antibodies were raised against the peptide CQALVLTGGEKKP-COOH (319–331)C^{319}.

2.3. Peptide sequencing

Proteins separated by SDS-PAGE were sequenced using a modified in situ proteolysis technique as described [14] using a Hewlett Packard G1000A Protein Sequencer and a PerSeptive Biosystems Voyager DE MALDI-TOF mass spectrometer.

2.4. Cloning of AMPK β2 cDNA

Total RNA was extracted from 5 g rat hind leg skeletal muscle using Total RNA Isolation Reagent (Advanced Biotechnologies, Surrey, UK) according to the manufacturer's instructions. One μg of RNA from this preparation was used to generate cDNA using the primer AATTCGCGGCCGC$(dT)_{15}$, containing a *Not*I restriction site, with Superscript II reverse transcriptase (Gibco) followed by RNAse H (Gibco) digestion. An initial 473 bp fragment was amplified by polymerase chain reaction (PCR) using degenerate primers based on the sequenced peptides MVGSTDDP and GQEMYVF (sense: CTGCAGGAATTCATGGTGGGGAGCACNGAYGAYCC containing an *Eco*RI site 5′, antisense: ATCGATAAGCTTRAANACRTACATYTCYTGNCC including a 5′ *Hin*dIII site. The product was cloned into the *Eco*RI and *Hin*dIII sites of pBluescript SK(−) (Stratagene) for sequence confirmation. To obtain the 5′ coding region, cDNA was synthesised using the primer GATATCGAATTCGACTCTTAATCAGAGGGATCTTGG, based on the 473 bp fragment. A 176 bp fragment was then PCR-amplified using a 5′-RACE strategy (Gibco) between the anchor primer (Gibco) and the nested reverse primer, GATATCGAATTCAGGGAGCTTGGAGTCCGGCAG. PCR conditions were 1 min at 94°C, 1 min at 55°C and 2 min at 72°C. The PCR product was ligated into pCR-SCRIPT for cloning and sequencing. 3′ Coding and untranslated residues were obtained using a nested PCR approach. The cDNA synthesis and reverse PCR primer was AATTAACCCTCACTAAAGGG$(dT)_{15}$ based on the T3 promoter, while forward primers were GCTGCAGAATTCGGAAAGCTCTGAGACATCATGTCG and GACATCGAATTCATGTCGAGACCTGTCCAGCTC for initial and nested PCRs, respectively. Both rounds of PCR were for 35 cycles of 94°C for 1 min, 50°C for 1 min and 72°C for 5 min. The 1225 bp fragment was ligated directly into *Sma*I-digested, alkaline phosphatase-treated pBluescript SK(−) for cloning and sequencing as above. Sequences of both strands of plasmids were determined using an ABI model 305 automated sequencer and ABI Prism sequencing kits as recommended by the manufacturer. The cDNA sequence was submitted to GenBank and has been assigned the GenBank accession number AF182717.

2.5. Immunoblotting

Anti-AMPK α2 (490–516) antibody Dynabeads (see above) were incubated with the partially purified AMPK α2 overnight with gentle mixing at 4°C. The magnetic beads were collected, washed with 2×1 ml phosphate-buffered saline, 3×1 ml 50 mM Tris pH 7.4, 1 mM EDTA, 0.5 M NaCl and 1% Triton X-100 and 2×1 ml with the same buffer containing 2% Triton X-100. The magnetic beads were boiled in SDS sample buffer containing 10 mM DTT, subjected to 12% SDS-PAGE and transferred to PVDF. The immunoblot was blocked in 5% milk powder, 0.1% Tween 20, in phosphate-buffered saline and incubated with the affinity-purified [12] (10 μg/ml) AMPK antibodies described in Fig. 2. Primary antibody was detected using goat anti-rabbit antibody conjugated to horseradish peroxidase (Dako) and the Amersham ECL detection system. All of the primary antibodies were prepared and affinity-purified as described previously [11]. The positive control (+) used in these blots was a partially purified fraction from rat liver containing AMPK isoforms α1β1γ1 and α2β1γ1 [17]. In addition, an antibody control consisting of rabbit IgG coupled at the same concentration as the α2 antibody to Tosyl-activated Dynabeads was used to verify α2 specificity.

2.6. Rat muscle immunoprecipitation and immunoblot

Male Sprague Dawley rats were anaesthetised with sodium pentobarbitone. The extensor digitorum longus (EDL) and soleus muscles were excised and snap frozen in liquid nitrogen. The muscles were homogenised in buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 50 mM NaF, 5 mM Na-pyrophosphate, 10% glycerol, 1% Triton X-100, 10 μg/ml trypsin inhibitor, 2 μg/ml aprotinin, 1 mM benzamidine and 1 mM PMSF). The homogenates were incubated with AMPK α1 (373–390) or AMPK α2 (490–516) antibody-bound protein A beads for 2 h at 4°C. The immunocomplex was washed with PBS and boiled in SDS sample buffer. The samples were separated by 10% SDS-PAGE, transferred to PVDF membranes and immunoblotted with antibodies specific for β1 and β2.

3. Results

The AMPK isoenzyme containing the α2 catalytic subunit was partially purified from rat skeletal muscle and isolated by immunoaffinity chromatography using an antibody against the AMPK α2 catalytic subunit (Fig. 1). The identity of the AMPK α2 catalytic subunit (63 kDa) was verified by peptide sequence analysis. For the predicted α2 band, 24 out of 34 masses were initially identified as α2 peptides based on expected tryptic digest masses. Three of the identified peptides were also confirmed by peptide sequencing, corresponding to sequences 270–299, 382–393 and 489–499, respectively (data not shown). The remaining masses could not be confidently matched to the α2 sequence and appear to be minor contaminants co-eluting with α2 peptides. Thus, 45% of the α2 catalytic subunit sequence was detected including seven peptides derived from the catalytic core and 10 peptides from the C-terminal domain. Immunoblot analysis also confirmed that the α2 catalytic subunit and not the α1 catalytic subunit immunoprecipitated with the α2 specific antibodies (Fig. 2).

In order to identify the proteins associated with the AMPK α2 catalytic subunit, the co-immunoprecipitated bands were also identified by a combination of mass spectrometry and Edman sequencing of tryptic peptides generated from 'in-gel' digests of individual bands (Fig. 1). Tryptic digestion of the 34 kDa band resulted in 13 peptides related to a human β2 EST consensus sequence previously reported [14] and accounted for 51% of the expected 271 residues of the β2 protein sequence (Fig. 3A). Two additional peptides sequenced corresponded to peptides from porcine trypsin and the IgG heavy chain. Immunoblot analysis of the AMPK α2 skeletal muscle immunoprecipitate also confirmed that it contained a 34 kDa band corresponding to the β2 subunit and negligible amounts of the β1 subunit (Fig. 2), consistent with the peptide sequencing data. In β1, the peptide containing Ser-182 is stoichiometrically phosphorylated [18]. In contrast, β2 was substantially dephosphorylated at this site and the dephosphorylated tryptic peptide, DLSS*S*PPGPYGQEMYVFR, identified by mass and Edman sequencing (5.2 pmol, 100% repetitive yield). The only phosphorylated form of the peptide detected was DLSSSPPGPYGQEMY, generated by a chymotryptic-like cleavage (2.8 pmol, 98% repetitive yield).

Tryptic peptide sequences derived from the 38 kDa protein

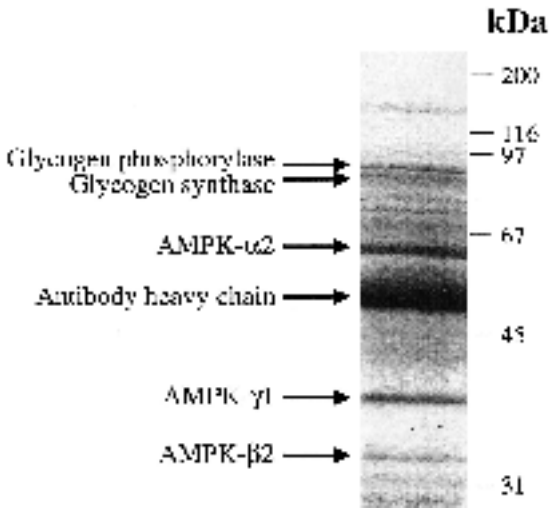

Fig. 1. Immunopurification of rat liver AMPK α2. AMPK α2 was purified as described in Section 2. Shown is an SDS-polyacrylamide gel (12%) containing the AMPK α2 catalytic subunit together with the AMPK non-catalytic subunits, β and γ. Antibody heavy chain, glycogen phosphorylase and glycogen synthase are also shown. Molecular weight standards are shown in kDa.

(Fig. 1) were identical to the γ1 isoform (data not shown) and accounted for 43% of the sequence. In addition, γ1 specific antibodies identified a 38 kDa band as γ1 in an AMPK α2 immunoprecipitate (Fig. 2). These results demonstrate that the skeletal muscle AMPK α2 catalytic subunit associates with the β2 and γ1 non-catalytic subunits. This contrasts with liver where the AMPK α2 catalytic subunit associates with the β1 and γ1 non-catalytic subunits [11].

The full length β2 sequence was obtained by a combination of reverse transcription (RT-) PCR and 5′ and 3′-RACE. An initial 473 bp PCR product (see Section 2) was used to design primers for both 5′-RACE and 3′-RACE strategies to obtain the complete coding sequence of β2. The 5′-RACE product was 176 bp and the 3′-RACE product was 1225 bp in length. The sequences of both fragments overlapped with the RT-PCR core fragment and were identical beyond the primer sequences in these overlaps. This demonstrates that all three fragments were derived from the same mRNA species. Combined, these clones covered an open reading frame, encoding a protein of 271 aa with a predicted myristoylation sequence at the N-terminus (Fig. 3A). Comparison with the β1 protein revealed 70% overall identity with only 43% aa identity in the N-terminal 72 residues of the protein while the remainder is 82% identical (Fig. 3B). The N-terminal 33 residues of the cDNA-predicted sequence would be expected to yield short tryptic peptides that would elute in the flow-through of the reversed phase column (Fig. 3A). The predicted mass of the myristoylated β2 is 30 306 compared to 34 kDa estimated by SDS-PAGE. When isolated from liver, the β1 subunit is present as the myristoylated mono-, di- and tri-phosphorylated forms with masses of 30 552, 30 365 and 30 722, respectively [18], and migrates on SDS-PAGE at approximately 40 kDa. Therefore, the slower than expected mobility of β1 in SDS-PAGE may be due to aberrant electrophoretic behaviour that is less pronounced for β2.

Immunopurified skeletal muscle AMPK α2β2γ1 also contained proteins in the 80–97 kDa range (Fig. 1). Tryptic peptide sequencing revealed that they corresponded to glycogen synthase (residues 40–50, 130–145, 211–230, 322–330 and 615–634) and glycogen phosphorylase (residues 193–206 and 492–307), respectively. Previously, glycogen synthase was reported to be a substrate of the AMPK [19]. Hence, the association of glycogen synthase observed here with the AMPK is consistent with it acting as a substrate in vivo. Glycogen phosphorylase associates with glycogen synthase but is not a substrate for the AMPK (Beyer et al., personal communication).

To test if the AMPK subunit composition differed according to the muscle type, fast and slow twitch muscle samples from normal rats were homogenised and immunoprecipitated with either AMPK α1 or α2 specific antibodies and immunoblotted for the presence of β1 or β2 specific isoforms. Fast twitch EDL muscle (E) was found to contain both isoforms β1 and β2 but slow twitch soleus muscle (S) only contained the β1 isoform (Fig. 4). The β2 subunit was only found associated with the AMPK α2 catalytic subunit and not with α1.

4. Discussion

The differential localisation of the two AMPK isoforms and activation provides an important basis for understanding their physiological roles in metabolic sensing in different muscle types. Previously, skeletal muscle exercise was reported to elevate AMPK activity [20] and more recently, electrical stim-

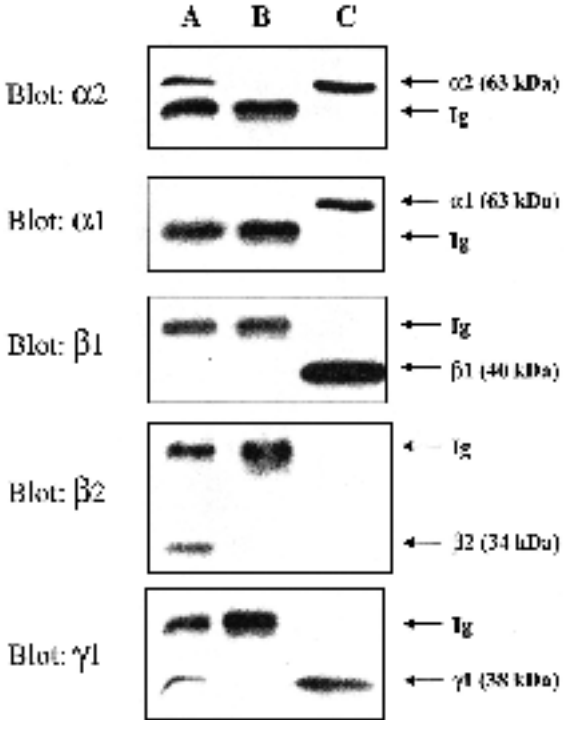

Fig. 2. Immunoblot analysis of skeletal muscle AMPK α2 immunoprecipitate. AMPK α2 was purified and blotted as described in Section 2. Lane A contains skeletal muscle AMPK α2 immunoprecipitates, lane B contains IgG control immunoprecipitates and lane C a partially pure AMPK preparation from rat liver that contains both α1β1γ1 and α2β1γ1 isoenzymes. Antibodies used for blotting are shown and the details of each can be found in Section 2. Arrows indicate subunits and IgG heavy chain.

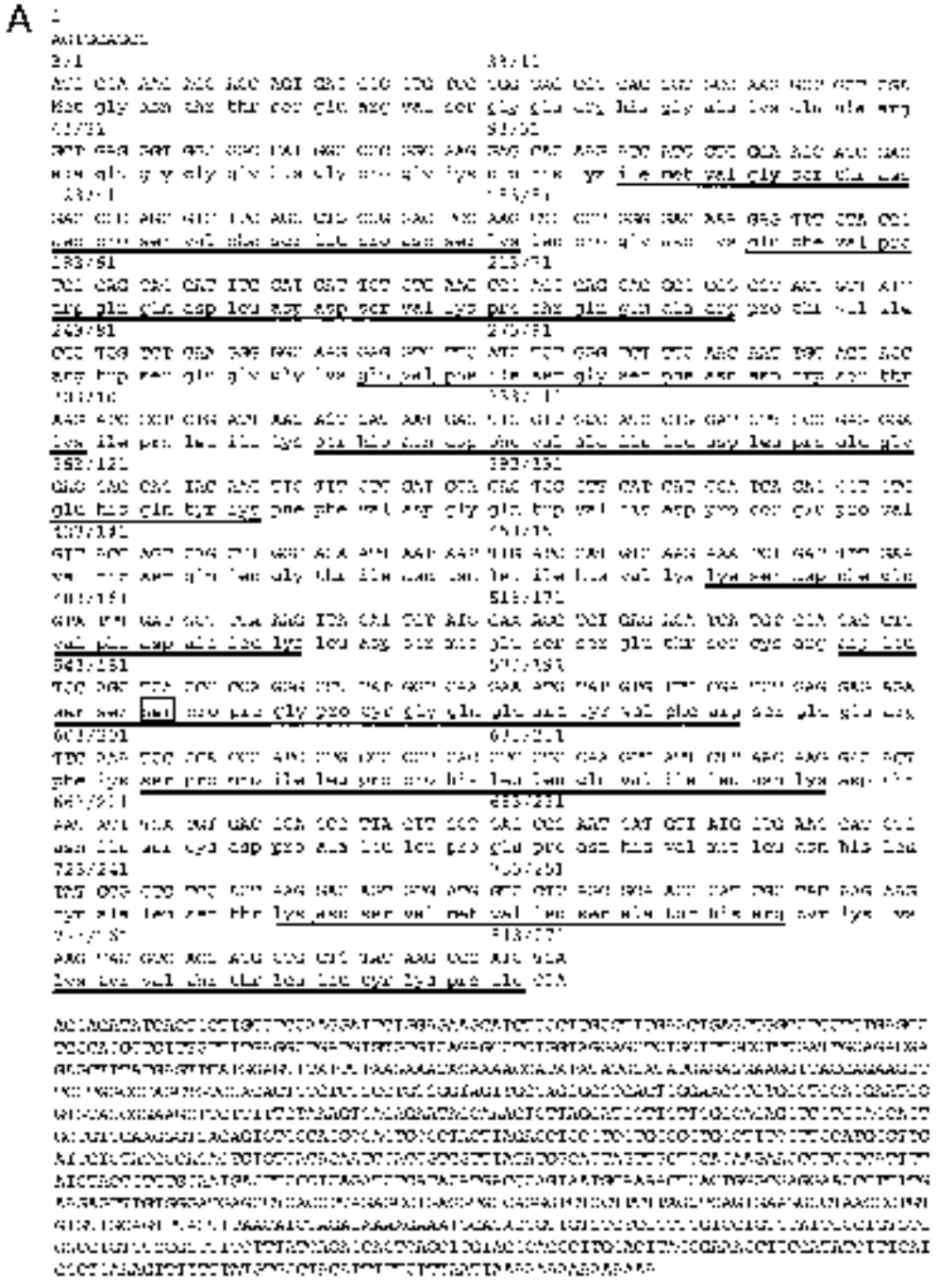

Fig. 3. A: AMPK β2 cDNA sequence. The sequence of the AMPK β2 cDNA and the deduced aa sequence are shown. Peptides that were sequenced from the 34 kDa band (Fig. 1) are underlined. The Ser-182 site that was found both phosphorylated and dephosphorylated is boxed. B: Comparison of AMPK β1 and β2 aa sequences. The sequences shown, human β1, accession number Y12556; rat β1, accession number P80386; *Caenorhabditis elegans* β1, accession number CAB04480; human β2, accession number AJ224538.1; rat β2, GenBank accession number AF182717 and *C. elegans* β2, accession number Y47D3A.q (found at http://wormserver.sanger.as.uk/cgi-bin/ace/sequence) were aligned with Clustalw and formatted [23]. Conserved aa are highlighted.

ulation of skeletal muscle was found to cause a rapid rise in activity of the AMPK α2 isoform [21]. Activation of the AMPK in skeletal muscle serves to stimulate fatty acid oxidation via phosphorylation of acetyl-CoA carboxylase [20] and increase glucose uptake [22] by translocation of GLUT4 to the plasma membrane.

The AMPK α2 catalytic subunit associates with both β1 and β2 in fast twitch muscle and β1 alone in slow twitch muscle using muscle lysates. Any β1 that may have been associated with α2 was essentially lost during the partial purification of the α2β2γ1 isoenzyme, though trace amounts can be observed in the immunoblot shown in Fig. 2.

Previously, we found that β1 isolated from rat liver was present in mono-, di- and tri-phospho forms. The phosphorylated residues detected were Ser-24, Ser-25 (either but not both), Ser-108 and Ser-182 [18]. In the case of β2, Ser-24-Ser-

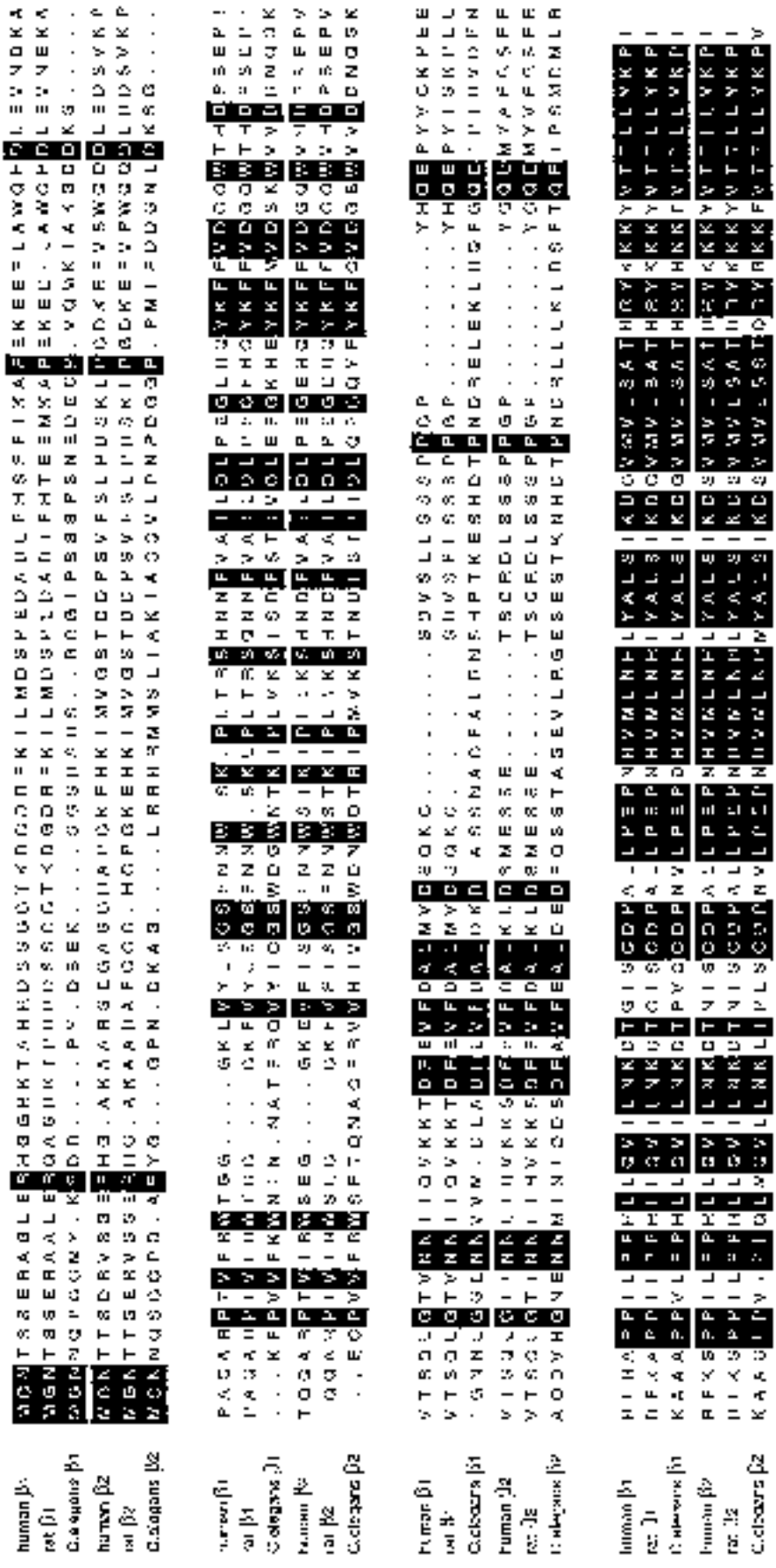

Fig. 3 (*continued*)

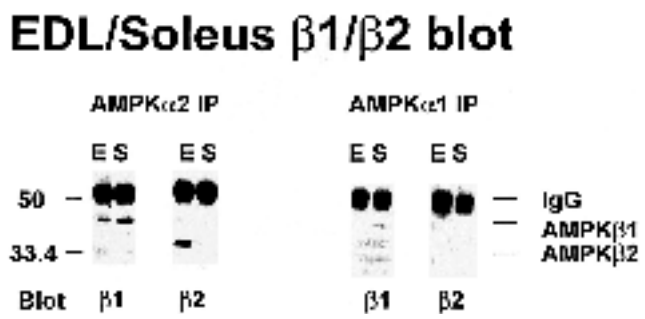

Fig. 4. Distribution of the AMPK β subunits in fast and slow twitch skeletal muscle. Rat EDL (E) and soleus (S) muscles were homogenised, immunoprecipitated (using AMPK α1 and α2 specific antibodies) and immunoblotted for both AMPK β1 and AMPK β2 as described in Section 2. IgG is indicated.

25 is replaced by Ala-Glu. The Ser-182 site in β2 is present as both the phosphorylated and dephosphorylated peptide based on the mass and sequence of the tryptic peptide, DLSSSPPGPYGQEMY. This suggests that in β2, the Ser-182-phosphate site is turning over whereas in the β1 isoform present in liver, it is stoichiometrically phosphorylated. Overall, β2 has 28 Ser residues compared to 21 for β1 and 14 versus 15 Thr residues, respectively (Fig. 3B). Comparing β2 and β1, there are 16 sites where Ser/Thr residues are substituted with non-hydroxyl containing aa, suggesting the potential for considerable diversity in the phosphorylation patterns of these proteins.

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