AMP-activated Protein Kinase β Subunit Tethers α and γ Subunits via Its C-terminal Sequence (186–270)*

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AMP-activated protein kinase (AMPK) is an important metabolic stress-sensing protein kinase responsible for regulating metabolism in response to changing energy demand and nutrient supply. Mammalian AMPK is a stable αβγ heterotrimer comprising a catalytic α and two non-catalytic subunits, β and γ. The β subunit targets AMPK to membranes via an N-terminal myristoyl group and to glycogen via a mid-molecule glycogen-binding domain. Here we find that the conserved C-terminal 85-residue sequence of the β subunit, β1-(186–270), is sufficient to form an active AMP-dependent heterotrimer α1β1-(186–270)-γ1, whereas the 25-residue β1 C-terminal (246–270) sequence is sufficient to bind γ1, γ2, or γ3 but not the α subunit. Deletion of the β C-terminal Ile-270 precludes βγ association in the absence of the α subunit, but the presence of the α subunit or substitution of Ile-270 with Ala or Glu restores βγ binding. Truncation of the α subunit reveals that β1 binding requires the α1-(313–473) sequence. The conserved C-terminal 85-residue sequence of the β subunit (90% between β1 and β2) is the primary αγ binding sequence responsible for the formation of the AMPK αβγ heterotrimer.

AMPK1 is a multi-substrate enzyme that is activated in response to both hormones and intracellular metabolic stress generated by exercise, hypoxia, and nutrient deprivation. There are multiple isoforms of each AMPK subunit, with α1, α2, β1, β2, γ1, γ2, and γ3 forming heterotrimers that differ in tissue and subcellular localization (reviewed in Ref. 1). The α subunit contains an N-terminal catalytic core (1–312) and a C-terminal sequence (313–548) responsible for autoregulation and binding the βγ subunits (2). Maximum activity requires all three subunits (3). The catalytic AMPK α1-(1–312) fragment is constitutively active whereas the α1-(1–392) fragment is autoinhibited, and neither bind βγ subunits (2). The three γ subunits each contain four CBS sequence repeats that were named after the corresponding sequences in cystathionine β-synthase (CBS) along with variable N-terminal extensions (4). AMP binds to the γ subunit and is responsible for the allosteric regulation of AMPK (5). There are two binding sites for AMP formed by the CBS1/2 and CBS3/4 sequence pairs (5), and because pairs of the CBS sequences form a discrete functional structure, they have now been termed Bateman modules (6). The β subunit N-terminal myristoyl group is responsible for targeting AMPK to the membrane (7), and an internal glycogen-binding domain (68–163) targets AMPK to glycogen (8, 9).

The AMPK α subunit is a homolog of yeast Snf1p kinase (10), which also binds βγ subunit homologs (11, 12). There are three β subunit homologs, Gal83p, Sip1p, and Sip2p, and a single γ subunit homolog, Snf4p (13). In contrast to mammalian AMPK, which requires all three subunits for optimal activity (3), Snf1p/Snf4p forms a stable active complex that can be readily isolated from bakers’ yeast without a β homolog (Gal83p, Sip1p, or Sip2p) (11, 14). Deletion of either Snf1p or Snf4p blocks growth on sucrose, as does deletion of all three β subunit homologs (15). Studies of the subunit interactions in yeast using the two-hybrid approach have identified two regions within the yeast AMPKs termed KIS (kinase interacting sequence) and ASC (association with Snf1p complex), respectively. The C-terminal 85-residue ASC sequence of Gal83p was shown to bind Snf4p by two-hybrid analysis (13). The internal KIS sequence of Gal83p, Sip1p, or Sip2p interacted with the non-catalytic C terminus of Snf1p (13). In contrast, studies on mammalian AMPK have shown that the corresponding internal KIS sequence in the β subunits is not sufficient for binding either α or γ subunits but instead corresponds to a glycogen binding sequence (8, 9). Because Snf1p/Snf4p forms an active heterodimer without a β homolog, it was of interest to undertake truncation mutagenesis of the AMPK α and β subunits in order to map the interaction sequences between the αβγ subunits. Our results show that an AMP-dependent heterotrimer can be formed with a C-terminal fragment of β encompassing residues 186–270, α1β1-(186–270)-γ1. We find the β C-terminal residues 246–270 alone are sufficient to bind γ1, γ2, or γ3, but additional residues contained within the β-(186–270) fragment are required for α binding. Furthermore, deletion of the C-terminal Ile (β-(186–269)) prevents βγ association, but the presence of the α subunit or the substitution of Ile-270 with Ala or Glu restores γ binding. Truncation of

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1 The abbreviations used are: AMPK, AMP-activated protein kinase; ASC, association with Snf1p complex; CBS, cystathionine β-synthase; GFP, green fluorescent protein; GST, glutathione S-transferase; HA, hemagglutinin.

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the α subunit reveals that the α1-(313–473) sequence contains the β binding interaction site.

EXPERIMENTAL PROCEDURES

Materials—COS-7 cells were purchased from the American Type Culture Collection. The polyclonal goat anti-GFP antibody was purchased from Rockland Immunochemicals (Gilbertsville, PA). The polyclonal goat anti-GFP antibody was generated as described previously (16). The rabbit polyclonal AMPK antibodies α1-(373–390), α2-(490–516), β1myr-(2–24), β1-(256–270), γ1-(319–331), γ2-(556–569), and γ3-(59–75) were generated and characterized as described previously (17).

Plasmid Constructs—Details of the mammalian expression constructs used in this study are summarized in Table I. Cloning was performed using standard molecular biology techniques or the Gateway cloning system (Invitrogen). Briefly, the coding sequences of the rat AMPK subunits α1, α2, β1, and γ1 were PCR-amplified from existing plasmids (3) using the relevant primers listed in Table I. The human AMPK β2 and γ3 coding sequences were obtained by reverse transcription of oligo(dT)-primed human liver and muscle total RNA using a SuperScript II first strand synthesis kit (Invitrogen). Second strand synthesis and amplification was performed with primers listed in Table I. The rat AMPK γ2 and γ3 cDNA were then cloned into pDONR201, sequence-verified, and shuttled into the relevant expression vector (e.g. pDEST27, 27, or 53) as per Table I. A Myc tag was inserted into the AMPK β1 sequence at β1 + 5 using standard cloning techniques. Alterations to coding sequences were performed by PCR using a QuickChange site-directed mutagenesis kit (Stratagene). These changes included point mutations to introduce residue substitutions or premature stop codons for C-terminal truncations. The sequences of oligonucleotides used for amplification or mutagenesis are also listed in Table I.

Cell Culture, Transfection, and Lysis—COS-7 cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum at 37 °C in 5% CO2 and transfected with plasmids using FuGENE 6 (Roche Applied Science) according to the manufacturer’s instructions. Briefly, 10-cm plates of COS-7 cells at 70% confluence were double or triple transfected with 1 μg of each DNA construct in FuGENE (2.5 μg/μl DNA). Combinations of the different expression plasmids were used as indicated. Approximately 24 h post-transfection, cells were harvested in 500 μl per plate of HT-lysis buffer (50 mM HEPES pH 7.5, 200 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM EGTA, 10 mM Na2PO4, 100 mM NaF, 5 μg/ml aprotinin, 1 mM diethiothreitol, and 1 mM Pefabloc (Roche Applied Science)). The lysis was cleared by centrifugation (13,000 rpm for 10 min at 4 °C), and the resulting supernatant was referred to here as the detergent-solubilized cell lysate.

Glutathione-Sepharose Precipitation—COS-7 cells were transiently transfected with GST-α fusion constructs together with other constructs as indicated. Approximately 4% of the detergent-solubilized cell lysate was analyzed directly by Western blot to monitor subunit expression levels (labeled in Figs. 2–5 as (L)), and the remaining cell lysate was incubated with Glutathione-Sepharose 4B (Amersham Biosciences) at 4 °C for ~2 h. After washing in lysis buffer (2× 50 volume) and phosphate-buffered saline (1× 50 volume), the Glutathione-Sepharose-bound AMPK was either directly analyzed or eluted with 10 mM reduced glutathione, 1 mM diethiothreitol in 50 mM Tris-Cl (pH 7.5) buffer for AMPK activity assays as indicated, and quantitated using scanning densitometry (Molecular Dynamics).

Immunoprecipitation—AMPK was immunoprecipitated from the detergent-solubilized cell lysate by incubation with covalently linked protein G-agarose (Sigma) pre-coupled to a primary polyclonal antibody and incubated at 4 °C for ~2 h. After washing in lysis buffer (2× 50 volume) and phosphate-buffered saline (1× 50 volume), the immunoprecipitated proteins were analyzed by SDS-PAGE and immunoblotting. Immunoreactivitiy was detected with horseradish peroxidase-conjugated protein G (BioRad) using enhanced chemiluminescence.

AMPK Assay—AMPK activity assays were performed in kinase assay buffer (50 mM Hepes, pH 7.5, 2 mM MgCl2, 5% glycerol, and 1 mM dithiothreitol) with 250 μM [γ-32P]ATP (1000 cpm/ml) containing 100 μM SAMS peptide with and without 100 μM AMP as described previously (18).
RESULTS

The AMPK β1 and β2 isoforms are conserved in the C-terminal (186–270) residues (90% identity) but differ in their N-terminal 62 residues (53% identity). The C-terminal sequences encompassing fragment 186–270 of β1, β2, and yeast Sip2p are illustrated in Fig. 1. The role of the β1 subunit in AMPK heterotrimer formation and the identification of important sequences that interact with the α and γ subunits was assessed using co-expression in transiently transfected COS-7 cells. Wild type β1 or mutant GFP-β1 subunits were expressed in COS-7 cells with wild type HA-γ1, Hisβ-γ2, or Hisβ-γ3, and the expressed AMPK β1γ was isolated by immunoprecipitation with either anti-β-(2–24), anti-GFP, or γ1 antibodies. The association of the γ subunits with the β truncations was assessed by Western blot analysis using specific GFP β1-(2–24) and γ subunit antibodies. The shorter β1-(186–270) fragment binds the γ subunit with similar efficiency to the full-length β1-(1–270) sequence, whereas the β1-(1–185) sequence does not bind γ (Fig. 2A), confirming that the C-terminal β1 sequence is

Fig. 1. AMPK β1 domain structure illustrated. The AMPK β1 domain structure featuring an N-terminal myristoyl group (myr), glycogen binding domain (GBD), αγ subunit binding sequence (αγ-SBS), and the phosphorylation sites is shown at the top. The single letter abbreviation for serine is used with position numbers. Amino acid alignment of the rat β1 subunit with rat β2 and yeast Sip2p is shown at the bottom. Identical residues are marked in black in reverse contrast, and conservative substitutions are marked in gray.

Fig. 2. β1 C-terminal sequence binds γ subunits. Using wild type β1 or mutants, β1-(1–185), GFP-β1-(186–270), -(186–245), and -(246–270) constructs were co-transfected with HA-γ1, Hisγ-γ2, or Hisγ-γ3 in COS-7 cells. A, HA-γ1 was immunoprecipitated (IP) and then analyzed for γ1 and associated β1 by Western blot (WB). Total β1 expression in lysates is indicated by (L). B–D, the β1 subunits were immunoprecipitated (IP) and analyzed for associated γ (-1, -2, or -3) and total γ expression in lysates (indicated by (L)) by Western blot (WB). All panels are representative of four independent experiments.

Fig. 3. Truncation of the β1 C-terminal Ile-270 prevents γ subunit interaction. A, wild type Myc-β1 and Myc-β1 substitutions (residues 186–270) constructs. B, wild type Myc-β1 and Myc-β1 substitutions (residues 186–270) and -(186–269) constructs were co-transfected with HA-γ1 and GST-α1 in COS-7 cells. The γ subunits were immunoprecipitated (IP) and analyzed for associated γ subunits and total γ subunit expression in lysates, indicated by (L), using Western blot (WB). Panels are representative of three independent experiments.
required for γ1 binding. Further truncations to the β1 sequence revealed that the shorter C-terminal 25-residue β1-(246–270) sequence, but not the 60-residue β1-(186–245) sequence, binds all three γ isoforms (γ1, γ2, and γ3) (Fig. 2, B–D). These data demonstrate that the extreme C-terminal 25-residue β1-(246–270) sequence contains the determinants for γ subunit binding.

The presence of a conserved Ile at the C terminus raised the possibility that it might be particularly important for subunit binding analogous to the C-terminal hydrophobic residue in target protein interactions with PDZ domains (19). We found that truncation of the C-terminal Ile-270 of the β1 subunit was sufficient to prevent γ subunit interaction. The full-length wild type β1 and β1-(1–269) constructs were expressed in COS-7 cells together with HA-γ1. Western blot analysis showed that truncation of Ile-270 abolished binding of β1 to γ1 (Fig. 3A) and γ2 (results not shown). To determine whether truncation of Ile-270 would impair binding of the β1-(1–269) fragment, this sequence was truncated to 186–269 and expressed in COS-7 cells together with HA-γ1. The β1-(186–269) fragment also did not associate with the γ1 subunit. However, binding of γ1 to the β1-(1–269) or β1-(186–269) fragment could be rescued by co-expression of γ1. The β1-(1–269) or the GFP-β1-(186–269) fragment was expressed in COS-7 cells together with HA-γ1 and GST-α1. Proteins were purified by anti-β1-(2–24) immunoprecipitation or GFP immunoprecipitation, as indicated (Figs. 2–5), for Western blot analysis. To test whether a C-terminal hydrophobic residue was important, Ile-270 was substituted with either Glu or Ala. For this experiment, Myc-β1-(1–270), Myc-β1-(1–269), and Myc-β1-(1–270), with point mutations I270E or I270A, were co-transfected with HA-γ1. Substitution of either Ala or Glu at the C terminus restored γ1 association (Fig. 5B). Thus although truncation of Ile-270 eliminates γ2 binding, these findings indicate that there is no specific requirement for the Ile side chain at this position.

To determine the sequence of the β subunit responsible for interaction with the α subunits, COS-7 cells were transfected with a series of β1 sequences including wild type β1-(1–270) and β1-(1–185) or GFP-β1-(186–270), GFP-β1-(186–245), and GFP-β1-(246–270) with GST-α1. The β1 sequence that binds α1 was identified by co-precipitation with glutathione-Sepharose and Western blot analysis, demonstrating that the β1-(186–270) sequence contains the α subunit binding determinants (Fig. 4A). The β1-(1–185), (186–245), and (246–270) fragments were not sufficient to bind the α1 subunit. To evaluate whether the β1-(186–270) sequence was sufficient to also bind α2, GFP β1 or GFP β1-(186–270), together with GST-α1 or GST-α2, was expressed in COS-7 cells. The α subunits were glutathione-Sepharose-affinity-purified, and associated β sequences were detected by Western blot analysis (Fig. 4B). The binding efficiencies of wild type β1 and β1-(186–270) to α1 and α2 were equivalent, confirming that the α2 binding determinants are conserved within the β1-(186–270) sequence. Both α subunits were always expressed at a higher level when co-transfected with the β1-(186–270) fragment compared with full-length β1-(1–270), but the reason for this observation is not known.

To determine whether the β subunit truncations supported AMP-dependent AMPK heterotrimer activity, COS-7 cells were
co-transfected with either β1 wild type-(1–270), β1-(1–269), GFP-β1-(186–270), or GFP-β1-(186–269), together with GST-α1 and HA-γ1. Following glutathione-Sepharose precipitation, the bound proteins were eluted in 10 mM reduced glutathione, assayed for AMPK activity, and subjected to SDS-PAGE and Western blot analysis. The activity of each construct was determined relative to the expression of the GST-α1 construct and standardized to wild type AMPK. All β1 mutant heterotrimerers showed similar AMPK activity and AMP de- pendeht to wild type αβγ (Fig. 4C), indicating that the β1-(186–270) fragment is sufficient to maintain an active hetero- trimer and that the β N terminal (residues 1–185) fragment does not contribute to heterotrimer formation or modulate AMP dependence. Previous studies have shown that the C terminus of the α catalytic subunit (residues 313–548) is responsible for βγ bind- ing (2). To determine the α1 sequence that binds the β subunit, wild type GST-α1, GST-α1-(1–312), or GST-α1-(313–548) was expressed in COS-7 cells with wild type β1. The association of β1 with the GST-α fragments was detected by Western blot analysis following glutathione-Sepharose affinity purification. As found previously (2), the important residues for β1 subunit binding are contained in the α-(313–548) C-terminal sequence and not the α-(1–312) catalytic domain (Fig. 5A). Further truncations of the α-(313–548) C-terminal sequence were tested for their effect on binding the β1 sequence. GST-α1-(313–548) and the C-terminal truncations GST-α1-(313–350), (313–392), (313–422), (313–473), (313–504), (313–524), and (313–548) were co-expressed with β1 wild type in COS-7 cells (Fig. 5B). The α1-(313–473) fragment was the shortest C-terminal truncation that retained the capacity to bind the β subunit.

**DISCUSSION**

The AMPK requires all three subunits (αβγ) for full activity (3), contrasting with yeast Snf1p kinase, which forms an active heterodimer, Snf1p/Snf4p (αγ) (11, 14). The AMPK β subunit acts as a targeting scaffold, tethering α and γ subunits via an overlapping C-terminal 85-residue sequence (residues 186– 270) that we have termed the αγ subunit binding sequence (Fig. 1). The C-terminal 25-residue β1-(246–270) sequence alone is sufficient to bind the γ subunits (γ1, γ2, and γ3 isoforms). There is cooperativity between α and γ binding to β, because we find that deletion of the C-terminal Ile-270 is suf- ficient to inhibit γ binding to β, but the presence of the α subunit restores γ binding to β-(1–269) and β-(186–269). Al- though the β C-terminal residue is required for γ binding to β in the absence of α, the side-chain of the β Ile-270 is not critical and can be replaced with either Ala or Gln. The reason for this effect is not yet known, but if the β C terminus is α-helical, then one interpretation is that Ile-270 or another C-terminal residue provides added stability to Pro-269 by acting as a helix-capping residue (20). The permissive effect of the α subunit in allowing γ to bind the β-(1–269) sequence may indicate that α enhances the β-(186–269) binding conformation for γ rather than α and γ interacting directly, or that it stabilizes the hypothetical C-terminal α helix in the absence of Ile-270. Previously, we detected traces of αγ binding (3), raising the possibility of some β subunit-independent αγ interaction. In the present study, however, we were unable to detect binding between α and γ in the absence of β. We cannot eliminate the possibility that endogenous β contributed to αγ binding in COS-7 cells expressing the tagged α and γ observed previously (3).

AMPK is primarily regulated by phosphorylation at Thr-172 in the kinase activation loop (14) by the upstream kinase LKB1 (21, 22). The β subunit is phosphorylated at multiple sites, autophosphorylated at Ser-108, Ser-24/25 (23), Ser-96, and Ser-101 (21), and also phosphorylated at Ser-182 in vivo (flank- ing the αγ subunit binding sequence) by an unidentified protein kinase (23). Mutation of Ser-108 contained within the glycogen- binding domain to Ala has no effect on glycogen binding (8) but inhibits activity (7), whereas mutation of the β Ser-24/25 and Ser-182 phosphorylation sites to alanine leads to nuclear re- distribution, suggesting a role in subcellular localization (7). These phosphorylation sites are located outside the αγ subunit binding sequence-(186–270) sequence and do not appear to play a direct role in subunit interactions.

The results obtained here are in broad agreement with the earlier studies in yeast showing that the C-terminal 84-residue ASC sequence (313–473) of the yeast β homologs (Gal83p, Sip1p, and Sip2p) is sufficient to bind Snf1p (24). However at first sight there are significant differences between the yeast and mammalian AMPK subunit interactions. For example, yeast β homologs have multiple interaction sites because deletion of the C-terminal 84-residue ASC sequence of Sip2p does not abolish its interaction with Snf1p, indicating that the ASC sequence was not solely responsible for Snf1p-Sip2p interaction (13). Furthermore the Sip2p-(1–336) N ter- minus binds to the Snf1p C termini (Fig. 6), as assessed by two-hybrid analysis. However this latter interaction appears weaker, because reliable co-immunoprecipitation of Sip2p and Snf1p depends on the C-terminal 84-residue ASC sequence Sip2p-(335–415) (24). In the present study we have mapped the β subunit-(186–270) interaction site on α to a 161-residue fragment, α-(313–473). This interaction is lost by further truncation to α-(313–422). The α-(313–473) site corresponds to Snf1p-(392–595) in the aligned sequences. There are apparent differences in the subunit interactions between yeast and mammalian αβ subunits, because the corresponding Sip2p-(332–415) interaction site on Snf1p maps to a shorter se-
sequence, Snf1p-(392–495). When Snf1p and α are aligned, the Sip2p-(332–415) ASC interaction site on Snf1p contains two insert sequences not present in the α subunit that may account for the differences in the β binding sequences between Snf1p and α (Fig. 6). Furthermore the Sip2p (332–415) interaction site on Snf1p overlaps with the Snf4p interaction site (13) in contrast to α, which does not bind γ. Again, the presence of the insert sequences in Snf1p-(392–495) may account for the capacity of Snf1p to form a stable active complex with Snf4p in contrast to AMPK αγ, which does not form a stable heterodimer. The interaction between Snf4p and Snf1p is also influenced by the catalytic activity of Snf1p. Whereas in active Snf1p the residues 392–495 bind Snf4p, the Snf1p residues 392–518 are required for kinase-dead Snf1p to bind Snf4 (25). The intervening Snf1p sequence 495–518 contains the highly conserved GIRSQSYPL sequence in α containing Ser-404 (underlined) as part of a putative 14.3.3-binding site (1), but it is not known whether this site is phosphorylated or influences Snf4p binding.

The N-terminal Sip2p-(1–336) residues also bind Snf1p but require the C-terminal snf1p sequence 515–633 for binding (Fig. 6). This overlaps the distal part of the equivalent α sequence (313–473) required to bind β (Fig. 6). Thus, if both reported Sip2p interaction sites on Snf1p are consolidated, there is more overlap with the sequence of α required to bind β than if only the C-terminal Sip2p-(332–415) ASC interaction is considered. In this way the aligned C termini of α and Snf1p provide clues to why Snf1p binds Snf4p, whereas α cannot bind γ, and Sip2p and β have similar binding requirements. Three-dimensional structural information will be required to fully reveal the nature of the subunit interaction architecture. The α Snf1p comparison shows that the α Ser-485 (26) phosphorylation site is outside the β binding sequence and is contained in a non-homologous loop not present in Snf1p (Fig. 6).

We have not undertaken detailed mapping studies of the β interaction site features on γ because truncation of one of any of the CBS sequences results in insolubility and AMPK inactivity when expressed in COS-7 cells.2 In summary, the AMPK β subunit is not only essential for formation of an active heterotrimer but acts as a scaffold for α and γ binding. The β (αγ subunit binding sequence) fragment, together with α and γ, is sufficient to form a heterotrimer with AMP dependence and activity similar to those of the wild type enzyme.

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