AMP-activated Protein Kinase Subunit Interactions

\[ \beta_1:\gamma_1 \text{ASSOCIATION REQUIRES } \beta_1 \text{Thr-263 AND Tyr-267}^* \]

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AMP-activated protein kinase (AMPK) plays multiple roles in the body’s overall metabolic balance and response to exercise, nutritional stress, hormonal stimulation, and the glucose-lowering drugs metformin and rosiglitazone. AMPK consists of a catalytic \( \alpha \) subunit and two non-catalytic subunits, \( \beta \) and \( \gamma \), each with multiple isoforms that form active 1:1:1 heterotrimers. Here we show that recombinant human AMPK \( \alpha_1\beta_1\gamma_1 \) expressed in insect cells is monomeric and displays specific activity and AMP responsiveness similar to rat liver AMPK. The previously determined crystal structure of the core of mammalian \( \alpha\beta\gamma \) complex shows that \( \beta \) binds \( \alpha \) and \( \gamma \). Here we show that a \( \beta_1(186–270)\gamma_1 \) complex can form in the absence of detectable \( \alpha \) subunit. Moreover, using alanine mutagenesis we show that \( \beta_1 \) Thr-263 and Tyr-267 are required for \( \beta_\gamma \) association but not \( \alpha_\beta \) association.

Mammalian AMP-activated protein kinase (AMPK) is a metabolite-sensing serine/threonine protein kinase that plays a central role in whole body energy homeostasis. The enzyme is activated in response to intracellular stresses that lead to increased AMP:ATP ratios (e.g. hypoxia, exercise, nutrient deprivation) and hormones that regulate whole-body energy metabolism, including adiponectin and leptin (1, 2). AMPK is also activated by metformin and rosiglitazone, glucose lowering drugs used to treat type II diabetes (3, 4). Activation of AMPK leads to phosphorylation of multiple downstream targets that restore energy imbalances broadly by switching off anabolic processes and stimulating energy producing pathways (2).

Fully functional AMPK is a heterotrimer composed of \( \alpha, \beta, \) and \( \gamma \) subunits (5), with distinct genes encoding each of the subunits (\( \alpha_1, \alpha_2, \beta_1, \beta_2, \gamma_1, \gamma_2, \) and \( \gamma_3 \)). The \( \alpha \) subunits each contain a highly conserved N-terminal catalytic core (1–312), an autoinhibitory sequence (313–335) (6, 7), and a less conserved C-terminal \( \beta \) binding sequence (313–473) with the remainder of the \( \alpha \) C terminus required for \( \gamma \) binding (7, 8). The three \( \gamma \) subunits each contain four conserved CBS sequence repeats, named after the corresponding regions in cystathionine-\( \beta \)-synthase. Pairs of CBS sequences (CBS1/CBS2 and CBS3/CBS4) form discrete functional structures termed Bateman domains (9) that bind nucleotides (10). Four nucleotide binding sites have been identified with each CBS sequence contributing one site (11). The mammalian \( \gamma_1 \) subunit crystal structure has 3 of these 4 sites occupied, with 2 sites capable of binding either AMP or ATP (12). It is presumed ligand binding to the Bateman domains induces conformational changes within the \( \alpha \) subunit and provides a mechanism for allosteric activation of the kinase. The \( \gamma \) subunits possess non-conserved N-terminal extensions of up to 94 residues, the functions of which are poorly understood but may be involved in subcellular targeting. The \( \beta \) subunits contain an N-terminal myristoylation site responsible for localization of AMPK to the membrane (13, 14) and an internal glycogen binding domain (residues 68–163) (15, 16).

Studies using AMPK subunits individually expressed in rabbit reticulocytes suggested that \( \beta \) subunit mediates the formation of a stable AMPK heterotrimer in vitro (17). However, until recently little was known about the subunit interacting regions. Studies on mammalian AMPK have focused on overexpression of truncated mutants of each subunit and assessment of their ability to form stable, functional AMPK complexes. Using this approach we previously found that the conserved \( \beta \) subunit C-terminal sequence \( \beta_1(186–270) \) was sufficient to form a stable AMP-activated heterotrimer (8). We termed this region the \( \alpha_\gamma \) subunit binding sequence. We also showed that the \( \beta_1 \) C-terminal 25-residue sequence is sufficient to bind \( \gamma_1, \gamma_2, \) or \( \gamma_3 \) subunits but not \( \alpha \). A conserved 20–25-residue sequence immediately N-terminal to the \( \gamma \) CBS1 domain is essential for...
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TABLE 1

Summary of plasmids

| Plasmid name | Protein expressed | Oligonucleotides | Reference |
|--------------|------------------|------------------|-----------|
| Baculovirus expression | | | |
| pFastBac1 | Human GST-α1 | Fwd 1: ATGACATTTGCTGGCCATGATGTAACATGTT | Invitrogen |
| pFastBac1-GST-TEV-α1 | Human GST-α1 | Rev 1: ATGACATTTGCTGGCCATGATGTAACATGTT | |
| FastBac1-β1 | Human β | Fwd 1: ATGACATTTGCTGGCCATGATGTAACATGTT | |
| pFastBac1-α1 | Human α1 | Rev 1: ATGACATTTGCTGGCCATGATGTAACATGTT | |
| pFastBac1-α1 | Human α1 | Fwd 2: ATGACATTTGCTGGCCATGATGTAACATGTT | |
| pFastBac1-β1 | Human β | Rev 2: ATGACATTTGCTGGCCATGATGTAACATGTT | |
| pFastBac1-β1 | Human β | Fwd 3: ATGACATTTGCTGGCCATGATGTAACATGTT | |
| pFastBac1-β1 | Human β | Rev 3: ATGACATTTGCTGGCCATGATGTAACATGTT | |
| pFastBac1-β1 | Human β | Fwd 4: ATGACATTTGCTGGCCATGATGTAACATGTT | |
| pFastBac1-β1 | Human β | Rev 4: ATGACATTTGCTGGCCATGATGTAACATGTT | |
| Mammalian expression | | | |
| pEBG | Rat GST-β1 | Fwd 1: ATGACATTTGCTGGCCATGATGTAACATGTT | 21 |
| pEBG-β1 | Rat GST-β1 | Rev 1: ATGACATTTGCTGGCCATGATGTAACATGTT | |
| pBKCMV-β1 | Rat β1 | Fwd 2: ATGACATTTGCTGGCCATGATGTAACATGTT | 5 |
| pBKCMV-β1 | Rat β1 | Rev 2: ATGACATTTGCTGGCCATGATGTAACATGTT | |
| pBKCMV-β1 | Rat β1 | Fwd 3: ATGACATTTGCTGGCCATGATGTAACATGTT | |
| pBKCMV-β1 | Rat β1 | Rev 3: ATGACATTTGCTGGCCATGATGTAACATGTT | |
| pBKCMV-β1 | Rat β1 | Fwd 4: ATGACATTTGCTGGCCATGATGTAACATGTT | |
| pBKCMV-β1 | Rat β1 | Rev 4: ATGACATTTGCTGGCCATGATGTAACATGTT | |
| pCDNA3.1-myc-β1 | Rat myc-β1 | Fwd 1: ATGACATTTGCTGGCCATGATGTAACATGTT | 8 |
| pCDNA3.1-myc-β1 | Rat myc-β1 | Rev 1: ATGACATTTGCTGGCCATGATGTAACATGTT | |
| pCDNA3.1-myc-β1 | Rat myc-β1 | Fwd 2: ATGACATTTGCTGGCCATGATGTAACATGTT | |
| pCDNA3.1-myc-β1 | Rat myc-β1 | Rev 2: ATGACATTTGCTGGCCATGATGTAACATGTT | |
| pCDNA3.1-myc-β1 | Rat myc-β1 | Fwd 3: ATGACATTTGCTGGCCATGATGTAACATGTT | |
| pCDNA3.1-myc-β1 | Rat myc-β1 | Rev 3: ATGACATTTGCTGGCCATGATGTAACATGTT | |
| pCDNA3.1-myc-β1 | Rat myc-β1 | Fwd 4: ATGACATTTGCTGGCCATGATGTAACATGTT | |
| pCDNA3.1-myc-β1 | Rat myc-β1 | Rev 4: ATGACATTTGCTGGCCATGATGTAACATGTT | |
| pMT2-HA-γ1 | HA-γ1 | Fwd 1: ATGACATTTGCTGGCCATGATGTAACATGTT | 5 |
| pMT2-HA-γ1 | HA-γ1 | Rev 1: ATGACATTTGCTGGCCATGATGTAACATGTT | |
| pMT2-HA-γ1 | HA-γ1 | Fwd 2: ATGACATTTGCTGGCCATGATGTAACATGTT | |
| pMT2-HA-γ1 | HA-γ1 | Rev 2: ATGACATTTGCTGGCCATGATGTAACATGTT | |
| pMT2-HA-γ1 | HA-γ1 | Fwd 3: ATGACATTTGCTGGCCATGATGTAACATGTT | |
| pMT2-HA-γ1 | HA-γ1 | Rev 3: ATGACATTTGCTGGCCATGATGTAACATGTT | |
| pMT2-HA-γ1 | HA-γ1 | Fwd 4: ATGACATTTGCTGGCCATGATGTAACATGTT | |
| pMT2-HA-γ1 | HA-γ1 | Rev 4: ATGACATTTGCTGGCCATGATGTAACATGTT | |

* Constructs prepared for this study.

the βγ interaction and the formation of an active trimeric complex (18). In contrast, Wong and Lodish (19) recently reported an alternative model for AMPK subunit interactions that claimed the subunit acted as the complex scaffold with no direct interaction between β and γ subunits.

A crystal structure of the regulatory fragment of mammalian (rat) AMPK, corresponding to α1-(396–548), β2-(187–272), and γ1 has recently been reported (12). The structure shows a direct βγ interaction mediated almost exclusively by hydrogen bonding and salt bridge interactions between three β-strands, two provided by the C terminus of the β subunit and the other overlapping the N-terminal region of the γ CBS1 domain.

We used a baculovirus-mediated recombinant expression system for human AMPK. We present conclusive evidence for a direct interaction between His-tagged human β1-(186–270) and γ1 using size exclusion chromatography in the absence of any α subunit. With the use of an alanine mutagenesis screen we demonstrate that two critical residues (Thr-263 and Tyr-267) within the rat β1 C terminus (260–270) are especially important for the βγ association.

EXPERIMENTAL PROCEDURES

Materials—SF21 cells and SF-900 II serum-free medium were purchased from Invitrogen. COS-7 cells were purchased from the American Type Culture Collection. Polyclonal goat anti-GST antibody, Western blot 2′-antibodies, glutathione-Sepharose 4B, Q-Sepharose FastFlow, and Superdex 200 and HisTrap FF 5-ml pre-packed columns were purchased from GE Healthcare. Rabbit polyclonal AMPK antibodies α1-(339–358), β1-(257–270), and γ1-(320–331) were generated and characterized as described previously (20). Glutathione-agarose was purchased from Sigma. Amicon Ultra centrifugal concentrators were purchased from Millipore. FLAG monoclonal antibody coupled affinity resin was obtained from Kem-En-Tec. All other materials were purchased from either Roche Applied Science or Sigma.

Plasmid Constructs—Details of the baculovirus and mammalian expression constructs used in this study are summarized in Table 1. Cloning was performed using standard molecular biology techniques. The full-length CaMKKβ construct was generated by overlapping PCR using partial human cortical brain cDNA clones obtained from Kristen Anderson and Anthony Meeks, Duke University. The resulting PCR product was cloned into pFastBac1-FLAG-N-TEV to generate FLAG-TEV-CaMKKβ. For mammalian expression vectors, point mutations were introduced into existing plasmids containing the coding sequence of the rat AMPK β1 subunit (pBKCMV-β1...
or pcDNA3.1-myc-β1) by PCR using a QuickChange site-directed mutagenesis kit (Stratagene) and the primers listed in Table 1. All point mutations along with the wild-type sequence, were cloned into the pE tagged mammalian expression vector to incorporate an N-terminal GST tag. All constructs described were sequence-verified.

SF21 Cell Culture, Expression, and Protein Purification—SF21 insect cells were grown in SF-900 II medium at 26 °C, 150 rpm. For expression of the αβγ heterotrimer, cells were triple-infected with GST-α1, β1, and γ1 baculovirus at an overall multiplicity of infection of 10. Cells were harvested 30 h post-infection by centrifugation at 1500 rpm for 15 min, and pellets were washed in phosphate-buffered saline before storage at −80 °C. For protein purification, cells were thawed and resuspended in 0.05x culture volume of ice-cold lysing buffer (50 mM Tris-HCl, pH 7.4, 200 mM NaCl, 1 mM dithiothreitol (DTT), 1 mM EDTA, 0.5 μM aprotinin, 20 μM leupeptin, 1 μg/ml pepstatin A, 2 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride). All subsequent steps were conducted at 4 °C. Cells were lysed using an Avestin EmulsiFlex C5 homogenizer, and cellular debris and membrane fragments were sedimented by centrifugation (40,000 × g, 60 min). Saturated (NH₄)₂SO₄ was added to the supernatant to a final concentration of 40% (v/v) with gentle stirring for 30 min, and precipitated protein was pelleted by centrifugation (5000 rpm, 30 min). Protein pellets were resuspended in 0.03x culture volume lysing buffer plus 0.1% (v/v) Triton X-100 and incubated with glutathione-agarose for 90 min. Glutathione-agarose was washed extensively in lysis buffer (5 × 10 volumes), and AMPK was eluted in lysis buffer containing 10 mM glutathione. GST constructs were cleaved by overnight incubation with TEV protease.

Recombinant AMPK was further purified using anion exchange chromatography. Samples were diluted in 50 mM Tris-HCl, pH 7.4, 1 mM DTT to a final NaCl concentration of 80 mM and loaded onto a Q-Sepharose FastFlow column (10/150 mm) at a flow rate of 1 ml/min. The column was washed with 5x column volumes of lysis buffer excluding protease inhibitors, and AMPK was eluted in 1-ml fractions using a NaCl gradient (100–500 mM). AMPK-containing fractions were pooled, concentrated to 500 μl (Amicon Ultra centrifugal concentrator, 30-kDa molecular mass cut-off membrane), and de-phosphorylated by treatment with λ-phosphatase for 30 min. Preparations were then subjected to size exclusion chromatography using a Superdex 200 (10/300 mm) GL column pre-equilibrated with 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM Tris[2-carboxyethyl] phosphate. AMPK was eluted at 0.5 ml/min, with the eluate monitored at 280 nm. The purity of AMPK preparations was confirmed by SDS-PAGE followed by either Coomassie Blue staining or Western blotting.

For expression of recombinant human His5-β1(186–270)y1 complex, SF21 cells were dually infected with His5-β1(186–270) (multiplicity of infection (m.o.i.) of 9) and y1 baculovirus (m.o.i. of 1). Cells were harvested, washed, and lysed as described above for the heterotrimer except that 30 mM imidazole and 0.1% (v/v) Triton X-100 were included in the lysis buffer. The clarified lysate was loaded onto a HisTrap FF 5 ml column (1 ml/min) and washed with 5x column volumes of lysis buffer (excluding protease inhibitors) containing 120 mM imidazole. A purified His5-β1(186–270)y1 complex was eluted with 500 mM imidazole. His5-β1(186–270)y1 preparations were concentrated and subjected to size exclusion chromatography as described above for the heterotrimer, with 0.1% (v/v) Triton X-100 included in the equilibration buffer. 0.5-ml fractions were analyzed directly by Western blotting, with immunoreactivity being detected by Alexafluor-680 anti-rabbit 2’ antibody.

For expression of FLAG-TEV-CaMKKβ, SF21 cells were infected at a multiplicity of infection of 10 and harvested 72 h post-infection. Cell lysates were prepared as for recombinant AMPK, and protein was purified on FLAG monoclonal antibody-coupled affinity resin. Protein was eluted with 0.25 mg/ml FLAG peptide (DYKDDDDK).

Recombinant AMPK Assay—Recombinant AMPK heterotrimer (α1β1γ1) was maximally phosphorylated using recombinant CaMKKβ in phosphorylation buffer: 20 mM Tris-HCl, pH 7.5, 0.1% Tween 20, 10 mM DTT, 8 mM MgCl₂, and 400 μM ATP for 30 min at 30 °C, then diluted 1/500 in the AMPK activity assay (as described for AMPKK in Chen et al. (22)). The AMPK activity was measured by the phosphorylation of the SAMS peptide (HMRSAMSGLHLKVRK) in 50 mM HEPES, pH 7.5, 2 mM MgCl₂, 5% glycerol, 1 mM DTT, 250 μM [γ-32P]ATP (500 cpm/pmol) containing 100 μM SAMS peptide and AMP at various concentrations as indicated in a reaction volume of 40 μl. After incubation for 10 min at 30 °C, 25-μl aliquots were applied to P81 papers, washed in 75 mM H₃PO₄, and quantified by liquid scintillation counting.

Sedimentation Velocity—Measurements were conducted on purified recombinant human AMPK preparations (0.5 mg/ml) in a Beckman-Coulter Optima XL-I analytical ultracentrifuge using an AnTi rotor and double sector 12-mm path length cells containing quartz windows and charcoal-filled Epon centerpieces. In a typical experiment the reference sector was filled with 400 μl of buffer and the sample sector with 390 μl of sample. Centrifugation was carried out at 20 °C using a rotor speed of 50,000 rpm. During centrifugation, the 280 nm absorbance of the sample was scanned from 5.8 to 7.3 cm at 0.003-cm increments. Scans were collected at 6-min intervals until the solution boundary had reached the cell bottom (7.22 cm). Scans 5–33 were imported into SEDFIT version 9.4 (23) and analyzed in terms of a continuous size-and-shape distribution (c(s,f(f₀))) (24). To obtain a model-independent estimate of the molar mass (i.e., buoyant molar mass), the density of the buffer solution was set to 0 g/ml. During the analysis the sedimentation coefficient distribution (c(s)) was calculated from 0.1 to 10.5 s using an S-grid increment of 0.25 s. Likewise, the frictional ratio distribution (c(f(f₀))) was calculated from 1.00 to 4.50 using an S-grid increment of 0.25. Data were fitted using maximum entropy regularization. The overall p value used for the regularization was 0.95, and the same amount of regularization was applied to the s- and f₀ directions. In addition, the radial position of the sample meniscus was optimized for best fit, and the data were corrected for both time-invariant and radial-invariant noise. Data were plotted using SigmaPlot Version 10 (SPSS).

Quadrupole-time of Flight Mass Spectrometry—Analyses were carried out on an Agilent 6510 quadrupole-time of flight
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liquid chromatograph/mass spectrometer coupled to an Agilent 1100 LC system (Agilent, Palo Alto, CA). All data were acquired, and reference mass was corrected via a dual-spray electrospray ionization source. Each scan or data point on the total ion chromatogram is an average of 10,000 transients, producing a scan every second. Spectra were created by averaging the scans across each peak. The mass spectrometer conditions were as follows: ionization mode, electrospray ionization, positive mode; drying gas flow, 7 liter/min; nebulizer, 30 psi; drying gas temp, 345 °C; Vcap, 4500 V; fragmentor, 250 V; skimmer, 65 V; octopole radio frequency voltage, 750 V; scan range acquired, 100–2000 m/z. Reversed phase chromatographic separation was carried out using a 5-μm Zorbax Poroshell SB300-C8 (2.1 × 75 mm) column using a gradient of 20% to 60% B (95% acetonitrile in water with 0.1% formic acid) for 100 min at 0.25 ml/min.

**Mammalian 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 according to the manufacturer’s instructions. Briefly, 10-cm plates of COS-7 cells at 70% confluence were dually transfected with 1 μg of each DNA construct in FuGENE 6. Combinations of the different rat subunit 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% (v/v) Triton X-100, 1 mM EGTA, 10 mM Na4P2O7, 100 mM NaF, 5 mM Pefabloc (4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride)). The lysate was cleared by centrifugation (13,000 rpm, 10 min, 4 °C), and the supernatant is referred to as the whole cell lysate.

**Glutathione-Sepharose Precipitation—**COS-7 cells were transiently transfected with GST-rat β1 fusion constructs together with pMT2.HA-rat α1. To monitor subunit expression levels, ~4% of the whole cell lysate was analyzed directly by Western blotting. To assess subunit binding the remaining cell lysate was incubated with glutathione-Sepharose at 4 °C for 2 h. After washing in lysis buffer (2 × 50 volumes) and phosphate-buffered saline (1 × 50 volumes), the glutathione-Sepharose-bound AMPK was analyzed directly by SDS-PAGE and Western blotting. Immunoreactivity was detected with horseradish peroxidase-conjugated protein G using chemiluminescence.

**RESULTS**

**Sf21-derived AMPK Possesses Similar Specific Activity and AMP Dependence to Native AMPK—**Recombinant human AMPK (α1β1γ1) was expressed in Sf21 cells and isolated using the protocol described under “Experimental Procedures.” The heterotrimeric complex eluted from an S200 gel filtration column at an elution volume of 11.75 ml, corresponding to a molecular mass of 207 kDa compared with molecular mass standards. Final yields of the purified heterotrimer from this source were modest, typically 0.5 mg/liter of culture. Densitometry analysis after SDS-PAGE and Coomassie staining indicated that recombinant AMPK preparations were ~95% pure (Fig. 1a). Western blotting using primary antibodies specific to phosphopeptides within the heterotrimer indicated that many of the phosphorylation sites already identified in mammalian and rodent studies, such as α1-Thr-172, α1-Ser-485, β1-Ser-108, and β1-Ser-182 are also phosphorylated in Sf21 cells (data not shown). Maximal phosphorylation on α1-Thr-172 was seen after treatment with CaMKKβ for 30 min (Fig. 1b), resulting in a 140-fold increase in activity as assessed by SAMS peptide assay. AMPK activity could be further stimulated by the addition of AMP up to ~80 μM to a maximum of 4.4-fold compared with levels without added AMP (Fig. 1b). This is comparable with the 2–3-fold stimulation by AMP previously seen in α1β1γ1 isolated from mammalian COS-7 cells (25) or rat liver (20). K_{	ext{A0.5}} for the recombinant AMPK was 7.6 ± 1.1 μM, comparable with values reported for COS-7 cell and rat liver AMPK preparations (10 and 15 μM, respectively). Maximum specific activity of fully phosphorylated recombinant AMPK was 6.2 μmol/min·mg⁻¹ protein, comparable with values reported for rat liver AMPK (8–25 μmol/min·mg⁻¹ protein (26)).

**Solution Properties of Sf21-derived Recombinant AMPK—**Sedimentation velocity measurements were carried out to determine the oligomeric state of recombinant human AMPK in solution. The open circles in Fig. 2a, upper panel, represent radial 280-nm absorbance scans of a 0.5 mg/ml AMPK solution taken at regular intervals during centrifugation at 50,000 rpm. The data are characteristically sigmoidal and exhibit a well defined solvent plateau, solution boundary, and solution plateau. The smooth lines in Fig. 2a, upper panel, represent the best fit of the experimental data to a continuous size-and-shape distribution model (23). The high quality of the fit is demonstrated by the randomness of the residuals (Fig. 2a, lower panel). The relative abundances of the sedimenting species recovered from the analysis (c(s,ff₀)) are plotted as a function of both sedimentation coefficient (s) and frictional ratio (ff₀) in Fig. 2b. It is evident that the sample sedimented essentially as a single species with a modal sedimentation coefficient of 5.8 s and a modal
frictional ratio of 2.15. Based on these values, the protein has a buoyant (reduced) molar mass of 32.3 kDa, consistent with a value of 33.9 kDa predicted for the \( \alpha_1, \beta_1, \gamma_1 \) heterotrimer.

Similarly purified AMPK preparations were subjected to electrospray ionization-quadrupole-time of flight mass spectrometry after separation of the subunits on a reversed phase column (Fig. 3a). Figs. 3, b–d, shows that each subunit possessed the expected molecular mass: \( \alpha_1 (2–550) \) plus residual Gly-Gly-Ser from TEV protease recognition site: theoretical mass 62,877.7 Da, recorded mass 62,878.7 Da; \( \beta_1 (2–270) \), theoretical mass 30,251.1 Da, recorded mass 30,251.3 Da; acetylated \( \gamma_1 (1–331) \), theoretical mass 37,621.3 Da, recorded mass...
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![Image](324x26 to 384x38)

**FIGURE 4.** Baculovirus-derived HIS-β1 (186–270) co-elutes with γ1 from a size exclusion column. A His-tagged C-terminal β1 fragment (186–270) was co-expressed with wild-type γ1 in Sf21 cells and purified using nickel-nitrotriacetic acid-agarose. Samples were injected onto a Superdex 200 column equilibrated with 50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 0.1% (v/v) Triton X-100 and 1 mM DTT and eluted at 0.5 ml/min in 0.5 ml fractions. Fractions indicated were Western-blotted for the presence of γ1 and C-terminal β1.

37,621.2 Da. The data also demonstrated that the α1 and γ1 subunits could be completely de-phosphorylated by treatment with λ-phosphatase (Fig. 3, b and d). One phosphorylation site in the β1 subunit (Ser-182 as determined by Western blotting) was resistant to λ-phosphatase treatment. The β subunit was also found to be partially myristoylated at the N terminus (Fig. 3c), although a short incubation with N-myristoyl transferase and myristoyl-CoA was sufficient to reduce the non-myristoylated species to negligible amounts (not shown). Minor peaks corresponding to sodium adducts are also evident in each spectrum.

**Hisγ β1(186–270)γ1 Heterodimer—** To test further the claim by Wong and Lodish (19) that βγ subunits do not form a stable complex in the absence of the α subunit, we co-expressed a Hisγ-tagged C-terminal fragment of human β1, encompassing the αγ subunit binding sequence, with wild-type human γ1 in Sf21 cells. The recombinant proteins were purified sufficiently by immobilized metal affinity chromatography to yield a single, symmetrical peak after Superdex 200 size exclusion chromatography (Fig. 4). Maximal absorbance at 280 nm was observed at an elution volume of 14.32 ml, corresponding to a molecular mass of 69 kDa compared with molecular mass standards. Analysis of the column fractions by Western blotting indicated that the two proteins eluted together, consistent with being a heterodimer. Immunoreactive bands at ~37 and 16 kDa represent γ1 and Hisγ β1(186–270), respectively (Fig. 4). The immunoreactive band at 16 kDa migrated as a smaller species at 13 kDa after treatment with TEV protease, consistent with removal of the N-terminal Hisγ tag from the β1 fragment (not shown). No endogenous α subunit was detectable in these fractions by Western blotting (not shown). These results demonstrate that the C terminus of β1 can form a stable complex with γ1 in the absence of α.

**β1-Thr-263 and β1-Tyr-267 Are Required for α1γ1 Subunit Association—** We previously showed that the β1 subunit C-terminal 25 residues (246–270) are required for βγ association in the absence of α. To determine specific residues involved in βγ association within this region, we individually substituted the C-terminal 11 residues of full-length rat β1 with alanine. N-terminal GST-tagged β1 wild-type and the mutations K260A, Y261A, V262A, T263A, T264A, L265A, L266A, Y267A, K268A, P269A, or I270A were co-expressed with HA-γ1 in COS-7 cells. The GST-β1 fusion proteins were precipitated with glutathione-Sepharose (GSH), and Western blots (WB) were analyzed for GST-β1, γ1, endogenous γ1 (endog), and expression levels in the whole cell lysate (L) of each. Data are representative of three independent experiments. The aromatic character of the β1 residue at position 267 is important for γ1 association. GST-tagged β1 wt and mutants β1(Y267A), β1(Y267F), β1(Y267F), β1(Y267F), and β1(Y267H) were co-expressed with HA-γ1 in COS-7 cells. The GST-β1 fusion proteins were precipitated and analyzed identically to the alanine mutation screen detailed above. Data are representative of three independent experiments.

**FIGURE 5.** The side chains of β1-Thr-263 and β1-Tyr-267 are critical for γ1, but not α1, binding. α, an alanine screen of the C terminus of rat AMPKβ1 subunit reveals the importance of Thr-263 and Tyr-267 to facilitate rat γ1 binding. GST-tagged β1 wild-type (wt) or β1 alanine substitution mutants K260A, Y261A, V262A, T263A, T264A, L265A, L266A, Y267A, K268A, P269A, or I270A were co-expressed with HA-γ1 in COS-7 cells. The GST-β1 fusion proteins were precipitated with glutathione-Sepharose (GSH), and Western blots (WB) were analyzed for GST-β1, γ1, endogenous γ1 (endog), and expression levels in the whole cell lysate (L) of each. Data are representative of three independent experiments.
CaMKKβ (Fig. 1b) and LKB1 (data not shown) and, unlike most recombinant AMPK preparations from bacteria (27–29), shows comparable AMP responsiveness and maximum specific activity to rat liver AMPK preparations (20, 30). We initially used a bacterial expression system for AMPK; however, samples rapidly aggregated after removal of affinity tags and readily lost the ability to be fully activated by CaMKKβ after storage. These stability problems are not evident with recombinant AMPK derived from Sf21 cells.

The crystal structures of truncated yeast Snf1 (31, 32) both revealed dimerization of the kinase domain within the unit cell. However, these studies provide conflicting evidence with regard to the presence of the dimer in solution. Townley and Shapiro (33) also observed dimerization in the crystal structure of the Schizosaccharomyces pombe Snf1 kinase heterotrimeric core.

Analytical ultracentrifugation of the purified, full-length protein revealed that recombinant human AMPK at 0.5 mg/ml exists as a monomer in solution, with a 1:1:1:1 y subunit ratio. This is consistent with the findings of Xiao et al. (12) who used multi-angle light scattering to show that the AMPK complex possesses a 1:1:1:1 stoichiometry and is monomeric in solution.

We consider that the early elution of AMPK during gel filtration chromatography may result in part to the elliptical structure of the γ subunit which possesses dimensions of ~60 × 60 × 30 Å (12).

Wong and Lodish (19) used deletion analysis and co-immunoprecipitation to provide evidence that the β and γ subunits do not interact directly and that the α subunit provides the “scaffold” requirements of the complex. These conclusions were inconsistent with our previous evidence and findings from crystallographic studies (12). Accordingly, we expressed the β and γ isoforms in insect cells. In this study we used immobilized metal affinity chromatography and gel filtration chromatography to purify recombinant human His-β1(186–270)/γ1 as a complex. Because this complex is stable in the absence of contaminating endogenous subunits, we consider that this provides further compelling evidence for a direct βγ interaction in mammalian AMPK. The complex eluted slightly earlier than expected from an S200 gel filtration column, which we again attribute to the elliptical structure of the γ subunit. In the Wong and Lodish (19) study high levels of detergent (1% Nonidet P-40, 0.5% deoxycholate and 0.1% SDS) were employed in cell
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![Diagram of AMPK subunit interactions](image)

侧链的β1-Tyr-267和γ1-Phe-51（人类序列编号）参与在一个T形π-堆积的相互作用，穿过βγ接口，可能稳定化了βγ相互作用的过程（图7b）。两个芳香环的距离被测量为5.4 Å，提供了一个约1.8 kcal/mol的键能，这几乎是最小的值，因为这种类型的相互作用（35）。γ1-Phe-51贡献了一个侧链的疏水口袋形成由γ1残基Val-49, Leu-55, Ala-60, and Ala-63，这可能会导致在附加稳定化作用中β1-Tyr-267。这个疏水口袋在酵母同源物中被发现（33, 36），尽管与Leu替换α1-Phe-51在Saccharomyces cerevisiae序列（Fig. 6b），这表明我们的发现与哺乳动物AMPK也适用于这些系统。

γ1-Lys-47在更高的组织中支持β1-Thr-263。不过，不太可能的是β1-T263A的突变对β1和Thr-263的相互作用有主要的扰动，因为其他大多数的突变对没有对亚单位的相互作用（Fig. 5a）。在结论中，这表明在加入一个网络的氢键之间β1的β1-T263和γ1(45–52)，至少一个的相互作用，描述了上述突变对稳定化β1γ1相互作用和他们这种破坏使β1γ1解离。

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