A Revised Model for AMP-activated Protein Kinase Structure

The α-subunit binds to both the β- and γ-subunits although there is no direct binding between the β- and γ-subunits

Received for publication, August 4, 2006, and in revised form, September 13, 2006. Published, JBC Papers in Press, September 29, 2006, DOI 10.1074/jbc.M607410200

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The 5′-AMP-activated protein kinase (AMPK) is a master sensor for cellular metabolic energy state. It is activated by a high AMP/ATP ratio and leads to metabolic changes that conserve energy and utilize alternative cellular fuel sources. The kinase is composed of a heterotrimeric protein complex containing a catalytic α-subunit, an AMP-binding γ-subunit, and a scaffolding β-subunit thought to bind directly both the α- and γ-subunits. Here, we use coimmunoprecipitation of proteins in transiently transfected cells to show that the α2-subunit binds directly not only to the β-subunit, confirming previous work, but also to the γ1-subunit. Deletion analysis of the α2-subunit reveals that the C-terminal 386–552 residues are sufficient to bind to the β-subunit. The γ1-subunit binds directly to the α2-subunit at two interaction sites, one within the catalytic domain consisting of α2 amino acids 1–312 and a second within residues 386–552. Binding of the α2 and the γ1-subunits was not affected by 400 μM AMP or ATP. Furthermore, we show that the β-subunit C terminus is essential for binding to the α2-subunit but, in contrast to previous work, the β-subunit does not bind directly to the γ1-subunit. Taken together, this study presents a new model for AMPK heterotrimer structure where through its C terminus the β-subunit binds to the α-subunit that, in turn, binds to the γ-subunit. There is no direct interaction between the β- and γ-subunits.

The 5′-AMP-activated protein kinase (AMPK) is a master metabolic regulator; it is activated by high intracellular AMP/ATP ratios to respond to diminishing cellular energy (1, 2) and by the adipokines leptin (3) and adiponectin (4). AMPK activation because of metabolic stress leads to serine/threonine phosphorylation of multiple targets reducing energy consuming processes, and increasing energy generating pathways, such as fatty acid oxidation (5). Furthermore, metformin, a drug in current clinical use to treat type II diabetes promotes glucose uptake at least in part through activation of AMPK (6, 7); how this activation occurs is still unclear. AMPK is reported to exist as a heterotrimer consisting of an α-catalytic subunit and two non-catalytic β- and γ-subunits. In mammals there are two α-subunits, three γ-subunits, and two β-subunits (α1, α2, γ1, γ2, γ3, β1, and β2). The γ-subunits each contain four tandem cystathionine β-synthase (CBS) domains that together bind two molecules of AMP and allow the kinase complex to sense adenine nucleotide levels. The β-subunits have been reported to serve a scaffolding function for the α- and γ-subunits as well as target the complex to intracellular sites such as the cell membrane through myristoylation of its N terminus (8, 9), and to intracellular glycogen through a glycogen binding domain (10). Binding of AMP to AMPK leads to phosphorylation of the α-subunit at Thr-172 by a family of upstream kinases, including LKB1 (11, 12) and increases the activity of AMPK by 50–100-fold (13, 14).

AMPK is functionally related to the yeast Snf1 (sucrose non-fermenting 1) protein kinase complex which plays a major role in regulating the nutritional stress response in Saccharomyces cerevisiae (2, 15). The Snf1 complex is also composed of three subunits; Snf1 (α-subunit), Snf4 (γ-subunit), and one of Sip1, Sip2, or Gal83 (β-subunits), which share limited primary amino acid sequence homology with their mammalian counterparts. Two regions in the yeast β-subunit homologs, the KIS (kinase interacting sequence) and ASC (association with Snf1 complex) domains interact with the α- and γ-subunits, respectively, in a yeast two-hybrid assay (16) implicating them as potential scaffolds. Initial characterization of the rat AMPK-β-subunit showed that, when synthesized in rabbit reticulocyte lysates, it bound individually to both the α- and γ-subunits, supporting a model where the β-subunit acts as a scaffold for the α- and γ-subunits (17).

The KIS and ASC domains present in the three yeast β-subunits (Sip1/Sip2/Gal83) were applied to the mammalian AMPK-β-subunits by sequence homology, and thus similar functional conservation of binding the α- and γ-subunits was inferred. This model was challenged recently when a β1-subunit mutant missing the KIS region was still able to co-immunoprecipitate with the α1-subunit (18) and with the identification of a glycogen binding domain overlapping the KIS domain (10). It was subsequently shown that the C-terminal 186–270 residues of AMPK-β1 were sufficient to tether the α- and γ-subunits (19). Another layer of complexity was uncovered by elucidation of the crystal structure of the yeast Snf1 catalytic domain. The catalytic domain of Snf1 was found to form dimers...
in solution and inhibit kinase activity (20). Furthermore, FLAG and HA epitope-tagged versions of full length Snf1 co-immunoprecipitated together and many residues that mediate the dimer interaction are conserved across species. These data suggest that AMPK may exist in a stoichiometry other than 1:1:1 (α:β:γ) with additional subunit interactions potentially involved in activation or repression of the kinase complex. These recent discoveries involving AMPK subunit interactions, particularly the role of the β-subunits, has led us to undertake a detailed examination of the subunit relationships of the AMPK heterotrimer. Here, we show that the full length AMPK-β-subunit does not bind directly to the γ1-subunit. Furthermore, we identify two interactions between the α- and γ-subunits, one of which is independent of the β-subunit. This study provides evidence for a new model of AMPK heterotrimer structure where the β-subunit binds to the α-subunit which, in turn, binds the γ-subunit but with no direct interaction between the β- and γ-subunits (Fig. 1).

**Structure of the AMPK Heterotrimer**

References: 16, 17, 21

**Current Study**

**TABLE 1**

| Oligonucleotide primers used for cloning of AMPK expression vectors |
|----------------------------------------------------------|
| Mammalian expression plasmids used in this study are listed in the first column. Oligonucleotide primers used to amplify specific cDNAs are listed in the second column, forward (Fwd) and reverse (Rev). All primers are displayed in the 5′ to 3′ orientation. DNA coding for the Myc and HA tags are in bold. Restriction endonuclease sites incorporated on the primers are underlined and listed in the third column. Species from which cDNA was derived is listed in the fourth column. |

**EXPERIMENTAL PROCEDURES**

**Materials**—HEK-293T cells were purchased from American Type Culture Collection. Anti-Myc tag 9B11 mouse monoclonal and Anti-AMPK-α were purchased from Cell Signaling Technology (Danvers, MA). Anti-FLAG M2 mouse monoclonal purchased from Sigma-Aldrich. Anti-HA rat monoclonal antibody purchased from Roche Applied Science (Indianapolis, IN). Anti-AMPK-β rabbit antibody was purchased from Upstate Cell Signaling Solutions (Charlottesville, VA).

**Expression Constructs**—Oligonucleotide primers used to amplify cDNAs for the AMPK expression constructs are detailed in Table 1. The coding sequence for mouse AMPK-β2 was amplified from total RNA extracted from mouse heart using an RNeasy kit (Qiagen, Valencia, CA) followed by a One-Step RT-PCR (Invitrogen, Carlsbad, CA) reaction and cloning into pCR4Blunt-Topo vector (Invitrogen). The cDNA was then cloned in-frame into a C-terminal FLAG expression vector, pCMV-Tag4A (Stratagene). Construction of the other AMPK-β expression plasmids used the full-length β2 cDNA as PCR template with primers listed in Table 1 and blunt-cloned into pCR4Blunt-Topo followed by subcloning into pCMV-Tag4A (C-term FLAG), pCMV-Tag5A (C-term Myc), or pCMV-Tag2A (N-term FLAG) with restriction sites in the primers (Table 1). The Myc-AMPK-α2 constructs were amplified from the rat cDNA for AMPK-α2 (14) with primers from Table 1 and were contributed by Dr. Tsu-Shuen Tsao. For the AMPK-γ1-HA construct, the HA tag was incorporated on the reverse primer and the cDNA amplified from a mouse E10.5 cDNA library. The PCR product was blunt end cloned into pCR4Blunt-Topo, followed by cloning into the pCMV-Tag4A expression construct. A stop codon was incorporated prior to the FLAG sequence in pCMV-Tag4A. GFP was expressed from pEGFP-N1 (Clontech, Mountain View, CA).

**Western Blotting**—Cells were collected and disrupted in lysis buffer (1× phosphate-buffered saline, 1% Nonidet P-40, 0.5%...
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sodium deoxycholate, 0.1% SDS, and complete protease inhibitor mixture (Roche Applied Science). Protein concentrations of lysates were determined using DC Protein Assay (Bio-Rad). 40 μg of total protein lysate was electrophoresed on 4–12% NuPage BisTris gradient gels (Invitrogen) followed by semi-dry transfer to nitrocellulose. Membranes were blocked in 5% non-fat dry milk in 50 mM Tris-Cl, 150 mM NaCl, 0.1% Tween 20 (TBST) overnight before incubation with primary antibodies for 1–3 h. Membranes were washed three times in TBST for 10 min each followed by incubation with anti-mouse, anti-rabbit, or anti-rat horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences). The membranes were washed three times in TBST, developed with Western Lightning Reagent Chemiluminescent detection (PerkinElmer Life Sciences, Boston, MA) and exposed to x-ray film.

Immunoprecipitations—Five hundred micrograms of total protein lysate was brought to a volume of 1 ml in lysis buffer and incubated with FLAG or Myc antibodies at 1:1000 at 4 °C on a rotator overnight. 50 μl of a 1:1 slurry of protein A/G-conjugated beads (Pierce) was added to the immunoprecipitation reactions and rotated for an additional 2 h. Immunoprecipitates were pelleted at 2500 rpm and washed three times with 0.5 ml of lysis buffer. Samples were boiled in LDS sample buffer for 4 min and centrifuged at 14,000 rpm for 4 min to pellet the beads. Supernatants were Western-blotted as described above.

Cell Culture and Transfection—HEK-293T cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, l-glutamine, and penicillin/streptomycin in a 37 °C incubator at 5% CO2. For transfections, cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, L-glutamine, and penicillin/streptomycin. Supernatants were Western-blotted as described above.

RESULTS

AMPK-β Binds Directly to AMPK-α2 but Not To AMPK-γ1—To examine the binding properties of the mammalian AMPK-β-subunit, expression constructs were created and tagged with a FLAG epitope at the C terminus to avoid disrupting the N-terminal myristoylation site. Additionally, a mutant AMPK-β2 construct was created that comprised only the first 203 residues, effectively deleting the ASC domain (residues 204–271). These AMPK-β2 constructs were used to test for binding to the Myc-AMPK-α2 subunit by co-expression in 293T cells followed by immunoprecipitation of β2 with anti-FLAG antibody and Western blotting for the AMPK-α2 subunit using the anti-Myc antibody. AMPK-α2 subunit bound to full-length AMPK-β2 (Fig. 2A, top blot, lane 4) but not to AMPK-β2-(1–203) (Fig. 2A, top blot, lane 5). As expected, the Myc-α2 band was not present in lysate from cells expressing GFP (Fig. 2A, top blot, lane 6). Western blotting for FLAG indicated that immunoprecipitation with FLAG antibody was successful (Fig. 2A, bottom blot, lanes 4 and 5). The same AMPK-β2 constructs were used to test for direct binding of the β2-subunit to the AMPK-γ1-subunit in a similar co-IP assay. In contrast to the AMPK-α2 subunit, the AMPK-γ1-HA subunit did not bind to full-length AMPK-β2-FLAG protein (Fig. 2B, top blot, lane 4), and as anticipated, γ1-HA did not bind to the β2-(1–203) mutant deleted for the ASC domain (Fig. 2B, top blot, lane 5). AMPK-β2-FLAG and β2-(1–203)-FLAG proteins were both present in the immunoprecipitate (Fig. 2B, bottom blot, lanes 4 and 5) indicating that these results were not caused by unsuccessful AMPK-β2 immunoprecipitation. To examine the interaction of the β-subunit with the α- and γ-subunits when expressed together, a triple transfection in 293T cells was performed using either full-length or ASC deleted β2-FLAG subunits together with full-length Myc-α2 and γ1-HA. Following FLAG-IP against the tagged β-subunits, the presence of the Myc-α2 and γ1-HA subunits in the immunoprecipitate was assessed by Western blot. Consistent with the results in Fig. 2A, the α2-subunit was communoprecipitated with the β2-FLAG subunit (Fig. 2C, top blot, lane 4). Importantly, the γ1-subunit was also present in the β-subunit IP (Fig. 2C, middle blot, lane 4) in contrast to the result seen when expressing the β2- and γ1-subunits alone. The β2-(1–203) deletion mutant did not bind either the α2- or γ1-subunit, consistent with previous results (Fig. 2C, lane 5). Similar results were obtained when AMPK-β1-FLAG and β1-(1–203)-FLAG subunits were used (data not shown). These data indicate that the AMPK-β2 subunit does not bind directly to the γ1-subunit. Fig. 2C suggests that the β2-subunit does bind to the α2-subunit which, in turn, binds directly to the γ1-subunit; below we show direct binding of α2- to γ1-subunit (Fig. 4).

To ensure that placement of the FLAG epitope at the β2 C terminus did not cause disruption of the γ1-subunit binding site, an N-terminal FLAG tagged β2-subunit expression construct was created (Table 1 and Fig. 3) and used in a similar co-immunoprecipitation assay. Furthermore, a FLAG-β2 (71–271) expression construct was also generated (Table 1) to express β-subunit protein similar to one used in an earlier AMPK subunit interaction study that found that the β-subunit bound to both α- and γ-subunits (17). Expression vectors for the full-length β2-subunit with either an N- or C-terminal FLAG tag and the deletion mutants FLAG-β2 (71–271) or β2-(1–203)-FLAG were transfected in 293T cells along with the γ1-HA vector together with, or without, the Myc-α2 expression vector (Fig. 3, left). Lysates prepared from these transfected cells were Western-blotted with anti-FLAG, Myc, and HA antibodies (Fig. 3A, top, middle, bottom, respectively), showing that all of the AMPK subunits were expressed. Immunoprecipitation with FLAG antibody was performed on these lysates and blotted with antibodies against Myc for the Myc-α2 subunit, HA for the γ1-HA subunit, and AMPK-α, for endogenous AMPK-α (Fig. 3B). In the absence of the Myc-α2 subunit, little γ1-HA co-immunoprecipitated with the β-subunit; whereas upon overexpression of the Myc-α2 subunit, a much stronger γ1-HA (~100-fold) co-immunoprecipitated with the β-subunit (Fig. 3B, lanes 1 and 2). This result was comparable to that observed for C-terminal FLAG-tagged AMPK-β2 (Fig. 3B, lanes 3 and 4). The FLAG-β2 (71–271) N-terminal deletion did not co-immunoprecipitate the γ1-HA subunit unless Myc-α2 subunit was co-expressed (Fig. 3B, lanes 5 and 6). Consistent with the results in Fig. 2, the ASC domain deleted β2-(1–203) did not co-immunoprecipitate either the α2- or γ1-subunits (Fig. 3B, lanes 7 and 8). A very small amount of AMPK-γ1-HA
AMPKα binds to both the β- and γ-subunits

AMPKα binds to both the β- and γ-subunits—To determine if AMPKα could bind directly to AMPKγ1, a series of AMPKα-δ2 deletion mutants (Table 1) was created and used in co-immunoprecipitation experiments with the γ1-HA subunit. Expression constructs for full-length and AMPKα-δ2 mutants containing only the catalytic domain (1–312), the catalytic plus the auto-inhibitory domain (1–398), the auto-inhibitory plus the β/γ binding domain (303–552), and the β/γ binding domain alone (386–552) all contained a Myc epitope tag on their N termini as depicted in Fig. 4. We co-expressed the series of Myc-α2 mutants with the γ1-HA subunit (Fig. 4, top) and performed co-immunoprecipitation with anti-Myc antibody. In this experiment, the β-subunit was not overexpressed. A series of negative controls for the co-immunoprecipitation was performed including expression of the γ1-HA subunit alone, β2-(1–203)-Myc with the γ1-HA subunit, full-length Myc-α2 and γ1-HA without anti-Myc antibody in the immunoprecipitation reaction, and GFP alone (Fig. 4, top). Western blotting of the input lysates with anti-Myc (Fig. 4, top blot), anti-HA (Fig. 4, middle blot), and anti-AMPK-β (Fig. 4, bottom blot) antibodies shows that all of the expected AMPK subunits were expressed. These lysates were subsequently used in immunoprecipitation reactions with anti-Myc antibody and blotted with anti-HA antibody for the presence of the γ1-HA subunit. Full-length AMPKα-δ2 was able to bind to the γ1-HA subunit (Fig. 4B, middle blot, lane 1). The C-terminal deletions of AMPKα-δ2 (1–312) and –(1–398) both bound the γ1-HA subunit (Fig. 4B, middle blot, lanes 2 and 3), as did the N-terminal deletions 303–552 and 386–552 (Fig. 4B, middle blot, lanes 4 and 5). Western blotting (Input, lanes 1–3) or immunoprecipitated with anti-FLAG antibody and Western-blotted for α2 (Myc), β2 (FLAG), and γ1-HA as indicated (lanes 4–6), β2-Flag and β2-(1–203)-FLAG immunoprecipitations were successful in all reactions as shown by Western blots using anti-FLAG antibody against the immunoprecipitates (A, B, and C; bottom panels, lanes 4 and 5). Asterisk indicates background immunoglobulin present in the IP. GFP was transfected as a negative control (A, B, and C, lanes 3 and 6). A, β2-Flag or β2-(1–203)-FLAG co-transfected with the Myc-α2 construct. Lanes 1–3, 40 μg of input lysate; lanes 4–6, FLAG IP then Western blot for Myc-α2 (top panel) and β2-FLAG (bottom panel). Arrow indicates the Myc-α2 polypeptide in the IP with full-length β2-FLAG (lane 4); note that this band is not present in the IP of β2-(1–203)-FLAG (lane 5). B, β2-FLAG or β2-(1–203)-FLAG co-transfected with the γ1-HA construct. Lanes 1–3, 40 μg of input lysate; lanes 4–6, FLAG IP then Western blot for γ1-HA (top panel) and β2-FLAG (bottom panel). Arrow indicates the absence of the γ1-HA polypeptide in the full-length β2-FLAG IP (lane 4), C, β2-FLAG or β2-(1–203)-FLAG co-transfected with the Myc-α2 and γ1-HA constructs. Lanes 1–3, 40 μg of input lysate; lanes 4–6, FLAG IP then Western blot for Myc-α2 (top panel), and β2-FLAG (bottom panel). Arrow indicates the γ1-HA polypeptide in the IP of full-length β2-FLAG (lane 4, middle panel) but when all three subunits are co-expressed. This result is representative of at least three independent experiments.
AMPK-α Binds to Both the β- and γ-Subunits

| Subunit: | β | α | γ | A. Input |
|---------|---|---|---|---------|
| 1.      | 01 | 0  | 1 |  |
| 2.      | 02 | 1  | 1 |  |
| 3.      | 03 | 0  | 1 |  |
| 4.      | 04 | 0  | 0 |  |
| 5.      | 05 | 0  | 0 |  |
| 6.      | 06 | 0  | 0 |  |
| 7.      | 07 | 0  | 0 |  |
| 8.      | 08 | 0  | 0 |  |
| 9.      | 09 | 0  | 0 |  |

**FIGURE 3.** AMPK-γ1 does not bind directly to N- or C-terminal FLAG-tagged AMPK-β2. cDNAs encoding the full-length AMPK-β2 with either C- or N-terminal FLAG tag, or FLAG-β2-(71–271), or β2-(1–203)-FLAG, and γ1-HA, together or not with Myc-α2 were transfected in 293T cells. Samples 1–8 also express the γ1-HA subunit. Sample 9 overexpresses GFP alone. The position of the FLAG tag is indicated on the β-subunits. Lysates were immunoprecipitated with anti-FLAG antibody and Western-blotted for α2(Myc), α1(HA), and AMPK-α. A. Western blots of input lysates (40 μg of total protein) blotted anti-FLAG for β-subunits, top panel; anti-Myc for α2-subunit, middle panel; and anti-HA for γ1-subunit, bottom panel. B. Anti-FLAG immunoprecipitation of the lysates from a Western-blotted with anti-Myc antibody (4), top blot, anti-HA antibody, middle blot, and anti-AMPK-α antibody, bottom blot. Asterisks indicate the position of the background immunoglobulin polypeptide in all of the immunoprecipitation samples including sample 9, which overexpresses GFP alone. This result is representative of two independent experiments.

and 5). The γ1-HA subunit alone did not immunoprecipitate with anti-Myc antibody (Fig. 4B, middle blot, lane 6) nor did it IP with the ASC deleted β2-(1–203)-Myc subunit (Fig. 4B, middle blot, lane 7). Furthermore, γ1-HA was not present in a control immunoprecipitation using full-length AMPK-α2 and γ1-HA and omitting the anti-Myc antibody, demonstrating that these results are not due to nonspecific binding (Fig. 4B, middle blot, lane 8). This result indicates that there are at least two binding sites for γ1-HA on the AMPK-α2 subunit because the γ1-HA subunit bound to both α2-(1–312) and α2-(386–552). Although no exogenous AMPK-β-subunit was expressed, we determined if AMPK-β subunit was present in these immunoprecipitations by blotting with antibody against the endogenous AMPK-β subunit. This experiment showed that AMPK-β subunit is present only in the immunoprecipitations of α2 constructs that contain the C-terminal β/γ binding domain of AMPK-α2 (Fig. 4B, bottom blot, lanes 1, 4, and 5). Despite binding of the α2 (1–312) and α2 (1–398) mutants to the γ1-HA subunit, no endogenous AMPK-β is detected in these immunoprecipitates (Fig. 4B, bottom blot, lanes 2 and 3). Taken together, these results indicate that AMPK-α2 can bind to the AMPK-γ1 subunit through at least two sites (1–312 and 386–552) and that at least the binding at the N-terminal site is independent of the β-subunit.

**The C-terminal 167 Residues of AMPK-α2 Are Sufficient to Bind AMPK-β2 and AMPK-γ1**—To map the domain of AMPK-α2 that is able to bind to both the β-subunit and the γ1-subunit, we co-expressed the series of Myc-α2 mutants with the β2-FLAG and γ1-HA subunits. Protein lysates from 293T cells transfected with expression constructs for the series of α2-subunits, β2-FLAG, and γ1-HA displayed high levels of exogenous protein as measured by Western blotting using anti-Myc, FLAG, and HA antibodies (Fig. 5A). These lysates were subsequently used in FLAG immunoprecipitation reactions for the β2-subunit and blotted for the presence of the α2- and γ1-subunits. Consistent with earlier results, in the absence of α2-subunit expression, γ1-HA did not co-immunoprecipitate with the β2-FLAG subunit (Fig. 5B, bottom blot, lane 1), while full-length AMPK-α2 did bind β2-FLAG (Fig. 5B, lane 2). In contrast to full-length α2, both the catalytic domain of AMPK-α2-(1–312) and the catalytic plus the auto-inhibitory domain of AMPK-α2-(1–398) did not bind the β2-subunit (Fig. 5B, top blot, lanes 3 and 4). Therefore, no γ1-HA was present in the β2-immunoprecipitate (Fig. 5B, bottom blot, lanes 3 and 4). The two C-terminal AMPK-α2 fragments, 303–552 and 386–552, both bind the β2-FLAG subunit (Fig. 5B, top blot, lanes 5 and 6) and to the γ1-subunit (Fig. 5B, bottom blot, lanes 5 and 6). Taken together, these data indicate that amino acid residues 386–552 of AMPK-α2-subunit are sufficient to bind to both the β2- and the γ1-subunits.
AMPK-α Binds to Both the β- and γ-Subunits

γ1-HA subunits were measured by Western blotting with anti-Myc and anti-HA antibodies (Fig. 6B, top and middle, lanes 1–9). Co-expression of the β2-(1–203)-Myc and the γ1-HA subunits was again used as a negative control for binding (Fig. 6B, lane 10). Immunoprecipitation with anti-Myc antibody followed by blotting with anti-HA antibody showed that the γ1-HA also bound to the Myc-α2-(386–552) mutant under all conditions tested (Fig. 6B, bottom, lanes 1–9) but did not bind the β2-(1–203)-Myc subunit. These results indicate that binding of γ1-HA to either the catalytic domain of AMPK-α2-(1–312), or the β/γ binding domain of AMPK-α2-(386–552), is not affected by a concentration of 400 μM AMP or ATP.

**DISCUSSION**

These experimental data present three major points that cannot be explained by the existing model for assembly of mammalian AMPK (Fig. 1, left). First, we show that AMPK-α2 does not bind to the KIS domain of AMPK-β, but instead to the C-terminal ASC domain of AMPK-β. The full-length β2-subunit binds to the α2-subunit, but the β2-(1–203) mutant, which lacks only the ASC domain, does not (Fig. 2). This result indicates that the C-terminal ASC domain of the β-subunit is critical for binding the AMPK-α2-subunit, and is consistent with results that show that the isolated C terminus of the β-subunit is sufficient to bind to the α1-subunit (18, 19). It is therefore unlikely that our lack of α-subunit binding to the β2-(1–203) mutant are caused by a general disruption of the β-subunit structure. Our result supports the abandonment of the KIS nomenclature as proposed in the study by Hudson et al. (18). Second, there are at least two binding sites on the α2-subunit for the γ1-subunit. Utilizing a series of AMPK-α2 deletion mutants with the γ1-subunit in co-immunoprecipitation experiments, distinct binding sites for the γ1-subunit were mapped to residues 1–312 and 386–552 of AMPK-α2 (Fig. 4). Third, the AMPK-β-subunit does not bind directly to the AMPK-γ1-subunit despite its reported role as a scaffold to bind directly both the α- and γ-subunits. This negative result is based on immunoprecipitation of the β-subunit followed by Western blotting for the γ1-subunit. Only upon co-expression of the α2-subunit, are all three subunits bound together in the immunoprecipitate (Fig. 2). Through deletion analysis of the α2-subunit, we showed that residues 386–552 of the α2-subunit were sufficient to bind to both the β- and γ-subunits (Fig. 5). These findings demonstrate that the catalytic α-subunit, not the β-subunit, binds both of the non-catalytic AMPK subunits. The results of these experiments led us to propose a new model for AMPK where the β-subunit binds to the α-subunit, which in turn binds the γ-subunit (Fig. 1, right).

In the existing model for AMPK heterotrimer structure, the β-subunit plays a central role in bringing the α- and γ-subunits together. With this in mind, we designed β-subunit expression constructs to control for potential artifactual effects of the epitope tags. We originally cloned a C-terminal FLAG epitope tag on the β-subunits because a previous report indicated that modification of the N terminus leads to a loss of myristoylation on glycine at position two (8). To ensure that the β-subunit C-terminal FLAG epitope tag itself did not disrupt binding to the γ-subunit, we performed similar experiments with an

| Subunit: | α | γ |
|---------|---|---|
| 1. Myc- | Catalytic | Al | β/γ |
| 2. Myc- | Catalytic | (1-312) | + |
| 3. Myc- | Catalytic | (1-398) | + |
| 4. (303-552) Myc | Al | β/γ |
| 5. (386-552) Myc | Al | β/γ |

**Negative Control:**

| | γ |
|---|---|
| 6. No other AMPK Subunit | + |
| 7. β2-KIS-Myc | + |
| 8. Myc | Catalytic | Al | β/γ |
| 9. GFP | | |

**A. Input**

![Image](attachment:image1)

**B. IP: Myc-α2**

![Image](attachment:image2)

**FIGURE 4.** AMPK-α2 can bind directly to the γ1-subunit. Co-immunoprecipitations of the AMPK-α2 subunit with the γ1-subunit were performed. CDAs encoding full-length AMPK-α2-(1–552), α2-(1–312), α2-(1–398), α2-(303–552), and α2-(386–552) were co-transfected with the γ1-subunit in 293T cells as indicated (top, lanes 1–5). Negative controls for the immunoprecipitations include expression of γ1-HA subunit alone (sample 8), β2-(1–203)-Myc and γ1-HA (sample 7), full-length Myc-α2 and γ1-HA without the anti-Myc IP antibody (sample 8), GFP alone (sample 9). A, Western blots of input lysates blotted with anti-Myc antibody for the α2-subunits (top blot), anti-HA antibody for the γ1-subunit (middle blot), and anti-AMPK-β for the endogenous AMPK-β protein (bottom blot). B, anti-Myc immunoprecipitations of lysates from A, blotted with anti-Myc antibody for the α2-subunits (top blot), anti-HA antibody for the γ1-subunit (middle blot), and anti-AMPK-β antibody for the endogenous AMPK-β protein. This result is representative of two independent experiments.
AMPK-α Binds to Both the β- and γ-Subunits

| Subunit:          | α | β | γ |
|-------------------|---|---|---|
| 1. No α2          | + | + | + |
| 2. Myc-Catalytic   | + | β/γ | + |
| 3. Myc-Catalytic(1-312) | + | + |
| 4. Myc-Catalytic(1-398) | + | + |
| 5. (303-552) Myc- | + | β/γ | + |
| 6. (386-552) Myc- | + | + |
| 7. No α2          | - | - | - |

A. Input

1 2 3 4 5 6 7

Myc-α2

β2-Flag

γ1-HA

Nucleotide: [MgCl2]:

AMP ATP None

B. IP: β-Flag

1 2 3 4 5 6 7

Myc-α2

β2-Flag

γ1-HA

Nucleotide: [MgCl2]:

AMP ATP None

N-terminal FLAG-tagged β2-subunit, despite concerns about altering myristoylation. Both N-terminal and C-terminal FLAG-tagged β2-subunits bound the α2- but not the γ1-subunit, indicating that the effects we observed were not caused by modification of the β-subunit C terminus (Fig. 3). Furthermore, an N-terminal 70 amino acid-deleted AMPK-β2(71–271) subunit still bound to the α2-subunit, and not the γ1-subunit. This particular deletion mutant was chosen based on a previous study performed by Woods et al. (17) to examine subunit interactions. In that study radiolabeled AMPK subunits were prepared from rabbit reticulocytes and immunoprecipitated with one another. They used a 70-amino acid N-terminal-deleted the α2-subunits (top blot), anti-FLAG antibody for the β2-subunit (middle blot), and anti-HA antibody for the γ1-subunit (bottom blot). Asterisks indicate the position of the two background immunoglobulin bands present in all of the immunoprecipitations including sample 7, which overexpresses GFP alone. This result is representative of two independent experiments.
AMPK-α Binds to Both the β- and γ-Subunits

β-subunit to distinguish it from the AMPK-γ1-subunit because they are both 38 kDa in size and found that the β-subunit bound to the α- and γ-subunits individually and together in a heterotrimer. Our results do not support binding of the β-subunit to the γ1-subunit independent of the α-subunit even when employing a similar β2-(71–271) mutant protein. The discrepancy observed may be caused by other differences between the programmed rabbit reticulocyte system versus the 293T overexpression system used in this study. The β2-FLAG immunoprecipitations in Fig. 3 show a very small amount of γ1-HA co-immunoprecipitating with the β2-subunit; however, re-blotting with antibody against AMPK-α clearly shows that endogenous AMPK-α is also present in the immunoprecipitate (Fig. 3B, bottom blot). Our interpretation of this result is that the small amount of γ1-HA seen in the immunoprecipitate is caused by binding to the endogenous AMPK-α expressed in 293T cells, and its subsequent interaction with the AMPK-β2-FLAG subunit. While we do not observe direct binding of the β-subunit to the γ1-subunit, we have not tested whether or not the γ2- or γ3-subunits can directly bind the β-subunit.

Utilizing a series of AMPK-α2 deletion mutants we determined that the C-terminal 167 residues of AMPK-α2 are sufficient to bind to both AMPK-β2 and AMPK-γ1. This region was previously identified to be critical for AMPK-α1 interaction with the β-subunit (21). However, in contrast to the report by Dyck et al., our finding is that the AMPK-β and γ-subunits do not interact with one another unless AMPK-α is present, specifically the C terminus of AMPK-α to act as a bridge between the β- and γ-subunits. The differences between these results may be due to the level of overexpression and/or the difference in cell type used for the subunit interaction experiments (293T versus COS7). Furthermore, our experiments show that the catalytic domain of AMPK-α2, and thus catalytic activity, is not required for forming a complex with the β- and γ-subunits, similar to conclusions made by Dyck et al. (21) using a kinase inactive AMPK-α1 mutant.

A significant finding of our work is that the α2- and γ1-subunits are able to interact independently of the β-subunit, and that there are at least two interaction sites on the α-subunit for the γ1-subunit. The catalytic domain of AMPK-α2 (residues 1–312) and the C-terminal 167 residues of AMPK-α2-(386–552) were both able to bind to AMPK-γ1. An important distinction between these two reactions is that the AMPK-β subunit did not bind to the α2-(1–312) catalytic domain but did bind to the C-terminal α2-(386–552) subunit (Fig. 4). This clearly demonstrates that AMPK-β is not required to join the α- and γ-subunits together as the current AMPK assembly model suggests. At this point, we do not know if the two γ-subunit binding sites on full-length AMPK-α are mutually exclusive. If they are not, then it is possible that AMPK contains more than one of each subunit rather than existing as a heterotrimer with 1:1 stoichiometry. This hypothesis is further supported by a recent report detailing the ability of the yeast Snf1 catalytic domain to form homodimers (20).

Because the γ1-subunit binds AMP and coordinates energy sensing, we sought to determine if the interactions we observed between the α- and γ-subunits are modulated by either 400 μM AMP or 400 μM ATP, a concentration of nucleotide expected to be saturating for binding to the enzyme (22). Our experiments showed that at this concentration, AMP and ATP did not affect the interaction of either the catalytic (1–312) or C-terminal (386–552) α2-domains to the γ1-HA subunit suggesting that their binding is not dependent on AMPK activation (Fig. 6). Furthermore, the α2-(386–552) C-terminal deletion does not contain the Thr-172 residue, critical for activation via phosphorylation by the upstream kinase LKB1, yet this construct still binds to the γ1-subunit. This demonstrates that this particular interaction is independent of the activation state of AMPK.

Understanding how mammalian AMPK is assembled is of great importance given its role as a master metabolic regulator. The identification of a family of upstream kinases able to phosphorylate and activate AMPK along with a newly discovered link to calcium dependent signaling (23–25) increases the complexity of AMPK regulation and activity. An accurate picture of how AMPK is assembled is essential to understanding its many functions. Based on the results presented here, we propose a new model for AMPK assembly where the β-subunit does not function as a “scaffold” but interacts with the α-subunit which in turn binds to the γ-subunit (Fig. 1). The α-subunits have a clear function as the catalytic subunit of AMPK, and the γ-subunits have an apparent role in sensing and binding AMP through their four CBS domains (22). Our new β–α–γ assembly model clearly introduces a new question, principally what is the function(s) of the β-subunit? One potential answer might be that instead of acting as a scaffold, the β-subunit targets the kinase to subcellular locations. For example, myristoylation of the β-subunit aids in localizing AMPK to cellular membranes (8, 9), and a glycogen binding domain localizes it to a site of a major energy store (10). The physiological importance of these properties and how they are regulated is currently unclear and awaits analysis in vivo with animal models. While AMPK has been an attractive drug target for many years, metformin has already been shown to mediate some of its beneficial effects through AMPK. Exactly how metformin activates AMPK is still unclear. An accurate AMPK model is critical for rationale design of therapeutics that can modulate AMPK function, and highlights the importance of our findings here.

Acknowledgments—We thank Dr. Tsu-Shuen Tsao and Sara Zarnegar for providing the α2 deletion expression vectors, and members of the laboratory for insightful discussions and critical reading of the manuscript.

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