A Conserved Sequence Immediately N-terminal to the Bateman Domains in AMP-activated Protein Kinase γ Subunits Is Required for the Interaction with the β Subunits*

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Mammalian AMP-activated protein kinase is a serine/threonine protein kinase that acts as a sensor of cellular energy status. AMP-activated protein kinase is a heterotrimer of three different subunits, i.e. α, β, and γ, with α being the catalytic subunit and β and γ having regulatory roles. Although several studies have defined different domains in α and β involved in the interaction with the other subunits of the complex, little is known about the regions of the γ subunits involved in these interactions. To study this, we have made sequential deletions from the N termini of the γ subunit isoforms and studied the interactions with α and β subunits, both by two-hybrid analysis and by co-immunoprecipitation. Our results suggest that a conserved region of 20–25 amino acids in γ1, γ2, and γ3, immediately N-terminal to the Bateman domains, is required for the formation of a functional, active αβγ complex. This region is required for the interaction with the β subunits. The interaction between the α and γ subunits does not require this region and occurs instead within the Bateman domains of the γ subunit, although the α·γ interaction does appear to stabilize the β·γ interaction. In addition, sequential deletions from the C termini of the γ subunits indicate that deletion of any of the CBS (cystathionine β-synthase) motifs prevents the formation of a functional complex with the α and β subunits.

Mammalian AMP-activated protein kinase (AMPK) is a serine/threonine protein kinase that acts as a sensor of cellular energy status. It is activated by cellular stresses that deplete ATP, either by inhibiting ATP production (e.g. hypoxia, glucose deprivation, heat shock, and mitochondrial inhibitors) or by accelerating ATP consumption (e.g. muscle contraction). Depletion of ATP is always accompanied by increases in AMP due to the reaction catalyzed by adenylate kinase, and the increase in AMP:ATP ratio activates AMPK in an ultrasensitive manner. Once activated it switches on catabolic pathways and switches off many ATP-consuming processes, including anabolic pathways (see Refs. 2–5, for reviews). AMPK is a heterotrimer composed of three different subunits, i.e. α, β, and γ. The α subunit is the catalytic subunit; it contains a highly conserved kinase domain at the N terminus and a less well conserved C-terminal regulatory domain. Two isoforms have been described, i.e. α1 and α2; both are localized in the cytoplasm, although α2 is also present in the nucleus (6). The γ subunits contain four tandem repeats of a structural module called a CBS motif (7), named after the enzyme cystathionine β-synthase, in which a pair of CBS motifs form a domain that binds the allosteric activator S-adenosyl methionine (8). In the AMPK γ subunits the four CBS motifs are known to act in two pairs, forming two domains (referred to as Bateman domains) that bind the regulatory nucleotides, AMP and ATP, in a mutually exclusive manner (8). Three isoforms of the γ subunit, i.e. γ1, γ2, and γ3, are encoded by distinct genes; they have poorly conserved N-terminal regions that in γ2 and γ3 are subject to alternate splicing, whereas the C-terminal regions, containing the two tandem Bateman domains, are conserved in all three isoforms. Two isoforms of the β subunit (β1 and β2) have been described; they differ at their N termini, but both appear to interact with the α and γ subunits with similar efficiency (9, 10). The β subunits have two conserved regions, a central glycogen-binding domain (11–13) and a C-terminal domain that is the only region required for the formation of the complex with α and γ (11).

All three subunits are required to form a functional AMPK complex (14, 15). Recent studies have begun to delineate the regions of the α and β subunits required for the formation of heterotrimeric complexes, although there have been some con-
flicting findings. Detailed mapping of the C-terminal domain of β1 suggested that the last 25 residues are sufficient to form a complex with γ1, γ2, and γ3, with the C-terminal residue (Ile270) being essential for the formation of a β-γ complex in the absence of α (16). By contrast, it was recently reported that β2 and γ1 do not form a complex in the absence of α2 (17). The C-terminal domain of the α subunit has been reported to be involved in the interaction with β (16) and γ subunits (10), although another study has suggested that γ1 interacts with α2 via both the N-terminal catalytic domain and the C-terminal domain of the latter (17).

As yet, little is known about which portions of the γ subunits are involved in the interaction with the α and β subunits. To study this, we have made deletions of the regions of the γ subunits N-terminal to the Bateman domains. Our results show that a conserved sequence of 20–25 residues, immediately N-terminal to the first Bateman domain (the “pre-CBS1 sequence”), is required for the binding of γ1, γ2, and γ3 to the β subunits and for the formation of an active αβγ complex. In addition, we have made C-terminal deletions of the γ subunits and observed that deletion of any of the CBS motifs prevents the formation of functional complexes with the α and β subunits.

### MATERIALS AND METHODS

**Microorganisms, Culture Conditions, and Genetic Methods—**

*Escherichia coli* DH5α was used as the host strain for plasmid constructions. It was grown in LB (1% peptone, 0.5% yeast extract, 1% NaCl, pH 7.5) medium supplemented with 50 mg/liter ampicillin.

*Saccharomyces cerevisiae* CTY10-5d (MATa ade2 his3 leu2 trp1 gal4 gal80 URA3::lexAop-lacZ) was used in the two-hybrid experiments. When indicated, the yeast strains FY250 and FY250 ssp1Δ, containing the pSH18-18 reporter plasmid (18), were also used in two-hybrid experiments. Yeast transformation was carried out using the lithium acetate protocol (19). Yeast cultures were grown in synthetic complete (SC) medium supplemented with 50 mg/liter ampicillin.

**Oligonucleotides—**

Oligonucleotides used in this work are described in Table 1.

**Plasmids—**

Plasmid pEG-AMPKγ3 (expressing LexA-AMPKγ3 fusion protein) was obtained by subcloning an EcoRI/XhoI fragment from plasmid pcDNA3-AMPKγ3 (containing the human cDNA of AMPKγ3) into plasmid pEG202 (21) digested with EcoRI/XhoI. Progressive deletions of the AMPKγ3 open reading frame were obtained by PCR using the forward oligonucleotides Gamma3-A, -B, -C, -D, and -F and the reverse oligonucleotide Gamma3-Sall (Table 1). The amplified fragments were sequenced to check the absence of undesired modifications and then digested with EcoRI/Sall and subcloned into pEG202 to obtain plasmids pEG-AMPKγ3A, -B, -C, -D, -E, and -F, respectively.

To construct plasmids pEG-AMPKγ1 (LexA-AMPKγ1) and pACT2-AMPKγ3, a 1.9-kb ApaI/NotI fragment from plasmid pcDNA3-AMPKγ1 (containing the human cDNA of AMPKγ1), was blunt-ended with Klenow enzyme and ligated into the SmaI site of pEG202 and pACT2 (22), respectively. Plasmid pEG-AMPKγ1-NtΔ was obtained by amplifying the corresponding fragment by PCR using oligonucleotides Gamma1-CBS1 and Gamma1-2 (Table 1). The fragment was then digested with SmaI/XhoI and subcloned into pEG202, previously digested with SmaI/XhoI. To obtain plasmid pACT2-AMPKγ1-CBS1, a BglII fragment from pACT2-AMPKγ1 (containing the N-terminal domain and the first CBS motif) was blunted into pACT2 digested with BglII and treated with alkaline phosphatase. Plasmid pACT2-AMPKγ1-CBS12 was obtained by eliminating an EcoRI fragment from pACT2-AMPKγ1 and re-ligating. Plasmid pACT2-AMPKγ1-CBS123 was obtained by amplifying the corresponding fragment by PCR using oligonucleotides Gamma1-1 and Gamma1-2CBS123 (Table 1). The fragment was then digested with NotI, filled with Klenow enzyme, digested with XhoI, and then subcloned into pACT2 digested with SmaI and XhoI.

To construct plasmids pEG-AMPKγ2 (LexA-AMPKγ2), an EcoRI/XhoI fragment from plasmid pcDNA3-AMPKγ2 (containing the human cDNA of AMPKγ2) was subcloned into plasmid pEG202 (21) digested with EcoRI/XhoI. Plasmid pEG-AMPKγ2 short (LexA-AMPKγ2 short) was constructed in a similar way from plasmid pcDNA3-AMPKγ2 short (expressing γ2 from residue 242 up to 569). Plasmid pEG-AMPKγ2-NtΔ was obtained by amplifying the corresponding fragment by PCR using oligonucleotides Gamma2-CBS1 and Gamma2-2 (Table 1). The fragment was then digested with SmaI/XhoI and subcloned into pEG202, previously digested with SmaI/XhoI. To obtain yeast plasmids expressing different mutated forms of AMPKγ2, EcoRI/XhoI fragments from plasmids pcDNA3-AMPKγ2 R302Q, L-ins, H383R, and T400N (containing the human cDNA of the corresponding mutated forms) were subcloned into pEG202.

To express the "short" and "long" truncations of γ subunits for co-precipitation studies in mammalian cells, DNAs encoding residues 22–331 (γ1) or 47–331 (γ1Δ) of human γ1 (P54619), 253–569 (γ2) or 279–569 (γ2Δ) of human γ2 (Q9UGJ0), or 177–489 (γ3) or 202–489 (γ3Δ) of human γ3 (Q9UG19) were cloned by reverse transcription-PCR from human brain mRNA (γ1 and γ2) or by PCR from Image clone clone 40005880 (γ3), and subcloned using EcoRI and KpnI sites into pFLAG-CMV5a (Sigma). The expressed proteins have a single methionine before the residues listed and a FLAG tag at the C terminus.

Other plasmids used in this study were pACT2 (22), pACT2-AMPKα2 (GAD-AMPKα2), pACT2-AMPKβ2 (GAD-AMPKβ2),

### Table 1

**Oligonucleotides used in this study**

New restriction sites are underlined.

| Name       | Sequence                                                                 |
|------------|--------------------------------------------------------------------------|
| Gamma3-Sall | 5'-GCACTTCTGCACTGACCCCGAGGATCGATGC-3'                                |
| Gamma3-A   | 5'-GCACTTCTGCACTGACCCCGAGGATCGATGC-3'                                  |
| Gamma3-B   | 5'-GCACTTCTGCACTGACCCCGAGGATCGATGC-3'                                  |
| Gamma3-C   | 5'-GCACTTCTGCACTGACCCCGAGGATCGATGC-3'                                  |
| Gamma3-D   | 5'-GCACTTCTGCACTGACCCCGAGGATCGATGC-3'                                  |
| Gamma3-E   | 5'-GCACTTCTGCACTGACCCCGAGGATCGATGC-3'                                  |
| Gamma3-F   | 5'-GCACTTCTGCACTGACCCCGAGGATCGATGC-3'                                  |
| Gamma1-1   | 5'-GAAATTTGAGATCGACCCCGAGGATCGATGC-3'                                  |
| Gamma1-2   | 5'-GAAATTTGAGATCGACCCCGAGGATCGATGC-3'                                  |
| Gamma1-CBS1| 5'-GCACTTCTGCACTGACCCCGAGGATCGATGC-3'                                  |
| Gamma1-CBS12| GAAATTTGAGATCGACCCCGAGGATCGATGC-3'                                   |
| Gamma1-CBS123| GAAATTTGAGATCGACCCCGAGGATCGATGC-3'                                  |
| Gamma2-CBS1| 5'-GCACTTCTGCACTGACCCCGAGGATCGATGC-3'                                  |
| Gamma2-CBS2| 5'-GCACTTCTGCACTGACCCCGAGGATCGATGC-3'                                  |
| Gamma2-1   | 5'-GCACTTCTGCACTGACCCCGAGGATCGATGC-3'                                  |
| Gamma2-2   | 5'-GCACTTCTGCACTGACCCCGAGGATCGATGC-3'                                  |

**Table 1**

**Interaction between AMPK β and γ Subunits**
pBTH-AMPKα2 (LexA-AMPKα2), and pBTH-AMPKβ2 (LexA-AMPKβ2) (10).

**Antibodies**—The antibodies used in this study were as follows: sheep polyclonal anti-panβ (23), sheep polyclonal anti-α1 and anti-α2 (14), mouse monoclonal anti-Myc (Sigma), mouse monoclonal anti-FLAG (Sigma).

**β-Galactosidase Assays**—β-Galactosidase activity was measured in yeast-permeabilized cells and expressed in Miller units as described by Ludin and collaborators (24).

**Preparation of Yeast Cell Extracts and Immunoblot Analysis**—Yeast cells corresponding to 1 unit of A<sub>600</sub> were collected by rapid centrifugation (14,000 rpm, 1 min), resuspended in 100 μl of Laemmli sample buffer, and boiled for 3 min. Glass beads (0.3 g, 450-μm diameter) were added to the suspension, and then the cells were vortexed at full speed for 30 s. The suspension was boiled again for 3 min and centrifuged at 14,000 rpm for 1 min. 20 μl of the supernatants was subjected to SDS-PAGE and immunoblotting using anti-LexA polyclonal antibodies (Invitrogen) or anti-HA polyclonal antibodies (Clontech).

**Cell Culture and Transfection**—HeLa and HEK293 cells were grown at 37 °C in a 5% CO<sub>2</sub> incubator. Cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. All transfections were carried out using SuperFect transfection reagent (Qiagen) following the manufacturer’s instructions.

**Co-immunoprecipitation Studies**—Cells were transiently transfected with combinations of plasmids encoding the α (N-terminal Myc-tagged), β (untagged), and γ (C-terminal FLAG-tagged) subunits of AMPK. Cell lysates were prepared 24 h post-transfection and incubated with anti-FLAG M2-agarose (Sigma) or anti-HA polyclonal antibodies (Clontech) and immunoblotting using anti-LexA polyclonal antibodies (Invitrogen) or anti-HA polyclonal antibodies (Clontech).

**Kinase Assays**—Activity of AMPK in immunoprecipitates was measured as described previously (25).

**Production and Growth of Mouse Embryo Fibroblasts**—AMPKα subunit knockout MEFs (α1<sup>–/–</sup> and α2<sup>–/–</sup>) and wild-type controls were constructed and grown as described previously (26). They were transfected using Lipofectamine (Invitrogen) according to the manufacturer’s instructions.

**RESULTS**

The γ Subunits Interact with α and β via Their N-terminal Regions by Two-hybrid Analysis—We commenced our study by analyzing two-hybrid interactions of mammalian AMPK subunits expressed in yeast. Because the α2β2γ3 complex appears to play an important role in skeletal muscle (27), we initially studied the interaction between β2 and γ3, which has an N-terminal extension of 201 amino acids prior to the first Bateman domain. An LexA-γ3 fusion protein interacted strongly with a GAD-β2 fusion (Fig. 1), so we made sequential deletions from the N terminus of γ3 and checked their interaction with GAD-β2. As shown in Fig. 1B, deletion of the first 33 amino acids of γ3 (γ3A) decreased the interaction slightly, whereas further successive deletions of 45 (γ3B) and 40 (γ3C) amino acids did not further modify the interaction. However, the deletion of the following 35 amino acids (γ3D) reduced the interaction, and the deletion of the next 47 amino acids (γ3E, truncated at the start of the first Bateman domain) completely abolished it.

FIGURE 1. AMPKγ3 interacts with AMPKβ2 through its N-terminal region.

A, diagram of the deletions constructed. Deletions were made as described under “Materials and Methods” and fused in-frame to LexA. The box drawn with a dashed line indicates the region in AMPKγ3 involved in the interaction. B, two-hybrid analysis of the AMPKγ3-AMPKβ2 interaction. Yeast CTY10.5d strain was transformed with plasmids expressing GAD-AMPKβ2 and different truncated forms of LexA-AMPKγ3. Transformants growing exponentially in 5C-4% glucose medium were harvested, and the β-galactosidase activity measured. Values correspond to means from four to six different transformants (Bars indicated ± S.D.). C, Western blot analysis. Crude extracts from the transformants described in section B were analyzed by Western blot using anti-LexA polyclonal antibodies. One representative transformant from each interaction is shown.
Interaction between AMPK β and γ Subunits

![Diagram showing interaction between AMPK β and γ subunits using two-hybrid analysis.](Image)

Additional deletion of the first CBS motif (γ3F) gave the same negative results as γ3E. Control experiments using the various LexA-γ subunits constructed and the empty vector pACT2 gave negligible (<1 unit) β-galactosidase activity in most cases. Western blot analysis indicated that all deleted forms were expressed at similar levels. These results suggested that the interaction between γ and β subunits requires the 47 amino acids immediately prior to the first Bateman domain, although residues N-terminal to that may improve the interaction. We also attempted to study the interaction between γ and α2 using the same approach, but there was a very low level of interaction evident even with the full-length construct (<1 unit of β-galactosidase).

We next studied γ1, the most abundant γ subunit in skeletal muscle (27). As shown in Fig. 2A, a fusion between LexA and full-length γ1 was able to interact strongly with both α2 and β2. However, the deletion of the first 41 amino acids from the N terminus (a truncation at the start of the first Bateman domain) completely abolished the interaction with both α2 and β2. Control experiments using both LexA-γ1 constructs and the empty vector pACT2 gave negligible (<1 unit) β-galactosidase activity (not shown). Western blot analysis indicated that the truncated LexA-γ1 protein was produced at similar levels to the full-length protein (Fig. 2A, right panel).

Similar results were obtained when we studied γ2. A fusion between LexA and full-length γ2, containing an N-terminal extension of 278 amino acids prior to the first Bateman domain, interacted with α2 and β2 (Fig. 2B). A truncated form containing only the first 37 amino acids prior to the first Bateman domain interacted strongly with α2 and β2 (Fig. 2B), perhaps because the truncated form was better expressed (see Fig. 2B, right panel). However, the deletion of these 37 amino acids from the N terminus (a truncation at the start of the first Bateman domain) completely abolished the interaction with both α2 and β2 (Fig. 2B). Control experiments using the different LexA-γ2 constructs and the empty vector pACT2 gave negligible (<1 unit) β-galactosidase activity (not shown). Therefore, our results suggest that 37–47 amino acids immediately prior to the first Bateman domain of the three γ subunits are necessary for their interaction with α2 and β2.

Because yeast contains orthologues to the three AMPK subunits (AMPKα, Snf1; AMPKβ, Gal83/Sip1/Sip2; and AMPKγ, Snf4), we studied whether the interaction between AMPKβ and the three γ subunits was dependent on the presence of the orthologous Snf1/AMPKα subunit. With this aim, we repeated the two-hybrid experiments in yeast cells lacking the SNF1 gene (snf1Δ mutant). As shown in Fig. 2C, the three γ subunits interacted with the β subunit in the absence of Snf1/AMPKα. These results indicated that the β and the γ subunits interacted directly. The lower levels of interaction observed in snf1Δ mutants may suggest that the presence of the α subunit stabilizes the β-γ interaction.

*Mutations in the γ2 Bateman Domains Do Not Affect Binding to α2 and β2—*Mutations in the PRKAG2 gene, encoding the γ2 subunit, cause heart diseases of varying degrees of severity that appear to be caused by excessive glycogen storage (28, 29). Several mutations have been described, e.g. R302Q (30), L-insert (an insertion of an extra Leu residue between the conserved Arg350Glu351) and H383R (31), T400N and N488I (28), R531G (32), and R531Q (33). In all cases, the described mutations affect critical residues in different CBS motifs of the γ2 subunit and, with the possible exception of the L-insert mutation, produce proteins with deficient AMP binding capacity (8, 33). We studied four of these mutations (R302Q, L-insert, H383R, and T400N (Fig. 3A)) and checked by two-hybrid analysis whether the mutated forms interact properly with α2 and β2. Compared with the wild type, all of the mutants interacted normally with α2, and the interaction was increased in the absence of glucose, as reported previously for the wild type (10) (Fig. 3B). None of the mutations affected the two-hybrid interaction of γ2 with β2 either (Fig. 3C).

*Alignment of γ Subunit Sequences—*We next aligned the sequences of human γ1, γ2, and γ3 with those of the orthologues from Drosophila melanogaster, Dictyostelium discoideum, S. cerevisiae (Snf4), and Schizosaccharomyces pombe (Fig. 4). According to the UNIPROT data base, the first Bateman domains of γ1, γ2, and γ3 begin at Lys92, Lys279, and
Interaction between AMPK β and γ Subunits

Requirement for the Pre-CBS1 Sequence Revealed by Co-precipitation—To test the importance of this pre-CBS1 sequence for complex formation and also to map the interactions using a method different from two-hybrid analysis, we made constructs of human γ₁, γ₂, and γ₃ that were either truncated at the start of the first Bateman domain (γ₁₁₀, γ₂₁₀, and γ₃₁₀; S represents short) or that contained the 25- to 26-residue pre-CBS1 sequence shown in Fig. 4 (γ₁₂₁, γ₂₂₁, and γ₃₂₁; L represents long). These were co-expressed in HeLa cells with β₁ and Myc-tagged α₁, and the γ subunits were immunoprecipitated with anti-FLAG antibody, making use of the FLAG tag at the C termini of all γ variants. Fig. 5A shows the results of Western blotting using anti-Myc and anti-FLAG antibodies to detect the α and γ subunits, respectively. A consistent finding was that Myc-α₁ (detected using anti-Myc antibody) only co-precipitated with γ₁, γ₂, and γ₃ when the latter contained the pre-CBS1 sequence (compare lane 1 (L variants) with lane 5 (S variants) for each γ subunit in the anti-Myc blot). Unfortunately, the γ₁₃ variant appeared to be expressed at a much lower level than the γ₁₁ variant. Also, for reasons that are unclear, it always migrated as a doublet, with one band migrating faster than γ₁₁ as expected, but the other migrating more slowly. Nevertheless, the Myc-tagged α₁ subunit (which was expressed equally well in both cases) only co-precipitated significantly with γ₁₁, γ₂₂, and γ₃₃ when the latter contained the pre-CBS1 sequence on γ₃, but Myc-α₁ only co-precipitated significantly with γ₁₃ and not with γ₂₃.

Very similar results were obtained when the constructs were expressed in HEK293 rather than HeLa cells (not shown). We also expressed Myc-α₁, β₁, and the γ-FLAG variants in both HeLa and HEK293 cells, immunoprecipitated the corresponding AMPK complexes using anti-Myc or anti-FLAG antibodies, and measured AMPK activity. Fig. 5B shows that active complexes were only obtained in anti-Myc immunoprecipitates from HEK293 cells using the long variants (γ₁₂₁, γ₂₂₁, and γ₃₂₁). The rather low level of activity obtained with γ₂₂ did not differ significantly from the background level in untransfected cells. Similar results were obtained by immunoprecipitation using anti-FLAG antibodies (not shown). To confirm that AMPK complexes containing the pre-CBS1 sequence were regulated normally in intact cells, we also treated HEK293 cells expressing Myc-α₁, β₁, and γ₁₁, or γ₁₃, γ₁₅-FLAG with 10 mM deoxyglucose. Fig. 5C shows that the γ₁₁-FLAG complex was activated by deoxyglucose as expected, whereas only background levels of activity were observed in the

FIGURE 3. Analysis of the interaction of different mutated forms of AMPKγ₂ and AMPKα₂ and AMPKβ₂. A, diagram of the position of the different AMPKα₂ mutations used in this study. B, interaction with AMPKα₂. Yeast CTY10.5d strain was transformed with plasmids expressing the indicated mutated forms of AMPKγ₂ (LexA-AMPKγ₂) and GAD-AMPKα₂. Transformants growing exponentially in SC-4% glucose medium were washed with water and shifted to SC-0.05% glucose medium for 3 h. Then samples were harvested and the β-galactosidase activity was measured. Values correspond to means from four to six different transformants (bars indicated ± S.D.). C, interaction with AMPKβ₂. Transformants growing exponentially in SC-4% glucose medium were harvested, and the β-galactosidase activity was measured. Values correspond to means from four to six different transformants (bars indicated ± S.D.).
Interaction between AMPK β and γ Subunits

The Pre-CBS1 Sequence of γ Interacts with the β Subunit—To test whether the pre-CBS1 sequence interacts with the α or the β subunits, we co-expressed the γ1, γ2, γ3, γ2, γ1, γ3, or γ3 subunits in HeLa cells together with plasmids encoding either Myc-α1, Myc-α2, β1, or β2 alone, i.e. without DNA encoding the third subunit. Cell lysates were immunoprecipitated with anti-FLAG, and the respective α or β subunit was detected in the immunoprecipitates with the corresponding antibodies. The experiments with β1 or β2 (detected using a pan-β antibody) showed that both isoforms were able to form a complex with γ1, γ3, or γ3, but not with γ1, γ3 (Fig. 6A). We also attempted the experiment with the γ2 construct but, probably because of the low level of expression, any complex formed with γ2 was not detectable (not shown). On the other hand, Myc-α1 or Myc-α2 appeared to form complexes with γ1 and γ3 variants, irrespective of the presence or absence of the pre-CBS1 sequence (Fig. 6B). These results suggest that, although the interaction between the β and γ subunits requires the pre-CBS1 sequence, α1 or α2 interact with γ subunits independently of this sequence, i.e. via the Bateman domains. Surprisingly, this is different from the results obtained in experiments when a β subunit was also expressed (e.g. Figs. 5A and 6C), where the association of α and γ is dependent on the presence of the pre-CBS1 sequence.

Recently, it has been proposed that the β and γ subunits of AMPK do not interact directly, but only indirectly via the α subunit (17). The results in Fig. 6A argue against that model, but a caveat was that HeLa cells do express low levels of endogenous α subunits. To rule out the possibility that endogenous α subunits could be providing a bridge between the overexpressed β and γ subunits, we co-expressed the γ-FLAG variants and β1, with or without Myc-α1, in wild-type or double knock-out (α1−/− and α2−/−) mouse embryo fibroblasts (WT or KO MEFs). Unfortunately, the MEF cell lysates contain an abundant protein that migrates just behind the α subunits on SDS-PAGE. This protein binds the anti-α1/α2 antibody and/or the second antibody used, and therefore almost obscures the α subunits when cell lysates were analyzed by Western blotting. However, this protein was not present in the immunoprecipitates. Endogenous α subunits from WT MEF cells could be seen to co-precipitate with γ1L (lane 8 in Fig. 6C), but no signal was obtained in the equivalent samples from KO MEFs (lane 8 in Fig. 6D), confirming the complete absence of α subunits from KO cells. Recombinant Myc-α1 was also observed to co-precipitate with γ1L when Myc-α1 was expressed in either WT or KO MEFs (lane 4 in Fig. 6, C and D). Despite the complication of the abundant protein in the MEF cell lysates, the results clearly show that β1 interacts with γ1L but not with γ1S, even in KO MEFs that completely lack both α subunits (anti-β blot, lane 8, Fig. 6, C and D). The presence of endogenous α1 in WT MEFs, or overexpressed Myc-α1 in either WT or KO MEFs does, however, appear to markedly increase the amount of β1 that co-immunoprecipitates with γ1L, but not with γ1S. These observations suggest that the α subunit does stabilize β-γ interaction, although it is not essential for the interaction to occur.

Both Bateman Domains of the γ Subunits Are Also Necessary to Allow the Formation of a Heterotrimeric Complex—We also made sequential deletions from the C terminus of γ1 and found that, upon elimination of the last CBS motif of the second Bateman domain, the two-hybrid interactions with α2 and β2 were completely lost (Fig. 7). Successive deletions of the remaining CBS motifs gave the same negative results. Control experiments using LexA-α2 or LexA-β2 and the empty vector pACT2 gave negligible (<1 unit) β-galactosidase activity (not shown). Western blot analysis indicated that the truncated GAD-γ1 proteins were produced at similar levels (Fig. 7, right panel).

We also made various truncations of human γ2 that contained only CBS motifs 3–4 (Bateman domain 2) or 2–4 (half Bateman domain 1 plus Bateman domain 2), as well as one that commenced at residue 244 (thus containing the pre-CBS1 sequence) but was truncated at the end of the first Bateman domain and did not contain the second domain. None of these constructs formed functional AMPK complexes when co-expressed with α1 and β1 (data not shown). These results indicated that the two Bateman domains may fold into a combined structure necessary to allow the formation of an active heterotrimeric complex. If this structure is altered, e.g. by deleting one of the CBS motifs, then the formation of a functional heterotrimeric complex is prevented.

DISCUSSION

AMPK exists as heterotrimeric complexes, and all three subunits are required to form a functional, AMP-activated complex when the full-length subunits are expressed (14, 15). Because the crystal structure of the complex has not been determined yet, alternative methods have been used to obtain information about its architecture. For example, co-expression and co-immunoprecipitation techniques have been used to show that only the C-terminal domain of β is required to form a functional complex with α and γ (11), whereas additional C-ter-
minal truncations have been used to more precisely map the regions of the C-terminal domain of β1 required for binding to α (residues 186–270) and γ (residues 246–270) (16). The same technique was used to show that a C-terminal region of α1 lacking the last 75 residues (313–473) was all that was required for binding to β1 (16). Two-hybrid analysis has also been used as an alternative technique to analyze the interactions among the three different subunits (34, 35). Recently, we reported that only the C-terminal domain of α2 (residues 313–552) was required for the interaction with γ1 (10). The two-hybrid technique has also been used extensively to study the architecture of the yeast orthologue of mammalian AMPK, i.e. the SNF1 complex (36). The results are broadly similar to those obtained in the mammalian system: (i) yeast α subunit (Snf1) interacts via its C-terminal domain with both the γ (Snf4) and β (Gal83/Sip1/Sip2) subunits (37); (ii) several deletions or point mutations in Snf1 also defined critical residues in the kinase.
Interaction between AMPK \( \beta \) and \( \gamma \) Subunits

![Graph](image)

**FIGURE 7.** The second Bateman domain in \( \gamma 1 \) is required to interact with \( \alpha 2 \) and \( \beta 2 \). Yeast CTV10.5d strain was transformed with plasmids expressing LexA-AMPK\( \alpha \)2 or LexA-AMPK\( \beta \)2 and C-terminal truncated forms of GAD-AMPK\( \gamma 1 \). Transformants growing exponentially in SC-4% glucose medium were harvested, and the \( \beta \)-galactosidase activity measured. Values correspond to means from four to six different transformants (S.D. < 15% in all cases; not shown). Crude extracts from these transformants were analyzed by Western blot using anti-HA polyclonal antibodies (plasmid pACT2-generated GAD-HA fusion proteins). One representative transformant from each interaction is shown.

Results indicate that the pathogenic defect associated with these mutations is not due to deficiencies in binding of \( \gamma 2 \) to \( \alpha 2 \) and \( \beta 2 \). This is consistent with findings that these \( \gamma 2 \) mutations give rise to complexes that are active, although in some cases defective in AMP activation, when co-expressed with \( \alpha \) and \( \beta \) subunits in mammalian cells (8, 33, 40). The findings that these mutant complexes were active suggested that they contained all three subunits, although this had not been directly addressed in the previous studies.

Recently, evidence was presented, using a co-precipitation approach, that mouse \( \beta 2 \) and \( \gamma 1 \) subunits do not interact directly (17). The authors proposed instead that the \( \alpha \) subunit bridges the interaction between \( \beta \) and \( \gamma \). Our results do not support this model. First, yeast two-hybrid analysis indicates that the interaction between \( \beta \) and \( \gamma \) subunits occurs in the absence of Snf1/AMPK\( \alpha \) subunit (Fig. 2C). Second, both \( \beta 1 \) and \( \beta 2 \) interacted with \( \gamma 1 \) and \( \gamma 3 \) when overexpressed together in HeLa cells without an \( \alpha \) subunit, and this was dependent on the pre-CBS1 sequence of the \( \gamma \) subunits, because it did not occur with the short variants (Fig. 6A). Both \( \alpha 1 \) and \( \alpha 2 \) also interacted with \( \gamma 1 \) and \( \gamma 3 \) in the absence of a co-expressed \( \beta \) subunit, but this was not dependent on the pre-CBS1 sequence, because the interaction occurred equally well with the short as the long \( \gamma \) variants (Fig. 6B). The fact that the \( \beta \) subunits require the pre-CBS1 sequence to interact with the \( \gamma \) subunits, whereas the \( \alpha \) subunits do not, argues against the idea that \( \alpha \) subunits provide a bridge between \( \beta \) and \( \gamma \). One caveat with our experiments in HeLa cells is that these cells do express low level of endogenous \( \alpha \) subunit. To rule out the possibility that the endogenous \( \alpha \) subunit might bridge the \( \beta-\gamma \) interaction, we repeated the experiment in WT MEFs and double knock-out cells that completely lacked \( \alpha \) subunits (KO MEFs). The results clearly showed that a \( \beta-\gamma \) interaction, which was dependent on the pre-CBS1 sequence, was still evident in the KO MEFs. However, the presence of \( \alpha \) subunits, either endogenous in the WT MEFs or recombinant Myc-\( \alpha 1 \) subunit in WT or KO MEFs, did appear to greatly increase the amount of \( \beta 1 \) recovered in the \( \gamma 1 \)-FLAG immunoprecipitates (Fig. 6, C and D). Thus, the presence of an \( \alpha \) subunit may stabilize the \( \beta-\gamma \) interaction. The lower levels of interaction observed between \( \beta \) and \( \gamma \) in snf1\( \Delta \) mutants are also consistent with this hypothesis.

Some rather surprising findings were that when \( \alpha \) and \( \gamma \) subunits were expressed on their own, they interacted even in the absence of the pre-CBS1 sequence (Fig. 6B), but when they were co-expressed with a \( \beta \) subunit, co-precipitation with the \( \gamma \) subunits was now dependent on the presence of the pre-CBS1 sequence on \( \gamma \) (Figs. 5A and 6C). Our interpretation of these somewhat puzzling findings is that the \( \alpha \) and \( \beta \) subunits may form a dimeric complex that interacts differently with the \( \gamma \) subunits than when the \( \alpha \) subunit is present on its own.
We have also made sequential deletions from the C terminus of γ1 and found that, upon elimination of even one of the four CBS motifs, the two-hybrid interactions with α2 and β2 were completely lost. In agreement with this observation, we also found that a C-terminal deletion of γ2 (lacking the second Bateman domain) was unable to form an active heterotrimeric complex. One plausible interpretation of these results is that both Bateman domains are required to form a functionally combined structure. If one of the CBS motifs or Bateman domains is deleted, then the structure does not fold properly, leading to an altered conformation of the protein that cannot bind to α and β even though the pre-CBS1 sequence is present.

Addendum—At the time that the manuscript was under revision, Townley and Shapiro (41) have defined the crystal structure of the AMPK complex from the yeast S. pombe. They demonstrate that the γ and subunits interact directly and that the pre-CBS1 sequence of the γ subunit participates in the binding to the β subunit.

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