AMP-activated protein kinase (AMPK) acts as an energy sensor, being activated by metabolic stresses and regulating cellular metabolism. AMPK is a heterotrimer consisting of a catalytic α subunit and two regulatory subunits, β and γ. It has been reported that the mammalian AMPK α subunit contained an autoinhibitory domain (α1: residues 313–392) and had little kinase activity. We have found that a conserved short segment of the α subunit (α1-(313–335)), which includes a predicted α-helix, is responsible for α subunit autoinhibition. The role of the residues in this segment for autoinhibition was further investigated by systematic site-directed mutation. Several hydrophobic and charged residues, in particular Leu-328, were found to be critical for α1 autoinhibition. An autoinhibitory structural model of human AMPK α1-(1–335) was constructed and revealed that Val-298 interacts with Leu-328 through hydrophobic bonding at a distance of about 4 Å and may stabilize the autoinhibitory conformation. Further mutation analysis showed that V298G mutation significantly activated the kinase activity. Moreover, the phosphorylation level of acetyl-CoA carboxylase, the AMPK downstream substrate, was significantly increased in COS7 cells overexpressing AMPK α1-(1–394) with deletion of residues 313–335 (Δα394) and a V298G or L328Q mutation, and the glucose uptake was also significantly enhanced in HepG2 cells transiently transfected with Δα394, V298G, or L328Q mutants, which indicated that these AMPK α1 mutants are constitutively active in mammalian cells and that interaction between Leu-328 and Val-298 plays an important role in AMPK α autoinhibitory function.

The AMP-activated protein kinase (AMPK)2 is a sensor of cellular energy state, being activated by a large variety of cellular stresses that increase cellular AMP and decrease ATP levels, such as glucose deprivation, hypoxia, oxidative stress, heat shock, and ischemia (1–3). AMPK is also activated by physiological stimuli, such as exercise, muscle contraction, hormones like leptin and adiponectin, pharmacological agents like thiazolidinediones and metformin, and a widely used AMPK activator, 5-aminoimidazole-4-carboxamide-1-β-d-ribofuranoside (4–10), modulating multiple metabolic pathways (11). Therefore, AMPK has been investigated for the treatment of type II diabetes, obesity, and even cancer (12).

AMPK is a heterotrimeric serine/threonine protein kinase consisting of a catalytic α subunit and two regulatory subunits, β and γ (13). In mammals, each AMPK subunit has multiple isoforms, α1, α2, β1, β2, γ1, γ2, and γ3 (14), suggesting that multiple heterotrimeric complexes may exist in different tissues and play different roles. Optimal kinase activity requires the formation of a heterotrimeric complex, involving AMP allosteric activation, and phosphorylation on Thr172 within the activation loop of the catalytic α subunit by an upstream kinase, AMPK kinase (AMPKK), identified as LKB1 or calcium/calmodulin-dependent protein kinase kinase β (CaMKKβ) (13, 15–25). In the yeast Saccharomyces cerevisiae, the Snf1p kinase, Gal83p/Sip1p/Sip2p, and Snf4p are the homologs of the mammalian AMPK subunits, respectively (26–29), which are involved in the induction of invertase (SUC2) under conditions of nutritional stress due to glucose starvation. Snf4p is sufficient to stimulate Snf1p kinase activity by binding to the Snf1p regulatory domain (residues 392–518) in glucose-limiting conditions (29–32). However, recent studies have showed that the three subunits of the SNF1 kinase heterotrimer are independent for association and assembly of a functional complex in vivo (33). Like Snf1p (33), the full-length α catalytic subunit possessed little kinase activity when expressed in mammalian cells without co-expression of the AMPK regulatory subunits β and γ (13, 34).

The mammalian AMPK α catalytic subunit contains an N-terminal kinase domain (residues 1–312) and a C-terminal domain (residues 313–548), which includes an autoinhibitory domain (AID) and a binding domain responsible for binding the α/γ subunits (34). Previous studies have identified residues 313–392 of the α1 subunit as the AID sequence and residues 313–473 of the α1 subunit as the α1 binding sequence (34, 35). Alignment analysis of the AID sequence indicates that this segment is highly conserved in the AMPK subfamily of kinases. However, there is little conservation between AMPK and AMPK-related protein kinases, except for several conserved residues located in the ubiquitin-associated (UBA) domain, which is located closely following the kinase domain in many
AMPK-related protein kinases and required for the activation and localization of these kinases (36). The mammalian AMPK α catalytic subunits do not possess this UBA domain within the reported sequence (313–392); instead, it plays an important role for α subunit autoinhibition. Although the AID is known to be responsible for α subunit inhibition, a more detailed autoinhibitory sequence and molecular basis for the autoinhibitory function of AMPK are unclear. Therefore, it was of interest to us to investigate which part of this domain (α1, residues 313–392) in the mammalian AMPK α catalytic subunit acts as the autoinhibitory sequence and determine how it exerts its autoinhibitory role. In this study, we identify that a conserved 23-residue sequence of the α subunit (α1, residues 313–335), including a predicted α-helix, acts as the autoinhibitory sequence in the AMPK α subunit by means of truncation, deletion, and point mutation of the α1 subunit. Hydrophobic interaction between Val-298 and Leu-328, using our hypothesized autoinhibitory structural model of α1-(1–335), was confirmed by mutation and kinase assay. The hydrophobic and charged residues in the conserved α-helix might play important roles for the autoinhibitory activity of the α subunit partly through tight packing of the α autoinhibitory domain and maintenance of the conformation interacting with the kinase domain.

EXPERIMENTAL PROCEDURES

Materials

The expression plasmid pGEMEX-1 was from Promega (Madison, WI). The pET28b and pETDuet vectors were purchased from Novagen (Milwaukee, WI). The plasmid pcdNA3.1 for transfection was from Invitrogen. The recombinant plasmid pMAL-CaM KK was a kind gift from Dr. Charles R. Mena (Duke University Medical Center). The SAMS peptide (HMRSAMSGHLHVLKRR, a synthetic substrate of AMPK) (2) was synthesized by SynPep Inc. (Dublin, CA). All restriction enzymes and T4 DNA ligase were from Takara (Dalian, China). The QIAquick spin plasmid kit and QIAquick gel extraction kit were from Qiagen. The Escherichia coli strain BL21 Codon Plus (DE3)-RIL and the QuikChange site-directed mutagenesis kit were purchased from Stratagene (La Jolla, CA). HiPrep Q-Sepharose column, chelating Sepharose column, scintillants [γ-33P]ATP and 2-deoxy-[1-3H]glucose, Western blotting detection kits (ECL), and Hyperfilm were purchased from Amersham Biosciences (Uppsala, Sweden). Anti-AMPK-α antibody, anti-phospho-AMPKα-(Thr-172) antibody, specific anti-phospho-ACC-ACC-Ser-79 antibody, anti-ACC antibody, anti-Myc, and β-actin antibodies were obtained from Cell Signaling Technology (Beverly, MA). The P30 filter paper was purchased from Wallac (Turku, Finland), and Liquid Scintillator (OptiPhase SuperMix) was obtained from PerkinElmer Life Sciences. Protein purification was carried out on an AKTA fast protein liquid chromatography system from Amersham Biosciences. Liquid scintillation counting was performed in Wallac MicroBeta TriLux (Wallac Oy, Turku, Finland). COS7 cells and human hepatoma HepG2 cells were purchased from ATCC (Manassas, VA). Dulbecco’s modified Eagle’s medium (DMEM), glucose-free DMEM, fetal bovine serum, and Lipofectamin 2000 transfection reagent were purchased from Invitrogen. All other reagents were purchased from Sigma.

Plasmid Construction

The construction of all recombinant plasmids used in this study is detailed in Table 1. Cloning was performed using standard molecular biology techniques. The coding sequences of all human AMPK α1 or α2 subunit truncations, deletions, and mutants were amplified from cDNA of the human AMPK α1 or α2 subunit using relevant primers listed in Table 1 and cloned into pGEMEX-1 or pET28b vector. For the trimer α1/β1/γ1 expression, the pETDuet-β1/α1 and pET28b-γ1 were constructed to co-transform into competent cells. The full-length genes of human AMPK α1 and γ1 were amplified and then subcloned into pET28b vectors to construct pET28b-α1 and pET28b-γ1 for expression of recombinant proteins with C-terminal polyhistidine tags, respectively. The human full-length AMPK β1 gene was cloned into the vector pETDuet-1 to form pETDuet-β1, and then the AMPK α1 subunit gene and its upstream T7 promoter were subcloned from pET28b-α1 and ligated into pETDuet-β1 to form pETDuet-β1/α1.

The deletions coding sequences were obtained by two-strand PCR amplification. Two strands were amplified with forward/ midantisense primers and midsense/reverse primers, respectively, and then this two-strand mixture was taken as a template to amplify relative deletions using corresponding forward/reverse primers. Both midsense and midantisense primers used for deletions are listed in Table 1. All mutants of AMPK-α1-(1–394) were prepared using the QuikChange site-directed mutagenesis kit for generating the single point mutants D320A, L322Q, A323R, A325E, Y326F, H327E, L328Q, I329N, and mutants were amplified from cDNA of the human AMPK α1 gene was cloned into the pETDuet-1 for expression of recombinant proteins with C-terminal His tag and Myc tag were incorporated into the truncations. All recombinant plasmids containing wild-type truncations, deletions, and mutations were verified by DNA sequencing.

Recombinant Protein Expression and Purification

E. coli cells containing the expression plasmids containing truncations, deletions, and mutant plasmids were cultured at 37 °C until the absorbance of 600 nm reached 0.6, and then 0.1 mM isopropyl-1-thio-β-D-galactopyranoside was added to induce protein expression at 22 °C overnight. The cells were spun down and sonicated in lysis buffer (TL for nonfusion proteins: 50 mM Tris, pH 7.5, 1 mM DTT, and 1% Triton X-100). The nonfusion truncated proteins, deletions, and mutants were purified by loading the lysate supernatants onto a 20-ml HiPrep Q-Sepharose column (preequilibrated with 50 mM Tris, pH7.5, 1 mM DTT) and eluted with a linear NaCl gradient (0–1 M) in 50 mM Tris, pH 7.5, 1 mM DTT. All purified proteins were stored at −70 °C.

The expression and purification of the trimer α1/β1/γ1 were carried out as described previously (38) with minor modifica-
of α1/β1γ1 expression, for which the recombinant plasmids pETDuet-β1/α1 and pET28b-γ1 were co-transformed into BL21 Codon Plus (DE3)-RIL. The purified complexes were dialyzed with buffer (50 mM Tris-HCl, pH 7.5, 1 mM β-mercaptoethanol, 1 mM EDTA) at 4 °C to remove imidazole and were stored at −70 °C until use. Recombinant CaMKKβ was expressed and purified as described previously (39).

**Measurement of Enzyme Activities**

In order to determine the phosphorylation time of kinase for an activity assay and due to inactive α1-(1–394), we chose α1-(1–312) as the active form incubated in a phosphorylation buffer containing 20 mM Tris-HCl, pH 7.5, 1 mM DTT, 5 mM MgCl₂, 200 μM ATP, activities of the phosphorylated α truncations or deletions or mutants were carried out under a typical assay in a 50-μl reaction mixture containing 20 mM Tris-HCl, pH 7.5, 1 mM DTT, 5 mM MgCl₂, 100 nm enzymes, 50 μM ATP (0.4 μCi of [γ-^{33}P]ATP/reaction), 50 μM SAMS. The reaction was initiated by the addition of enzyme, incubated at 30 °C for 10 min, and terminated by the addition of 50 μl of 1% H₃PO₄. The reaction mixture was then transferred to P30 filter paper and washed three times with 0.1% H₃PO₄. Radioactivity that had been incorporated in the proteins was determined by liquid scintillation counting in a Wallac Microbeta plate counter. Background radioactivity estimated from reactions conducted without enzyme was subtracted from sample radioactivities. All reactions were repeated in three independent experiments.

**Cell Culture, Transfection, and Activity Analysis of Expressed AMPK in COS7**

COS7 cells were maintained in DMEM supplemented with 10% fetal bovine serum, penicillin, and streptomycin at 37 °C in 5% CO₂. Transfection with plasmids was performed using Lipofectamine 2000 transfection reagent in cells that were
about 80% confluent. Briefly, COS7 cells in a 6-well plate were transiently transfected with 8 µg of each DNA construct in Lipofectamine 2000 transfection reagent (0.5 µl/µg of DNA), using a GFP vector of pEGFP-C1 to monitor transfection efficiency when required and still cultured for about 24 h, allowing for AMPK expression. Before Western blotting analysis, the medium was aspirated, and the cells were rinsed with PBS and then lysed directly by 1× SDS-PAGE loading buffer.

**Glucose Uptake Assay in Human Hepatoma HepG2 Cells**

HepG2 cells were cultured in DMEM containing 10% fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin, and 5.5 mM glucose in 5% CO2 at 37 °C. For experiments, HepG2 cells were split into a 24-well plate, and the next day the cells, when grown to 70% confluence, were transiently transfected with 2 µg of each plasmid/well in Lipofectamine 2000 transfection reagent (0.5 µl/µg DNA). A GFP vector of pEGFP-C1 was used as a control to monitor transfection efficiency. After transfection for 24 h, cells were washed once with PBS and then incubated in either glucose-containing or glucose-free DMEM for 24 h. To determine the glucose uptake, the medium was switched to glucose-free HEPES-buffered saline (HBS buffer: 136 mM NaCl, 4.7 mM KCl, 1.25 mM MgSO4, 1.2 mM CaCl2, 0.2% bovine serum albumin, 20 mM HEPES, pH 7.4). Glucose uptake was measured by adding a 50-µl glucose mixture (1 mM 2-deoxyglucose and 0.5 µCi of 2-[^3]H]2-deoxy-[1-14C]glucose in HBS) to 450 µl of HBS followed by incubation for 3 min at 37 °C. Nonspecific glucose uptake was measured by parallel incubations in the presence of 10 µM cytochalasin B, which blocks transporter-mediated glucose uptake, and was subtracted from the total uptake in each assay. Uptake was terminated by quickly washing the cells three times with ice-cold PBS, and the cells were mixed with 200 µl of cell lysis solution (0.1% Triton X-100 in phosphate-buffered saline). A portion of the cell lysate was used in a radioactivity assay performed using a liquid scintillation counter.

**Western Blotting and Other Analytical Procedures**

The above prepared samples were subjected to 8–12% SDS-PAGE and transferred onto polyvinylidene difluoride membrane. The quantities of the protein and phosphorylation were detected by the following primary antibodies: anti-AMPK-α antibody and anti-phospho-AMPKα-Thr-172 antibody (1:1,000 dilution), anti-phospho-ACC-Ser-79 antibody (1:1,000 dilution), anti-ACC antibody (1:1,000 dilution), anti-Myc antibody (1:2,000 dilution), and anti-β-actin antibody (1:5,000 dilution). The total protein of each sample was quantified by β-actin. The signal was detected by a chemiluminescence kit and analyzed by densitometric evaluation using an imaging system and analyzing software (Shanghai Furi Science & Technology Co.). Protein concentration was measured using the Bradford method.

**Modeling of AMPK α1 Autoinhibitory Structure**

**For Homology Modeling**—The crystal structure of yeast AMP-activated protein kinase homologous Snf1p (Protein Data Bank code 2EUE) was used to construct the kinase domain (residues 1–280) of human AMPK α1 by utilizing the program MODELLER. The sequence identity between these two proteins is 56%, and the positive score is 71%, whereas for the autoinhibitory domain (residue 290–335), the sequence searching against the Protein Data Bank did not result in any informative alignment. Therefore, the secondary structure prediction was conducted within the server (available on the World Wide Web at www.compbio.dundee.ac.uk/~www-jpred/) performed by the built in program Jpred, which suggested that this domain contained three α-helices. Then functional search found that the ubiquitin-associated domains of many kinases contain such structural features. Also, as provided by Jaleel et al. (36), the sequence alignments between AMPKα1 and microtubule affinity-regulating kinases (MARKs) were adopted to build this autoinhibitory domain. Due to the low sequence identity, after the comparative modeling by MODELLER, the CHARMM program was used to solvate this domain in a water box extending 10 Å from any atoms of the domain. Then the energy minimization was performed in about 1000 steps with the conjugate gradient method to relax the autoinhibitory domain. Following this, 10-ps molecular dynamics was carried out to further explore the conformation of the domain by constraint of the backbone atoms with a force constant of 10 Kcal/mol Å².

**For Docking**—As suggested in the crystal structure of protein kinase MARK/Par-1, the ubiquitin-associated domain in MARK (equivalent to the autoinhibitory domain in AMPK) is located at the back side of the N-terminal lobe of the kinase domain. Thus, the complex of autoinhibitory domain and kinase domain of AMPK was formed according to the coordinates of the crystal structure of MARK (Protein Data Bank code 1Y8G). To release the crash between these two domains, the CHARMM program was used again to minimize the complex with the procedure based on the program RDOCK, first by only minimizing the van der Waals interaction and keeping each domain as a rigid body and then by adding the electrostatic force to minimize the complex while the distances of each domain were still fixed; after that, the side chains of the two domains were allowed to move under the minimization of the bonded and nonbonded interactions as normally conducted in the CHARMM force field. Finally, the complex was used to analyze the interaction between the two domains and explain the experiment results at the atomic basis.

**Statistical Analysis**

The results were expressed as the means ± S.E. Significance was analyzed using a two-tailed unpaired Student’s t test. p < 0.05 was considered significant.

**RESULTS**

**Analysis of Truncated α1 Subunit of Human AMPK—**Alignment of all available DNA sequences of the AMPK subfamily within the residue sequence α1-(271–394) of human AMPK, which succeeds the kinase catalytic domain, indicated that this segment was highly conserved. There were 62% identity and 77% positives between human AMPK α1 and α2 isoforms, with 100% identity between human AMPK α1 and rat AMPK α1 and 16% identity between human AMPK α1 and the yeast Snf1p kinase homolog (Fig. 1A). There are several conserved segments among members of the AMPK subfamily within this...
region. Due to the conserved autoinhibition in AMPK subfamily kinases, the detailed mechanism must rely on conserved residues or dimensional structure. Sequence alignments suggest that these segments might play an important role for the autoinhibitory function of the AMPK α catalytic subunit. Although the catalytic domain between AMPK and the AMPK-related protein kinases is much conserved, there is only weak conservation within the residue sequence equivalent to α1(1–394) of human AMPK.

In previous studies, Crute et al. (34) identified residues 313–392 in the mammalian AMPK α1 catalytic subunit as an autoinhibitory sequence and α1(1–312) as constitutively active, whereas α1(1–392) and full-length α1 catalytic subunit have little activity. A more detailed autoinhibitory sequence and mechanism remains unclear. In order to define the location of the autoinhibitory domain more precisely, we constructed a series of truncations of human AMPK α1-(1–394) under the strategy to gradually remove the conserved sequence and assessed their activity. Seven α1 C-terminal deletion constructs were created in pGEMEX-1 plasmid in order to truncate the human AMPK α1 subunit at residues 394, 377, 367, 351, 341, 335, or 312 (Fig. 2). The truncations were purified to near homogeneity by Q-Sepharose anion exchange chromatography (Fig. 3). All truncated proteins were fully phosphorylated at Thr-172 after incubation with CaMKKβ to a similar extent, and then the kinase activity was assessed by incorporation of [33P]phosphate into the SAMS peptide under a typical assay described under “Experimental Procedures.”

![FIGURE 1. Sequence alignment of AMPK subfamily and AMPK/AMPK-related protein kinases. A, alignment of human AMPK α1-(271–394) sequence with other subfamily members. Shown are alignments of AMPK α1 (human (h) sequence: aa 271–394; rat (r) and mouse (m) sequences: aa 269–392) and α2 (human, rat, and mouse sequences: aa 269–398), the Xenopus kinase homolog (aa 280–404; GenBank Accession number AF340021.1), and the yeast Snf1p kinase from S. cerevisiae (aa 289–439; GenBank Accession number NC_001136.6). B, alignment of members of human AMPK-related protein kinases (hMARK1, hMARK2, hMELK, and hSIK) with human AMPK α1-(18–335) sequence. The identical residues are shown in black, and the conserved residues are shown in gray.](image-url)
**α-Helix Responsible for AMPK Autoinhibition**

**A.**

- M 1 2 3 4 5 6 7 8 9 10 11
- 97 kDa—
- 66 kDa—
- 43 kDa—
- 31 kDa—

**B.**

- anti-AMPKα

**FIGURE 3.** Purification and identification of the truncations and deletions. *A*, samples were analyzed on a 12% SDS-polyacrylamide gel, and then the gel was stained with Coomassie Blue. *M*, protein molecular weight markers; lanes 1–11, purified α1-(1–312), α1-(1–335), α1-(1–341), α1-(1–351), α1-(1–367), α1-(1–377), α1-(1–394), α1-(1–394, Δ313–335), α2-(1–398), α2-(1–398, Δ311–333), and α1-(1–550, Δ313–335), respectively. *B*, purified truncations and deletions of human AMPKα subunit as described above were analyzed by immunoblotting with anti-AMPKα antibody.

The activity of the full-length AMPKα1 subunit was examined. The homogeneous α1-(1–312) truncation, purified to near homogeneity (data not shown), showed little kinase activity (13, 34). In this study, the α1-(1–394) or full-length α1 subunit did increase activity, ~2-fold higher than α1-(1–394) but only one-third lower than that of α1-(1–394, Δ313–335) (Fig. 5).

According to the sequence alignments, residues 313–335 among members of the AMPK kinase family showed 86% homology (78% identity) between AMPKα1 and α2 isoforms (Fig. 1). Thus, we further examined the effect of deletion of residues 311–333 on α2-(1–398) activity. As shown in Fig. 5, deletion of residues 311–333 did activate α2-(1–398) and increased kinase activity above 10-fold that of α2-(1–398) without deletion of residues 311–333.

**Effect of Mutation of Residues 320–335 on α1-(1–394) Autoinhibitory Activity**—According to the enzyme's secondary structure prediction using the Jpred software (available on the World Wide Web at www.cmpbio.dundee.ac.uk/~www-jpred/), the 23 residues of α1-(313–335) might contain a conserved α-helix structural fold between residues 320 and 335. The presence of more conserved charged residues and hydrophobic residues in this predicted α-helix raised the possibility that these residues might be responsible for the enzyme's autoinhibitory activity. In order to test which residues were critical, we next assessed how mutation of each of the residues affected the catalytic activity of α1-(1–394). Each mutant of α1-(1–394) was purified to near homogeneity (data not shown). Several mutations were chosen to examine their phosphorylation level by immunoblotting with phospho-AMPKα-Thr-172 antibody, and they were phosphorylated by CaMKKβ to a similar extent after incubation with phosphorylation buffer for 4 h (Fig. 6A). It was found that these mutants, except for α323R, H327E, and I335N, had higher kinase activities than wild-type α1-(1–394) by measuring the kinase activity toward AMPK peptide substrate SAMS (Fig. 6B). Interestingly, mutation of the conserved Asp-320, Leu-328, Arg-333, or Arg-334, especially the hydrophobic residue Leu-328, markedly enhanced the catalytic activity of the enzyme, indicating that the charged and hydrophobic residues in 320–335 of the α1 subunit might play important roles for the autoinhibition.

**Construction of a Structural Model for Human AMPK α1 Subunit, Including the Kinase Domain and Autoinhibitory Domain**—Recently, the homologous yeast Snf1 kinase domain was crystallized, and the structure was determined by x-ray crystallography. It provided an opportunity to model the kinase domain of human AMPKα1 as described under “Experimental Procedures.” The secondary structure prediction of residues 280–335 suggested that this segment has several α-helix structures. Then a structural homology search was conducted using the model of the AMPK kinase domain (residues 1–280) against the AMPK-related kinases in the Protein Data Bank. It was found that the structure of human MARK2 (Protein Data Bank code 1Y8G) contained a similar scaffold to the kinase catalytic domain and also contained a three-α-helix UBA domain. This is consistent with other studies, such as the UBA domain of HHR23A. Then the structure of residues 290–335 of AMPKα1 was constructed based on the MARK2 UBA domain and presented in Fig. 7. We modeled two structures rather than one partly due to the sequence conservation and the lack of the α1-(280–290) homologous region in MARK2. To form a com-

---

The text above contains a detailed analysis of AMPK autoinhibition, focusing on the α1 subunit. It discusses the purification and identification of various truncations and deletions of the AMPKα1 subunit. The study examines the effect of deletion of residues 311–333 on α2-(1–398) activity, finding that it activates the enzyme and increases kinase activity. The α-helix responsible for AMPK autoinhibition is identified, with a focus on residues 320–335, which are critical for enzyme activity. The construction of a structural model for human AMPKα1, including the kinase domain and autoinhibitory domain, is also discussed.
plex with the kinase domain and autoinhibitory domain of AMPK \(\alpha1-(1–335)\), the two built models were adapted and superimposed on the structure of MARK2. Similar to the MARK2 protein, the autoinhibitory sequence (residues 290–335) was formed by three \(\alpha\)-helices and located on the backside of the N-terminal lobe of the kinase catalytic domain. Due to the impact of residue Leu-328 of the human AMPK \(\alpha1\) catalytic subunit on the autoinhibitory function and its conservation in most of AMPK-related kinases (36), its location was assessed by our complex model of AMPK \(\alpha1\). As Fig. 8 shows, Leu-328 might interact with Val-298, which is located in the noncatalytic domain according to their distance of around 4.3 Å between the first helix and the second helix of the AID. In order to determine whether the Val-298 amino acid interacts with Leu-328 to play a similar role for autoinhibition, Val-298 was mutated to glycine in \(\alpha1-(1–394)\). It was confirmed that V298G weakened the autoinhibition by enhancing the enzyme activity, similar to that of L328Q (Fig. 8). Another two mutants were also suggested by the complex model, being residues located on the interface of kinase domain and autoinhibitory domain. However, the point mutation results showed that these two mutants had only low levels of kinase activity similar to that of wild-type \(\alpha1-(1–394)\), which indicates that Leu-39 and Tyr-82 are not involved in the interactions between the kinase domain and the AID (Fig. 8).

**Overexpression of \(\alpha1-(1–394, \Delta313–335)\) (\(\Delta\alpha394\)), V298G, or L328Q in COS7 Cells Stimulates ACC Phosphorylation**—We then investigated whether the constitutively active \(\Delta\alpha394\), V298G, and L328Q indeed had higher kinase activities similar to that of wild-type \(\alpha1-(1–394)\), which indicates that Leu-39 and Tyr-82 are not involved in the interactions between the kinase domain and the AID (Fig. 8).

![Activities of the truncated human AMPK \(\alpha1\) subunit proteins against the SAMS peptide](image-url)
**α-Helix Responsible for AMPK Autoinhibition**

**DISCUSSION**

Although the exact mechanism of autoinhibition in the AMPK α subunit is unclear, it is possible that the autoinhibitory domain binds to the kinase domain in an inactive conformation, with the α subunit interfering with kinase substrate binding and catalytic function. Similar autoregulation has been shown to exist in other protein kinases, such as the Ca\(^{2+}\)/calmodulin-dependent protein kinases, whose C-terminal domain binds into the catalytic cleft (46, 47), cAMP-dependent...
protein kinase, protein kinase C, mitogen-activated protein kinases (48–50), or MARK isoforms, which have an insertion of a third helix of the UBA domain through interaction with the N-terminal lobe (51). The results obtained from the present studies using truncation, deletion, and mutagenesis methods provide important information about the mechanism of AMPK\(\alpha_1\) subunit autoinhibition.

An important observation made in this study is that, in the truncated form of the human AMPK\(\alpha_1\) subunit, 1-(1–335) is sufficient to maintain an inactive, autoinhibited state, suggesting that residues 313–335 play an autoinhibitory role. This result extends the findings of previous truncation studies that had indicated a role for residues 313–392 in autoinhibition of the mammalian AMPK\(\alpha\) catalytic subunit (19, 34). The 1-(313–335) residue sequence is highly conserved in the AMPK subfamily (Fig. 1). The removal of this segment results in human AMPK\(\alpha_1\)-(1–394) or \(\alpha_2\)-(1–398) being constitutively active (Fig. 5). However, deletion of residues 313–335 in the full-length \(\alpha_1\) subunit did not fully activate the kinase domain. We speculate that the extreme C-terminal sequence 394–550 of the \(\alpha_1\) subunit might also interact with the kinase domain, resulting in a partially active conformation of \(\alpha_1\)-(1–550, 313–335), but this detailed model was not constructed. Surprisingly, the \(\alpha_1\) subunit without residues 313–335 was obtained without degradation when expressed in bacterial cells (Fig. 3). Previous studies have shown that the full-length \(\alpha_1\) monomer of mammalian AMPK, when expressed in mammalian cells, had a more rapid turnover than the \(\alpha_1\) fragment truncated to 312 or to 392, whereas binding of the \(\beta/\gamma\) regulatory subunits to the C-terminal 313–548 sequence of \(\alpha_1\) catalytic subunit stabilizes the \(\alpha_1\) subunit, resulting in increased expression and decreased turnover (34). It is possible that residues 313–548 may be targeted by cellular proteins, such as ubiquitin, for rapid degradation. We have also found this degradation of full-length \(\alpha_1\) subunit when expressed in bacterial cells lacking the ubiquitin-mediated pathway, so there may also exist an autodegradation mechanism within the cellular AMPK\(\alpha\) catalytic subunits. However, the degradation mechanism remains unclear and will be further investigated. These data demonstrate that residues 313–335 comprise the exact autoinhibitory sequence in the human AMPK\(\alpha_1\) subunit and not only are responsible for the kinase autoinhibitory function but may also be responsible for full-length kinase degradation.

Secondary structure prediction indicates that this autoinhibitory sequence region forms an \(\alpha\)-helix between residues 321 and 329. The results obtained from the present studies using truncation, deletion, and mutagenesis methods provide important information about the mechanism of AMPK\(\alpha\) subunit autoinhibition.

FIGURE 7. Structural model of human AMPK\(\alpha_1\) subunit, including the kinase domain and autoinhibitory domain constructed by utilizing the Snf1 (Protein Data Bank code 2EUE) as the structural template. A, overall view of the kinase domain shown in green and autoinhibitory domain shown in blue and two important residues, Val-298 and Leu-328, shown in red. B, the close view of the interface between kinase domain and autoinhibitory domain. The color scheme is same as in A. C, superimposition of the MARK2 (Protein Data Bank code 1Y8G, shown in yellow) and AMPK kinase domains (shown in green), which demonstrate that these two domains are very similar and conserved although AMPK was constructed from Snf1.
α-Helix Responsible for AMPK Autoinhibition

A.

1 2 3 4 5 6

---endogenous AMPKα

AMPKα

c-myc

β-Actin

Lane 1

504

Lane 2

Lane 3

Lane 4

Lane 5

Lane 6

pACC

ACC

B.

FIGURE 9. Overexpression of the deletion AMPK α mutant (Δα394), V298G, and L328Q stimulates ACC phosphorylation in COS7 cells, respectively. A. COS7 cells were transiently transfected with α1-(1–394), Δα394, V298G, or L328Q for 24 h, using empty vector of pcDNA3.1 as a control and using pEGFP-C1 to monitor transfection efficiency. The phosphorylation level of ACC, AMPK downstream substrate, was detected by immunoblotting with specific anti-phospho-ACC-Ser79 antibody. The expression levels of ACC and AMPKα, including transfected proteins were also monitored by immunoblotting with anti-ACC, anti-AMPKα, and anti-Myc antibodies, respectively. Lane 1, COS7 cells transfected with pEGFP-C1; lane 2, COS7 cells transfected with pcDNA3.1; lane 3, cells transfected with pcDNA3.1-α1 (1–394); lane 4, cells transfected with pcDNA3.1-α1-(1–394, Δα313–335); lane 5, cells transfected with pcDNA3.1-α1-(1–394, L328Q); lane 6, cells transfected with pcDNA3.1-α1-(1–394, V298G). This experiment was repeated three times with similar results. Representative immunoblots were shown. B. Immunoblots like those shown in A were scanned, quantified, and normalized by total levels and plotted. The quantified values represent the means ± S.E. of three individual experiments, where control (pEGFP-C1) is assigned a value of 1. *, p < 0.05; **, p < 0.01 compared with cells transfected with α1-(1–394).

through interaction with the first α-helix. The removal of this α-helix as an autoinhibitory sequence might alter the open (inactive) conformation into an active conformation and significantly enhance the kinase catalytic ability. It is also speculated by the structural model that this α-helix might be associated with the large lobe of the kinase domain to interfere with substrate binding. The α1-(313–473) sequence, including residues 313–335, is required for β1 binding (35). Once it is binding with the AMPK regulatory subunits β/γ to detach the autoinhibitory domain from the kinase domain, the full-length α catalytic subunit would exhibit the maximal kinase activity. Therefore, the autoinhibitory conformation of the full-length α subunit induced by the α1 (313–335) sequence will be disrupted through interaction between the AMPK regulatory subunits β/γ and the α1-(313–335) sequence. These results provide insight into the interrelationship between the autoinhibition of the AMPK α subunit and the activation of the AMPK α subunit by AMPK regulatory subunits.

The hydrophobic and charged residues in the conserved α-helix play important roles for the autoinhibitory activity of the α subunit, as indicated by the point mutation experiment. Many constitutively active mutants underscore that this conserved α-helix is the determinant of the autoinhibition (Fig. 6). Most of the mutations resulted in the generation of a modest level of the kinase activity, but mutation of the charged residues to alanine resulted in a significant increase in the kinase activity. It is possible that these charged residues are involved in mediating charge-based interactions between the autoinhibitory domain and the kinase domain and serve to anchor the α-helix in its correct position. However, this interaction was not a pseudosubstrate interaction like cAMP-dependent protein kinase, protein kinase C, or Ca2+/calmodulin-dependent protein kinase regulation (46, 52–54), because the isolated sequence α1-(313–335) did not inhibit the kinase domain activity (data not shown). Among all point mutations, the maximum activity of the L328Q mutant reaches about three-quarters the activity of α1-(1–394, Δα313–335), suggesting that the Leu-328 residue is the key residue responsible for autoinhibition of the α1 subunit activity. However, it is not responsible for full-length α1 subunit degradation due to the marked degradation of L328Q-α1-(1–550) when expressed in bacterial cells (data not shown).

From our speculative model constructed by homology modeling, it was demonstrated that Leu-328 has a hydrophobic interaction with Val-298 within the link of the kinase catalytic domain and autoinhibitory domain (Figs. 7 and 8). Through the kinase assay of the V298G mutant of AMPK α1 subunit, it strongly weakened the autoinhibition by enhancing the enzyme activity similar to that of L328Q. This reinforced that Leu-328 forms hydrophobic intra-AID interactions directly with Val-298 to further stabilize the AID internal cohesion, which retains it in the autoinhibitory conformation to interact with the kinase domain, as shown in our autoinhibitory structural model (Fig. 7). Based on the alignment of the autoinhibitory sequence (residues 313–335) of the human AMPK α1 subunit with that of AMPK-related kinases (Fig. 1), it was found the Leu-328 is one
of the most conserved residues across the whole superfamily, as was Val-298. Similar to the UBA domain of human MARK2, three conserved leucines near the end of helix α3 of UBA also play critical roles in MARK2 stability, whereas the functions of the AID in AMPK catalytic subunits are absolutely distinct from the UBA domain of AMPK-related kinases, which is required for the activation of kinases. The kinase activity assay of the mutants of L328Q and V298G shed light on the autoinhibitory mechanism and encourage us to use the complex model to assess the interacting residues in the kinase catalytic domains. Because the L328Q or V298G mutants could not fully abolish the kinase inhibition to the same extent as Δα394, it is suggested that these charged residues, such as Asp-320, Arg-333, Arg-334, or others in this α-helix, may make hydrogen bond contacts with the kinase domain, which could be concluded from the activity of the mutants in contrast to wild-type. From the superimposition of the AMPK α1 model on the MARK2 structure, it is clearly demonstrated that the residues Leu-39 and Tyr-82 are located on the interface between the kinase catalytic domain and AID and would interact strongly with the AID. Our attempts to demonstrate the anticipated interaction residues by mutation of these residues (L39G and Y82G) in the kinase domain, however, were frustrated by the kinase inhibition activities from these mutants (Fig. 8). One possibility is that these mutation sites in the kinase domain might not interact with the autoinhibitory domain. As studied by Jaleel et al. (36), small angle x-ray scattering experiments showed that the UBA domain of MARK2 was located close to the substrate binding site to inactivate the kinase. This may indicate that the conformation of the kinase domain is different in solution than in crystal lattice. Other possibilities may also exist; e.g. the mutation on these residues may destroy the integrity of the kinase domain, so the mutants could not fold properly and therefore were functionally inept. Recently, Nayak et al. (37) have reported a crystal structure of the kinase domain of inactive yeast Snf1 presented as a homodimer in solution and in yeast cells whose interface involved the kinase domain segment through hydrophobic interactions resulting in the inhibition of substrate binding. The high conservation of most of the dimer interface residues within the Snf1/AMPK and AMPK-related kinase family indicates that the kinase domain of AMPK or AMPK-related kinases would also form dimers. However, the conserved α-helix in the AID of AMPK α subunit, located at the back side of the cleftlike UBA domain of human MARK2, may not be involved in the dimer interaction. These constitutively active deletions or mutations might not disrupt the α subunit dimer conformation.

Further cellular experiments provide confirmation that the constitutively active deletion AMPK α mutant (Δα394), V298G, and L328Q function as in vitro. When transfected into COS7 cells, Δα394, V298G, and L328Q maintain constitutive activity, resulting in an increase in the phosphorylation level of the AMPK downstream substrate, ACC, compared with control, respectively (Fig. 9). When transfected into HepG2 cells, following glucose deprivation, which normally activated AMPK, Δα394, V298G, and L328Q significantly enhanced cellular glucose uptake, compared with control, respectively (Fig. 10). The above cellular results confirmed that deletion of this conserved α-helix is sufficient to keep the AMPK α catalytic subunit active, in which the conserved Leu-328 residue has strong interaction with the residue Val-298, which is outside of that autoinhibitory domain. As our speculative modeling suggested in Fig. 7, the strong hydrophobic Val-298/Leu-328 interaction may stabilize the conformation of the three α-helices in the autoinhibitory domain and may aid the interaction between the N-terminal kinase domain and the autoinhibitory domain.

In summary, we have for the first time elucidated the exact autoinhibitory sequence, a conserved α-helix in the AMPK α catalytic subunit isoforms, and our results support the autoinhibitory model we constructed, in which the autoinhibitory domain folds into an autoinhibitory conformation predominantly by hydrophobic interactions between Leu-328 and Val-298 residues or others. This special conformation will open a new regulatory mechanism for kinase autoinhibition. Furthermore, three-dimensional structural studies will hopefully reveal additional details about the AMPK autoinhibition mechanism and apply this mechanism to the autoregulation of Snf1p kinase and the regulation by the β/γ subunit.

REFERENCES

1. Hardie, D. G., and Carling, D. (1997) Eur. J. Biochem. 246, 259–273
2. Davies, S. P., Carling, D., and Hardie, D. G. (1989) Eur. J. Biochem. 186, 123–128
3. Kemp, B. E., Stapleton, D., Campbell, D. J., Chen, Z. P., Murthy, S., Walter, M., Gupta, A., Adams, I. J., Katsis, F., van Denderen, B., Jennings, I. G., Iseli, T., Michell, B. I., and Witters, L. A. (2003) Biochem. Soc. Trans. 31, 162–168
4. Winder, W. W., and Hardie, D. G. (1996) Am. J. Physiol. 270, E299–E304
5. Hutter, C. A., Hardie, D. G., and Winder, W. (1997) Am. J. Physiol. 272, E262–E266
6. Minokoshi, Y., Kim, Y. B., Peroni, O. D., Fryer, L. G., Muller, C., Carling, D., and Kahn, B. B. (2002) Nature 415, 339–343
7. Yamauchi, T., Kamon, J., Minokoshi, Y., Ito, Y., Waki, H., Uchida, S., Yamashita, S., Noda, M., Kita, S., Ueki, K., Eto, K., Akamura, Y., Froguel, P., Foufelle, F., Ferre, P., Carling, D., Kimura, S., Nagai, R., Kahn, B. B., and
