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Identification of Phosphorylation Sites in AMP-activated Protein Kinase (AMPK) for Upstream AMPK Kinases and Study of Their Roles by Site-directed Mutagenesis*

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Bacterially expressed heterotrimeric (α_1, β_1, and γ_1) wild-type, catalytically inactive, and constitutively active forms of AMP-activated protein kinase (AMPK) were used to study phosphorylation by an upstream AMPK kinase preparation. Here, we report the identification of two new phosphorylation sites in the α-subunit, *viz.* Thr^{258} and Ser^{485} (Ser^{491} in the α_2-subunit) by mass spectrometry, in addition to the previously characterized Thr^{172} site. Also, autophosphorylation sites in the β_1-subunit were identified as Ser^{96}, Ser^{101}, and Ser^{108}. Mutagenesis of Thr^{172}, Thr^{258}, and Ser^{485} to acidic residues to mimic phosphorylation in the recombinant proteins indicated that Thr^{172} was involved in AMPK activation, whereas Thr^{258} and Ser^{485} were not. Transfection of the non-phosphorylatable S485A and T258A mutants in CCL13 cells subjected to stresses known to activate AMPK either by increasing the AMP:ATP ratio (slow lysis) or without changing adenine nucleotide concentrations (hyperosmolarity) resulted in no significant differences in AMPK activation. All three sites within the α-subunit were phosphorylated *in vivo*, as seen in AMPK immunoprecipitated from anoxic rat liver. In transfected CCL13 cells, the level of Ser^{485} phosphorylation did not change upon AMPK activation. The newly identified phosphorylation sites could play a subtle role in the regulation of AMPK, *e.g.* in subcellular localization or substrate recognition.

The AMP-activated protein kinase (AMPK)[1] is a serine/threonine protein kinase that is highly conserved in higher eukaryotes, yeast, and plants (1–3). AMPK is activated by changes in the AMP:ATP ratio, although activation of AMPK has been observed in response to stimuli that do not change intracellular adenine nucleotide concentrations (4). Under stress conditions, for example, during anoxia or exercise-induced skeletal muscle contraction, the ATP:ADP ratio falls, and there is a subsequent rise in intracellular AMP (5, 6), which activates AMPK. AMPK can also be activated by hyperosmotic stress (4), leptin (7), and adiponectin (8); by the antidiabetic drugs metformin (4, 9) and rosiglitazone (4); and by the compound 5-amino-4-imidazolecarboxamide ribonucleoside, which, upon entering cells, is converted into an AMP analog (10). Once activated, AMPK acts to down-regulate ATP-consuming pathways such as fatty acid synthesis by phosphorylating and inactivating acetyl-CoA carboxylase (11) and protein synthesis by promoting the phosphorylation of eukaryotic elongation factor-2 (12). AMPK also activates pathways involved in ATP production. For example, in heart, AMPK activation stimulates glycolysis by increasing glucose uptake (13) and by activating 6-phosphofructo-2-kinase (14). In yeast, the AMPK homolog Snf1 kinase is activated by low glucose concentrations and increases the transcription of genes for growth on alternative carbon sources (2). Therefore, AMPK can be regarded as both an energy and a nutrient sensor.

AMPK is a heterotrimer consisting of a catalytic α-subunit and two regulatory subunits, β and γ. Each subunit exists as multiple isoforms (α_1, α_2, β_1, β_2, γ_1, γ_2, and γ_3), giving 12 different possible combinations of holoenzyme with different tissue distribution and subcellular localization (2, 3). AMPK is allosterically stimulated by AMP and is itself regulated by phosphorylation via an upstream AMPK kinase (AMPKK) (2, 3). The major regulatory phosphorylation site has been identified as Thr^{172} within the activation loop between the DFG and APE motifs of the α-subunits (15, 16). The upstream kinase has been partially purified from rat liver and was reported to contain an 58-kDa catalytic subunit in a 195-kDa complex (15). AMPK can also be phosphorylated at Thr^{172} by the surrogate AMPKK, Ca^{2+}/calmodulin-dependent protein kinase I (17), leading to activation (17, 18). AMP has been reported to activate AMPKK (18), although others have reported that AMPKK is constitutively active (17). Site-directed mutagenesis showed that phosphorylation at Thr^{172} accounts for most of the activation of AMPK by AMPKK, but that other sites were implicated

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[1] The abbreviations used are: AMPK, AMP-activated protein kinase; AMPKK, AMP-activated protein kinase kinase; HPLC, high pressure liquid chromatography; PP, protein phosphatase; ESI-MS/MS, electrospray ionization tandem mass spectrometry.

TABLE I
Nucleotide sequences of the primers used for unique site mutagenesis
Nucleotides in boldface indicate the mismatches causing the mutation.

Primer	Sequence
Selection primers	
*Nhe*I → *Kpn*I (elimination of a *Nhe*I site and introduction of a *Kpn*I site)	5′-CAA CGC ATA TAG CG**G** TA**C** CAG CAC GCC ATA G-3′
*Kpn*I → *Nhe*I (elimination of a *Kpn*I site and introduction of a *Nhe*I site)	5′-CAA CGC ATA TAG CG**C** TA**G** CAG CAC GCC ATA G-3′
Mutagenesis primers	
α_1-T172E	5′-GGT GAA TTT TTA AGA **GA**G AGC TGT GGC TCG CCC AAT TAT G-3′
α_1-T258D	5′-CCT ATG AAG AGG GCC **GAT** ATA AAA GAT ATC AGG-3′
α_1-S485D	5′-CCA CAG AGA TCG GGA **GA**C ATC AGC AAC TAT CG-3′

(19). The native β_1-subunit was found to be phosphorylated *in vivo* at Ser^{24}/Ser^{25}, Ser^{108}, and Ser^{182} (16). Moreover, mutation of Ser^{108} to alanine leads to the inhibition of AMPK, whereas the S24A/S25A and S182A mutants have no effect on activity, but lead to the nuclear redistribution of the holoenzyme (20). AMPK autophosphorylates mainly on the β-subunits (21), but these sites have not been identified.

Here, we have used recently available bacterially expressed AMPK heterotrimers (22) together with mass spectrometry to investigate phosphorylation of the kinase by an AMPKK preparation. By comparing the phosphorylation of the wild-type complex with that of a catalytically inactive mutant complex (D157A in the α_1-subunit) (19), we were able to distinguish between autophosphorylation sites and AMPKK phosphorylation sites. This approach allowed us to identify, in addition to Thr^{172}, two new AMPKK phosphorylation sites in the α-subunit and three autophosphorylation sites in the β_1-subunit.

EXPERIMENTAL PROCEDURES

Materials—The protease inhibitor mixture was from Sigma. Sequencing-grade trypsin and chymotrypsin were from Promega and Roche Diagnostics, respectively. All other materials were from ICN or Sigma. HPLC-grade solvents and acids were from Acros Organics. Anti-phospho-Thr^{172} α-subunit antibody was obtained from Cell Signaling Technology (Beverly, MA). Anti-phospho-Ser^{485} α-subunit antibody was kindly provided by Prof. Grahame Hardie (University of Dundee). The antibody was raised in sheep against the peptide PQRSCpSAAGLHR plus an N-terminal cysteine for coupling to keyhole limpet hemocyanin. Anti-FLAG monoclonal antibody M2 was obtained from Sigma.

Expression and Purification of Recombinant AMPK—Expression and purification of wild-type and mutant AMPKs in bacteria were carried out as described (22). The coding regions of mutants α_1-T172D and α_1-D157A were subcloned from pcDNA3 constructs (19) as described for the construction of wild-type plasmid p$\gamma_1\beta_1$His-α_1 (22). All other mutations were introduced directly into p$\gamma_1\beta_1$His-α_1 by unique site-directed mutagenesis, which allows selection by simultaneous introduction of the mutation together with a new unique restriction site (23). With the first mutation (T172E, T258D, or S485D) in wild-type p$\gamma_1\beta_1$His-α_1, the unique *Nhe*I site was exchanged with a unique *Kpn*I site, whereas the double mutant T172E/S485D was obtained from the single mutant T172E vector with *Kpn*I reverted to *Nhe*I, and the triple mutant from the double mutant vector by reverting again to *Kpn*I. Mutagenic primers were synthesized by Microsynth (Balgach, Switzerland) and are listed in Table I. The complete AMPK coding region of all plasmids was sequenced to verify the newly introduced mutations and the absence of random mutations utilizing the BigDye™ terminator cycle sequencing kit (PE Applied Biosystems, Rotkreuz, Switzerland) in an Applied Biosystems Prism 310 Sequencer according to the manufacturer's instructions.

Phosphorylation of Bacterially Expressed AMPK Heterotrimers by AMPKK—Purified recombinant AMPK (2–50 μg) was incubated in the presence or absence of a preparation of AMPKK (0.8–10 μg) partially purified from rat liver up to the Q-Sepharose step as described previously (15). The incubations (20 μl) contained 5 mM $MgCl_2$ in 50 mM Hepes (pH 7.4) and 0.1 mM [γ-^{32}P]ATP (1000 cpm/pmol) with or without 0.2 mM AMP and were conducted for 30 min at 37 °C. Following dilution, AMPK was repurified by binding to nickel-nitrilotriacetic acid magnetic agarose beads (QIAGEN Inc.) that were washed with buffer containing 50 mM Hepes (pH 7.4), 150 mM NaCl, 500 mM sucrose, 0.5% (v/v) Triton X-100, and 25 mM imidazole. SDS sample buffer was added directly to the beads, and the mixture was subjected to SDS-PAGE in gels containing 10% (w/v) acrylamide.

Treatment of AMPK from Anoxic Liver with Protein Phosphatases—AMPK purified from anoxic rat liver up to the DEAE-Sepharose step as described previously (24) was dialyzed against buffer without phosphatase inhibitors (50 mM Tris (pH 7.4), 1 mM EDTA, 10% (v/v) glycerol, and 1 mM dithiothreitol). $MgCl_2$ was added to a final concentration of 10 mM, and a mixture of protein phosphatase (PP)-1 and PP2A purified from bovine heart as described previously for rabbit muscle (25) was added to a final concentration of 10 milliunits/ml. Alternatively, recombinant PP2C (a generous gift from Dr. R. Beri) (26) was added at a final concentration of 0.16 mg/ml. Samples were incubated for 20 min at 37 °C before addition of SDS sample buffer.

Phosphorylation Site Identification by Mass Spectrometry and Edman Sequencing—Phosphorylated bands corresponding to the α-subunit (63 kDa) and β-subunit (40 kDa) were cut from Coomassie Blue-stained gels and concentrated in 2% (w/v) agarose in Pasteur pipettes (27, 28). The phosphorylated proteins were melted out of the agarose and digested with 1 μg of sequencing-grade trypsin or chymotrypsin as described (27). Peptides were separated by reverse-phase narrow-bore HPLC at a flow rate of 200 μl/min (27). Radioactive peaks were analyzed (29) by nanoelectrospray ionization tandem mass spectrometry (nano-ESI-MS/MS) in an LCQ Deca XP Plus ion-trap mass spectrometer (ThermoFinnigan, San Jose, CA). Spectra were taken in full MS and Zoom scan mode to determine parent ion monoisotopic masses and their charge states. The source voltage was set at 0.8 kV, and the collision-induced dissociation energy was adjusted to the minimum needed for fragmentation. Phosphopeptides were identified in MS^2 mode by the loss of H_3PO_4 (98 Da) under low collision-induced dissociation energy, and the phosphorylated residue was pinpointed in MS^3 mode. In some cases, radioactive peaks were further purified for Edman sequencing (30).

For *in vivo* phosphorylation site analysis, AMPK was immunoprecipitated from anoxic rat liver with anti-α_1- or α_2-AMPK antibodies (31) and subjected to electrophoresis in SDS gels containing 10% (w/v) acrylamide. Protein bands were excised from the gel, concentrated, and digested as described above for on-line capillary HPLC-ESI-MS/MS. Peptides were separated by reverse-phase HPLC on a C_{18} PepMap capillary column (0.3 mm × 25 cm; LC Packings) as described (29). For phosphorylation site mapping, the mass spectrometer was operated in fully automated mode with an ion source at 4.8 kV using a data-dependent acquisition routine. This routine consisted of a full MS scan, followed by a high resolution Zoom scan and a full MS/MS scan of the most intense ion selected from the previous MS spectrum. Normalized collision-induced dissociation energy was set at 28%, and dynamic exclusion ensured collection of data on coeluting peptides. After data acquisition, a specific ion chromatogram based on the phosphopeptide masses observed *in vitro* was extracted from the total ion chromatogram. If a peak was observed in this chromatogram, the identity of the phosphopeptide was confirmed in the Zoom and MS/MS scans.

Immunoblotting—The amounts of protein indicated in the figure legends were separated by SDS-PAGE in 12% (w/v) polyacrylamide and transferred onto a polyvinylidene fluoride membrane. The membrane was blocked in Tris-buffered saline (10 mM Tris-HCl (pH 7.4) and 0.5 M NaCl) with 5% (w/v) low fat milk powder. The membrane was incubated with various dilutions of primary antibody in the same buffer for 2–16 h. After extensive washing with Tris-buffered saline, the membrane was incubated for 1 h at room temperature with the appropriate secondary antibody coupled to horseradish peroxidase. After further extensive washing with Tris-buffered saline, the blots were developed

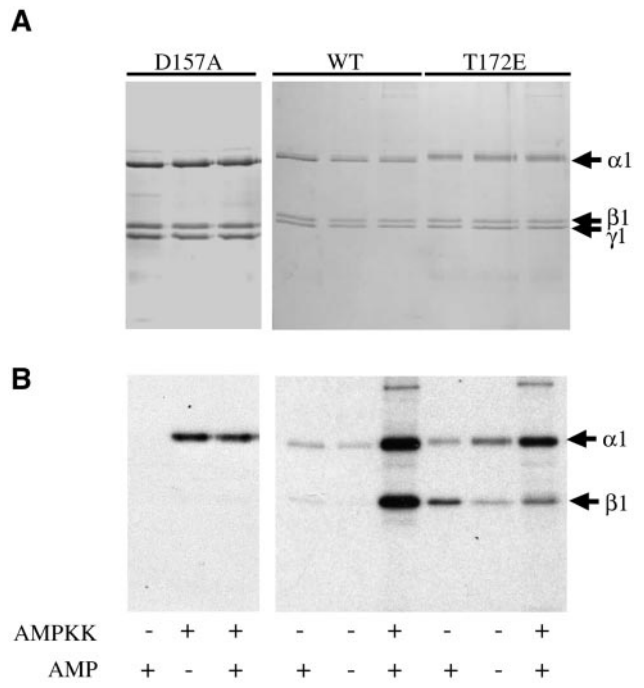

FIG. 1. **AMPKK phosphorylation of bacterially expressed AMPK complexes.** Purified bacterially expressed AMPK complexes (2 μg; wild-type (*WT*) $\alpha_1\beta_1\gamma_1$, catalytically inactive mutant α_1(D157A)$\beta_1\gamma_1$, or constitutively active mutant α_1(T172E)$\beta_1\gamma_1$) were incubated in the presence or absence of partially purified rat liver AMPKK (0.8 μg) with or without 0.2 mM AMP and [γ-^{32}P]ATP. A Coomassie Blue-stained gel of the complexes after repurification on nickel-agarose (*A*) and the corresponding autoradiogram (*B*) are shown.

using enhanced chemiluminescence (Amersham Biosciences). For quantitation of AMPK phosphorylation, the blots were visualized by enhanced chemiluminescence using a 16-bit CCD cooled camera (Gene Gnome, Syngene, Cambridge, UK). Quantification was achieved using GeneTools software (Syngene).

Activation of Bacterially Expressed AMPK—Purified recombinant AMPK (0.1 μg) was activated with the preparation of AMPKK (0.8 μg) in buffer consisting of 5 mM $MgCl_2$ in 50 mM Hepes (pH 7.4) and 0.1 mM ATP for 30 min at 37 °C (in the presence or absence of 0.2 mM AMP, as described in the figure legends). Samples were diluted in assay buffer (50 mM Hepes (pH 7.4), 1 mM EDTA, and 10% (v/v) glycerol) and assayed for AMPK activity by phosphorylation of the SAMS peptide (32) using 0.2 mM [γ-^{32}P]MgATP (specific radioactivity of 200 cpm/pmol). Partially purified AMPK from anoxic rat liver purified up to the DEAE-Sepharose step as described previously (24) was also assayed by phosphorylation of the SAMS peptide.

Transient Transfection of CCL13 Cells and Measurement of AMPK Activity—Plasmid DNA was prepared using a QIAGEN maxiprep kit according to the manufacturer's instructions. CCL13 cells were cotransfected with cDNA (10 μg of plasmid) encoding the wild-type α_1-subunit, S485A, or T258A and the β_1- and γ_1-subunits (all cDNAs cloned into pcDNA3) by calcium phosphate precipitation (33). The α_1-subunit cDNAs encode an N-terminal Myc epitope tag (EQKLISEEDL) (34), and the γ_1-subunit cDNA encodes an N-terminal FLAG epitope (DYKDDDDK). Cells were harvested 72 h post-transfection by one of two methods (rapid or slow lysis) (35). AMPK activity was determined in immune complexes isolated by immunoprecipitation from cell lysates using anti-Myc monoclonal antibody (clone 9E10) (34). The lysate volume was normalized according to the amount of expression of the γ-subunit, as assessed by quantitation of blots probed with anti-FLAG antibody. Cell lysates were incubated for 2 h at 4 °C with anti-Myc antibody bound to protein G-Sepharose. Immune complexes were harvested by centrifugation at 6000 × *g* for 1 min and washed with lysis buffer prior to assay. AMPK activity was measured as described above. Activities are expressed as nanomoles of phosphate incorporated into the SAMS peptide/min/ml of cell lysate.

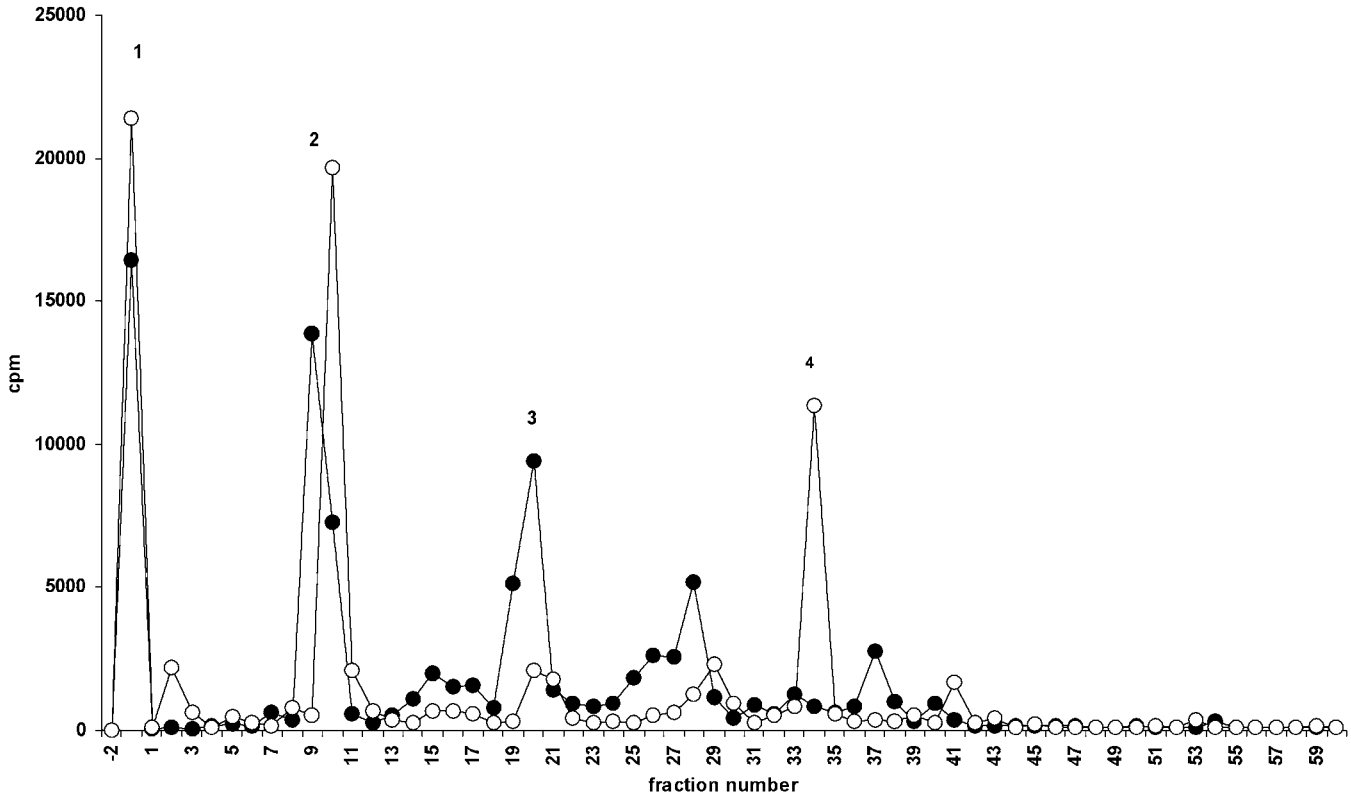

FIG. 2. **HPLC profiles of ^{32}P-labeled peaks from the α_1-subunits phosphorylated by AMPKK and [γ-^{32}P]ATP and digested with trypsin.** Bacterially expressed wild-type (*open circles*) and catalytically inactive (*closed circles*) AMPK heterotrimers were phosphorylated *in vitro* by AMPKK. The α_1-subunits were cut from an SDS-acrylamide gel, concentrated on agarose, and digested with trypsin. Peptides were separated by reverse-phase narrow-bore HPLC in a linear acetonitrile gradient. The *numbered peaks* are discussed under "Results," and phosphopeptide amino acid sequences are summarized in Table II.

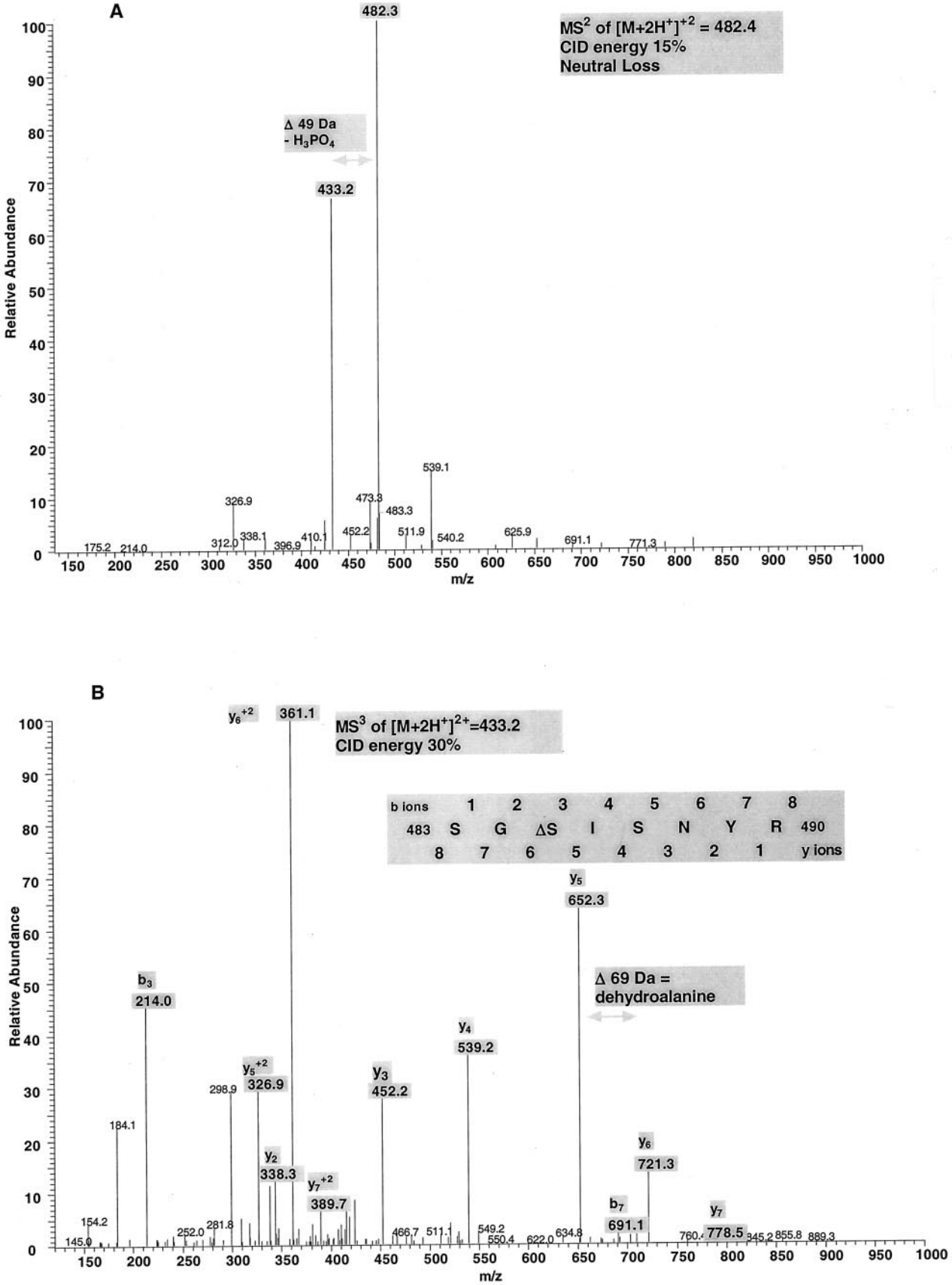

FIG. 3. **Identification of Ser^{485} as an *in vitro* phosphorylation site in the α_1-subunits by nano-ESI-MS/MS.** A major ^{32}P-labeled peak (peak 2 in Fig. 2) from the tryptic digest of AMPK α_1-subunits phosphorylated by AMPKK was analyzed by nano-ESI-MS/MS and found to contain one phosphopeptide ion (P1 in Table II). *A*, the MS^2 spectrum of the double-charged phosphopeptide ion (*m/z* 482.4) is shown. A loss of 98 Da was observed (H_3PO_4) under low collision-induced dissociation (*CID*) energy to produce an ion at *m/z* 433.2. *B*, the MS^3 spectrum of the *m/z* 433.2 daughter ion is shown. The y_5 and y_6 fragments have a mass difference of 69 Da, corresponding to dehydroalanine, identifying the product of phosphoserine after losing H_3PO_4. Δ*S* denotes dehydroalanine, and *b* and *y* refer to ions containing the N- or C-terminal end of the peptide, respectively.

TABLE II
Sequences of AMPK phosphopeptides determined by ESI-MS/MS

After *in vitro* phosphorylation or immunoprecipitation from anoxic rat liver AMPK (*in vivo*), AMPK preparations were submitted to tryptic or chymotryptic digestion as described under "Experimental Procedures." *In vitro* or *in vivo* sites were analyzed by nano-ESI-MS/MS or by on-line capillary HPLC-ESI-MS/MS, respectively. Phosphopeptides were indentified by loss of 98 Da upon collision-induced dissociation, and the phosphorylated residue was further identified by fragmentation in MS^3 mode. ND, not detected; c, acrylamide-modified cysteine residue; p, phosphorylated residue.

Phosphopeptide	Measured average masses [M + H^+]		Theoretical average masses [M + H^+]	Sequence
	In vitro sites	*In vivo* sites		
P1 (α_1)	963.3	963.8	963.9	^{483}SGpSISNYR^{490}
P2 (α_2)	1305.5	1305.8	1306.4	^{489}ScpSAAGLHRPR^{499}
P3 (α_1/α_2)	1860.2	ND	1860.9	^{172}pTScGSPNYAAPEVISGR^{188}
P4 (α_1/α_2)	3745.9	ND	3746.1	^{155}IADFGLSNMMSDGEFLRpTScGSPNYAAPEVISGR^{188}
P5 (α_1/α_2)	3353.2	3353.9	3353.8	^{250}QVDPMKRApTIKDIREHEWFKQDLPKY^{275}
P6 (β_1)	637.1	ND	637.7	^{101}pSKLPL^{105}
P7 (β_1)	1485.6	ND	1485.6	^{101}SKLPLTRpSQNNF^{112}
P8 (β_1)	1004.2	ND	1004.9	^{93}LSGpSFNNW^{100}
P9 (β_1)	946.4	947.2	946.8	^{106}TRpSQNNF^{112}

RESULTS

Phosphorylation of Recombinant AMPK—Phosphorylation of AMPK by both autophosphorylation and AMPKK was investigated by [^{32}P]phosphate incorporation from [γ-^{32}P]ATP into the α_1-, β_1-, and γ_1-subunits of wild-type, catalytically inactive (α_1-D157A), and constitutively active (α_1-T172E) AMPKs. Fig. 1*A* shows the Coomassie Blue-stained gel, and Fig. 1*B* shows the corresponding autoradiogram of the repurified AMPK complexes. That the D157A mutant was catalytically inactive was confirmed by the fact that there was no phosphorylation of its AMPK subunits in the absence of AMPKK. Incubation of the D157A mutant with AMPKK led to phosphorylation of the α-subunit, which was apparently insensitive to AMP (see below). For the D157A mutant, there was no phosphorylation of the β-subunits, even in the presence of AMPKK, suggesting that phosphorylation of the β-subunits represents autophosphorylation. Phosphorylation of the wild-type AMPK complex was very low in the absence of AMPKK. In the presence of AMPKK, both the α- and β-subunits were phosphorylated. The T172E mutant underwent autophosphorylation of the α-subunit and, to a lesser extent, of the β-subunit in the absence of AMPKK and AMP. However, autophosphorylation of the β-subunit was enhanced in the presence of AMP, reflecting an increased enzymatic activity of the T172E mutant. The α-subunit of the T172E mutant was further phosphorylated by AMPKK, implying the existence of additional sites on the α-subunit for AMPKK other than Thr^{172}. However, β-subunit phosphorylation remained unchanged. The stoichiometry of phosphorylation in all cases was rather low. For example, for phosphorylation by AMPKK, ^{32}P incorporation was ~0.5 mol of phosphate/mol of D157A mutant. The reason for such a low incorporation is not clear.

Identification of Phosphorylation Sites for AMPKK in the AMPK α-Subunits—Bacterially expressed wild-type and catalytically inactive (α_1-D157A) AMPK heterotrimers were incubated with purified AMPKK and [γ-^{32}P]ATP as described above. The α_1- and α_2-subunits were separated by SDS-PAGE and concentrated in agarose for digestion with trypsin or chymotrypsin. The resulting peptides were separated by reverse-phase HPLC. Tryptic digestion of the α_1- and α_2-subunits gave rise to four major radiolabeled peaks (labeled *1–4* in Fig. 2), with peak 1 appearing in the flow-through fractions. Chymotryptic digestion gave rise to two major radioactive peaks (data not shown). These peaks were absent when AMPK was incubated without AMPKK and therefore represent sites for the upstream kinase rather than autophosphorylation. No differences in the HPLC profiles were observed between wild-type and catalytically inactive AMPKs (Fig. 2), although some minor

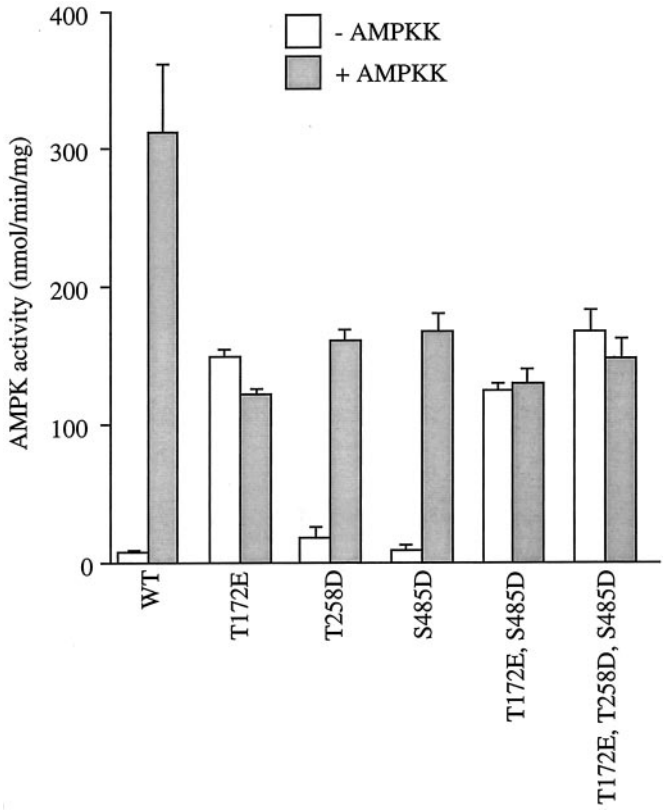

FIG. 4. **Activity measurements of mutant AMPK complexes.** Purified bacterially expressed AMPK complexes (0.1 μg) were incubated in the presence or absence of AMPKK (0.8 μg) prior to the measurement of AMPK activity by ^{32}P incorporation into the SAMS peptide. The results are the means ± S.E. from four independent experiments. *WT*, wild-type AMPK.

radioactive peaks were absent in the latter and probably contain autophosphorylation sites. Similar results were obtained in six separate experiments with different preparations of both AMPK and AMPKK, except that the distribution and extent of radioactivity between peaks were somewhat variable. Each radioactive fraction was screened for phosphopeptides by neutral loss of H_3PO_4 (98 Da) by nano-ESI-MS/MS. Peak 2 (Fig. 2) contained a phosphopeptide of $[M + 2H^+]^{2+} = 482.4$, whose mass decreased to 433.2 Da upon low energy collision (Fig. 3*A*), consistent with a single phosphate addition to a predicted tryptic fragment of the α_1-subunit (P1 in Table II). The difference of 98 Da corresponds to the loss of H_3PO_4 via β-elimina-

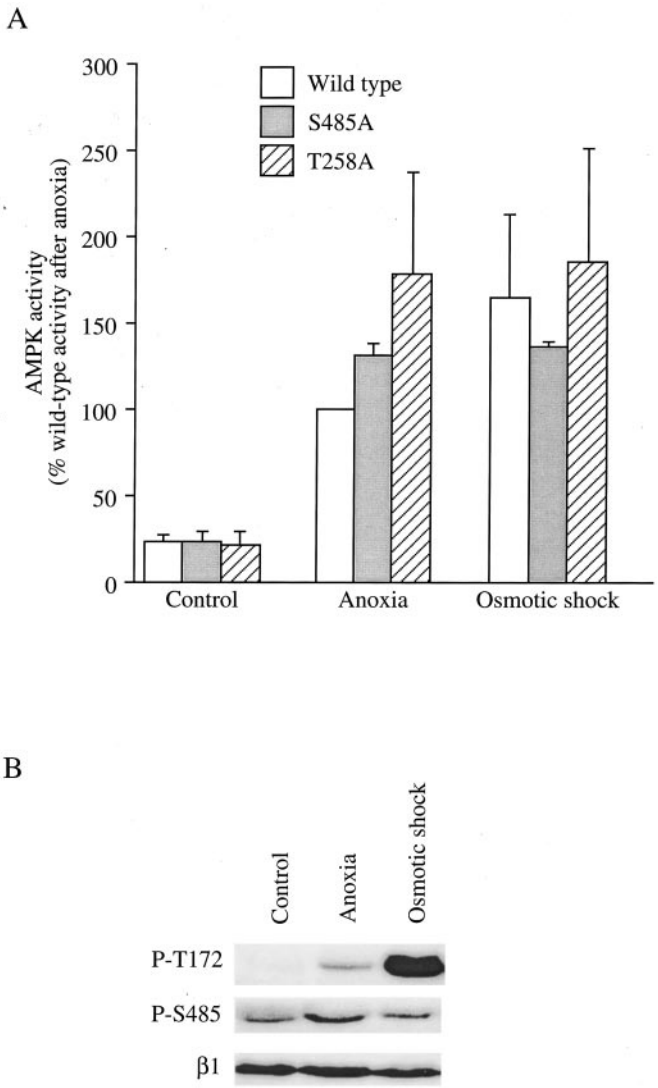

FIG. 5. **Effect of hyperosmotic stress or anoxia on the activity and phosphorylation of α_1-subunit complexes overexpressed in mammalian cells.** *A*, the wild-type and mutant α_1-subunit isoforms were coexpressed with the β_1- and γ_1-subunits in CCL13 cells. At 48 h post-transfection, cells were incubated in the absence (*Control*) or presence of 0.5 M sorbitol (*Osmotic shock*) for 30 min prior to rapid lysis or allowed to go anoxic by slow lysis (*Anoxia*). AMPK complexes were isolated by immunoprecipitation, and activity was measured using the SAMS peptide. Activities are shown as a percentage of the activity measured in the wild-type complex after anoxia. Analysis of the individual non-normalized data indicated that the extent of activation of AMPK of the T258A and S485A mutants by anoxia or osmotic stress was not significantly different from the control. The results are the means ± S.E. from four independent experiments. Immunoblotting confirmed that each complex was similarly expressed. *B*, immunoblots of the cell lysates probed with anti-phospho-Thr^{172} (*P-T172*) or anti-phospho-Ser^{485} (*P-S485*) antibody or anti-β_1-subunit antibody as a loading control are shown.

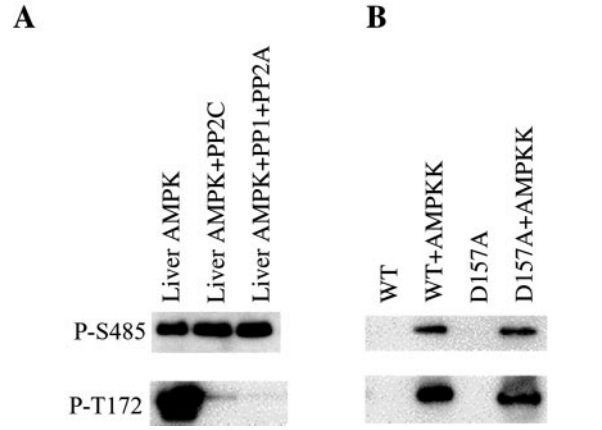

FIG. 6. **Phosphorylated Ser^{485} is resistant to protein phosphatases.** *A*, AMPK from anoxic rat liver was treated with either PP2C or a mixture of PP1 and PP2A for immunoblotting with anti-phospho-Thr^{172} (*P-T172*) or anti-phospho-Ser^{485} (*P-S485*) antibody. *B*, wild-type (*WT*) or catalytically inactive (D157A) AMPK complexes expressed in bacteria were incubated in the presence or absence of AMPKK prior to immunoblotting with anti-phospho-Thr^{172} or anti-phospho-Ser^{485} antibody. The results from a representative experiment are presented.

tion, leaving dehydroalanine in place of the phosphorylated serine residue (36). When the ion at *m/z* 433.2 was fragmented in MS^3 mode, the sequence of the tryptic fragment was confirmed, and the phosphorylated residue was identified as Ser^{485} (Fig. 3*B*). The equivalent residue in the α_2-subunit (Ser^{491}) was also found to be phosphorylated (P2 in Table II). Phosphorylation of Ser^{485} was also confirmed by Edman sequencing.

Peak 3 (Fig. 2) contained a phosphopeptide of $[M + 2H^+]^{2+}$ = 930.6, which lost 98 Da upon low energy collision to give a product ion of *m/z* 881.6 (data not shown). This ion was further fragmented, allowing us to identify Thr^{172} as the phosphorylated residue (P3 in Table II). Another peptide, presumably containing phosphorylated Thr^{172}, was sometimes observed later in the HPLC gradient (peak 4 in Fig. 2) and corresponds to a missed cleavage peptide, probably due to the fact that Arg^{171} was followed by the phosphorylated Thr^{172} (P4 in Table II). Peak 1 (Fig. 2) in the HPLC flow-through fractions was not seen when the α_1- or α_2-subunit bands were digested with chymotrypsin and therefore cannot be explained by radioactive phosphate lost by hydrolysis. A radioactive fraction from a chymotryptic digest gave a phosphopeptide of $[M + 2H^+]^{2+}$ = 1677.8, whose mass decreased by 98 Da upon low energy collision. This mass was assigned to a monophosphorylated peptide in the α-subunits (P5 in Table II) that contains two potential phosphorylated residues (Thr^{258} and Tyr^{275}) and two missed cleavages. Fragmentation in the mass spectrometer could not unambiguously pinpoint the modified residue. However, Thr^{258} resides in a small tryptic peptide (^{257}ATIK^{260}) that would not be expected to be retained on the C_{18} column, thus explaining the presence of peak 1 in Fig. 2. Therefore, Thr^{258} is most likely to be the phosphorylated residue in peptide P5.

Identification of Phosphorylation Sites in the AMPK β-Subunits—Bacterially expressed wild-type AMPK heterotrimer was phosphorylated, and the β_1-subunit was purified and digested as described above. Chymotryptic digestion gave rise to four major radiolabeled peaks containing phosphopeptides P6–P9 in Table II. These peaks were not observed with catalytically inactive AMPK with or without AMPKK, suggesting that they represent autophosphorylation. Peak 1 contained one phosphopeptide of $[M + H^+]$ = 637.1, consistent with a single phosphorylated residue in a β_1-subunit chymotryptic peptide (P6 in Table II). Fragmentation confirmed the sequence and identified Ser^{101} as the modified residue (data not shown). Peak 2 contained one phosphopeptide of $[M + 2H^+]^{2+}$ = 743.3. This mass can be assigned to a monophosphorylated peptide in the β_1-subunit (P7 in Table II) that contains three potential phosphorylated residues and one missed cleavage. Fragmentation led to the identification of Ser^{108} as the phosphorylated residue. Although peptide P7 contains both Ser^{101} and Ser^{108}, no double phosphorylated ions could be detected in this peak. Peak 3 contained an ion of $[M + 2H^+]^{2+}$ = 502.6, which lost H_3PO_4 upon low energy collision. This mass can be assigned to a phosphorylated peptide in the β_1-subunit (P8 in Table II) that contains two serine residues and two missed cleavages. Fragmentation identified Ser^{96} as the phosphorylated residue. An additional peak (peak 4) was sometimes observed earlier in the

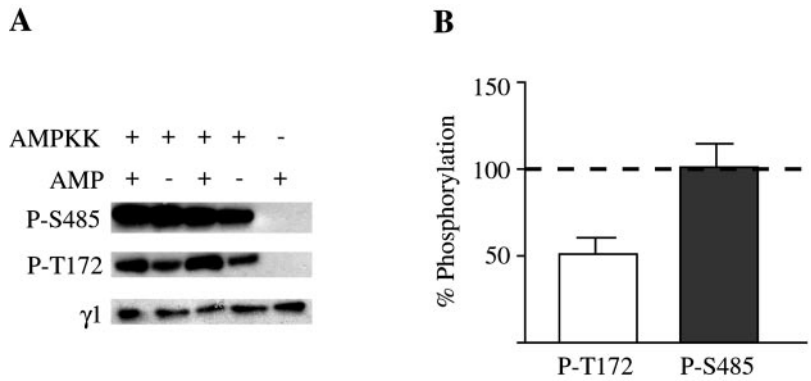

FIG. 7. **Effect of AMP on the phosphorylation of AMPK by AMPKK as assessed by immunoblotting with phospho-specific antibodies.** The α_1(D157A)$\beta_1\gamma_1$ complex was incubated in the presence or absence of AMPKK with or without 0.2 mM AMP for 30 min at 37 °C before adding SDS sample buffer. The samples were analyzed by immunoblotting, and the *upper blots* were probed with either anti-phospho-Thr^{172} (*P-T172*) or anti-phospho-Ser^{485} (*P-S485*) antibody. The *lower blot* was probed with anti-γ_1-subunit antibody as a control for protein loading. *A* shows a typical blot, and *B* shows the quantitation of four independent blots by direct measurement of chemiluminescence using a 16-bit CCD cooled camera. The results are the means ± S.E. and are plotted as a percentage of the value in the presence of AMP.

TABLE III
Alignment of sequences surrounding phosphorylation sites in the AMPK α- and β_1-subunits
In the Thr^{258}/Ser^{485}/Ser^{491} consensus, **B** represents a basic residue, and ***ϕ*** represents a hydrophobic residue.

		P		**B**				***ϕ***			***ϕ***	**B**			Consensus
		−5		**−3**				**+1**			**+4**	**+5**			
S	T	**P**	Q	**R**	S	C	**S**	**A**	A	G	**L**	**H**	R	P	Ser^{491} α_2
A	T	**P**	Q	**R**	S	G	**S**	**I**	S	N	**Y**	**R**	S	C	Ser^{485} α_1
V	D	**P**	L	**K**	R	A	**T**	**I**	K	D	**I**	**R**	E	H	Thr^{258} α_2
V	D	**P**	M	**K**	R	A	**T**	**I**	K	D	**I**	**R**	E	H	Thr^{258} α_1
S	D	G	E	F	L	R	**T**	S	C	G	S	P	N	Y	Thr^{172} α_1/α_2
S	G	S	F	N	N	W	**S**	K	L	P	L	T	R	S	Ser^{101} β_1
S	K	L	P	L	T	R	**S**	Q	N	N	F	V	A	I	Ser^{108} β_1
K	E	V	Y	L	S	G	**S**	F	N	N	W	S	K	L	Ser^{96} β_1

HPLC profile. It contained phosphorylated Ser^{108} in a peptide with no missed cleavage (P9 in Table II).

Identification of in Vivo Phosphorylation Sites in Immunoprecipitated AMPK—To determine whether the AMPK phosphorylation sites identified *in vitro* could also be detected *in vivo*, we analyzed AMPK α_1- or α_2-subunit complexes immunoprecipitated from anoxic rat liver by on-line capillary liquid chromatography-ESI-MS/MS. This allowed us to confirm that Ser^{485}, Ser^{491}, and Thr^{258} in the AMPK α-subunits and Ser^{108} in the β-subunits were phosphorylated *in vivo* (Table II). However, the remaining β-subunit sites identified *in vitro* or those previously described in the literature (16) were not detected, perhaps due to poor ionization/ion suppression of the phosphorylated peptides. Alternatively, metabolic conditions may not have been such that phosphorylation at these sites could occur.

Effect of Mutation of Phosphorylated Residues on AMPK Activity, Activation by AMPKK, and AMP Sensitivity—Recombinant AMPK has similar properties to those of the native holoenzyme purified from rat liver (22) and is therefore suitable to analyze the effects of phosphorylation. The AMPK activities of various bacterially expressed α_1-subunit phosphorylation site mutants were studied both after autophosphorylation and after phosphorylation by AMPKK. Fig. 4 shows that the wild-type AMPK complex was essentially inactive against the SAMS peptide, unless phosphorylated by AMPKK or mutated to mimic phosphorylation at Thr^{172} by introduction of a glutamate or aspartate residue. Similar mutations of Thr^{258} and Ser^{485} to aspartate yielded inactive enzymes, unless phosphorylated by AMPKK. Thus, only mutation of Thr^{172} to glutamate or aspartate (data not shown) caused constitutive activation, as this activity was not further increased following phosphorylation by AMPKK. The T172E mutant was less active than wild-type AMPK that had been activated with AMPKK, presumably indicating that the glutamate residue only partially mimics phosphorylation. The T172E/S485D double mutant and the T172E/T258D/S485D triple mutant were not significantly more active than the T172E single mutant and were not further activated upon phosphorylation by AMPKK. Taken together, these results confirm that T172 phosphorylation is important for activation and that phosphorylation at Ser^{485} or Thr^{258} does not lead to a significant further activation.

The AMPK activities of the T172E, T172E/S485D, and T172E/T258D/S485D mutants were assessed in the presence of varying AMP concentrations. The half-maximal stimulation of the T172E-containing complex was 55 ± 15.6 μM. This value is similar to the previously reported value for the T172D mutant expressed in mammalian cells (19). Introduction of the additional phosphorylation site mutations S485D and T258D did not significantly change the value for half-maximal stimulation (data not shown).

Transient Transfection of Phosphorylation Site Mutants in CCL13 Cells—Activation of AMPK is known to occur via both an AMP-dependent mechanism (*e.g.* anoxia) and an AMP-independent mechanism (*e.g.* hyperosmotic shock). To determine whether Ser^{485} and Thr^{258} are important for activation by either of these routes, AMPK activity was measured in cells transiently transfected with AMPK mutants with an alanine substitution at these sites. After 48 h of transfection, cells were rapidly lysed (control) and treated in such a way as to cause the cells to become either anoxic (slow lysis) or osmotically stressed (sorbitol treatment). The extent of AMPK activation of the non-phosphorylatable S485A or T258A mutant by either anoxia or osmotic shock was not different from that of the wild type (Fig. 5, *upper panel*).

Cell lysates were blotted with either anti-phospho-Thr^{172} or anti-phospho-Ser^{485} antibody. Unfortunately, attempts to raise

antibodies against phospho-Thr^{258} were unsuccessful. The extent of phosphorylation of Thr^{172} reflected the degree of AMPK activation, regardless of whether AMPK was activated via raised AMP levels (slow lysis) or otherwise (sorbitol) (Fig. 5, *lower panel*). However, in this system, Ser^{485} was phosphorylated even when AMPK was relatively inactive in the unstressed control cells. Moreover, the extent of phosphorylation of Ser^{485} was unchanged in anoxia and appeared to decrease under osmotic stress (Fig. 5, *lower panel*), suggesting that Ser^{485} is constitutively phosphorylated and has no direct effect on AMPK activation.

Active AMPK from anoxic rat livers was treated with either PP2C or a mixture of PP1 and PP2A, resulting in a >95% loss of activity (data not shown). The samples were then blotted using phospho-specific antibodies (Fig. 6*A*). Treatment with PP2C or PP1/PP2A completely abolished the signal detected using anti-phospho-Thr^{172} antibody. However, there was no significant change in the signal detected with anti-phospho-Ser^{485} antibody, indicating that this site is resistant to dephosphorylation by the protein phosphatases used. To test whether anti-phospho-Ser^{485} antibody was contaminated with antibodies that recognize non-phosphorylated Ser^{485}, bacterially expressed wild-type and D157A complexes were treated with AMPKK and immunoblotted with anti-phospho-Ser^{485} antibody. Anti-phospho-Ser^{485} antibody recognized only these proteins that were treated with AMPKK (Fig. 6*B*) at the concentrations used here. Taken together, these results suggest that AMPK is constitutively phosphorylated at Ser^{485} and that once phosphorylated, this residue is resistant to dephosphorylation *in vitro*.

Since AMPKK phosphorylates multiple sites within the α-subunit, we decided to re-examine the effect of AMP on the phosphorylation of individual sites. This was made possible by the availability of antibodies specific for phospho-Thr^{172} and phospho-Ser^{485}. Fig. 7 shows that phosphorylation of Thr^{172} was stimulated ~2-fold by 0.2 mM AMP (by direct measurement of chemiluminescence using a 16-bit CCD cooled camera), whereas phosphorylation of Ser^{485} was unaffected by AMP. The reason why we did not detect an effect of AMP on the phosphorylation by AMPKK as assessed by [^{32}P]phosphate incorporation (Fig. 1) could have been due to the fact that only a small part of the overall phosphorylation is AMP-sensitive.

DISCUSSION

Expression of AMPK in bacteria provides access to large quantities of heterotrimeric enzyme of defined subunit composition (22). Since the recombinant enzyme is inactive and probably exists in an entirely unphosphorylated form, it is an especially useful tool to study phosphorylation. Here, we investigated the phosphorylation of bacterially expressed recombinant wild-type, catalytically inactive, and constitutively active forms of AMPK by an upstream AMPKK preparation. This approach allowed us to identify two new AMPKK-specific phosphorylation sites in the α-subunit and their initial characterization.

In addition to Thr^{172}, the new AMPKK phosphorylation sites in the α-subunits were identified as Thr^{258} and Ser^{485} (α_1)/Ser^{491} (α_2) both *in vitro* and *in vivo*. Inspection of the sequences surrounding Thr^{258}, Ser^{485}, and Ser^{491} revealed a similar consensus not found around Thr^{172} (Table III). This suggests that Thr^{258} and Ser^{485}/Ser^{491} could be phosphorylated by the same AMPKK that would be distinct from the AMPKK(s) that phosphorylate Thr^{172} for activation. The existence of more than one AMPKK could also account for the AMP stimulation we observed upon phosphorylation of AMPK: a Thr^{172} AMPKK sensitive to AMP, but an AMP-insensitive Ser^{485}/Ser^{491}/Thr^{258} AMPKK. AMP could be acting in one of two ways to enhance phosphorylation of Thr^{172}. Binding of AMP to AMPK could make it a better substrate for Thr^{172} AMPKK; or alternatively, AMP could allosterically activate AMPKK directly, as previously reported (18).

Mutation of the phosphorylation sites to acidic residues to mimic phosphorylation in the bacterially expressed proteins indicated that Thr^{172} phosphorylation was sufficient for AMPK activation, but that Thr^{258} and Ser^{485} phosphorylation was not essential for activity *in vitro*. Similarly, phosphorylation at the additional sites had no influence on the AMP sensitivity of the enzyme. These conclusions are based on the presumption that a charged residue introduced at these sites mimics phosphorylation. However, following treatment with AMPKK, the activity of the wild-type complex was far greater than that of any of the constitutively active complexes. This could be accounted for if mutation of Thr^{172} to glutamate only partly mimics phosphorylation at this site. Alternatively, AMPK activation could depend on phosphorylation at sites other than those detected or on protein-protein interactions between AMPK and AMPKK.

Mutation of Thr^{258} and Ser^{485} to a non-phosphorylatable alanine residue and transfection in CCL13 cells did not significantly affect AMPK activation by AMP-dependent and AMP-independent pathways. If anything, activation of the T258A construct tended to be greater, which would be consistent with an inhibitory role of Thr^{258} phosphorylation. The role of Ser^{485}/Ser^{491} phosphorylation is unclear at present. However, Ser^{485} phosphorylation seems to be constitutive (not present in bacterially expressed kinase) and resistant to dephosphorylation by protein phosphatases. It could therefore play a structural role or be involved in intermolecular interactions. Although our data suggest that the new phosphorylation sites are not involved in activation or AMP sensitivity of AMPK, they may contribute to specificity in the increasingly complex AMPK signaling network, *e.g.* via subcellular localization or substrate recognition.

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