Post-translational modifications of the β-1 subunit of AMP-activated protein kinase affect enzyme activity and cellular localization

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The AMP-activated protein kinase (AMPK) is a ubiquitous mammalian protein kinase important in the adaptation of cells to metabolic stress. The enzyme is a heterotrimer, consisting of a catalytic α subunit and regulatory β and γ subunits, each of which is a member of a larger isoform family. The enzyme is allosterically regulated by AMP and by phosphorylation of the α subunit. The β subunit is post-translationally modified by myristoylation and multi-site phosphorylation. In the present study, we have examined the impact of post-translational modification of the β-1 subunit on enzyme activity, heterotrimer assembly and subcellular localization, using site-directed mutagenesis and expression of subunits in mammalian cells. Removal of the myristoylation site (G2A mutant) results in a 4-fold activation of the enzyme and relocalization of the β subunit from a particulate extranuclear distribution to a more homogenous cell distribution. Mutation of the serine-108 phosphorylation site to alanine is associated with enzyme inhibition, but no change in cell localization. In contrast, the phosphorylation site mutations, SS24,25AA and S182A, while having no effects on enzyme activity, are associated with nuclear redistribution of the subunit. Taken together, these results indicate that both myristoylation and phosphorylation of the β subunit of AMPK modulate enzyme activity and subunit cellular localization, increasing the complexity of AMPK regulation.

Key words: AMP-activated protein kinase, enzyme subunit structure, myristoylation and phosphorylation.

INTRODUCTION

The AMP-activated protein kinase (AMPK) is member of a metabolite-sensing protein kinase family found in all eukaryotes including plants, yeast, *Drosophila*, *Caenorhabditis elegans* and mammals [1–3]. AMPK is activated in response to exercise, ischaemia, nutrient starvation and hypoxia, and has been implicated in the regulation of several aspects of cell metabolism, including fatty acid and sterol synthesis, fatty acid oxidation, glucose transport and gene regulation [1–4]. Its substrate targets include acetyl-CoA carboxylase, 3-hydroxy-3-methylglutaryl-CoA reductase, hormone-sensitive lipase, endothelial nitric oxide synthase and malonyl-CoA decarboxylase [1–3,5,6]. Purification of pig and rat liver AMPK revealed a heterotrimeric kinase structure, consisting of a 63 kDa α catalytic subunit and non-catalytic, regulatory β (40 kDa) and γ (38 kDa) subunits [7,8]. The α catalytic subunit of AMPK is a member of the *Saccharomyces cerevisiae* SNF1 protein kinase subfamily, whereas the AMPK-γ subunit is homologous with the yeast protein, Snf4p, and the AMPK-β subunit is related to the yeast Sip1p/Sip2p/Gal83p family of proteins [9–12].

AMPK subunits have wide tissue distribution, and each is a member of an isoform family. In mammals, two α isoforms have been identified, with α-1 shown to localize in the cytoplasm of cells, whereas α-2 is found to have some nuclear localization [11,13,14]. Two isoforms of β and three isoforms of γ have also been characterized [10,12,15–17]. These isoforms interact to form different heterotrimers in various tissues. AMPK α-1 and α-2 subunits in rat liver associate with β-1 and γ-1, whereas α-2 associates with β-2 and γ-1 (or γ-3) in skeletal muscle [15]. AMPK is regulated by its own phosphorylation by an 'upstream' kinase/kinase (AMPKK). Phosphorylation occurs on the α subunit activation loop on threonine-172, and is essential for AMPK activation [1–3,18–20]. All three subunits must be expressed for full activation of the enzyme. Binding of the regulatory β and γ subunits results in the relief of the autoinhibition of the catalytic α subunit, as well as reduced α turnover in the cell [18,19].

The two isoforms of β, β-1 and β-2, display significant variation in their expression levels in different tissues. Whereas β-1 is highly expressed in the liver and has low expression in skeletal muscle, the β-2 isoform has the opposite expression pattern [10,15,16]. However, fast-twitch rat muscle expresses both isoforms, while slow-twitch rat muscle contains only the β-1 isoform [15]. In the brain, the expression of individual β isoforms is quite heterogeneous; the β-2, but not the β-1 subunit, appears to be developmentally regulated in brain neurons [14]. In this same study, we found that the β-1 isoform shows a heavy nuclear preponderance in neurons, whereas the β-2 subunit in neurons and the β-1 and β-2 subunits in astrocytes is predominantly cytoplasmic. The two β isoforms are 71 % identical in amino acid sequence, with the greatest variation in the N-terminal region; the sequences in the C-terminal region, shown to be important for α and γ binding, are nearly identical [15,16]. The β-1 subunit has been found to undergo significant post-translational modification, including myristoylation and phosphorylation [9,20]. The N-terminus of β-1 (MGNTSSERAA...) contains a penultimate glycine (G2) in an appropriate context of downstream sequence (MGNXXS) of other myristoylation sites [9]. MS analysis of liver β-1 subunit reveals the presence of a myristate group in the N-terminus, which appears to affect enzyme association with a cellular particulate fraction, as judged by membrane isolation [9]. Four native phosphorylation sites have been identified on the β-1 subunit of AMPK, located at serine-24, -25, -108 and -182 [9]. The phosphorylation of serine-24 or serine-25 appears to be mutually exclusive. Based on ^{32}P-

Abbreviations used: AMPK, AMP-activated protein kinase; AMPKK, AMPK kinase; CMV, cytomegalovirus; DMEM, Dulbecco's minimal essential medium; GFP, green fluorescent protein; GST, glutathione S transferase; YFP, yellow fluorescent protein.

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incorporation studies with the isolated enzyme, the serine-108 and serine-24/25 residues are partially phosphorylated *in vivo*, but can be further phosphorylated on incubation *in vitro* with ATP, consistent with an intramolecular autophosphorylation [9]. On the other hand, phosphorylation at serine-182 is probably catalysed by a separate protein kinase with serine/proline specificity.

In the present paper, we have investigated the functional roles for myristoylation and phosphorylation of the β-1 subunit, using site-directed mutagenesis and assessment of expressed enzyme by activity assay, heterotrimer composition analysis and cellular localization of green fluorescent protein (GFP)-fusion proteins.

EXPERIMENTAL

Cell culture

COS or HEK-293 cells were grown at 37 °C and 5% CO_2 in Dulbecco's minimal essential medium (DMEM) with 10% fetal bovine serum and penicillin/streptomycin. Cells were grown in T-75 culture flasks and plated for transfection in six-well plates (Corning Inc., Corning, NY, U.S.A.).

AMPK cDNA plasmid vectors

For mammalian cell expression, AMPK subunit cDNAs were cloned into either pEBG vector (α_1 and α_2 subunits), pMT2 vector (γ-1 subunit) or pBK-cytomegalovirus (CMV) (β-1, β-2, β-1 mutants) [9,18,19]. In the pEBG vector, the cloned α insert is preceded by a glutathione S-transferase (GST) sequence, which enables isolation of expressed protein on GSH–agarose [18,19]. The pMT2 vectors incorporates an N-terminal epitope tag (haemagglutinin) on the γ-1 subunit. For cellular localization studies, wild-type β-1 and β-2 and the β-1 mutants were cloned into the pEGFP-N vector (ClonTech Laboratories); full-length α-1 (amino acid residues 1–548) and a truncated α-1 (amino acid residues 1–312) were cloned into pEYFP-C or pEGFP-C vectors.

Mutagenesis of cDNAs

We have previously reported the construction of the β-1 G2A myristoylation mutant [9]. The three β-1 phosphorylation mutants (SS25,25AA, S108A and S182A) were created using the Transformer site-directed mutagenesis kit with slight modifications (ClonTech Laboratories). (Since there was variability in whether serine-24 or serine-25 was phosphorylated *in vivo*, we elected to make the double mutant for analysis in these studies.) A single-stranded DNA copy of the pBK-CMV β plasmid was obtained through phage infection of XL-1 Blue bacteria expressing the vector. Two primers were then annealed to the single-stranded DNA template; a mutagenic primer that included the serine-to-alanine change, as well as a selection primer that changed a unique *Sna*b1 restriction site to *Nar*1. Each mutagenic primer altered the DNA to result in one amino acid change (except for the double amino acid change for the SS24/25AA primer), as well as introduce a diagnostic restriction enzyme site (silent mutation). The three mutagenic primers were as follows, with the diagnostic restriction enzyme sites introduced in parentheses: S108A, 5′-CTCACTAGAGCCCAAAACAACTTCG-TAGCCATCCTAGATCTGCGG-3′ (*Bgl2*); S182A, 5-'atggtc-gactcccaaaagtgctccgatgtatctgagctgtccagtgccccc-3′ (*Sac*1); SS24/25AA, 5′-CCGCGGAGGGACGCCGCGGAGGGTACCAA-GGATG-3′ (*kpn*).

The selection primer was the same for all three mutants and changed a *Sna*b1 site to a *Nar*1 site, apart from the β-1 insert: 5′-GCTCATCCGGAATGGCGCCTGGCAATGAAAGACG-3′. After elongation with T4 DNA polymerase, the double- stranded plasmid pool was cut with *Sna*b1 to linearize the wild-type plasmids. Circular mutant vectors were then transformed into a repair-deficient BMH 71 *mutS* bacterial strain. Final screening was performed through restriction enzyme cutting of the silent diagnostic site introduced with the mutagenic primers. The new β constructs were then sequenced over the entire β cDNA insert to verify the mutation as well as to confirm that no other mutations were introduced.

Transient transfection

For enzyme isolation and analysis, COS and HEK-293 cells were plated at a density of 3.5×10^5 cells/well in six-well culture plates. For microscopy studies with HEK-293 cells, cells were plated on glass coverslips in these wells and the cell density was set at 5×10^5 cells/well. The following day, cells were washed with serum-free, antibiotic-free DMEM. Individual wells were transfected with a total of 2 μg of DNA in 10 μl of LIPOFECTAMINE™ reagent (Life Technologies, Inc./BRL) in 1 ml of serum-free, antibiotic-free DMEM. Cells were then incubated for 5 h at 37 °C and 5% CO_2. The medium was then replaced with 2 ml of DMEM with 10% fetal bovine serum, and the cells were incubated overnight. The following day, the medium was again replaced with the standard growth medium, and the cells were grown for another 24 h.

Cell lysis and isolation and analysis of AMPK heterotrimers

Cells were lysed and enzyme heterotrimers isolated by GSH–agarose adsorption, as previously detailed [18,19]. AMPK activity was assayed by *in vitro* phosphorylation of the SAMS, the synthetic peptide HMRSAMSGLHLVKRR [21]. Enzyme activity was normalized between samples by quantification of the amount of α subunit recovered by immunoblotting. Isolates were immunoblotted, as described in [18,19], and the α content estimated by scanning densitometry. Care was taken to keep the amount of α within a load-linear range. The enzyme activity (as pmol of [^{32}P]P_i incorporated/min) was divided by the measured pixel density to give an arbitrary enzyme specific activity unit. Immunoblots, after separation of the enzyme isolates by SDS/PAGE (9% gel), were performed to assess enzyme heterotrimer composition, and were individually probed with anti-GST (for α-1), an anti-β (raised against a bacterially expressed β-1 protein) and anti-haemagglutinin (for γ-1). The primary antibody was detected with the correct secondary antibody conjugated with horseradish peroxidase and ECL®. Developed films were then quantified using a scanning densitometer and IPLab Gel software.

Microscopy

For immunofluorescence studies of AMPK-β-1 subunit, cells, plated on coverslips, were fixed using 3.5% formaldehyde and permeabilized using 0.2% Triton X-100. AMPK-β-1 was detected using a primary antibody raised against a synthetic peptide corresponding to amino acids 2–24 of the β-1 sequence and a secondary antibody conjugated to alexa 488 (Molecular Probes, Eugene, OR, U.S.A.). For the co-localization studies, mitochondria were labelled using Mitotracker® dye (Molecular Probes) for 30 min at 37 °C prior to permeabilization. Membrane labelling of the endoplasmic reticulum was identified in these studies with immunofluorescence of calnexin, using an anti-calnexin-specific primary antibody (Stressgen Biotechnologies,

Victoria, BC, Canada) and a secondary antibody labelled with alexa 568 (Molecular Probes). Confocal microscopy was carried out using a Bio-Rad MRC1024 confocal laser scanning microscope (Bio-Rad Laboratories). Images were analysed using Laser Sharp software (Bio-Rad) for image acquisition and Adobe Photoshop 5.0 for image processing and presentation. For the study of GFP-labelled fusion proteins, cells were either fixed or viewed as living cells using either confocal imaging or with a Nikon Optiphot microscope equipped for epifluorescence.

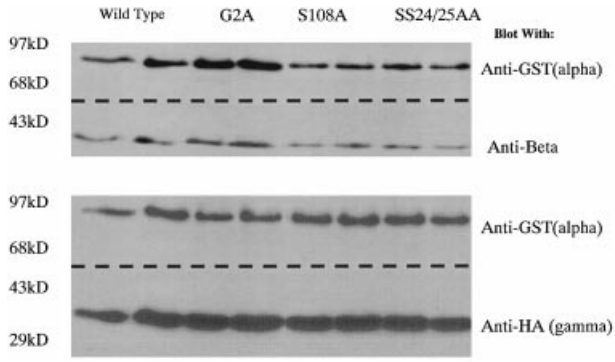

Figure 1 Composition of isolated AMPK heterotrimers

A representative experiment is shown in which AMPK heterotrimers were isolated by GSH–agarose absorption and GSH elution after triple transfection of COS-7 cells with AMPK subunits. In this experiment, cells were triply transfected (in duplicate) with pEBG-α-1, pMT2-haemagglutinin-γ-1 and pBK-CMV β-1 plasmids (wild-type, G2A, S108A and SS24,25AA). At 48 h after transfection, cell lysates were prepared and heterotrimers isolated, as described in the Experimental section. The isolates were then immunoblotted with anti-GST (for α-1) and anti-β-1 (membrane divided prior to blotting; upper panel) and anti-GST and anti-haemagglutinin (for γ) antibodies (lower panel). Identical results were obtained with the S182A β-1 mutant (results not shown).

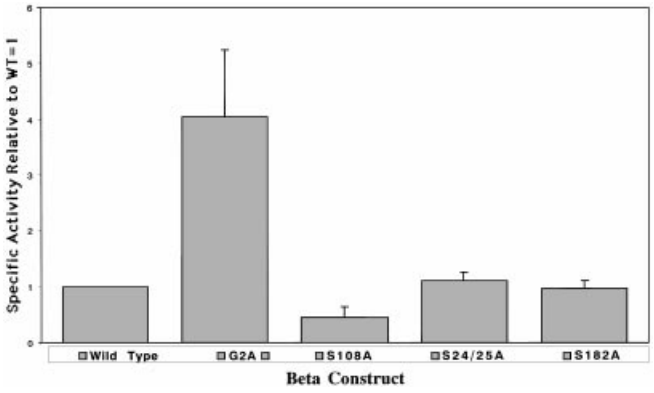

Figure 2 Activity of isolated AMPK heterotrimers

Collective data from separate enzyme isolations ($n = 4$–9) are shown in which the specific activities of various AMPK heterotrimers at V_{max} conditions were compared. Following triple transfection of COS cells and heterotrimer isolation, enzyme activity (in triplicate) was determined in the presence of saturating concentrations of AMP (200 μM) against the SAMS peptide substrate. The same samples were subjected to immunoblotting to determine the content of the α-1 subunit in the isolates by scanning densitometry (in duplicate for each sample). Activity was then corrected within each experiment for small differences in α subunit recovery by dividing the measured activity (pmol/min) by the pixel density on scanning. The data are displayed normalized to the wild-type (WT) value (set at 1) for each experiment $\pm$ S.D. The G2A and S108A activities are statistically significantly different from control at $P < 0.01$, as determined by a two-tailed t test.

RESULTS

Effects of β-1 mutation on AMPK activity and heterotrimer composition

The mutant AMPK-β-1 cDNAs were co-expressed with AMPK-α-1 and AMPK-γ-1 in COS-7 cells, and heterotrimeric AMPK was isolated by GSH–agarose absorption and GSH elution [18,19]. Analysis of these heterotrimers by immunoblotting showed that none of the β-1 subunit mutations had any effect on the formation of heterotrimers with similar mass of α-1, β-1 and γ-1 expressed in the various isolates (Figure 1).

Assay of these same isolates under V_{max} conditions (saturated with respect to SAMS peptide substrate and in the presence of 200 μM AMP) revealed that the G2A mutation led to a 4-fold increase in AMPK specific activity (i.e. corrected for α subunit recovery), whereas the S108A mutation reduces activity by approx. 60 % (Figure 2). Neither the SS24,25AA double mutant

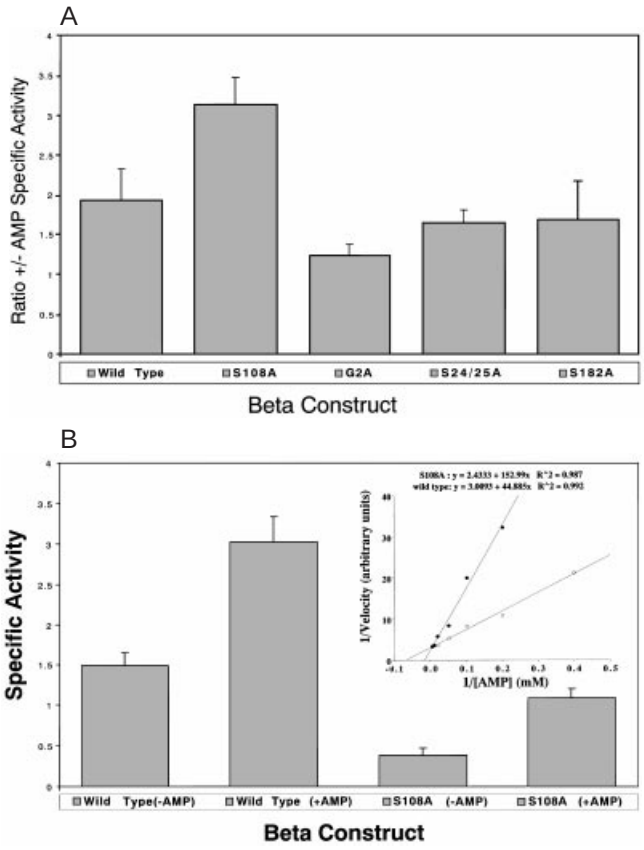

Figure 3 Effects of AMP on AMPK heterotrimers

(**A**) Collective data are shown for experiments ($n = 5$–9) in which the AMP dependence of isolated AMPK heterotrimer activity was determined in isolates after triple transfection and enzyme isolation. All samples were also immunoblotted for α content. The data are displayed as the ratio of an arbitrary specific activity (activity/pixel density of α) of assays conducted in the presence of AMP (200 μM) to that in its absence ($\pm$S.D.). The S108A activity is statistically different from the control values at $P < 0.05$ by a two-tailed t test. (**B**) In these studies ($n = 4$ experiments), the activity of the wild-type heterotrimer to that of heterotrimer containing the S108A β-1 are compared after assay in the presence and absence of AMP. The activity (means $\pm$ S.D.) is represented as an arbitrary specific activity (activity/pixel density). The S108A activities are statistically different from the control values at $P < 0.01$ by a two-tailed t test. Inset: Lineweaver–Burk plot derived from the analysis of replicate preparations of wild-type (○) and S108A-containing (●) heterotrimers (data here not corrected for α content) assayed at various concentrations of AMP allowing calculation of the K_a for AMP (see the Experimental section).

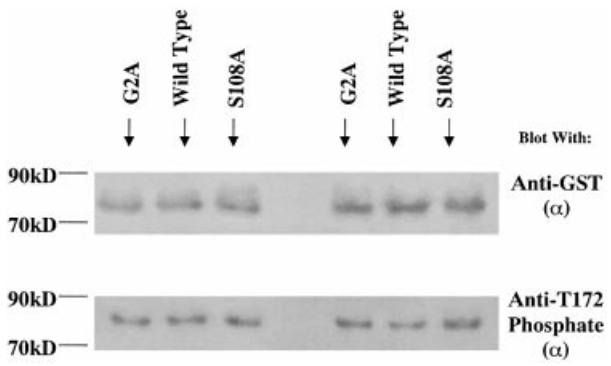

Figure 4 Phosphorylation of the AMPK α-1 subunit

A representative blot is shown in which AMPK heterotrimers (wild-type, G2A and S108A), isolated after triple transfection of COS cells, were blotted for α-1 content (anti-GST; upper panel) and threonine-172 phosphorylation (lower panel), the latter with an antibody specific for the phosphorylated peptide surrounding the T172 sequence on α1. kD, kDa.

nor the S182A mutation had any effect on enzyme activity. The effects of the G2A and S108A mutations did not alter the K_m for the SAMS peptide substrate (results not shown). The S108A mutation caused a modest increase in the apparent allosteric activation by AMP (portrayed as the ratio of activity in the presence to absence of AMP), that was unaffected in the other mutants (Figure 3A). However, the effect of this mutation on activity accounted for both a reduction in AMP-independent activity as well as a decrease in V_{max} activity, measured in the presence of a saturating concentration of AMP (Figure 3B). Indeed, this mutation caused a 4-fold increase in the K_a for AMP, increasing it from 15 to 63 μM (Figure 3B, inset).

The effects of the S108A and G2A mutations on AMPK heterotrimer activity might be accounted for by the altered activation of AMPK in the intact cell through altered phosphorylation of the activation-loop threonine-172 by AMPKK(s). However, immunoblotting with an anti-threonine-172P antibody reveals that there is no difference in the phosphorylation state of the α-1 subunit in these preparations (Figure 4), suggesting that the differences in the activity of the catalytic subunit reflect differences in subunit interactions in the enzyme heterotrimer. These changes in AMPK activity seen on co-expression with the α-1 subunit were also observed in α-2/β-1G2A/γ-1 and α-2/β-1S108A/γ-1 heterotrimers, indicating that the effects are not α-specific (results not shown).

Effects of β-1 mutation on cellular localization

Immunostaining of HEK-293 cells with a β-1-specific antibody shows predominant extranuclear staining of the β-1 subunit with a particulate pattern of distribution (Figure 5). No β-2 staining was visible in these cells (results not shown). To ascertain the nature of this distribution and the effects of the β-1 mutations on it, plasmids were constructed with GFP fused to the C-terminus of the β-1 protein. Expression of the wild-type β-1-GFP fusion protein in HEK-293 cells reveals the same pattern of extranuclear particulate distribution seen with the endogenous β-1 protein (Figures 6A and 6C, Figure 7A, and Figures 8B and 8F). The wild-type β-2-GFP fusion protein showed a similar distribution (Figure 7B). It was also shown that these fusion proteins were competent to support heterotrimer formation with either plasmid-expressed or endogenous α/γ subunits in a fashion indistinguishable from the native β-1 protein (results not shown).

We attempted to co-localize the wild-type β-1-GFP fusion protein to either an endoplasmic reticulum or mitochondrial fraction by confocal microscopy of the expressed fusion protein. As shown in Figure 8, the fusion protein does not precisely co-localize to either fraction, as determined by co-localization studies with a mitochondrial redox dye (Figures 8A–8D) or calnexin (endoplasmic reticulum) (Figures 8E–8G), leaving uncertain the nature of its associations.

We have previously shown by analysis of cell homogenates that a significant fraction of expressed β-1 protein (and its heterotrimer partners, α and γ) sediments with a particulate fraction, and that this association is reduced either in the G2A mutant or by the addition of an N-terminal epitope to the protein (abrogating the myristoylation site) [9]. As shown in Figures 6(B) and 6(D) and Figure 7(C), mutation of this myristoylation site is associated with an alteration in the distribution of the β-1 protein, now seen as non-particulate and generally homogeneous throughout the cell in an extranuclear pattern.

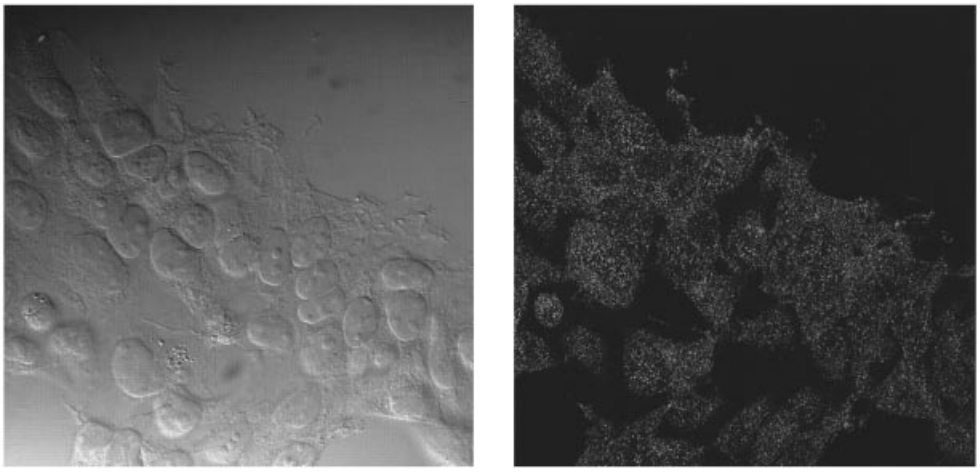

Figure 5 Subcellular localization of endogenous β-1 subunit in HEK-293 cells

Representative confocal microscopy is shown of HEK-293 cells on immunostaining with an anti-β-1 antibody (right panel). Left panel: the differential interference contrast image of the same cells for comparison. Non-immune IgG at the same concentration gave no discernable staining (results not shown)

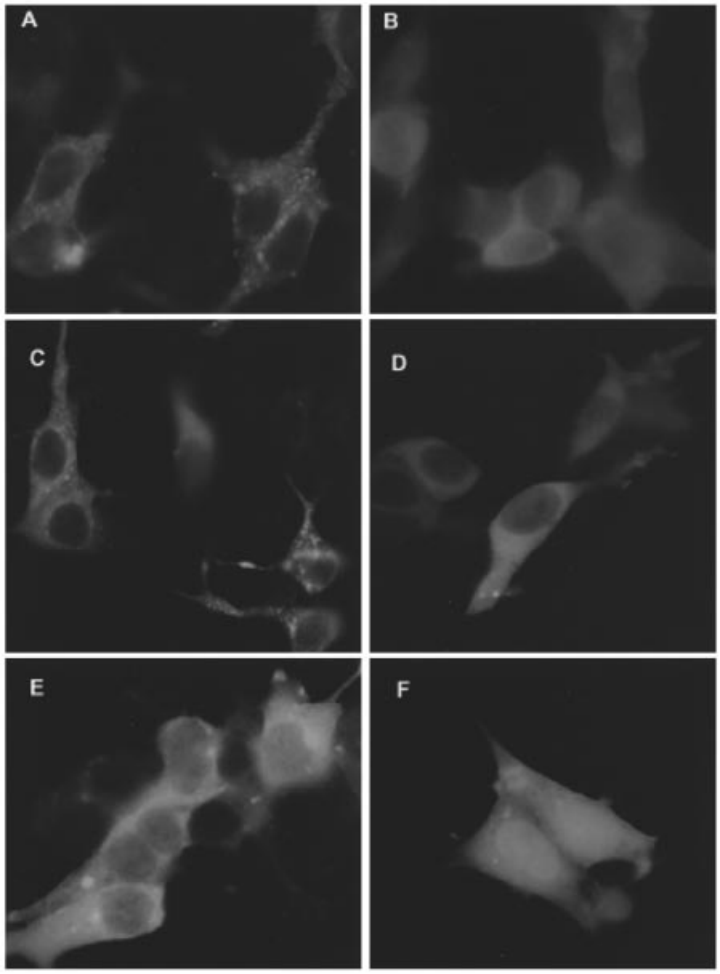

Figure 6 Subcellular localization of β-1 and α subunits by GFP-fusion protein fluorescence

Representative panels of HEK-293 cells following transient transfection is shown, as viewed by standard epifluorescence microscopy. Cells were transfected with wild-type β-1-GFP (**A** and **C**), G2A-β-1-GFP (**B** and **D**), full-length α-1(1–548)-YFP (**E**) and truncated α-1(1–312)-YFP (**F**).

Protein phosphorylation is well known to play a role in subcellular localization of phosphoproteins. We therefore sought to determine whether any of the β-1 phosphorylation sites had any impact on β-1 localization. As can be seen in Figure 7(D), the S108A–GFP fusion protein has a cellular distribution identical with that of the wild-type subunit (Figure 7A and Figures 6A and 6C). However, mutation of either S24/S25 or S182 phosphorylation sites is associated with a dramatic alteration in subcellular distribution (Figures 7E and 7F respectively). The pictures in these panels indicate a distinct nuclear shift in their distribution, with some continued homogenous extranuclear expression.

It was of interest to see how the distribution of the α-1 subunit might be changed as a function of its capacity to bind the β-1 subunit. An α-1-yellow fluorescent protein (YFP) fusion protein shows a wide, generally non-particulate distribution predominantly in the extranuclear space, with a tendency to a perinuclear pattern (Figure 6E). Truncation of the α-1 subunit (amino acid residues 1–312), which removes its ability to bind to the β and γ subunits [19], is associated with a 'nuclear shift' in its distribution pattern (Figure 6F), suggesting the possibility that cytoplasmic β-1 subunit might tether the α-1 subunit outside of the nucleus. It is also possible that the reduced size of the YFP-α-1-1-312 fusion protein (61 kDa versus 87 kDa for the full-length α) affects the localization, and further studies are needed to support this role for the β-1 subunit.

DISCUSSION

The results of this investigation indicate the importance of post-translational modification of the non-catalytic β-1 subunit of AMPK in the determination of enzyme activity and subcellular localization. While not specifically studied in this investigation, it seems likely that the β-2 subunit will share similar features, in that the glycine-2 myristoylation site and two of the four phosphorylation sites (serine-24/serine-25 being the exception) are preserved in this isoform. The effects of these modifications do not appear, however, to alter the ability of the β subunit to associate with the α and γ subunits.

N-myristoylation of target proteins can play a structural role in stabilizing three-dimensional protein conformation, in membrane binding and in membrane targeting [22]. Of interest, the myristate group of the catalytic subunit of the cyclic AMP-dependent protein kinase is required for structural and thermal

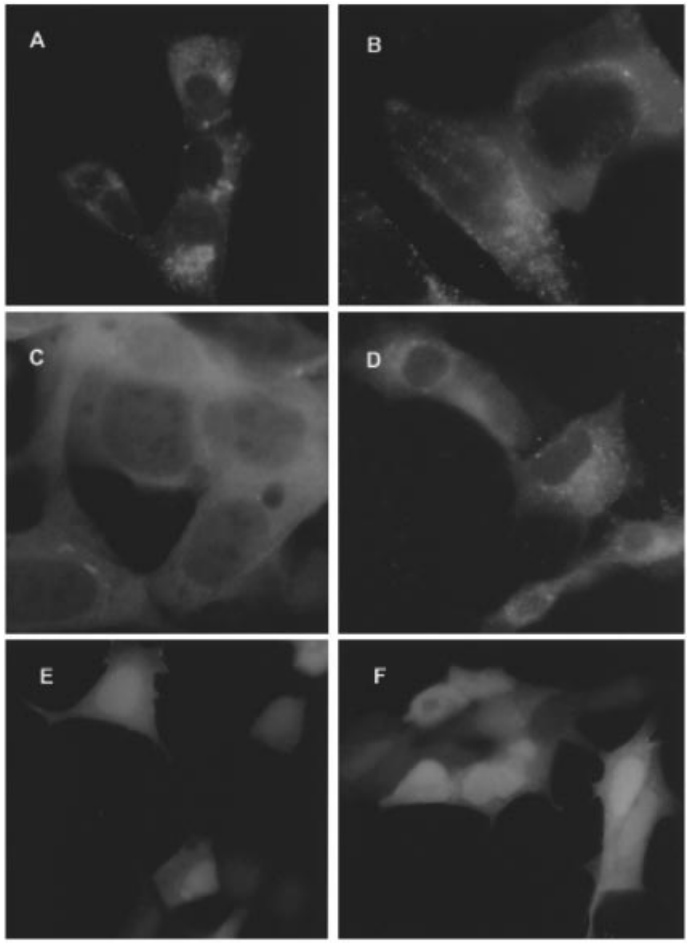

Figure 7 Subcellular localization of wild-type and phosphorylation site mutant β-1 subunits

Representative panels of HEK-293 cells are shown following transient transfection, as viewed by standard epifluorescence microscopy. Cells were transfected with wild-type β-1-GFP (**A**), wild-type β-2-GFP (**B**), G2A-β-1-GFP (**C**), S108A-β-1-GFP (**D**), SS24,25AA-β-1-GFP (**E**) and S182A-β-1-GFP (**F**).

stability of that enzyme [23]. We have previously identified, by MS, myristoylation of the β-1 subunit from rat liver, and found that mutagenesis of glycine-2 to alanine or the addition of an N-terminal epitope (removing the glycine from position 2) is associated with increasing recovery of expressed AMPK heterotrimer in a soluble cell fraction [9]. The results of the present study confirm this particulate extranuclear association of both the endogenous β-1 and GFP–β-1 fusion protein, although they do not clarify the actual site of that association. Our results also show a marked redistribution of the enzyme on removal of the myristoylation site. Myristoylation alone of proteins is not sufficient for membrane anchoring; a polybasic sequence or palmitoylation is often necessary for this association [22]. However, the β-1 subunit does not have either an upstream polybasic sequence or a Met-Gly-Cys N-terminal sequence seen in myristoylated, palmitoylated proteins. Membrane interactions of single acylated proteins may be enhanced by protein–protein interactions, and it seems possible that this mechanism might account for the particulate association and perhaps targeting of the β-1 subunit (and AMPK heterotrimer).

In the present paper, mutagenesis of glycine-2 to alanine is associated with a dramatic 4-fold enhancement of AMPK activity. We have no evidence for demyristoylated forms of the β-1 subunit in intact tissue or cells, although there is precedent for two pools of myristoylated and demyristoylated forms of the same protein (e.g. myristoylated alanine-rich C-kinase substrate protein in brain, Gpa1p in yeast and a 68 kDa protein in *Dictyostelium discoideum*) [22]. The myristate group of a modified protein does not necessarily 'stick out' of the protein, but may be buried in a hydrophobic pocket (examples include recoverin, the cAMP-dependent protein kinase and Arf proteins) [22]. In these proteins the myristate group functions as a switch mechanism for reversible membrane binding, moving out of the hydrophobic pocket on ligand binding to interact with the membrane phospholipids. One possibility is that the movement of the myristate group of β-1 from a hydrophobic pocket of AMPK on membrane association might mimick demyristolyation and thus serve an activating function for the kinase.

In our initial studies of the β-1 and β-2 subunits, we identified several sites of phosphorylation, likely catalysed both by autophosphorylation and exogenous AMPK kinases active on the β subunit [9,15]. The β subunit isolated from liver was largely a mixture of di- and tri-phosphorylated species. Serine-24/serine-25 and serine-108 were substoichiometrically phosphorylated in the isolated enzyme; however, these sites could be autophosphorylated *in vitro*. Interesting, the phosphorylation of serine-24 and serine-25 appeared to be mutually exclusive, as either one or the other were phosphorylated *in vivo* in different

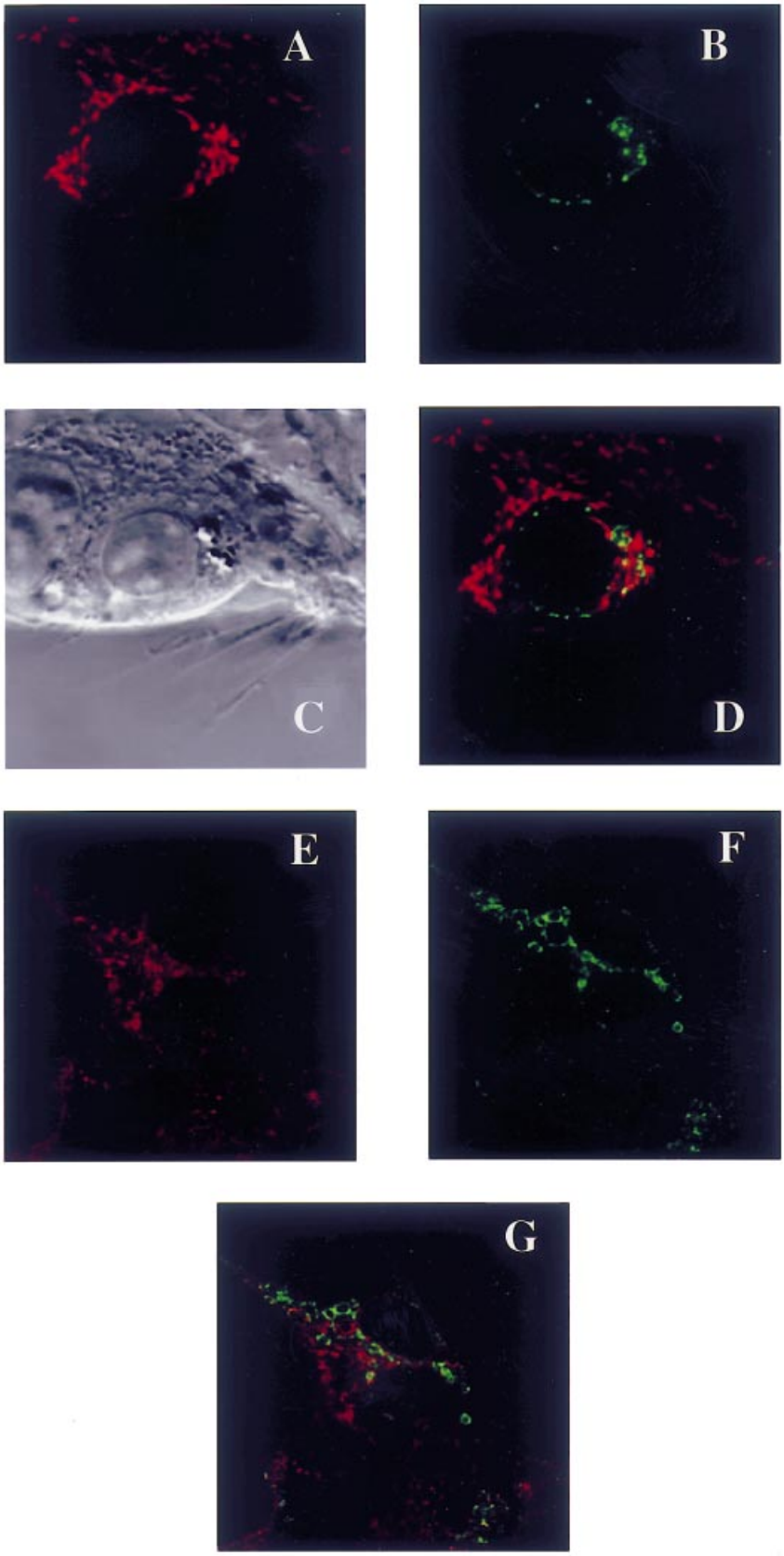

Figure 8 Co-localization studies of β-1 subunit

(**A–D**) Representative photos of HEK-293 cells by confocal microscopy after transfection with wild-type-β-1–GFP and incubation with Mitotracker® to identify mitochondria. (**A**) Mitochondrial stain, (**B**) β-1–GFP fluorescence and (**D**) the merged images. (**C**) DIC image of the same cells. (**E–G**) Representative photos of HEK-293 cells by confocal microscopy after transfection with wild-type-β-1–GFP and counter-staining with anti-calnexin antibody. (**E**) Anti-calnexin stain, (**F**) wild type-β-1–GFP fluorescence and (**G**) the merged images.

preparations, but never both. However, there was some preference for serine-24. In contrast to the β-1 subunit sequence around serine-24/serine-25 (RRDSSGG), the corresponding sequence in the β-2 subunit, ARAEGGG, contains an acidic glutamic acid at the serine-24-equivalent position, but no phosphorylatable residue [15,16]. In the β-1 subunit, serine-182 appears to be fully phosphorylated *in vivo*, whereas the serine-182 site in the β-2 subunit is only partially phosphorylated in skeletal muscle, suggesting a more robust turnover of that phosphate in the β-2 isoform [15]. Total β-1 subunit phosphorylation increases on activation of the recombinant heterotrimer with sodium azide in ^{32}P-labelled COS cells, though the site(s) of this phosphorylation event was not determined [9]. Others have indicated that activating AMPKKs, which phosphorylate the α subunit, are also active on the β subunit [20], consistent with the overall notion that a concerted phosphorylation of both the α and β subunits might be necessary for maximum AMPK activation.

In our studies of the phosphorylation sites, only the mutagenesis of serine-108 had any effect on AMPK activity, decreasing it by approx. 60–70 %, implying that the phosphorylation of this site is associated with enzyme activation, either in a concerted autophosphorylation activating mechanism and/or by unidentified AMPKK(s) active on this site. Of interest, this mutation also alters the reactivity of AMPK heterotrimer with its allosteric activator, AMP, increasing the apparent K_a 4-fold. The allosteric site for AMP on the AMPK heterotrimer has not been identified. Recently, it was reported that the reactive AMP analogue, 8-azido-AMP, labels the γ subunit, and that the γ subunits can contribute to AMP reactivity [17]. The α subunit does not contain any AMP-binding site, and truncation of the α subunit, which removes the β/γ binding sites, renders the enzyme AMP-insensitive, indicating that an intact holoenzyme may be required to form the AMP-binding pocket [19,20]. The present data indicate that the β subunit also contributes to AMP sensitivity in a mechanism that might involve β subunit phosphorylation.

As revealed by immunofluorescence studies, the β-1 subunit in HEK-293 cells is almost entirely cytoplasmic in a particulate distribution, and this pattern is mimicked by the wild-type β-1–GFP fusion protein. Though in these latter studies we only transfected the β-1 subunit, it was fully capable of recombining with the endogenous α and γ subunits, and the formation of heterotrimer with triple transfection in COS cells was not altered by the addition of the GFP fusion sequence to the β subunit (results not shown). Because of the ability of AMPK to regulate fatty acid oxidation and/or sterol synthesis, we expected that we might encounter association with either mitochondria or endoplasmic reticulum. However, we were unable to localize the β subunit to either of these structures by confocal microscopy, leaving uncertain its localization. Electron microscopic studies are in progress to further delineate the nature of the subcellular distribution of AMPK subunits.

Mutation of SS24,25 and serine-182, but not serine-108, to alanine is associated with a dramatic redistribution of β subunit subcellular localization, which assumes a largely nuclear distribution. This implies that a phosphorylation event, catalysed either by autophosphorylation and/or by AMPKK(s) active on the β subunit, somehow tethers the β-1 subunit (and perhaps a portion of the AMPK heterotrimer) to an extranuclear distribution. Consistent with this, truncation of the α subunit, removing its β/γ-binding region, was shown to alter α-1-YFP-fusion protein distribution, indicating that the binding of α to β (and γ) not only regulates enzyme activity, but also cellular localization.

AMPK regulates both cytoplasmic and nuclear events, and AMPK subunits have been shown to have a quite heterogenous subcellular distribution, depending on the cell type and the subunit under examination [9,13–15]. The post-translational modification of the β subunit, by both phosphorylation and myristoylation, may therefore be central to a process of regulated cellular distribution in the actions of AMPK, increasing the complexity of this important metabolic enzyme.

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