A homologue of AMP-activated protein kinase in *Drosophila melanogaster* is sensitive to AMP and is activated by ATP depletion

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We have identified single genes encoding homologues of the α, β and γ subunits of mammalian AMP-activated protein kinase (AMPK) in the genome of *Drosophila melanogaster*. Kinase activity could be detected in extracts of a *Drosophila* cell line using the SAMS peptide, which is a relatively specific substrate for the AMPK/SNF1 kinases in mammals and yeast. Expression of double stranded (ds) RNAs targeted at any of the putative α, β or γ subunits ablated this activity, and abolished expression of the α subunit. The *Drosophila* kinase (DmAMPK) was activated by AMP in cell-free assays (albeit to a smaller extent than mammalian AMPK), and by stresses that deplete ATP (oligomycin and hypoxia), as well as by carbohydrate deprivation, in intact cells. Using a phosphospecific antibody, we showed that activation was associated with phosphorylation of a threonine residue (Thr-184) within the 'activation loop' of the α subunit. We also identified a homologue of acetyl-CoA carboxylase (DmACC) in *Drosophila* and, using a phosphospecific antibody, showed that the site corresponding to the regulatory AMPK site on the mammalian enzyme became phosphorylated in response to oligomycin or hypoxia. By immunofluorescence microscopy of oligomycin-treated *Dmel2* cells using the phosphospecific antibody, the phosphorylated DmAMPK α subunit was mainly detected in the nucleus. Our results show that the AMPK system is highly conserved between insects and mammals. *Drosophila* cells now represent an attractive system to study this pathway, because of the small, well-defined genome and the ability to ablate expression of specific gene products using interfering dsRNAs.

Key words: cell signalling, glucose deprivation, hypoxia, interfering RNA, oligomycin.

INTRODUCTION

The AMP-activated protein kinase (AMPK) is the downstream component of a protein kinase cascade that acts as a sensor of cellular energy charge in mammalian cells [1–3]. AMPK is a heterotrimeric complex comprising a catalytic α subunit and regulatory β and γ subunits [4–6], with co-expression of all three subunits being essential for the formation of an active, stable complex [7,8]. In mammals each subunit is encoded by multiple genes (α1, α2, β1, β2, γ1, γ2, γ3), and these all appear to form complexes, such that there are twelve possible heterotrimeric combinations [9–11].

AMPK is activated by increases in AMP, coupled with decreases in ATP, via a mechanism involving both allosteric activation and increased phosphorylation by upstream kinase(s) at a threonine residue (Thr-172) that lies within the activation loop of the kinase domain on the α subunit [12–14]. This complex activation mechanism results in an ultrasensitive response, such that there can be a large activation for a rather small increase in AMP [15]. AMP increases inside the cell, via the adenylate kinase reaction, whenever ATP is depleted. The AMPK cascade is therefore switched on by either cellular stresses that inhibit ATP production (e.g. heat shock or metabolic poisons [16], glucose deprivation [17], hypoxia or ischaemia [18]), or stresses that accelerate ATP consumption (e.g. exercise in muscle [19]). Once activated, the cascade switches on ATP-producing catabolic pathways while switching off ATP-consuming processes, both via direct phosphorylation of proteins involved in the target process and via indirect effects on gene expression (reviewed in [20]).

To fully elucidate the physiological roles of the AMPK system, genetic approaches to knock out the kinase are required. Recently, Birnbaum and co-workers [21] were successful in ablating AMPK activity in muscle of transgenic mice by overexpressing a kinase-inactive α subunit from a creatine kinase promoter. This acted as a dominant negative mutant due to competition for the limiting amount of β and γ subunits. Analysis of the mice confirmed that AMPK is wholly responsible for the effect of hypoxia, and partially responsible for the effect of contraction, on muscle glucose uptake. Expression of dominant negative mutants in mammalian cell culture has also been an informative approach [18,22,23]. Work is also in progress on the development of AMPK knock-out mice, although a complicating factor in mammals is the existence of multiple AMPK subunit isoforms encoded by distinct genes. We therefore turned our attention to the fruit fly *Drosophila melanogaster*, where the essentially complete genome sequence indicates that there are fewer than half as many genes in total as in mammals [24], and where reliable double stranded RNA (dsRNA) interference methods are available to knock out expression of specific proteins [25]. In this paper we identify the single α, β and γ subunit genes of *Drosophila* AMPK and show that expression of the α subunit, and kinase activity, can be ablated using dsRNAs targeted at any of the three subunits. We also show that *Drosophila* AMPK is activated by AMP, that it can be activated by ATP-depletion in intact cells, and that the downstream target, acetyl-CoA carboxylase, appears to be conserved between flies and mammals.

MATERIALS AND METHODS

Molecular biology materials

The RNeasy kit was from Qiagen (Crawley, West Sussex, U.K.), the Titan One Tube RT-PCR kit from Roche (Lewes, East

Abbreviations used: AMPK, AMP-activated protein kinase; DAPI, 4,6-diamidino-2-phenylindole; DmACC, a *Drosophila* homologue of acetyl-CoA carboxylase; DmAMPK, a *Drosophila* homologue of AMPK; dsRNA, double stranded RNA; dsRNAi, interfering dsRNA; HRP, horseradish peroxidase.

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Sussex, U.K.), and the T7 *in vitro* transcription kit (MEGAscript T7) was from Ambion (Austin, TX, U.S.A.). The gene-specific primers were synthesized by Sigma-Genosys (Cambridge, U.K.) and are listed below, all in the 5′ to 3′ direction. For PCR cloning, we utilized forward primers (αF, βF, γF) that placed a suitable restriction site upstream of the start codon, and reverse primers (αR, βR, γR) that placed a suitable restriction site downstream of the stop codon. The primers were: αF, CCAGGATCCATGCCCCAGATGAGGGCT; αR, CGTCTAGATTAGCGAGCCAGTTGAAT; βF, TACGAATTCATGGGCAACGCCAGCTCC; βR, GGCTCTAGACTAAATGGGCTTGTACAG; γF, GCCGAATTCATGAACTCCATGAAGGTG; γR, GCCTCTAGATTATTCACTAACCAACGC. For synthesis of dsRNA, we used oligonucleotides containing a T7 polymerase binding site (GAATTAATACGACTCACTATAGGGAGA) at the 5′ end, with gene-specific sequences at the 3′ end. The gene-specific sequences were: αF, TTCGGCAAGGTGAAGATC; αR, CACTTGCAGCATCTGACA (producing a dsRNA from nucleotides 115 to 789 of the α subunit coding sequence); βF, CATACGGCAGTCTTGAAC; βR, GGCGTACAAGTGGTTAAG (producing a dsRNA from nucleotides 229 to 936 of the β subunit coding sequence); γF, AACGTCGTGCATCAGTTG; γR, GCGGTCTCGATGTTGTTA (producing a dsRNA from nucleotides 379 to 1078 of the γ subunit coding sequence).

Proteins, antibodies and other immunological reagents

Rat liver AMPK was purified as far as the Q-Sepharose step [13]. The anti-PT172 antibody has been described previously [26]. For the anti-QSSM antibody, the peptide CQSSMDHQAPLATVT (residues 375–388 of the putative *Drosophila* α subunit, plus N-terminal cysteine for coupling; amino acids denoted using one-letter symbols) was synthesized. This region of sequence is rather poorly conserved between *Drosophila* and mammals, and between the two mammalian α subunits, and peptides derived from these regions of the mammalian α subunits generated useful isoform-specific antibodies [7]. For the anti-PS93 antibody, the peptide CTLKPSMSRGTGLG [residues 86–99 of *Drosophila* acetyl-CoA carboxylase (DmACC), plus N-terminal cysteine; amino acids denoted using one-letter symbols] was synthesized with or without phosphate on the underlined serine. Immunization of sheep was performed by Diagnostics Scotland (Carluke, Lanarkshire, U.K.). Anti-peptide antibodies were affinity purified using methods described previously [7,26]. Horseradish peroxidase (HRP)-linked goat anti-(rabbit IgG) secondary antibody was from Sigma, UK. Donkey anti-(sheep IgG) (AffinitiPure) and donkey serum were from Stratech Scientific (Luton, U.K.).

Construction of dsRNAs

Total RNA was isolated from the *Dmel2* cells using the RNeasy kit (Qiagen), treated with DNase I (Roche) and subsequently used in reverse transcription-PCR (Titan One Tube RT-PCR, Roche). The gene-specific primers for each of the α, β and γ subunits are listed above. The products were cloned into the pcDNA3 plasmid (Invitrogen, Carlsbad, CA, U.S.A.) to give pcDNA3-α, pcDNA3-β and pcDNA3-γ. dsRNA was synthesized according to the method of Clemens et al. [25]. Briefly, PCR was carried out with the pcDNA3-α, -β or -γ plasmids as the template, and forward and reverse primers containing a 5′ T7 polymerase binding site and a 3′ gene-specific sequence (see above) was used to generate fragments of ≈ 700 bp. The purified PCR products were then used as templates for an *in vitro* T7 transcription reaction. The complementary RNA strands were precipitated, resuspended in RNase-free water, annealed together, and diluted to a final concentration of 3 μg/μl. The dsRNA was stored at −20 °C until further use. No loss of efficacy was evident over 6 months of storage.

Cell culture and treatment with dsRNA and stress conditions

Dmel2 cells were maintained at 25 °C in *Drosophila* serum-free medium supplemented with 2 mM L-glutamine (Life Technologies, Paisley, U.K.). For the carbohydrate-deprivation experiments, the manufacturers made a custom batch of identical medium lacking all carbohydrate. Treatment with dsRNA was based on the procedure of Dixon and co-workers [25]. Cells were seeded in 6-well trays at 1×10^6 cells/well in 1 ml of serum-free medium, with 37 μM dsRNA. Control wells were incubated without dsRNA or, where indicated, with a dsRNA targeted at the *Escherichia coli lacZ* gene product. After 1 h, wells were supplemented with an additional 2 ml of serum-free medium and incubated for a further 95 h. Stress treatments were then carried out as specified in the text or Figure legends. Oligomycin was added in DMSO and an equal volume of solvent was added to controls.

Cell lysis

The cell were lysed *in situ* on the culture plates by pouring off the medium and adding 300 μl of ice-cold lysis buffer [50 mM Tris/HCl (pH 7.4 at 4 °C), 50 mM NaF, 1 mM Na pyrophosphate, 1 mM EDTA, 1 mM EGTA, 1 % (v/v) Triton X-100, 1 mM dithiothreitol, 0.1 mM benzamidine, 0.1 mM PMSF and 5 μg/ml soybean trypsin inhibitor]. Protein concentrations were determined using the Bradford assay [27]. Lysates were frozen in liquid nitrogen and stored at −80 °C until analysed.

Kinase assays

AMPK activity was determined via phosphorylation of the SAMS peptide as described previously [28]. One unit of AMPK incorporates 1 nmol of phosphate into the peptide per min at 30 °C. In some cases, where stated in the text, the kinase was partially purified by poly(ethylene glycol) precipitation prior to assay [29].

Western blotting and immunoprecipitation

Protein samples were resolved by SDS/PAGE using NuPAGE (Invitrogen) gels, and transferred electrophoretically to nitrocellulose. Membranes were blocked by incubation in TBS-Tween [20 mM Tris/HCl (pH 7.4), 137 mM NaCl, 0.1 % (v/v) Tween-20] supplemented with 5 % (w/v) low-fat milk powder for 1 h at 20–22 °C. Primary antibodies were applied in the same buffer supplemented with 1 % (w/v) low-fat milk powder and incubated overnight at 4 °C. After extensive washing with TBS-Tween, the blots were incubated for 1 h at 20–22 °C with HRP-linked rabbit anti-(sheep IgG), or goat anti-(rabbit IgG), as appropriate. After further extensive washing, blots were developed using enhanced chemiluminescence (Amersham Biosciences, Little Chalfont, Bucks., U.K.). Immunoprecipitation using Protein G–Sepharose was essentially as described previously [29].

Fluorescence microscopy

Dmel2 cells were seeded as described above, but with a glass coverslip (pre-washed in 1 M HCl) in each well of the 6-well dish. Following 96 h with or without dsRNA treatment, cells were fixed for 20 min in 4 % (w/v) paraformaldehyde in PBS. The cells were washed with PBS and excess fixative quenched by incubation for 15 min in 100 mM glycine. Cells were permea-

bilized in PBS containing 0.1 % (v/v) Triton X-100 for 15 min, then blocked with PBS containing 5 % (v/v) donkey serum for 90 min at 20–22 °C. They were washed extensively with PBS, followed by incubation at 37 °C in a humid chamber for 90 min in PBS containing primary antibody and supplemented with 1 % (v/v) donkey serum. The cells were again washed extensively in PBS, followed by a 1 h incubation in PBS containing FITC-conjugated donkey anti-(sheep IgG), supplemented with 1 % (v/v) donkey serum. Cells were again extensively washed in PBS. Nuclei were stained using 4,6-diamindino-2-phenylindole (DAPI, 1 μg/ml) (Sigma) dissolved in PBS. The cells were then visualized using a Deltavision wide-field deconvolution microscope [30].

RESULTS

AMPK homologues in the *Drosophila* genome

BLAST [31] searches of predicted proteins from the *Drosophila* genome database with the amino acid sequences of the α1, β1 and γ1 subunits of rat AMPK revealed single homologues in each case (FlyBase IDs: FBgn0023169, FBgn0033383, FBgn0025803 respectively). The P values for the α, β and γ homologues were 1.4×10^{-161}, 8.9×10^{-57} and 6.4×10^{-102} respectively, with the P values for the second best matches being 2×10^{-66}, 0.4 and 0.078 (P values represent the significance of the alignment; the most highly significant P values are those close to 0). The second best match for the α subunit sequence was clearly a distinct protein kinase and not another α subunit isoform: if the search was repeated using only the regulatory domain sequence of rat α1 (residues 269–548, which excludes the kinase domain), the only significant match was FBgn0023169 ($P = 1.7 \times 10^{-47}$, next best match $P = 0.63$). Phylogenetic trees created using CLUSTALX [32], showing how the *Drosophila* α, β and γ subunits are related to homologues in other species, are shown in Figure 1. For each of the three subunits, the *Drosophila* sequences cluster most closely with the mammalian (rat or human) sequences. Given the clear similarity of these putative α, β and γ subunits between mammals and *Drosophila*, it seemed likely that the *Drosophila* proteins will form a heterotrimeric complex *in vivo*; further evidence for this is provided below. We will utilize the term DmAMPK to refer to this complex.

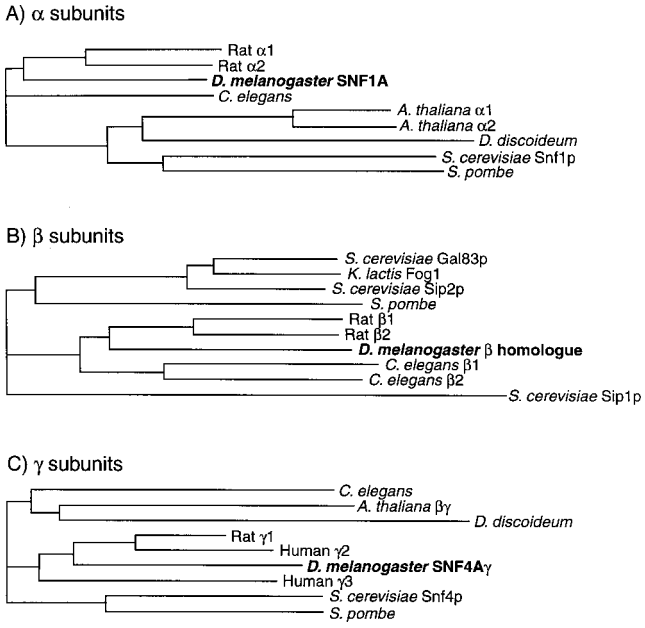

Figure 1 Phylogenetic trees of putative or known α, β and γ subunits of AMPK/SNF1 complexes from different eukaryotic species

Amino acid sequences were initially aligned using PILEUP [47] and the alignments refined and trees constructed by neighbour-joining analysis using CLUSTALX [32]. Sequences from the public databases (accession numbers or database IDs are given) were: rat α1 (U40819), rat α2 (Z29486), *D. melanogaster* SNF1A (AF181649), *Caenorhabditis elegans* α (CE07458), *Arabidopsis thaliana* α1 (M93023) and α2 (X94755), *Dictyostelium discoideum* α homologue (AF118151), *Saccharomyces cerevisiae* Snf1 (M13971), *Schizosaccharomyces pombe* Snf1 homologue (SPCC74.03C), *S. cerevisiae* Gal83 (L13599), *Kluyveromyces lactis* Fog1 (X75408), *S. cerevisiae* Sip2 (L31592), *S. pombe* β homologue (SPCC1919.03C), rat β1 (X95577) and β2 (AF182717), *D. melanogaster* β homologue (TrEMBL:Q9V541), *C. elegans* β1 (CE22044) and β2 (CE18744) homologue, *S. cerevisiae* Sip1 (M90531), *C. elegans* γ homologue (CE29350), *A. thaliana* $\beta\gamma$ homologue (AF250335), *D. discoideum* γ homologue (contig 15687), rat γ1 (X95578), human γ2 (AJ249976) and γ3 (AJ249977), *D. melanogaster* SNF4Aγ (TrEMBL:096613), *S. cerevisiae* Snf4 (M30470) and *S. pombe* Snf4 homologue (Q10343). The *A. thaliana* $\beta\gamma$ homologue is an interesting case where the γ subunit also seems to contain an N-terminal KIS domain, normally found on the β subunit [48].

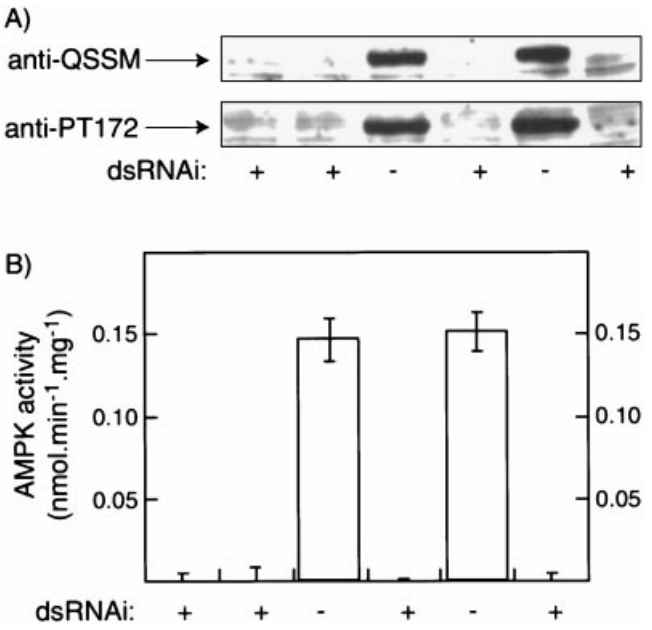

Figure 2 (A) Western blots and (B) kinase assays of *Dmel2* cell lysates

Cells had been treated for 4 days with or without four different preparations of dsRNA targeted against the DmAMPK α subunit. For Western blots, 30 μg, and for kinase assays, 1.8 μg of lysate protein was analysed.

dsRNA inactivation of DmAMPK

We used PCR to clone full length cDNAs from the coding regions of the putative DmAMPK α, β and γ subunits, and utilized the protocol of Dixon and co-workers [25] to construct interfering dsRNAs of about 700 bp targeted at each subunit. To test the efficacy of the dsRNAs, we utilized the *Drosophila* embryonal cell line, *Dmel2*. To monitor expression of DmAMPK, we utilized two different antibodies, namely anti-QSSM, an anti-peptide antibody raised against residues 375–388 of the DmAMPK α subunit, and anti-PT172, a phospho-specific antibody raised against the sequence around the activating phosphorylation site, Thr-172, on the rat enzyme. This sequence is perfectly conserved in the DmAMPK α subunit, although the residue equivalent to Thr-172 is Thr-184 in *Drosophila*. Figure 2(A) shows that a polypeptide of the expected size ($\approx$ 60 kDa)

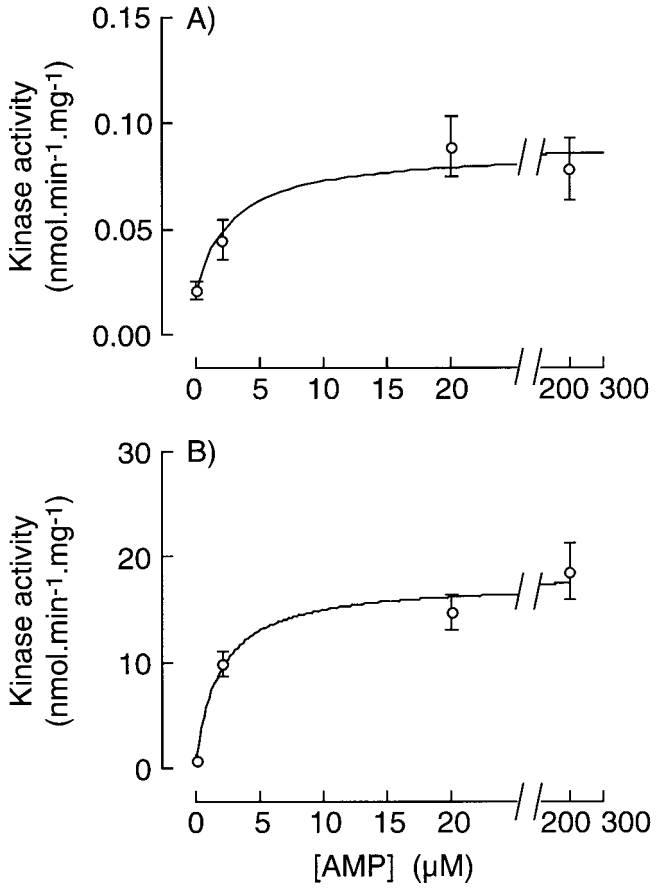

Figure 3 AMP-dependence of (A) DmAMPK and (B) rat liver AMPK after immunoprecipitation using the anti-PT172 antibody

The resuspended immunoprecipitates were divided into aliquots and assayed at 0, 2, 20 and 200 μM AMP. Data were fitted to the equation: activity = basal + {(activation × basal − basal) × [AMP]/(K_a + [AMP])} using Graphpad Prism software, where basal is the activity in the absence of AMP, activation is the degree of stimulation by AMP, and K_a is the concentration of AMP giving half-maximal activation. The curves were generated using the best fit values of these parameters given in the text.

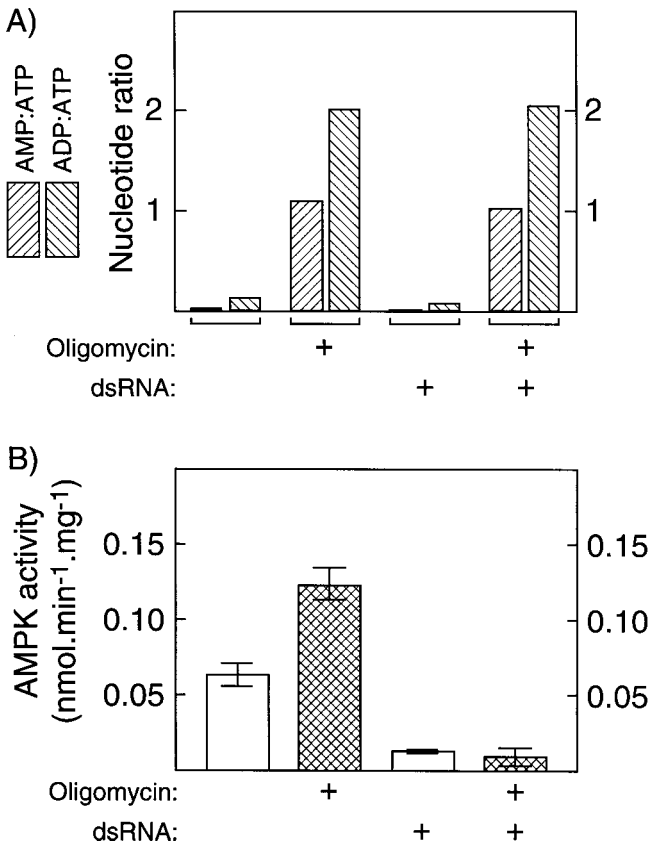

Figure 4 Nucleotide ratios (A) and DmAMPK activity (B) of *Dmel2* cells treated with dsRNA against the DmAMPK α subunit

Dmel2 cells incubated for 4 days in the presence or absence of dsRNA targeted against the DmAMPK α subunit were incubated with 100 nM oligomycin for 1 h. DmAMPK was assayed after poly(ethylene glycol) precipitation.

was detected using either of these antibodies, and that the expression of this polypeptide was eliminated using four different preparations of dsRNA targeted at the α subunit.

Measurement of DmAMPK activity and effect of AMP

To monitor the activity of DmAMPK, we utilized the SAMS peptide, which we have shown to be a relatively specific substrate of AMPK in mammalian cells [28], and the SNF1 complex in budding yeast [33]. Figure 2(B) shows that in extracts from *Dmel2* cells there was an activity of around 0.15 nmol · min^{-1} · mg^{-1}, which is somewhat lower than, although of the same order of magnitude as, the AMPK activity obtained using the same assay in a range of mammalian cell extracts [28]. The activity was clearly due to DmAMPK because it was totally abolished by treatment with four different preparations of dsRNA targeted at the DmAMPK α subunit. We routinely used cells that had been treated with dsRNA for 4 days, although pilot experiments showed that the effect was almost maximal by 2 days. A control dsRNA targeted at the *E. coli lacZ* gene product had no effect (results not shown).

Since mammalian AMPK is activated by AMP [34] but the budding yeast SNF1 complex is not [33], we were interested to find out whether DmAMPK was AMP-sensitive. This could not be studied in crude cell lysates because of contamination with endogenous AMP and/or adenylate kinase (which can generate AMP from ADP during the assay). We therefore immunoprecipitated the protein and measured the activity in the resuspended pellet after extensive washing. During initial attempts using the anti-QSSM antibody we were unable to demonstrate any AMP-dependence. However, after immunoprecipitation with the anti-PT172 antibody, the kinase was activated 4.5-fold by AMP with a half-maximal effect at 3 μM (Figure 3A). As a positive control, the activation of purified rat liver AMPK was also determined after precipitation with the anti-PT172 antibody. The mammalian kinase was activated by AMP with a half-maximal effect at 2 μM (Figure 3B). Surprisingly, however, the maximal stimulation of the rat liver enzyme by AMP was 22-fold. Since this was a much larger effect than we had observed previously (e.g. [35]), we wondered whether it was connected with the use of the anti-PT172 antibody for immunoprecipitation. We therefore immunoprecipitated the rat liver enzyme in parallel using the anti-PT172 antibody and a mixture of anti-α1 and -α2 antibodies. The degree of stimulation was much greater in the former case (16-fold versus 2.6-fold), although the activity obtained at maximal AMP concentrations (200 μM) was 3-fold higher using the anti-α1 and -α2 antibodies. There was no significant activity remaining in the

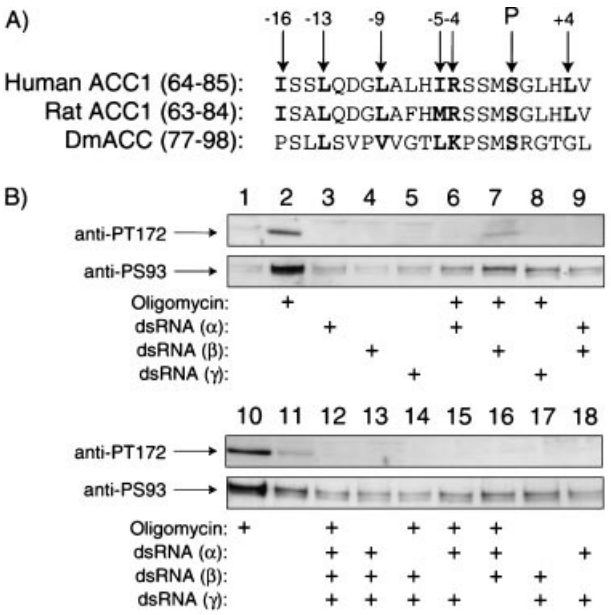

Figure 5 (A) Alignment of acetyl-CoA carboxylase sequences and (B) effect of oligomycin on the phosphorylation of DmAMPK and DmACC

(**A**) Alignment of sequences from the N-terminal regions of human, rat and *Drosophila* acetyl-CoA carboxylases containing the known or putative regulatory phosphorylation site for AMPK. The phosphorylated serine is labelled 'P' and residues believed to be important in recognition by AMPK are highlighted with bold type and residue number relative to the serine. (**B**) Effect of oligomycin on phosphorylation of Thr-184 on the DmAMPK α subunit (assessed using anti-PT172 antibody) and Ser-93 on DmACC, in *Dmel2* cells treated with different combinations of dsRNAs targeted against the putative α, β and γ subunits of DmAMPK.

supernatants after immunoprecipitation (results not shown) ruling out the possibility that the anti-PT172 antibody was selectively precipitating a more AMP-dependent fraction of the kinase.

DmAMPK is activated and phosphorylated in response to oligomycin treatment

If DmAMPK is sensitive to AMP, it should be activated in intact cells by treatments that inhibit ATP synthesis, such as oligomycin, an inhibitor of the mitochondrial ATP synthase. Figure 4(A) shows that treatment of *Dmel2* cells with 100 nM oligomycin for 1 h caused very large increases in the cellular AMP:ATP and ADP:ATP ratios (40-fold and 14-fold respectively), and that this was unaffected by the dsRNA treatment. Figure 4(B) shows that DmAMPK activity in the cell lysates was increased 2-fold by oligomycin, and that both the basal and oligomycin-stimulated activity was almost completely abolished by pre-treatment with dsRNA targeted against the α subunit.

Figure 5(B) (lanes 1 and 2) show that oligomycin also stimulated the phosphorylation of Thr-184 on the α subunit of DmAMPK, as judged by Western blotting using the anti-PT172 antibody. The signal obtained using this antibody was abolished using dsRNAs targeted against the α or γ subunits of DmAMPK, or any combination of two or three dsRNAs ($\alpha+\beta$, $\alpha+\gamma$, $\beta+\gamma$, $\alpha+\beta+\gamma$, Figure 5B). In the particular experiment shown in Figure 5, treatment with dsRNA targeted against the β subunit alone did not completely abolish the signal obtained after oligomycin treatment (Figure 5B, lane 7), although in experiments with other preparations of this dsRNA there was a complete ablation of the signal (results not shown).

Activation of DmAMPK is associated with phosphorylation of acetyl-CoA carboxylase

Another question was the identity of the downstream targets for DmAMPK. One of the best established substrates for AMPK is acetyl-CoA carboxylase, which in the case of the ACC-1/-α isoform, is inactivated by phosphorylation at Ser-79 [36,37]. BLAST searches of predicted proteins in the *Drosophila* genome using the sequence of rat ACC-1 revealed a single match (FlyBase: FBgn0043811) that we will term DmACC. Studies of the alignment revealed that the sequence around Ser-79/80 on ACC-1/α in the rat/human enzymes was highly conserved (Figure 5A), although because of the presence of a few extra amino acids at the N-terminus, the serine residue equivalent to Ser-79/80 in DmACC is Ser-93. Surrounding Ser-93 are residues (indicated by arrows and bold type in Figure 5A) of the type shown to be positive determinants for recognition by AMPK [38,39], including a basic residue at position -4, and regularly spaced hydrophobic residues at positions -5, -9 and -13. To test whether this site was a target for DmAMPK in intact cells, we made a phosphospecific antibody against this sequence. Figure 5(B) (lanes 1 and 2) show that treatment of *Dmel2* cells with 100 nM oligomycin caused a large increase in the signal obtained with this antibody for a polypeptide that migrated with the expected size (>200 kDa). This signal was reduced, but not completely eliminated, by pre-treatment with dsRNAs targeted against the α or γ subunits of DmAMPK, or any combination of two or three dsRNAs ($\alpha+\beta$, $\alpha+\gamma$, $\beta+\gamma$, $\alpha+\beta+\gamma$). Similar to the results obtained with the anti-DmAMPK (anti-PT172) antibody, in this experiment the dsRNA targeted against the β subunit alone seemed to be slightly less effective than those targeted against α or γ. With every combination of dsRNA used, a low level of residual phosphorylation of DmACC was seen, but this was no longer affected by oligomycin. A faint signal for a polypeptide migrating just ahead of DmACC was also detected. This was not affected by dsRNA treatment, and may represent a non-specific interaction of the antibody with another protein.

Activation of DmAMPK by hypoxia and glucose removal

We also studied two other treatments known to activate mammalian AMPK, namely hypoxia [18] and glucose deprivation [17]. To examine the effect of hypoxia, we flushed the *Dmel2* cell medium with nitrogen gas, tightly sealed the tissue culture flask, and continued incubation. Figure 6(A) shows that this treatment caused a 2-fold increase in DmAMPK activity after 2 h and 4 h. Figure 6(B) shows that treatment under hypoxic conditions for 4 h also caused increased phosphorylation of Ser-93 on DmACC. Another treatment that activated DmAMPK was carbohydrate deprivation: replacement of the medium with carbohydrate-free medium for 2 h caused a 2.5 ± 0.5-fold activation (mean $\pm$ S.E.M., $n=5$).

Subcellular localization of DmAMPK

To investigate the subcellular localization of DmAMPK, we used our antibodies to probe fixed *Dmel2* cells, with detection using fluorescein-labelled second antibodies in the Deltavision fluorescence microscope. With the anti-CQSS antibody, a rather diffuse cellular fluorescence was obtained, but this was not completely abolished by treatment with dsRNAi (interfering dsRNA) targeted against the α subunit, so this antibody appears not to be sufficiently specific for this purpose. Using the anti-PT172 antibody, there was an intense punctate fluorescence

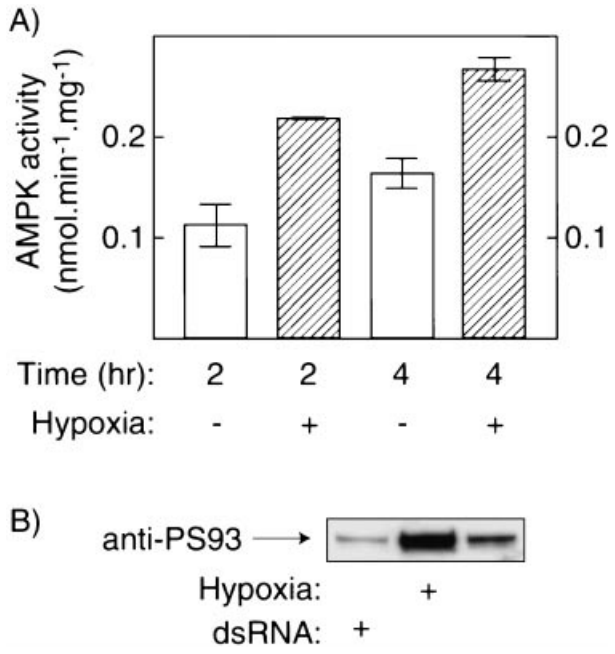

Figure 6 (A) Activation of DmAMPK, and (B) phosphorylation of Ser-93 on DmACC in *Dmel2* cells in response to hypoxia

In (**A**) the activities were measured in the crude cell lysate. In (**B**) the duration of hypoxia was 4 h.

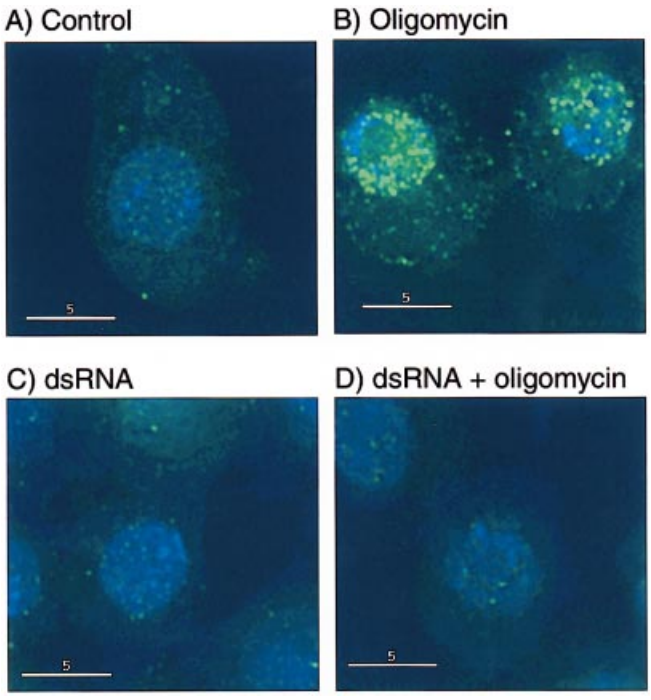

Figure 7 Immunohistochemical detection of DmAMPK using the anti-PT172 antibody in control cells (A), cells treated with 100 nM oligomycin for 1 h (B), cells pre-treated with dsRNA targeted at the DmAMPK α subunit (C), and dsRNA pre-treated cells treated with oligomycin (D)

Cells were fixed, permeabilized and probed with anti-PT172, with indirect detection using FITC-labelled donkey anti-(sheep IgG). Cells were also stained with DAPI to reveal the nuclei. The bars show the scale in μm. Images were deconvolved [30] and are optical sections.

associated with the nucleus, and a much weaker signal in the cytoplasm. As expected, the fluorescence signal with the anti-PT172 antibody was only seen to a significant extent when the cells had been treated with oligomycin (cf. Figures 7A and 7B). The signal obtained after oligomycin treatment was almost totally abolished by pre-treatment with dsRNAi targeted against the DmAMPK α subunit (cf. Figures 7B and 7D). This confirms that this antibody is specific for DmAMPK when used for immunohistochemical detection.

DISCUSSION

Although there have been previous studies of the homologous SNF1 system in yeast [1] and the SNF1-related protein kinases in higher plants [40], to our knowledge, this is the first study of AMPK in the animal kingdom outside of mammals. Figure 1 shows that the α, β and γ subunits of DmAMPK are more closely related to the mammalian homologues than to those of fungi or plants. DmAMPK and the rat homologue were activated by similar concentrations of AMP (half-maximal effect at 2–3 μM), although the degree of stimulation of the *Drosophila* kinase was lower (4.5-fold compared with 22-fold). Both DmAMPK and the rat liver kinase were activated to much greater extents by AMP when they were purified by immunoprecipitation with the anti-PT172 antibody rather than other antibodies, and in fact we were unable to demonstrate any AMP dependence for DmAMPK after immunoprecipitation using the anti-QSSM antibody. The explanation for this curious behaviour remains unclear, although it is interesting that Stein et al. [14] reported that Thr-172 $\rightarrow$ Asp mutant versions of recombinant $\alpha1\beta1\gamma1$ and $\alpha2\beta1\gamma1$ complexes were activated to much greater extents by AMP than the wild-type complexes (62-fold versus 3-fold for $\alpha1$; 43- versus 13-fold for $\alpha2$). The binding of the antibody to phospho-Thr-172 may produce a subtle conformational change that accentuates the effect of AMP, and this effect is perhaps mimicked by the T172D mutation.

In several respects the biochemical properties of the insect system are closely related to those of the mammalian system. (1) Like mammalian AMPK, DmAMPK was allosterically activated by AMP, albeit to a lower extent. (2) Like the mammalian kinase, the insect kinase was activated by treatments that depleted cellular ATP and caused increases in AMP, such as oligomycin and hypoxia. This was associated with phosphorylation of Thr-184 within the activation loop, a regulatory feature exhibited by all AMPK/SNF1-related protein kinases. The present results also show that DmAMPK is activated by glucose deprivation, as are its homologues in budding yeast [33] and mammalian cells [17]. (3) The phosphorylation of acetyl-CoA carboxylase by AMPK at a homologous site near the N-terminus (Ser-79/Ser-93) is also conserved between mammals and insects.

Our results strongly suggest that the putative α, β and γ subunit sequences identified by homology with the mammalian homologues do indeed correspond to the subunits of a heterotrimeric DmAMPK complex in *Drosophila* cells. In the case of the α subunit, we were able to immunoprecipitate kinase activity detectable using the SAMS peptide (a rather specific substrate for mammalian AMPK) with either of two antibodies (anti-CQSS and anti-PT172) made against synthetic peptides derived from the sequence. The detection of the 60 kDa α subunit by Western blotting with the anti-PT172 antibody after oligomycin treatment, as well as phosphorylation of Ser-93 on DmACC, was also reduced or abolished by treatment with dsRNA targeted against the putative β or γ subunits, as well as dsRNA targeted against the α subunit itself. This provides evidence that all three subunits are required to form a functional complex in

insect cells. Very similar findings have been reported in the mammalian and yeast systems. In mammals, significant expression of the recombinant α subunit is not seen unless DNAs encoding a β and γ subunit are co-transfected with that encoding the α subunit [7,8]. In budding yeast, disruption of the genes encoding the γ subunit (*SNF4*) [41], or those encoding all three β subunits (*SIP1*, *SIP2*, *GAL83*) [42] results in the same phenotype as disruption of the gene encoding the catalytic subunit, *SNF1* [43].

Using the anti-PT172 antibody, active DmAMPK appears to be largely confined to the nucleus of *Dmel2* cells (Figure 7). In that respect, it is similar to the α2 isoform of mammalian AMPK, which is located in the nucleus in the pancreatic β cell line, INS-1 [35], as well as in neurons in the hippocampus and cortex of rat brain [44], and in skeletal muscle [45]. In budding yeast, the Gal83p isoform of the β subunit appears to target the SNF1 complex to the nucleus [46]. It should be noted that the anti-PT172 antibody only detects the phosphorylated, activated form of DmAMPK-α, and we cannot rule out the possibility that there is a pool of inactive kinase in the cytoplasm. As expected, the nuclear fluorescence obtained with the anti-PT172 antibody was greatly enhanced if the cells had been treated with oligomycin. This difference, together with the abolition of the signal in cells pre-treated with dsRNA targeted at the α subunit (Figure 7), confirmed that the antibody is specific for the phosphorylated α subunit of DmAMPK.

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