A novel physical and functional association between nucleoside diphosphate kinase A and AMP-activated protein kinase $\alpha 1$ in liver and lung

Russell M. CRAWFORD*, Kate J. TREHARNE*, O. Giles BEST*, Richmond MUIMO†, Claudia E. RIEMEN* and Anil MEHTA*¹

*Department of Maternal and Child Health Sciences, Ninewells Hospital, University of Dundee, Dundee DD1 9SY, U.K., and †Academic Unit of Child Health, Stephenson Wing, Sheffield Children's Hospital, Sheffield S10 2TH, U.K.

Nucleoside diphosphate kinase (NDPK, NM23/awd) belongs to a multifunctional family of highly conserved proteins (~16–20 kDa) containing two well-characterized isoforms (NM23-H1 and -H2; also known as NDPK A and B). NDPK catalyses the conversion of nucleoside diphosphates into nucleoside triphosphates, regulates a diverse array of cellular events and can act as a protein histidine kinase. AMPK (AMP-activated protein kinase) is a heterotrimeric protein complex that responds to cellular energy status by switching off ATP-consuming pathways and switching on ATP-generating pathways when ATP is limiting. AMPK was first discovered as an activity that inhibited preparations of ACC1 (acetyl-CoA carboxylase), a regulator of cellular fatty acid synthesis. We report that NM23-H1/NDPK A and AMPK $\alpha 1$ are associated in cytosol from two different tissue sources: rat liver

and a human lung cell line (Calu-3). Co-immunoprecipitation and binding assay data from both cell types show that the H1/A (but not H2/B) isoform of NDPK is associated with AMPK complexes containing the $\alpha 1$ (but not $\alpha 2$) catalytic subunit. Manipulation of NM23-H1/NDPK A nucleotide transphosphorylation activity to generate ATP (but not GTP) enhances the activity of AMPK towards its specific peptide substrate *in vitro* and also regulates the phosphorylation of ACC1, an *in vivo* target for AMPK. Thus novel NM23-H1/NDPK A-dependent regulation of AMPK $\alpha 1$ -mediated phosphorylation is present in mammalian cells.

Key words: acetyl-CoA carboxylase, AMP-activated protein kinase (AMPK), catalytic subunit, fatty acid synthesis, nucleoside diphosphate kinase (NDPK), nucleotide, SAMS peptide.

INTRODUCTION

Nucleoside diphosphate kinase (NDPK, NM23/awd) has been recognized for over 40 years as a ubiquitous enzyme that catalyses the transfer of the γ -phosphate of a (deoxy)nucleoside triphosphate to a different (deoxy)nucleoside diphosphate [1]. This versatility allows NDPK to function as a nucleotide converter, balancing different cytosolic nucleotide pools. To date, nine human isoforms of NDPK (NM23-H1–NM2-H9), transcribed from different NM23 genes, have been identified and constitute a family of structurally and functionally conserved proteins consisting of four to six identically folded monomers of approx. 16–20 kDa [2]. NDPK is also reported to be a protein histidine kinase [3]. The most widely expressed isoforms of NDPK (NM23-H1 and -H2; also known as NDPK A and B) regulate a diverse array of cellular events including growth and development, tumour metastasis and transcriptional regulation [3,4]. Despite sharing 88% amino acid sequence identity, these two isoforms are reported to have distinct cellular functions [5]. Accumulating evidence indicates that protein–protein interactions modulate the specific molecular actions of NDPK, with new binding partners being identified at an increasing rate [4]. For convenience, we will use the term NDPK A/B to refer to NM23-H1/-H2 and its rat homologue throughout the present paper. In contrast, when we refer to the NDPK antibody raised against the human isoform, we will use its commercial designation, NDPK-H1 or -H2.

AMPK (AMP-activated protein kinase) is a heterotrimeric protein with a 63 kDa catalytic α subunit and two regulatory β and γ subunits (38 and 36 kDa respectively), each of which is encoded by distinct genes ($\alpha 1$ and $\alpha 2$; $\beta 1$ and $\beta 2$; $\gamma 1$, $\gamma 2$ and $\gamma 3$) [6,7]. AMPK is reported to be a sensor of cellular energy status, re-

sponding to the cytosolic AMP/ATP ratio that itself varies as the square of the ADP/ATP ratio, the whole process being equilibrated by the adenylate kinase reaction [8]. Once activated by a rise in cellular AMP concentration, AMPK phosphorylates several downstream substrates. The net effect switches off ATP-consuming pathways (e.g. fatty acid synthesis and cholesterol synthesis) and switches on ATP-generating pathways (e.g. fatty acid oxidation and glycolysis) [9]. AMPK was first discovered as an activity that inhibited preparations of ACC1 (acetyl-CoA carboxylase), a major enzyme in the regulation of cellular fatty acid synthesis [10]. This enzyme was the first described *in vivo* target for AMPK, with phosphorylation resulting in a decline in cytosolic malonyl-CoA concentration, that switches off fatty acid synthesis (ATP conserving) and augments mitochondrial uptake of fatty acids for β -oxidation (ATP generation) [11]. AMPK has other longer-term effects in addition to the more short-term conservation of ATP, by altering both protein and gene expressions, although the physiological consequences of such effects are not fully understood [12,13].

Paradoxically, recent results have indicated that AMPK is able to respond to stimuli that do not cause a detectable change in the AMP/ATP ratio, suggesting that other signals can feed into the AMPK system allowing for a more complex mechanism of cellular energy control [14,15]. In the present study, we add to this notion by demonstrating that the AMPK $\alpha 1$ (but not $\alpha 2$) catalytic subunit is associated with NDPK A (but not B). We provide *in vitro* evidence that local channelling of ATP produced from GTP by an NDPK-catalysed reaction is capable of altering the activity of AMPK in an AMP-independent manner, as determined by specific AMPK assay. Further, we show that manipulation of NDPK activity regulates AMPK-dependent phosphorylation

Abbreviations used: ACC1, acetyl-CoA carboxylase; AMP-CP, ADP[α, β -CH₂]; AMPK, AMP-activated protein kinase; AMPKK, AMPK kinase; DMP, dimethyl pimelimidate; NDPK, nucleoside diphosphate kinase.

¹ To whom correspondence should be addressed (email a.mehta@dundee.ac.uk).

of ACC1, an *in vivo* AMPK target, independent of the upstream AMPKK (AMPK kinase). We propose that a functional association between these two enzymes allows nucleotide concentration to be efficiently sensed and responded to, within this novel protein complex.

EXPERIMENTAL

NDPK/AMPK cDNA constructs and purification

pcDNA3.1 constructs containing cDNA encoding for human AMPK $\alpha 1$ and AMPK $\alpha 2$ (a gift from D. Carling, Imperial College London) and NDPK A and NDPK B (a gift from M. L. Lacombe, INSERM U402, Faculté de Médecine Saint-Antoine, Paris, France) were grown in bacterial culture using an ampicillin resistance selection and affinity-purified using a poly-His tag following the Lebediker method (Protein Purification Facility, Hebrew University of Jerusalem). Briefly, 1 litre of LB (Luria–Bertani) broth was inoculated with pcDNA3.1-His⁺ plasmid cDNA containing sequence-verified AMPK $\alpha 1$, AMPK $\alpha 2$, NDPK A or NDPK B and grown for 10–12 h in the presence of ampicillin. The bacteria were pelleted with a 10 min spin at 4000 rev./min and resuspended in 10 ml of lysis buffer [50 mM NaPO₄, 0.3 M NaCl, 8 M urea and 10 mM imidazole (pH 8), with 2% (v/v) Tween 20 and protease inhibitors added fresh] and sonicated for 3 × 15 s bursts. The lysate was then passed through a high-gauge needle and DNA/insoluble proteins were pelleted by rigorous centrifugation for 5 min. The supernatant from this spin was added to 5 ml of start buffer (0.2 M NaPO₄, 0.5 M NaCl and 10 mM imidazole, pH 7.4) and applied to a 5 ml Ni²⁺ Sepharose affinity-purification column (Bio-Rad, Hemel Hempstead, Herts., U.K.), pre-equilibrated in start buffer. The sample was applied three times through the column and then washed with a further 10 column volumes of start buffer prior to elution of His-tagged proteins with 7 ml of elution buffer (0.2 M NaPO₄, 0.5 M NaCl and 500 mM imidazole, pH 7.4) collected in 0.5 ml fractions by gravity flow. Column fractions were screened for target protein by Bradford analysis and Western blotting using isoform-specific antibodies. Purity of the eluted protein was verified by both Blue and silver staining and appeared more than 90% pure.

Antibodies used in the present study

Anti-NDPK-H1 (NM301) mouse monoclonal and anti-NDPK-H2 (L-15) goat polyclonal antibodies were supplied by Santa Cruz Biotechnology (Autogen Bioclear, Wiltshire, U.K.). Rabbit anti-MEK/MAPKK antibody [where MEK stands for MAPK (mitogen-activated protein kinase)/ERK (extracellular-signal-regulated kinase) kinase] was supplied by Sigma, and the acetyl-CoA, phospho-ACC1, AMPK $\alpha 1$ and $\alpha 2$ and phospho-T172-specific antibodies and other reagents were kindly provided by D. G. Hardie (University of Dundee) and D. Carling.

Cytosolic protein extraction

Fractionation of tissue/cells was carried out as reported previously [16]. Briefly, homogenization of tissue or cell extracts was achieved by using mechanical lysis into 3 vol. of ice-cold buffer I (10 mM Tris, 20 mM NaH₂PO₄ and 1 mM EDTA, pH 7.8, containing a fresh protease-inhibitor cocktail). Osmolarity was restored by adding 1/20 volume of 2.4 M KCl, 1/40 volume of 1.2 M NaCl and 1/5 volume of 1.25 M sucrose. Mixtures were centrifuged for 5 min at 500 g. The pellets obtained contain nuclei. Supernatants were diluted in buffer II (30 mM imidazole, 120 mM KCl, 30 mM NaH₂PO₄ and 250 mM sucrose, pH 6.8, with protease inhibitor as above) to a protein concentration of

approx. 0.7 mg/ml. Samples were then spun down at 7000 g for 15 min to remove mitochondria. The supernatant was then subjected to a 30000 g spin for 30 min. The resultant pellets contain the remaining membrane proteins and the supernatants contain cytosolic protein.

Immunoprecipitation

Prior to precipitation, DMP (dimethyl pimelimidate) linking was performed by the method of Harlow and Lane [16a], thereby eliminating both the antibody heavy and light chains from detection and non-specific cross-reactivity in the system. Briefly, excess antibody was added to Protein A (in the case of mouse antibody) or Protein G (in the case of goat/sheep antibody) beads and incubated at room temperature (25°C) for 1 h with gentle rocking. The beads were then washed twice with 10 vol. of 3 M NaCl and 50 mM sodium borate (pH 9.0) by centrifugation and aspiration. Beads were then resuspended in 10 vol. of 3 M NaCl, 0.2 M sodium borate (pH 9.0) and DMP added to a final concentration of 20 mM. This was mixed at room temperature for 30 min whilst rocking and the reaction was stopped by washing the beads once in 0.2 M ethanolamine (pH 8.0) and further incubating the beads in 0.2 M ethanolamine (pH 8.0) for 2 h at room temperature with gentle mixing. Before use, the beads were resuspended in standard assay buffer (50 mM Hepes, pH 7.0, 50 mM NaF, 1 mM EGTA, 1 mM EDTA, 0.2% Tween 20 and 10%, v/v, glycerol).

We used a 7 μ l slurry of either DMP-antibody-linked Protein A or DMP-antibody-linked Protein G beads and added cytosolic protein as detailed in each Figure legend. Samples were then shake-incubated for 60 min at 4°C. The samples were pelleted in a desktop centrifuge, followed by 3 × 1 ml washes with standard assay buffer containing 1 M NaCl and 3 × 1 ml washes into standard assay buffer. Precipitation pellets were resuspended in 20 μ l of assay buffer and assayed/probed as described below.

Western-blot analysis

Protein concentrations were determined by the method of Bradford. SDS/PAGE was performed using the Novex (Invitrogen) system on 4–12% Bis-Tris polyacrylamide gels and Mes buffer, allowing for good separation of proteins between 17 and 180 kDa. Western-blot analysis was performed as detailed in [17]. Briefly, nitrocellulose membranes were blocked in TBS-Tween (0.5% Tween 20) plus 5% milk powder for 30 min followed by 4 × 15 min washes in TBS-Tween. Primary antibodies were incubated for 90 min followed by a further 4 × 15 min washes. Species-specific secondary antibodies were used according to the manufacturer's instructions and incubated for 45 min, followed by 4 × 15 min washes. ECL[®] (enhanced chemiluminescence) reagent (Amersham Biosciences) was used for visualization. Specificity of the antibodies to their targets is described elsewhere [5,18] and confirmed here in Figure 1.

AMPK activity (SAMS) assay

AMPK activity was assayed by measuring the incorporation of ³²P from 500 nM [γ -³²P]ATP into 1 mM of the synthetic peptide substrate 'SAMS' (HMRSAMSGSLHLVKRR) exactly as detailed by Sullivan et al. [19]. In detail, samples were suspended to a final volume of 25 μ l in standard assay buffer and assays were carried out at 30°C for 10 min and terminated by spotting a 15 μ l aliquot on to a 1 cm² piece of P-81 phosphocellulose paper and washing for 3 × 5 min in 1% phosphoric acid. Samples were then air-dried and incorporation of γ -³²P was quantified using a Packard Instant Imager [20]. Additionally, to investigate the effects of bulk medium ATP concentrations, in Figure 5, we performed

Figure 1 Co-precipitation of AMPK and NDPK

(A, B) Calu-3 cytosolic protein extract (20 μ g) immunoprecipitated using 10 μ g of NDPK-H1 (A) or AMPK α 1 (B) antibody. Each precipitate was probed with a 1/1000 dilution of one among NDPK-H1, NDPK-H2, AMPK α 1 and AMPK α 2 to test the isoform specificity of each antibody. BA, beads alone precipitation control (no primary antibody) probed with NDPK-H1 (A) or AMPK α 1 (B). UA, unrelated antibody control (1/1000 dilution of anti-MEK antibody) probed with NDPK-H1 (A) or AMPK α 1 (B). Positive controls: the AMPK α 2 positive control was an equivalent AMPK α 2 precipitation probed with a 1/1500 dilution of AMPK α 2 to verify the presence of α 2 in the extract. Likewise, the NDPK-H2 positive control was an NDPK-H2 precipitation probed with a 1/1000 dilution of NDPK-H2. (C) Bacterially expressed recombinant AMPK α 1 and α 2 (5 μ g) were spotted on to nitrocellulose membrane and overlaid with 5 μ g of bacterially expressed recombinant NDPK-H1 or -H2 as indicated. Positive control (+ Ct) was 10 μ g of each recombinant NDPK protein overlaid on to itself, negative control (- Ct) was simply a blocked membrane without prebound sample loaded to control for non-specific interactions.

AMPK-SAMS assays in the presence of two 'bulk-solution' ATP concentrations, 500 nM (standard) or 1 mM (excess) ATP, as described in the legend to Figure 5.

Densitometry

The Quantiscan densitometry software package from Biosoft (Cambridge, U.K.) was used with data expressed in AU (arbitrary units) (mean $\pm$ range) corrected for background.

RESULTS AND DISCUSSION

We investigated two sources of cytosolic NDPK and AMPK protein: rat liver tissue extracts and Calu-3 human lung-derived epithelial cells.

A novel NDPK A-AMPK α 1 interaction

First, we confirmed that the NDPK-H1, NDPK-H2, AMPK α 1 and AMPK α 2 isoform-directed antibodies were specific and that there were no cross-reactive or non-specific precipitants. We combined this approach with a Western blotting and immunoprecipitation method and observed that, in both rat liver and Calu-3 cytosolic extracts, an NDPK-H1 antibody precipitated the

AMPK α 1 protein and vice versa. Figure 1 shows representative immunoprecipitates from 20 μ g of Calu-3 cytosolic protein extracts, using 10 μ g of DMP-linked NDPK-H1 (Figure 1A) or AMPK α 1 (Figure 1B) antibody (results were identical in rat liver; results not shown). Each precipitate was probed with one of the following antibodies: NDPK-H1, NDPK-H2, AMPK α 1 or AMPK α 2, to confirm the isoform specificity of each. A beads-alone (BA) precipitation control (no primary antibody) was added and probed with NDPK-H1 (Figure 1A) or AMPK α 1 (Figure 1B) antibodies. An unrelated antibody (UA) control (anti-MEK antibody) was also used and probed with NDPK-H1 (Figure 1A) or AMPK α 1 (Figure 1B) antibodies. To verify the presence of AMPK α 2 and NDPK B in the extracts, positive controls were included in the last two lanes using precipitations of AMPK α 2 and NDPK B probed with AMPK α 2 and NDPK-H2 respectively. Thus the data in lanes 3 of Figures 1(A) and 1(B) specify the association with NDPK A and AMPK α 1, which survives stringent salt washes and is not present in either the NDPK B or AMPK α 2 precipitates (lanes 4).

To confirm this, Figure 1(C) shows the isoform-selective binding of NDPK A to AMPK α 1 using purified, bacterially expressed recombinant NDPK A/B and AMPK α 1/ α 2 isoforms in an overlay matrix, each detected by its specific antibody. Positive control was 10 μ g of each recombinant protein overlaid on to itself (taking advantage of the fact that NDPK forms multimers) and negative control was simply a blocked membrane without prebound protein to control for non-specific binding. The recombinant interaction mirrors the isoform selectivity witnessed in the tissue/cell samples and provides a control for non-specific association during high stringency immunoprecipitation.

NDPK A and AMPK α 1 are specifically and quantitatively associated in both liver and lung

We quantified the association between NDPK A and AMPK complexes containing the α 1 catalytic subunit from rat liver and Calu-3 cytosol by a titration-precipitation method. Figure 2 shows Western blots where 20 μ g of either Calu-3 (Figure 2A) or rat liver cytosolic (Figure 2B) protein extract was immunoprecipitated with increasing amounts (1, 3, 5 and 10 μ g) of either NDPK-H1 or AMPK α 1 isoform-specific antibody irreversibly linked to protein Sepharose beads as detailed in the Experimental section. In each case, the positive control was 20 μ g of protein extract without precipitation. Each NDPK-H1 antibody precipitate pellet (Figures 2A1 and 2B1) and residual supernatant (Figures 2A2 and 2B2) was probed with a 1/1000 dilution of AMPK α 1 antibody. The reciprocal condition was also analysed, i.e. pellets (Figures 2A3 and 2B3) and supernatants (Figures 2A4 and 2B4) were probed with NDPK-H1. Precipitation pellets from each antibody (NDPK-H1 or AMPK α 1) were also probed with NDPK-H2 (Figures 2A5 and 2B5) and AMPK α 2 (Figures 2A7 and 2B7) antibodies. These were blank, providing negative controls. Residual precipitation supernatants were further probed with NDPK-H2 (Figures 2A6 and 2B6) and AMPK α 2 (Figures 2A8 and 2B8) antibodies, thereby not only confirming that both AMPK α 2 and NDPK B are present in our cytosolic extract, but also confirming that equivalent amounts of each are present across the protein samples.

These results demonstrate that only NDPK A is able to precipitate specifically and quantitatively almost all the detectable AMPK α 1 protein and vice versa, from both Calu-3 lung cells and rat liver cytosol extract, as corroborated by the quantitative removal of each respective protein from the residual supernatants. This result was unexpected because gross quantities of the abundant protein NDPK A should exceed those of AMPK by

Figure 2 Quantitative co-precipitation of NDPK and AMPK from both Calu-3 and liver cytosolic extract

Calu-3 (A) or liver (B) cytosolic protein extract (20 μ g each) was immunoprecipitated with increasing amounts (1, 3, 5 and 10 μ g) of either NDPK-H1 or AMPK α 1 isoform-specific antibody irreversibly linked to protein Sepharose beads as detailed in the Experimental section. In each case, the positive control (+Ct) was 20 μ g of protein extract without precipitation. Increasing NDPK-H1 antibody precipitate pellets (A1, B1) and residual supernatants (A2, B2) were probed with a 1/1000 dilution of AMPK α 1 antibody. The reciprocal condition was also analysed; thus pellets (A3, B3) and supernatants (A4, B4) were probed for NDPK-H1. Precipitation pellets from each antibody (NDPK-H1 or AMPK α 1) were also probed for NDPK-H2 (A5, B5) and AMPK α 2 (A7, B7) as indicated. Residual precipitation supernatants were also probed for NDPK-H2 (A6, B6) and AMPK α 2 (A8, B8), thereby confirming that both AMPK α 2 and NDPK-H2 are present in the cytosolic extracts and that equivalent amounts of each are present across the protein samples, confirming equal loading.

several orders of magnitude. To investigate this anomaly, we measured precipitable AMPK activity.

An NDPK-H1 antibody is able to precipitate AMPK α 1-SAMS activity from Calu-3 and liver cytosolic extracts

The ordinates in Figures 3(A) and 3(B) quantify AMPK activity data from three independent experiments using 20 μ g of either Calu-3 (Figure 3A) or rat liver (Figure 3B) cytosolic protein immunoprecipitated using an excess (10 μ g) of one among either NDPK-H1, NDPK-H2, AMPK α 1 and AMPK α 2 antibodies as indicated. Assays were performed in triplicate and data are presented as c.p.m. $\pm$ range. The total detectable AMPK-SAMS activity from 20 μ g of either Calu-3 or rat liver cytosol without precipitation is presented (Figures 3A and 3B, TOTAL). The suffix 'p' demonstrates the amount of detectable AMPK-SAMS activity recovered in each precipitating pellet under these conditions.

Excess NDPK-H1 antibody specifically precipitates AMPK α 1-directed SAMS activity from cytosolic extracts of both cell types. We observed a relatively small proportion ($\sim$ 15–20%) of the total AMPK activity attributable to AMPK α 2, which was not precipitated by either NDPK- or AMPK α 1-specific antibodies (columns 3 in Figures 3A and 3B, and results not shown). We further observed that NDPK B was unable to precipitate AMPK-SAMS activity from any extract (last lane). The selectivity of this activity data is consistent with our observations using the Western-blot and overlay data, further demonstrating that NDPK A alone is quantitatively and specifically associated with AMPK α 1 (but not α 2) in Calu-3 and rat liver cytosol.

Figure 3(C) shows a Western blot using NDPK-H1 and AMPK α 1 antibodies to probe 10 μ g of total cell lysate, nuclear, cytosolic and membrane fractions from rat liver. The differential distribution of NDPK A and AMPK α 1 throughout these subcellular fractions indicates that almost all the AMPK α 1 is

Figure 3 NDPK and AMPK precipitates from Calu-3 and rat liver cytosolic extracts assayed for AMPK-SAMS activity

Calu-3 (**A**) or rat liver (**B**) cytosolic protein extract (20 μ g each) was immunoprecipitated using an excess (10 μ g) of one among NDPK-H1, NDPK-H2, AMPK α 1 and AMPK α 2 antibodies as indicated (Figure 1 confirms that 10 μ g of antibody precipitates all the detectable AMPK-SAMS activity from this amount of starting sample). Assays were performed in triplicate and corrected for background phosphorylation, presented as c.p.m. $\pm$ range. 'TOTAL', the total AMPK-SAMS activity that was detected in 20 μ g of the starting material (either Calu-3 or rat liver cytosol extract), without precipitation. AMPK α 1 pⁱ, AMPK α 2 pⁱ, NDPK-H1 pⁱ and NDPK-H2 pⁱ detail the amounts of detectable AMPK-SAMS activity that was recovered in the precipitating pellets under these experimental conditions. (**C**) Left panel: Western blots using NDPK-H1 and AMPK α 1 antibodies, probing 10 μ g of total lysate (Lys), nuclear (Nuc), cytosolic (Cyto) and membrane (Memb) fractions. The histogram presents the data from three independent experiments, the arrow highlighting that whilst approx. 90% AMPK α 1 is present in the cytosolic fraction, only approx. 5% of total cellular NDPK A is accounted for in this fraction.

present in the cytosol, whilst a relatively small proportion of cellular NDPK A is present in this fraction, most being nuclear. The arrow in the accompanying quantification (Figure 3C, right panel) demonstrates that whilst approx. 90% of AMPK α 1 is present in the cytosol, only approx. 5% of total cellular NDPK A is present therein. NDPK is one of the most abundant proteins in the cell present in submicromolar amounts; AMPK is also abundant, estimated to lie in the nanomolar range (D. G. Hardie, personal communication). Taken into account, firstly, that there are multiple isoforms of NDPK making up the submicromolar total and, secondly, that we demonstrate that only approx. 5% of this pool is available to bind AMPK α 1 in cytosol, this suggests that the amount of NDPK and AMPK in liver cytosol lie approximately within the same order of magnitude and rationalizes the quantitative association reported in Figures 2 and 3. As proof of the concept, we investigated a functional correlate of this association using a well-characterized AMPK α 1 target protein.

NDPK is able to regulate AMPK-dependent phosphorylation of an *in vivo* AMPK target, ACC1

ACC1 is an established *in vivo* target for AMPK and forms the basis for the SAMS peptide assay with respect to the AMPK phosphorylation consensus motif [21]. We investigated whether the phosphorylation of ACC1 by AMPK was regulated by NDPK

using a phospho-specific ACC1 antibody. Figure 4 demonstrates NDPK-regulated, AMPK-dependent phosphorylation of ACC1. Figure 4(A, lane 1) shows a positive control using an anti-ACC1 antibody to probe 20 μ g of rat liver cytosol and confirms that the ACC1 antibody is functioning specifically, recognizing a polypeptide of approx. 265 kDa, corresponding to the established molecular mass of liver ACC1. Co-precipitation (using ACC1) samples were from the same amount of input cytosol (Figure 4A, lanes 2–4) and show that, in rat liver, AMPK α 1 and NDPK A are both present in an ACC1 precipitate whilst NDPK B is not. We speculated that NDPK A could regulate the phosphorylation status of ACC1 by acting on AMPK α 1. We therefore added different nucleotide combinations [22] to test our hypothesis that NDPK phosphotransferase activity (i.e. NDPK-dependent synthesis of ATP from ADP and GTP, or GTP from ATP and GDP) could affect AMPK phosphorylation.

The upper Western blot in Figure 4(B) also shows ACC1 phosphorylation as a marker for AMPK enzyme activity using a phospho-specific ACC1 antibody [11]. The lanes show identical anti-ACC1 precipitates exposed to various nucleotides as follows: lane A, positive control (ACC1 maximally phosphorylated using pure active AMPK); lane B, GDP + ATP; lane C, ATP alone; lane D, ADP + GTP; lane E, ADP alone; and lane F, GTP alone (all nucleotides added at 500 nM final concentration). The histogram represents densitometry from three independent

Figure 4 NDPK activity regulates AMPK phosphorylation of ACC1, an *in vivo* AMPK target

(A) Positive control was 20 μ g of rat liver cytosol probed using the specific anti-ACC1 antibody (+Ct). To establish specificity, we used the anti-ACC1 antibody to precipitate from 20 μ g of rat liver cytosol, and probed for AMPK α 1 (α 1), NDPK-H1 (NDPK A) and NDPK-H2 (NDPK B). (B) Anti-ACC1 precipitates treated with positive control (lane A), GDP + ATP (lane B), ATP alone (lane C), ADP + GTP (lane D), ADP alone (lane E) and GTP alone (lane F), subjected to SDS/PAGE (4–12% polyacrylamide) and transferred and probed with a 1/2000 dilution of phospho-ACC1 antibody and parallel samples were probed with a 1/2000 dilution of the phospho-T172 AMPK antibody (B, Western blot). Densitometry was performed on the ACC1 bands and expressed in AU corrected for background and plotted as means $\pm$ range (B, histogram).

experimental ACC1-probed samples. All lanes were probed with a 1/2000 dilution of anti-phospho-ACC1 antibody and identical samples were probed with a 1/2000 dilution of the anti-phospho-T172 AMPK antibody as a marker for upstream AMPKK activity [15].

We find that NDPK-generated trinucleotides could change AMPK activity towards a physiologically relevant *in vivo* target (ACC1; Figure 4B, lanes B and D, histogram). This changed AMPK activity either positively or negatively (NDPK-dependent ATP or GTP generation respectively), and requires further investigation. We observe that NDPK is able to exert its effects on an AMPK target, independent of the significantly changing AMPKK activity, the implications of which will be discussed below.

NDPK regulates AMPK phosphorylation of SAMS peptide substrate

In light of the NDPK-dependent regulation of ACC1 phosphorylation by the NDPK–AMPK interaction observed in Figure 4, we quantified the significance of NDPK-dependent nucleotide turnover on AMPK's ability to phosphorylate its synthetic peptide substrate, the SAMS peptide [19]. We manipulated nucleotide levels *in vitro* to determine whether NDPK turnover, with respect to ATP or GTP production, was able to alter AMPK–SAMS activity.

Figure 5(A) shows a standard SAMS assay (using [γ - 32 P]ATP) performed on AMPK α 1 precipitations (black bar) or NDPK-H1 precipitations (grey middle bar) from 20 μ g of rat liver cytosol, alongside identical assays using pure AMPK (open bar). The background condition (triplet 1 in Figure 5A) denotes the standard background AMPK turnover without enzyme activation

(no AMP) in the presence of substrate. We incubated our precipitates with 500 nM of one among AMP, ADP, non-hydrolysable AMP-CP, AMP + AMP-CP, GTP, GTP + ADP and GTP + AMP-CP (where AMP-CP stands for ADP[α,β -CH₂]), as indicated in Figure 5(A). The arrow highlights that the greatest increase in SAMS phosphorylation upon incubation with GTP and ADP is only observed when NDPK is present (prior experimentation confirmed that NDPK was not present in the pure AMPK samples; results not shown). In this experiment, we also included a specificity control using AMP-CP, a non-hydrolysable ADP analogue. This additional control was included to ensure that ADP was not being broken down to AMP in this system by contaminating/co-precipitating enzyme(s). Neither did we observe an increase in AMPK–SAMS phosphorylation upon incubation of ADP alone, indicating that the activation of AMPK was not caused by non-specific metabolism of ADP to AMP within the precipitates. Finally, to test whether substrate channelling was present, we performed an additional experiment increasing bulk medium ATP concentration to the mM range.

Normally, the SAMS assay is performed with submicromolar ATP in the bulk solution. Figures 5(B) and 5(C) show NDPK-H1 antibody precipitations from 20 μ g of rat liver (filled bars) or Calu-3 (open bars) cytosolic fraction, assayed for AMPK activity, expressed as a percentage of maximum SAMS phosphorylation (100%). The standard assay in Figure 5(B) was performed using a final ATP concentration of 500 nM, whilst Figure 5(C) shows equivalent data at a final ATP concentration of 1 mM. In each last lane, we also generated an 'ATP-local' condition using [32 P]GTP + ADP to generate AMPK-localized ATP via NDPK for further transfer on to the SAMS substrate via AMPK, once again at the two different bulk solution ATP concentrations.

Figure 5 NDPK regulates AMPK phosphorylation of SAMS peptide substrate

(A) Standard SAMS assays (using [γ - 32 P]ATP) performed on AMPK α 1 precipitations (black bar) or NDPK-H1 precipitations (grey bar) from 20 μ g of rat liver cytosol, alongside identical assays using pure AMPK (open bar). The background condition denotes the standard AMPK phosphorylation of SAMS peptide without activation in this *in vitro* system, followed by incubation with 500 nM of one among AMP, ADP, AMP-CP (a non-hydrolysable ADP analogue), AMP + AMP-CP, GTP, GTP + ADP and GTP + AMP-CP as indicated. The arrow highlights that the increase in SAMS phosphorylation observed upon incubation with GTP and ADP is only observed when NDPK is present (NDPK is not present in the pure AMPK sample). (B, C) NDPK-H1 immunoprecipitations from 20 μ g of rat liver (closed bars) or Calu-3 (open bars) cytosolic fraction, assayed for AMPK activity, expressed as a percentage of maximum SAMS phosphorylation (with 100% denoting maximal AMPK phosphorylation of the SAMS peptide). We added 6.7 nM of either [32 P]ATP [supplemented with 500 nM unlabelled ATP (B) or 1 mM unlabelled ATP (C)] or [32 P]GTP, either alone or in combination with 500 nM of ADP, GDP or GDP β S as indicated.

We observed that, under conditions where NDPK could make ATP (from the combination of ADP and GTP, Figures 5B and 5C, last lanes), we found a significant 2.5-fold increase above ATP-alone baseline (lane 1) SAMS phosphorylation ($P < 0.0001$). Conversely, we also observed that under conditions where NDPK could make GTP from bulk ATP and GDP (i.e. sequester bulk ATP), AMPK-SAMS phosphorylation decreased by 50% suggesting that 'substrate steal' could also occur away from AMPK.

Importantly, we observe that AMPK uses the γ - 32 P phosphate from the ATP that is generated by NDPK to transfer to the SAMS peptide substrate, despite the presence of 1 mM ATP in the bulk solution (Figure 5C). Furthermore, and consistent with this notion, the failure to significantly change AMPK activity using the hydrolysis-resistant, GDP analogue compound [22], GDP β S (lane 3), in the presence of 1 mM bulk ATP is consistent with the idea that a local supply of ATP is provided to AMPK α 1 by NDPK via substrate channelling within the NDPK A-

AMPK α 1 complex. We also confirmed that GTP alone is not utilized by AMPK (penultimate lane) and we observed no significant AMPK-SAMS activity. Conversely, under conditions where GTP was made by NDPK from ATP (lane 2), we observed a significant decrease in AMPK-SAMS activity despite the presence of mM bulk ATP (Figures 5B and 5C, compare lanes 1 and 2). This suggests that NDPK actually sequesters substrate ATP from AMPK, probably due to its much higher turnover rate ($\sim 1000/\text{min}$) [23], even in the presence of 1 mM ATP in the surrounding solution. It is interesting to note that, in [24], the authors were able to demonstrate that GDP β S is a substrate for pure NDPK with a higher efficacy for the B isoform, but in our experience in the tissue extracts and precipitates used here, the GDP β S compound has not been a substrate for NDPK [22] and this is confirmed in Figures 5(B) and 5(C).

The simplest interpretation of our findings is that under conditions where AMPK is active, NDPK-mediated generation or sequestration of ATP within the complex is able to bidirectionally

Figure 6 NDKP substrate channelling hypothesis

ATP produced by NDKP, from GTP and ADP, is used by AMPK as a substrate in the AMPK-mediated phosphotransfer reaction. AMPK then uses this NDKP-mediated substrate to phosphorylate its downstream targets, one example of which is ACC1. Accumulation of ADP as a result of AMPK phosphotransfer could potentially feed back to NDKP in the microenvironment and fuel the generation of more ATP. By this rationale, even under extremely limiting ATP concentrations, NDKP would provide substrate ATP to AMPK by proxy of their tight association, allowing AMPK to remain functional despite a general shortage of cellular ATP. Thus NDKP has a role to play in the AMPK signalling cascade. The schematic diagram is based on the observations in Figures 4 and 5, hypothesizing the interaction of NDKP with AMPK taking into account the fates of ADP, GTP and ATP pertaining to the ability of AMPK to phosphorylate ACC1, a major downstream component of the AMPK signalling cascade.

influence the supply of ATP for use as phosphate donor by AMPK acting as either a positive (i.e. activating AMPK) or negative (i.e. decreasing the AMPK activity) regulator. We further tested whether manipulation of the NDKP reaction with the nucleotide treatments detailed above changed the phosphorylation status of AMPK itself (Figure 4B, lower panel). AMPK is regulated by an upstream kinase, AMPKK, by phosphorylation of Thr¹⁷² [15,25]. We probed NDKP-H1 antibody precipitates treated with nucleotides as detailed in Figure 4, with a specific AMPK anti-phospho-Thr¹⁷² antibody. We observed no gross changes in Thr¹⁷² phosphorylation under conditions where NDKP was able to alter SAMS-directed AMPK activity (Figure 5B). Thus we believe that the upstream AMPKK is unlikely to be involved in this phenomenon.

The NDKP A/AMPK $\alpha 1$ association: a substrate-channelling hypothesis

Our data suggest a novel functional association between two enzymes critical for cellular energy supply, with NDKP acting as a regulator to maintain AMPK activity towards a metabolically important target in fatty acid synthesis, ACC1 [10]. First, we propose that the NDKP A–AMPK $\alpha 1$ interaction provides AMPK with NDKP-synthesized local ATP, and furthermore, show that NDKP A, but not the closely related NDKP B, can undertake this role, suggesting specificity of function. Secondly, we suggest that the proximity of these enzymes could facilitate efficient nucleotide availability to both GTP- and ATP-utilizing enzymes within the same protein complex, depending on the immediate needs of the cell. For example, under cellular stress, where ATP declines, our observed association could create a bypass to maintain AMPK activity by NDKP generating ATP from GTP local to AMPK, despite critically low cellular ATP.

Figure 6 presents our working hypothesis for the association of NDKP with AMPK. Compared with AMPK, NDKP is a rapidly acting enzyme that balances nucleotide levels, efficiently maintaining that balance. For illustration purposes alone, NDKP is shown utilizing either (i) GDP and ATP to form GTP or (ii) ADP and GTP to form ATP. Under conditions of cellular stress, when AMPK is activated [8,9], the ATP/ADP ratio changes in favour of ADP production. NDKP works to balance nucleotide levels; thus a rise in ADP levels and a fall in ATP levels would prompt NDKP to utilize cellular GTP (and other available trinucleotides) to

generate ATP until balance is re-established. We speculate that the ATP generated from other trinucleotides by NDKP can be utilized by AMPK in the short term to keep this critical enzyme functional under stress conditions. NDKP-mediated substrate channelling could permit efficient, undisrupted AMPK phosphorylation of its downstream targets (e.g. ACC1) despite cellular ATP shortage by transphosphorylating its breakdown product, ADP [26].

We also provide evidence that AMPK-dependent phosphorylation of a known *in vivo* target, ACC1, in rat liver cytosol can be regulated by NDKP. NDKP is also reported to phosphorylate ATP-citrate lyase, the primary source of cytosolic acetyl-CoA, for ACC1 and other biosynthetic pathways, including lipogenesis and cholesterol synthesis [27]. Since phosphorylation of ATP-citrate lyase by NDKP reduces the amount of available acetyl-CoA, this, taken together with our reported NDKP-dependent AMPK phosphorylation of ACC1, could explain how NDKP plays such an important role in the regulation of fatty acid synthesis and membrane biosynthesis [27].

It has been previously suggested that ATP inhibits AMPK by competitively binding at its AMP-binding site [28]. This produces a potential problem in that AMPK requires ATP to function as a kinase. Furthermore, recent publications also indicate that an AMP and/or ATP ligand-interaction site lies on the regulatory AMPK γ subunit [29]. We hypothesize that because NDKP A (but not B) is associated in an AMPK $\alpha 1$ (catalytic subunit)-dependent manner, ATP generated by NDKP A is channelled to AMPK $\alpha 1$ and could thus bypass the previously reported inhibitory effect of ATP on AMPK activity. This idea is supported by our findings that local generation of [³²P]ATP by NDKP results in an increased AMPK-SAMS phosphorylation with no detectable inhibition of AMPK activity despite the presence of 1 mM ATP (Figure 5) or a significant change in AMPK phosphorylation status at Thr¹⁷² (Figure 4).

We conclude that a novel physical and functional association exists between the A isoform of NDKP and AMPK complexes containing the $\alpha 1$ catalytic subunit from two different sources, liver and lung. We propose that NDKP can act to maintain AMPK activity by providing an alternative source of substrate ATP to AMPK. Thus, if AMPK is a cellular fuel gauge [8], then NDKP may provide a reserve power supply to keep the gauge working in times of crisis. We believe that our reported association may provide a novel hypothesis to explain how AMPK is able to function under conditions where cellular ATP is low and describes a new regulatory role for NDKP in the well-established AMPK signalling cascade.

We are grateful to Professor D. G. Hardie, Professor D. Carling, Dr A. Woods and Professor M.-L. Lacombe for constructs, antibodies and reagents. We thank the Wellcome Trust, Cystic Fibrosis Trust (Bromley, Kent, U.K.) and Anonymous Trust (Ninewells Hospital Medical School, University of Dundee) for their support.

REFERENCES

- Heidbuchel, H., Callewaert, G., Vereecke, J. and Carmeliet, E. (1992) Membrane-bound nucleoside diphosphate kinase activity in atrial cells of frog, guinea pig, and human. *Circ. Res.* **71**, 808–820.
- Postel, E. H. (1998) NM23-NDP kinase. *Int. J. Biochem. Cell Biol.* **30**, 1291–1295.
- Engel, M., Veron, M., Theisinger, B., Lacombe, M. L., Seib, T., Dooley, S. and Welter, C. (1995) A novel serine/threonine-specific protein phosphotransferase activity of Nm23/nucleoside-diphosphate kinase. *Eur. J. Biochem.* **234**, 200–207.
- Roymans, D., Willems, R., Van Blockstaele, D. R. and Slegers, H. (2002) Nucleoside diphosphate kinase (NDPK/NM23) and the waltz with multiple partners: possible consequences in tumor metastasis. *Clin. Exp. Metastasis* **19**, 465–476.
- Bosnar, M. H., De Gunzburg, J., Bago, R., Brecevic, L., Weber, I. and Pavelic, J. (2004) Subcellular localization of A and B Nm23/NDPK subunits. *Exp. Cell Res.* **298**, 275–284.

- 6 Stapleton, D., Mitchelhill, K. I., Gao, G., Widmer, J., Michell, B. J., Teh, T., House, C. M., Fernandez, C. S., Cox, T., Witters, L. A. et al. (1996) Mammalian AMP-activated protein kinase subfamily. *J. Biol. Chem.* **271**, 611–614
- 7 Nielsen, J. N., Mustard, K. J., Graham, D. A., Yu, H., MacDonald, C. S., Pilegaard, H., Goodyear, L. J., Hardie, D. G., Richter, E. A. and Wojtaszewski, J. F. (2003) 5'-AMP-activated protein kinase activity and subunit expression in exercise-trained human skeletal muscle. *J. Appl. Physiol.* **94**, 631–641
- 8 Hardie, D. G. and Carling, D. (1997) The AMP-activated protein kinase—fuel gauge of the mammalian cell? *Eur. J. Biochem.* **246**, 259–273
- 9 Hardie, D. G. and Hawley, S. A. (2001) AMP-activated protein kinase: the energy charge hypothesis revisited. *BioEssays* **23**, 1112–1119
- 10 Hardie, D. G. and Pan, D. A. (2002) Regulation of fatty acid synthesis and oxidation by the AMP-activated protein kinase. *Biochem. Soc. Trans.* **30**, 1064–1070
- 11 Winder, W. W., Wilson, H. A., Hardie, D. G., Rasmussen, B. B., Hutber, C. A., Call, G. B., Clayton, R. D., Conley, L. M., Yoon, S. and Zhou, B. (1997) Phosphorylation of rat muscle acetyl-CoA carboxylase by AMP-activated protein kinase and protein kinase A. *J. Appl. Physiol.* **82**, 219–225
- 12 Barnes, K., Ingram, J. C., Porras, O. H., Barros, L. F., Hudson, E. R., Fryer, L. G., Foulfelle, F., Carling, D., Hardie, D. G. and Baldwin, S. A. (2002) Activation of GLUT1 by metabolic and osmotic stress: potential involvement of AMP-activated protein kinase (AMPK). *J. Cell Sci.* **115**, 2433–2442
- 13 Holmes, B. and Dohm, G. L. (2004) Regulation of GLUT4 gene expression during exercise. *Med. Sci. Sports Exerc.* **36**, 1202–1206
- 14 Hawley, S. A., Gadalla, A. E., Olsen, G. S. and Hardie, D. G. (2002) The antidiabetic drug metformin activates the AMP-activated protein kinase cascade via an adenine nucleotide-independent mechanism. *Diabetes* **51**, 2420–2425
- 15 Hawley, S. A., Boudeau, J., Reid, J. L., Mustard, K. J., Udd, L., Makela, T. P., Alessi, D. R. and Hardie, D. G. (2003) Complexes between the LKB1 tumor suppressor, STRAD alpha/beta and MO25 alpha/beta are upstream kinases in the AMP-activated protein kinase cascade. *J. Biol.* **2**, 28
- 16 Ranki, H. J., Crawford, R. M., Budas, G. R. and Jovanovic, A. (2002) Ageing is associated with a decrease in the number of sarcolemmal ATP-sensitive K⁺ channels in a gender-dependent manner. *Mech. Ageing Dev.* **123**, 695–705
- 16a Harlow, E. and Lane, D. (1998) *Antibodies – A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Plainview, NY
- 17 Crawford, R. M., Ranki, H. J., Botting, C. H., Budas, G. R. and Jovanovic, A. (2002) Creatine kinase is physically associated with the cardiac ATP-sensitive K⁺ channel *in vivo*. *FASEB J.* **16**, 102–104
- 18 Woods, A., Salt, I., Scott, J., Hardie, D. G. and Carling, D. (1996) The alpha1 and alpha2 isoforms of the AMP-activated protein kinase have similar activities in rat liver but exhibit differences in substrate specificity *in vitro*. *FEBS Lett.* **397**, 347–351
- 19 Sullivan, J. E., Carey, F., Carling, D. and Beri, R. K. (1994) Characterisation of 5'-AMP-activated protein kinase in human liver using specific peptide substrates and the effects of 5'-AMP analogues on enzyme activity. *Biochem. Biophys. Res. Commun.* **200**, 1551–1556
- 20 Muimo, R., Hornickova, Z., Riemen, C. E., Gerke, V., Matthews, H. and Mehta, A. (2000) Histidine phosphorylation of annexin I in airway epithelia. *J. Biol. Chem.* **275**, 36632–36636
- 21 Davies, S. P., Carling, D. and Hardie, D. G. (1989) Tissue distribution of the AMP-activated protein kinase, and lack of activation by cyclic-AMP-dependent protein kinase, studied using a specific and sensitive peptide assay. *Eur. J. Biochem.* **186**, 123–128
- 22 Muimo, R., Banner, S. J., Marshall, L. J. and Mehta, A. (1998) Nucleoside diphosphate kinase and Cl[−]-sensitive protein phosphorylation in apical membranes from ovine airway epithelium. *Am. J. Respir. Cell Mol. Biol.* **18**, 270–278
- 23 Kowluru, A., Tannous, M. and Chen, H. Q. (2002) Localization and characterization of the mitochondrial isoform of the nucleoside diphosphate kinase in the pancreatic beta cell: evidence for its complexation with mitochondrial succinyl-CoA synthetase. *Arch. Biochem. Biophys.* **398**, 160–169
- 24 Schaertl, S., Konrad, M. and Geeves, M. A. (1998) Substrate specificity of human nucleoside-diphosphate kinase revealed by transient kinetic analysis. *J. Biol. Chem.* **273**, 5662–5669
- 25 Hawley, S. A., Selbert, M. A., Goldstein, E. G., Edelman, A. M., Carling, D. and Hardie, D. G. (1995) 5'-AMP activates the AMP-activated protein kinase cascade, and Ca²⁺/calmodulin activates the calmodulin-dependent protein kinase I cascade, via three independent mechanisms. *J. Biol. Chem.* **270**, 27186–27191
- 26 Hardie, D. G. (2003) Minireview: the AMP-activated protein kinase cascade: the key sensor of cellular energy status. *Endocrinology* **144**, 5179–5183
- 27 Wagner, P. D. and Vu, N. D. (1995) Phosphorylation of ATP-citrate lyase by nucleoside diphosphate kinase. *J. Biol. Chem.* **270**, 21758–21764
- 28 Cheung, P. C., Salt, I. P., Davies, S. P., Hardie, D. G. and Carling, D. (2000) Characterization of AMP-activated protein kinase gamma-subunit isoforms and their role in AMP binding. *Biochem. J.* **346**, 659–669
- 29 Adams, J., Chen, Z. P., Van Denderen, B. J., Morton, C. J., Parker, M. W., Witters, L. A., Stapleton, D. and Kemp, B. E. (2004) Intrasteric control of AMPK via the gamma1 subunit AMP allosteric regulatory site. *Protein Sci.* **13**, 155–165

Received 11 February 2005/8 June 2005; accepted 18 July 2005

Published as BJ Immediate Publication 18 July 2005, doi:10.1042/BJ20050269