5′-AMP inhibits dephosphorylation, as well as promoting phosphorylation, of the AMP-activated protein kinase. Studies using bacterially expressed human protein phosphatase-2Cα and native bovine protein phosphatase-$2A_C$

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Abstract Human protein phosphatase-2Cα (PP2Cα) was purified to homogeneity after expression in *Escherichia coli*. AMP inhibited the dephosphorylation of AMP-activated protein kinase (AMPK), but not phosphocasein, by PP2Cα. The concentration dependence and the effects of other nucleotides (ATP and formycin A-5′-monophosphate) suggest that AMP acts by binding to the same site which causes direct allosteric activation of AMPK. A similar, although less pronounced, effect was observed with another protein phosphatase ($PP2A_C$). We have now shown that AMPK activates the AMPK cascade by four mechanisms, which should make the system exquisitely sensitive to changes in AMP concentration.

Key words: AMP-activated protein kinase; Adenosine monophosphate; Adenosine triphosphate; Protein phosphatase-2C; Protein phosphatase-2A

1. Introduction

The AMP-activated protein kinase (AMPK) is the central component of a protein kinase cascade which is activated by cellular stresses, particularly those which deplete ATP levels and hence (by means of the adenylate kinase reaction) elevate AMP [1]. AMPK phosphorylates multiple targets, particularly key enzymes of biosynthetic pathways, and appears to be the mammalian homologue of the yeast SNF1 protein kinase, which is involved in the response to the stress of glucose starvation [2–6].

AMPK is phosphorylated and activated by an upstream protein kinase termed AMPK kinase (AMPKK). We have previously demonstrated that 5′-AMP activates the AMPK cascade via at least three mechanisms: (i) directly activating AMPKK; (ii) binding to AMPK and making it a better substrate for AMPKK; (iii) directly activating AMPK [7–9]. Some, if not all, of these effects of AMP are antagonized by high (mM) concentrations of ATP [8], so that the system responds to the AMP:ATP ratio rather than to AMP per se. Since the AMP:ATP ratio is a sensitive indicator of the energy status of the cell [1,10], the AMPK cascade can be regarded as a cellular 'fuel gauge'.

The active, phosphorylated form of AMPK can be inactivated in cell-free assays by either protein phosphatase-2A (PP2A) or protein phosphatase-2C (PP2C), the latter enzyme accounting for the majority of the AMPK phosphatase in a rat liver extract [11]. Experiments in isolated rat hepatocytes also suggest that an okadaic acid-insensitive protein phosphatase, such as PP2C (but not PP2A, which is potently inhibited by okadaic acid) is responsible for dephosphorylation of AMPK in intact cells [12].

In this paper we report that, in addition to the three effects of AMP on the AMPK cascade described above, the nucleotide has a fourth effect, i.e. inhibition of the inactivation of active, phosphorylated AMPK by PP2C and PP2A. To facilitate the studies with PP2C, the human α isoform was expressed in *Escherichia coli*.

2. Materials and methods

2.1. Materials

Oligonucleotides were synthesised by Miss Audrey Gough on an ABI 394 DNA synthesizer. The pCW vector was a gift from Dr. A. Roth and Prof. R.W. Dalquist. AMPK (435 U/mg) was purified from rat liver as far as the gel filtration step [13]. The catalytic subunit of protein phosphatase-2A (1100 mU/mg) was purified to homogeneity from bovine heart using the method previously described for rat liver [14].

2.2. Enzyme assays and units

AMPK was assayed using the 'SAMS' peptide as substrate [15], PP2C was assayed using [^{32}P]phosphocasein after phosphorylation by cyclic AMP-dependent protein kinase [16] and PP2A using [^{32}P]phosphorylase *a* [14], all at 30°C. One unit of AMPK phosphorylates 1 nmole of peptide per min, whereas 1 unit of protein phosphatase releases 1 μmol of phosphate per min.

2.3. Bacterial expression and purification of PP2Cα

The human PP2Cα coding sequence [17] was amplified by polymerase chain reaction using the oligonucleotides 5′-CGCG-CAT*ATG*GGAGCATTTTTAGACAAGC-3′ and 5′-GCGC-AAGC*TTA*CCACATATCATCTGTTGATG-3′. The first oligonucleotide creates an *Nde*I site (underlined) at the initiating methionine codon (italicised), whereas the second creates a *Hind*III site (underlined) at the terminating codon (italicised) of the open reading frame (ORF). Digestion of the PCR product with restriction endonucleases *Nde*I and *Hind*III allowed the entire ORF to be cloned into the *Nde*I-*Hind*III sites of the plasmid pCW [18] to generate the plasmid pCW-PP2Cα. The sequence of the ORF within plasmid pCW-PP2Cα was confirmed by sequencing both DNA strands using *Taq* dye terminator

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Abbreviations: AMPK, AMP-activated protein kinase; AMPKK, AMP-activated protein kinase kinase; PP2Cα, protein phosphatase-2C (α isoform); $PP2A_C$, protein phosphatase-2A catalytic subunit; S.E.M., standard error of the mean.

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cycle sequencing on an Applied Biosystems model 373 A automated DNA sequencer.

E. coli strain DH5α {(*sup*E44, Δ(lacZYA-*arg*F)U169, φ80d*lac*ZDM15, *hsd*R17, *rec*A1, *end*A1, *gyr*A96, *thi*-1, *rel*A1, *deo*R} was transformed with plasmid pCW-PP2Cα by electroporation using an 'Easyject plus' electroporator (Equibio). Transformants were plated onto Luria Broth LB plates containing ampicillin (100 μg/ml). After verifying the sequence of the expression construct, single colonies of *E. coli* containing it were grown to an A_{600} of 0.5 at 37°C, and expression of PP2Cα was induced at 28°C in the presence of 1 mM $MnCl_2$, by addition of IPTG to a final concentration of 100 μM for 20 h.

2.4. Purification of protein phosphatase-2Cα

PP2Cα was purified by a modification of the method of McGowan and Cohen [19]. Bacteria were harvested by centrifugation (5000 × *g*; 10 min; 4°C) then resuspended in 20 ml of buffer A (50 mM Tris-HCl pH 7.5, 1 mM DTT, 1 mM EDTA, 0.1 mM EGTA, 100 mM NaCl, 2 mM $MnCl_2$, 1 mM benzamidine, 0.1 mM PMSF, 0.1 mM TPCK, 0.03% (v/v) Brij-35) and sonicated for 4 × 30 s on ice using a Jencons probe sonicator (model GE 50). The lysate was centrifuged at 48,000 × *g* for 20 min at 4°C, the supernatant decanted, and protein precipitating between 30% and 55% saturation with $(NH_4)_2SO_4$ was resuspended in 5 ml of buffer B (20 mM triethanolamine pH 7.0, 1 mM EGTA, 2 mM $MnCl_2$, 0.1% (v/v) β-mercaptoethanol, 5% (v/v) glycerol, 0.03% (v/v) Brij-35). Following dialysis against 1 litre of this buffer for 2 h at 4°C with one change of buffer, the sample was loaded onto a Hiload 16/10 Q-Sepharose column which was developed with a linear gradient (0–400 mM NaCl, 400 ml). Active fractions (eluting at ≈230 mM NaCl) were pooled and dialysed against 1 litre of buffer B at 4°C for 2 h, then applied to a Mono-Q column which was developed with a linear gradient (0–400 mM NaCl, 40 ml). Active fractions were pooled, dialysed for 2 h at 4°C against buffer B containing 50% (v/v) glycerol, and stored at −20°C.

3. Results

3.1. Expression of PP2Cα in Escherichia coli

The entire coding region for human PP2Cα was cloned into the *E. coli* expression vector pCW, allowing expression of the full-length protein. Under the conditions used, PP2Cα protein constituted ≈5–10% of soluble protein (Fig. 1). Mn^{2+} ions were included in the culture medium and purification buffers since this led to increased specific activity of PP2C (data not shown) as reported for human protein phosphatase 1 isoforms expressed in *E. coli* [20]. A simple purification procedure, requiring only two chromatographic steps, produced essentially pure PP2Cα protein migrating on SDS-PAGE with an apparent molecular mass of 42 kDa (Fig. 1), as obtained for native PP2Cα isolated from mammalian tissues [19]. The specific activity of the purified enzyme against ^{32}P-labelled casein was 100 mU/mg, which is comparable with that of PP2C purified from mammalian tissues (140 mU/mg [19]).

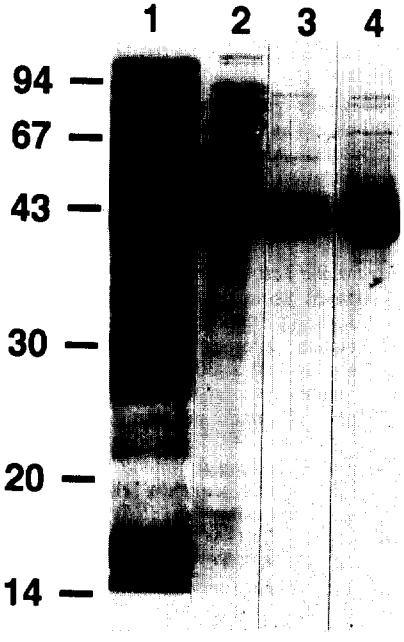

Fig. 1. Purification of PP2Cα from *E. coli*. Fractions from various stages of the purification were separated by SDS-PAGE in 12.5% gels and stained with Coomassie Blue. Key: 1, crude soluble extract; 2, ammonium sulphate; 3, Q-Sepharose; 4, Mono-Q. Mobility of marker proteins (molecular masses in kDa) are indicated on the left.

3.2. AMP inhibits inactivation of AMPK by PP2C and PP2A

AMPK was purified from rat liver in its phosphorylated, active form [13] except that protein phosphatase inhibitors were omitted from the final gel filtration column. Fig. 2A shows that homogeneous, bacterially expressed PP2Cα caused a rapid inactivation of AMPK. When added together with PP2Cα, AMP (200 μM) produced a severe inhibition of this inactivation, whereas GMP at the same concentration caused only a slight inhibition (<15%). The AMPK assays were conducted at a saturating AMP concentration (200 μM), so that the effect of carryover of AMP from the preincubation with PP2C would be negligible. This can also be seen from the finding that neither AMP nor GMP had significant effects on controls incubated without the essential cofactor for PP2C, Mg^{2+} ions (Fig. 2, open symbols). At a concentration of 200 μM, both AMP and GMP produced a small (≈10%) inhibition of the dephosphorylation of [^{32}P]casein by PP2Cα (not shown).

Fig. 2B shows that AMP, but not GMP, also protected AMPK against inactivation by the homogeneous catalytic subunit of PP2A ($PP2A_C$), although in this case protection was only partial. Expressed per mole of protein phosphatase, AMPK was inactivated ≈3-fold more rapidly by PP2Cα than by $PP2A_C$.

3.3. Dose-response for effects of AMP and other nucleotides on inactivation of AMPK

The inhibition of inactivation of AMPK by PP2Cα at different concentrations of AMP (Fig. 3A) was fitted to a single-site model as discussed in the legend to Fig. 3. In the absence of ATP, AMP gave maximal inhibition of 84 ± 2% (± S.E.M.), with a half-maximal effect ($I_{0.5}$) at 3.1 ± 0.3 μM. Using $PP2A_C$ in the absence of ATP, the $I_{0.5}$ for AMP (3.2 ± 0.5 μM) was very similar, but the maximal inhibition was lower at 47 ± 2% (not shown). Using PP2Cα in the presence of 4 mM ATP, the dose-response curve for the effect of AMP was shifted to the right (Fig. 3A), and the $I_{0.5}$ for AMP was now increased 10-fold to 34 ± 7 μM, with a maximal inhibition of 93 ± 6%.

Formycin A-5′-monophosphate (FMP) is an analogue of 5′-AMP which mimics the effect of AMP on allosteric activation of AMPK, albeit with lower potency [7]. Fig. 4 shows that FMP also inhibits the inactivation of AMPK by PP2Cα. FMP gave 99 ± 3% inhibition with a half-maximal effect at 30 ± 4 μM (cf. half-maximal effect of AMP: 3.1 ± 0.3 μM). Using the same preparation of AMPK the $A_{0.5}$ values for the allosteric activation of AMPK by AMP and FMP were 3.2 ± 0.4 and 20 ± 2 μM, respectively (not shown).

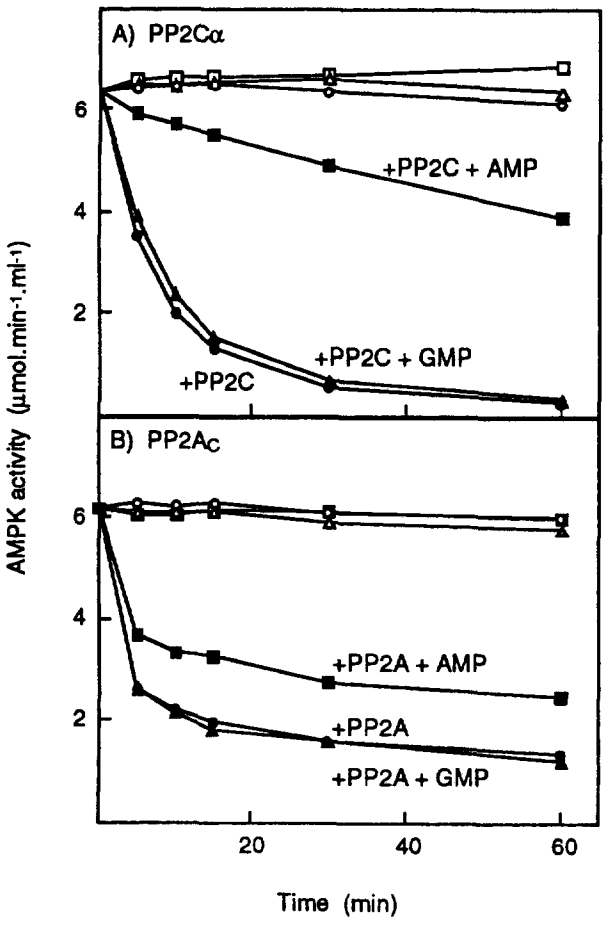

Fig. 2. Time course of inactivation of AMP-activated protein kinase by (A) PP2Cα and (B) $PP2A_C$. AMPK (60 U/ml) was incubated in 50 mM Na-HEPES, pH 7.0, 1 mM DTT, 0.02% Brij-35 at 30°C with PP2Cα (0.1 mU/ml) or $PP2A_C$ (3.2 mU/ml). PP2Cα incubations were performed in the presence of 20 mM $MgCl_2$ (filled symbols) with controls (open symbols) in the absence of $MgCl_2$. $PP2A_c$ incubations were performed in the absence of okadaic acid (filled symbols) or in the presence of 100 nM okadaic acid (open symbols), all in the absence of $MgCl_2$. Further additions were: none (circles), 5′-AMP (200 μM; squares) or 5′-GMP (200 μM; triangles). At various times 5 μl aliquots were withdrawn for AMPK assay in a total volume of 25 μl.

4. Discussion

Expression of human PP2Cα in *E. coli* provides an abundant source of active enzyme, which can be purified to homogeneity more rapidly and in higher yield than from rabbit liver or skeletal muscle [19]. The expression vector encodes the entire open reading frame, and the protein purified is very similar to rabbit PP2Cα both in mobility on SDS-PAGE and in specific activity against [^{32}P]casein [19]. Unlike PP2A, where the catalytic subunit exists in vivo as complexes with other subunits which modify its properties [21], native PP2C appears to be a monomer [19]. The expressed PP2Cα should therefore be a useful tool for further studies of PP2C and its substrates.

In the present paper we analyse dephosphorylation of AMPK indirectly by monitoring changes in kinase activity. Inactivation was completely blocked in controls (Fig. 2) carried out in the absence of Mg^{2+} (PP2Cα) or in the presence of okadaic acid ($PP2A_C$), demonstrating that it was a function of the protein phosphatase activities in the preparations. Inactivation was also reversed by incubation with Mg-ATP and AMPKK (not shown, but see e.g. [8]). It is therefore clear that the inactivation was due to dephosphorylation. We considered monitoring dephosphorylation more directly via a ^{32}P-release assay, after labelling of dephosphorylated AMPK using [γ-^{32}P]ATP and AMPKK. However such analyses would be complicated by the fact that under these conditions the catalytic (α) subunit of AMPK is autophosphorylated at several sites which do not regulate kinase activity, in addition to the single site at which it is phosphorylated and activated by AMPKK (D.G.H. and S.A. Hawley, unpublished data). Dephosphorylation at these other sites may not be regulated by AMP. The activity assay is therefore a more reliable quantitative measure of the state of phosphorylation at the AMPKK site.

Using the inactivation assay as the criterion, dephosphoryla-

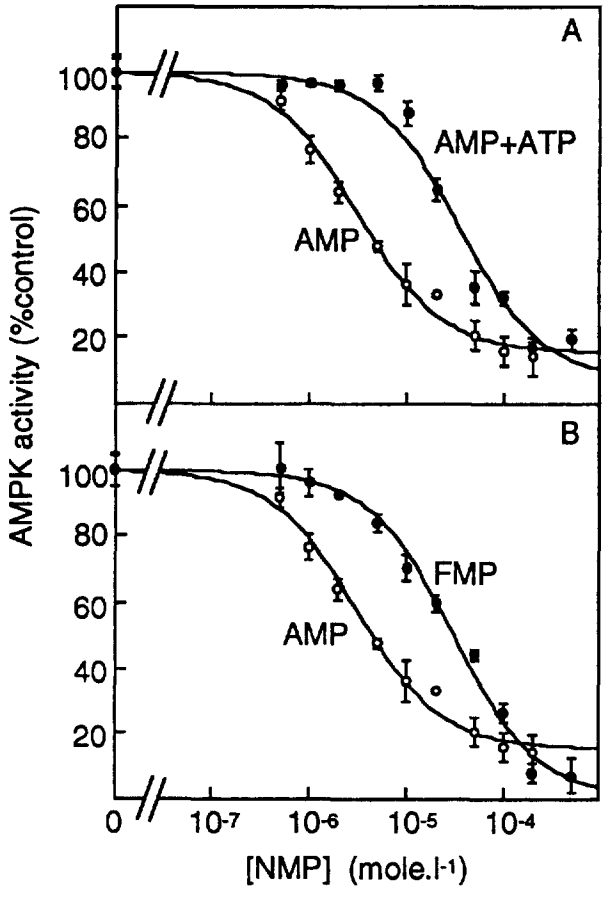

Fig. 3. Dose–responses for effects of nucleotides on dephosphorylation of AMPK by PP2Cα. (A) Effects of AMP in the presence (filled circles) and absence (open circles) of 4 mM ATP. (B) Effects of FMP (filled circles) with results for AMP (open circles) reproduced from (A) for comparison. Results are expressed as the% activity of control incubations without nucleotide (100% = 50 U/ml). AMPK was incubated with PP2Cα as described for Fig. 2 but for a fixed time of 7 min and at different concentrations of nucleoside monophosphate (NMP). Data were fitted to the equation: Activity = $100 - (Max \times [NMP])/(I_{0.5} + [NMP])$, where Max = maximal inhibition, [NMP] = concentration of NMP, and $I_{0.5}$ is the concentration of NMP giving half-maximal inhibition. Data were fitted using Kaleidagraph (Abelbeck software) on a Macintosh SE/30, and the curves are theoretical curves obtained using values for Max and $I_{0.5}$ quoted in the text.

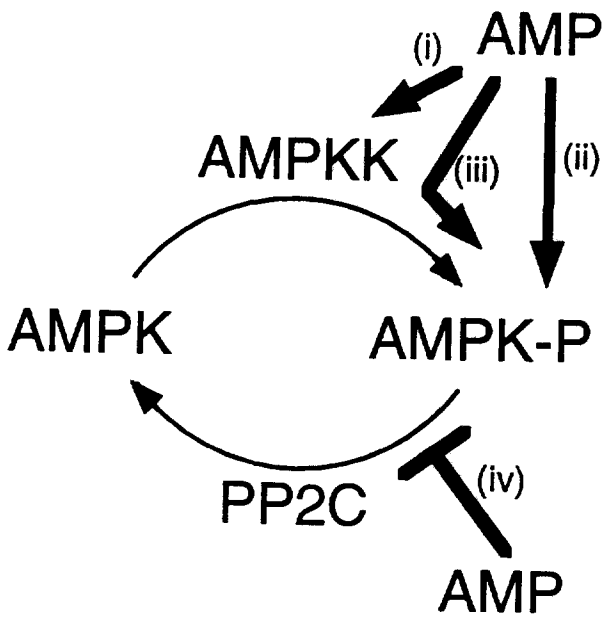

Fig. 4. Model showing the four mechanisms by which AMP promotes activation of the AMPK cascade. AMP causes: (i) direct activation of AMPKK; (ii) direct activation of AMPK; (iii) promotion of phosphorylation of AMPK by AMPKK; (iv) inhibition of dephosphorylation of AMPK.

tion of AMPK by both PP2Cα and $PP2A_C$ is inhibited by 5′-AMP. Many phosphorylated compounds have previously been shown to cause non-specific inhibition of protein phosphatases. This probably explains the small (< 15%) inhibition of PP2Cα dephosphorylation of AMPK by 200 μM GMP, and the ≈ 10% inhibition of [^{32}P]casein dephosphorylation by both AMP and GMP at the same concentration. However, the much larger and more potent effect of AMP on dephosphorylation of AMPK is due to binding of the nucleotide to the substrate (AMPK) rather than to the protein phosphatase. In addition, the effect appears to be due to binding of AMP to the same site at which it causes allosteric activation of AMPK. The evidence for these views may be summarised as follows.

(1) The $I_{0.5}$ for the effect of AMP on dephosphorylation by PP2Cα (3.1 ± 0.3 μM) was identical to the $A_{0.5}$ for the direct effect of AMP on kinase activity (3.2 ± 0.4 μM).

(2) In the presence of 4 mM ATP, the $I_{0.5}$ for AMP was increased 10-fold to 34 ± 7 μM: this concentration of ATP also increases the $A_{0.5}$ for the direct effect of AMP on kinase activity to ≈ 30 μM [8].

(3) Formycin A-5′-monophosphate, an analogue of AMP which activates AMPK with a slightly lower potency ($A_{0.5}$ = 20 ± 2 μM), also inhibits dephosphorylation by PP2Cα with an $I_{0.5}$ of the same order of magnitude (30 ± 4 μM).

(4) The effect is not specific for a particular protein phosphatase, because AMP also inhibited dephosphorylation of AMPK by $PP2A_C$ with an $I_{0.5}$ of 3.2 ± 0.5 μM AMP, almost identical to that obtained with PP2Cα. In this case inhibition was only partial, with a maximal inhibition of 47 ± 2%.

This study now increases the number of individual mechanisms by which AMP activates the AMPK cascade to four: i.e. (i) direct activation of the upstream protein kinase, AMPKK; (ii) direct activation of the downstream protein kinase, AMPK; (iii) promotion of phosphorylation of AMPK by AMPKK; (iv) inhibition of dephosphorylation of AMPK at the AMPKK site (Fig. 4). We believe that these multiple mechanisms would ensure that the system responded in an exquisitely sensitive manner to any changes in cellular AMP. At least two of these mechanisms, i.e. ((ii): [8]) and ((iv): this study) are antagonized by millimolar concentrations of ATP. The phosphorylation of AMPK by AMPKK is also inhibited by high concentrations of ATP (D.G.H. and S.A. Hawley, unpublished) and studies are currently in progress to determine whether this is mediated by binding of AMP to AMPKK (mechanism (i)) or to AMPK (mechanism (iii)), or both. Whichever is correct, it is clear that the system reponds to changes in the AMP:ATP ratio rather than simply to AMP.

The molecular mechanisms by which AMP has these multiple effects on AMPK will be an intriguing subject for future structural studies. However, it would appear that the kinase must exist in at least four different conformational states: (i) dephospho-, no AMP; (ii) dephospho-, AMP bound; (iii) phospho-, no AMP; (iv) phospho-, AMP bound. Binding of AMP to the dephospho form causes a shift from state (i) to state (ii) and exposes the site for phosphorylation by AMPKK. By contrast, binding of AMP to the phospho- form causes a shift from state (iii) to state (iv) and shields the phosphorylation site against protein phosphatase action. This shielding is more complete for PP2Cα than it is for $PP2A_C$, which is interesting given the evidence that PP2C rather than PP2A is responsible for dephosphorylation of AMPK in intact cells [12].

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References

[1] Corton, J.M., Gillespie, J.G. and Hardie, D.G. (1994) Curr. Biol. 4, 315–324.
[2] Hardie, D.G., Carling, D. and Halford, N.G. (1994) Semin. Cell Biol. 5, 409–416.
[3] Carling, D., Aguan, K., Woods, A., Verhoeven, A.J.M., Beri, R.K., Brennan, C.H., Sidebottom, C., Davison, M.D. and Scott, J. (1994) J. Biol. Chem. 269, 11442–11448.
[4] Mitchelhill, K.I., Stapleton, D., Gao, G., House, C., Michell, B., Katsis, F., Witters, L.A. and Kemp, B.E. (1994) J. Biol. Chem. 269, 2361–2364.
[5] Stapleton, D., Gao, G., Michell, B.J., Widmer, J., Mitchelhill, K., Teh, T., House, C.M., Witters, L.A. and Kemp, B.E. (1994) J. Biol. Chem. 269, 29343–29346.
[6] Woods, A., Munday, M.R., Scott, J., Yang, X., Carlson, M. and Carling, D. (1994) J. Biol. Chem. 269, 19509–19515.
[7] Weekes, J., Hawley, S.A., Corton, J., Shugar, D. and Hardie, D.G. (1994) Eur. J. Biochem. 219, 751–757.
[8] Corton, J.M., Gillespie, J.G., Hawley, S.A. and Hardie, D.G. (1995) Eur. J. Biochem. 229, 558–565.
[9] Hawley, S.A., Selbert, M.A., Goldstein, E.G., Edelman, A.M., Carling, D. and Hardie, D.G. (1995) J. Biol. Chem. 270, 27186–27191.
[10] Hardie, D.G. and MacKintosh, R.W. (1992) BioEssays 14, 699–704.
[11] Clarke, P.R., Moore, F. and Hardie, D.G. (1991) Adv. Protein Phosphatases 6, 187–209.
[12] Moore, F., Weekes, J. and Hardie, D.G. (1991) Eur. J. Biochem. 199, 691–697.
[13] Carling, D., Clarke, P.R., Zammit, V.A. and Hardie, D.G. (1989) Eur. J. Biochem. 186, 129–136.
[14] Cohen, P., Alemany, S., Hemmings, B.A., Resink, T.J., Strålfors, P. and Lim, T.H. (1988) Methods Enzymol. 159, 390–408.

[15] Davies, S.P., Carling, D. and Hardie, D.G. (1989) Eur. J. Biochem. 186, 123–128.
[16] MacKintosh, C. (1993) in: (Hardie, D.G. eds.) pp. 214–216, Oxford University Press, Oxford.
[17] Mann, D.J., Campbell, D.G., McGowan, C.H. and Cohen, P.T.W. (1992) Biochim. Biophys. Acta 1130, 100–104.
[18] Muchmore, D.C., McIntosh, L.P., Russell, C.B., Anderson, D.E. and Dahlquist, F.W. (1989) Methods Enzymol. 177, 44–73.
[19] McGowan, C.H. and Cohen, P. (1988) Methods Enzymol. 159, 416–426.
[20] Alessi, D.R., Street, A.J., Cohen, P. and Cohen, P.T.W. (1993) Eur. J. Biochem. 213, 1055–1066.
[21] Wera, S. and Hemmings, B.A. (1995) Biochem. J. 311, 17–29.