FEBS Letters 403 (1997) 254–258 FEBS 18207

Identification of Raf-1 Ser^{621} kinase activity from NIH 3T3 cells as AMP-activated protein kinase

Amy B. Sprenkle[a], Stephen P. Davies[b], David Carling[c], D. Grahame Hardie[b], Thomas W. Sturgill[a,*]

[a] *Howard Hughes Medical Institute, Departments of Medicine and Pharmacology, University of Virginia, Charlottesville, VA 22908, USA*
[b] *Department of Biochemistry, The University of Dundee, Dundee, DD1 4HN, UK*
[c] *Department of Molecular Medicine, Royal Postgraduate Medical School, MRC Center for Clinical Science, London, UK*

Received 17 December 1996; revised version received 10 January 1997

Abstract Raf-1 is extensively phosphorylated on Ser^{621} in both quiescent and mitogen-stimulated cells. To identify the responsible kinase(s), cytosolic fractions of NIH 3T3 cells were analyzed for Ser^{621} peptide kinase activity. One major peak of activity was detected and identified as AMP-activated protein kinase (AMPK) by immunodepletion experiments. AMPK phosphorylated the catalytic domain of Raf-1, expressed in *Escherichia coli* as a soluble GST fusion protein, to generate a single tryptic [^{32}P]phosphopeptide containing exclusively phospho-Ser^{621}. AMPK also phosphorylated full-length, kinase-defective Raf-1 (K375M) to generate two [^{32}P]phosphopeptides, one co-migrating with synthetic tryptic peptide containing phospho-Ser^{621} and the other with phospho-Ser^{259}.

© Federation of European Biochemical Societies.

Key words: Raf-1; AMP-activated protein kinase; cAMP-dependent protein kinase; 14-3-3 protein

1. Introduction

The Raf-1 serine/threonine protein kinase is a key effector for Ras, relaying signals from receptors to the nucleus via stimulation of MEK and the MAP kinases p44^{mapk}/ERK1 and p42^{mapk}/ERK2 [1]. Ras-GTP binds the NH_2-terminal domain of Raf-1 and recruits Raf-1 to the plasma membrane [1] where Raf-1 is activated by processes that are still the subject of intense investigation.

Phosphorylation of Raf-1 is important for its regulation [2]. Activation of Raf-1 in vitro by membranes from v-Ras and v-Src transformed cells requires ATP [3]. Raf-1 is phosphorylated at Y^{340}, and possibly also at Y^{341}, and enzymatically activated in *Sf9* cells and NIH 3T3 cells expressing Ras and mutationally activated Src (Src^{Y527F}), whereas doubly mutated Raf-1 (F^{340,341}) is not [2,4]. In addition to Y^{340,341}, several serine/threonine residues have been studied as sites of potential regulatory phosphorylation [2]. The studies described herein concern Ser^{621} near the COOH-terminus.

Mutational and biochemical studies of Raf-1 indicate an important role of phosphorylated Ser^{621} in Raf-1 structure and regulation (see Section 4). Ser^{621} was identified as an in vivo site of Raf-1 phosphorylation in *Sf9* cells and NIH 3T3 cells [5]. Ser^{621} is likely to be phosphorylated by kinase(s) other than Raf-1 since phosphopeptide maps of kinase-defective Raf-1(K375M) contain [^{32}P]Ser^{621} phosphopeptide. However, treatment of NIH 3T3 cells with platelet-derived growth factor, a potent Raf activator, did not detectably alter abundance of Ser^{621} [^{32}P]phosphopeptide under the experimental methods used [5]. In this paper, we identify the principal Raf-1 Ser^{621} kinase activity present in cytosolic extracts of NIH 3T3 cells as AMP-activated protein kinase (AMPK). Identification of AMPK as a Ser^{621} kinase has important implications for studies of Raf-1 regulation.

2. Materials and methods

2.1. Materials

Synthetic Raf-1 Ser^{621} peptide SLPKINRSAS^{621}EPSLHR*R* (*R*, added) was used for peptide kinase assays, and peptide KINRSASEPSLHR for peptide mapping after phosphorylation and tryptic cleavage. The Raf-1 259 peptide RQRSTS^{259}TPNVHMVSTTLPVDSRMIE was similarly used. AMPK was purified from rat liver as far as the gel filtration step [6]. C-subunit of PKA was purified from bovine heart [7]. GST-22W Raf-1 [8] was expressed in *E. coli* and purified. FLAG-tagged K375M Raf-1 was expressed in *Sf9* cells and partially purified essentially as described [9].

2.2. Cell culture and preparation of cell extracts

NIH 3T3 cells (ATCC CRL-1658) were grown to confluency in DMEM supplemented with 5% calf serum (Gibco-BRL). Cells (6×150 mm plates) were starved overnight in serum-free DMEM. Plates were washed twice with cold PBS and placed on ice. Cells were scraped from the plate in 1 ml/plate lysis buffer (25 mM Tris-HCl, pH 7.9, at 4°C, 0.5 mM EDTA, 0.5 mM EGTA, 0.3 M sucrose, 1 mM DTT, 1 μM MCLR-LR, 200 μM Na_2VO_4, 5 mg/ml pepstatin, 1 mg/ml aprotinin, 5 mg/ml E64, 1 mM Pefabloc-SC (Boehringer-Mannheim)). Cells were homogenized in a Teflon/glass homogenizer and centrifuged at 3000×*g*. The supernatant was centrifuged at 100 000×*g* for 20 min (S100).

2.3. Column chromatography

S100 (above) was diluted to 10 ml in buffer B (25 mM Tris-HCl, pH 7.9 at 4°C, 1 mM EDTA, 1 mM EGTA, 0.1% (v/v) NP-40, 10 mM benzamidine) and applied to a MonoQ HR5/5 column (LKB-Pharmacia). The column was developed at 0.5 ml/min with a NaCl gradient from 0 to 0.5 M over 50 ml, following collection of the flow-through (fractions 1–10). One milliliter fractions were collected into tubes already containing glycerol and protease and phosphatase inhibitors for final concentrations of: 10% (w/v) glycerol, 1 μg/ml aprotinin, 1 μg/ml pepstatin, 1 mM Pefabloc-SC, 0.5 μM MCLR.

2.4. Kinase assays

Peptide kinase assays (25 μl, final volume) were performed in duplicate by mixing on ice 15 ml of the indicated fractions with 5 ml of the peptide stock (1 mg/ml) and 2.5 μl of 10× kinase assay buffer (0.2 M Tris-HCl, 0.2 M β-glycerol phosphate, 5 mM EGTA, 0.1 M $MgCl_2$, 10 mM $MnCl_2$ (pH 7.8 at 22°C)). Reactions (30°C, 20 min) were initiated by addition of 2.5 μl of [γ-^{32}P]ATP (final concentration,

*Corresponding author. Fax: (804) 924-9659

Abbreviations: AMPK, AMP-activated protein kinase; MAP, mitogen-activated protein; PKA, cAMP-dependent protein kinase; GST, glutathione S-transferase; DMEM, Dulbecco's modified Eagle's medium; MCLR, microcystin-LR; DTT, dithiothreitol; 2-ME, 2-mercaptoethanol; HVE, high-voltage electrophoresis; FSBA, *p*-fluorosulfonylbenzoyl 5′-adenosine

0014-5793/97/$17.00 © 1997 Federation of European Biochemical Societies. All rights reserved.
PII S0014-5793(97)00062-8

50 μM (5 cpm/fmol)). Portions (15 μl) were transferred to P81 paper for determination of ^{32}P incorporation.

2.5. Immunodepletion of AMPK from MonoQ fractions

Immunoprecipitations were performed in duplicate with 300 μl of indicated fractions, 20 μl of protein A agarose (Pierce) and 1 μl of pre-immune serum or antiserum to the C-terminal 217 residues of the AMPKβ subunit [10]. Binding was carried out for 2 h at 4°C. Beads (washed twice with 1× kinase assay buffer) and the initial supernatant were separately assayed.

2.6. Phosphopeptide mapping and Edman degradation of GST-22W

Bacterially expressed GST-22W Raf-1 (aa 306–648) [8] was partially purified (fusion protein ~20% of total) and ^{32}P-phosphorylated to ~0.2 mol/mol by rat liver AMPK. [^{32}P]GST-22W (63 kDa) was excised from the gel after SDS-PAGE and digested with trypsin. The digest was analyzed by HPLC [11]. Radioactive peaks were subjected to Edman sequencing to determine the cycle of ^{32}P release [12].

2.7. Phosphopeptide mapping of K375M Raf-1

Partially purified K375M Raf-1 was treated sequentially with FSBA [13], and catalytic subunit of PP2A prior to use. PP2A-treated K375M Raf-1 (1.5 μg) was ^{32}P-labeled with AMPK, resolved by SDS-PAGE (10% gel), and transferred to an Immobilon-CD membrane (Millipore). The [^{32}P]K375M Raf-1 band was excised, and the protein was digested on the matrix with sequencing grade modified-trypsin (Promega) according to the manufacturer's instructions. Membrane pieces were pre-treated and peptides were recovered as described [14]. Recovery of ^{32}P in the supernatant was ≥80%. The supernatant was lyophilized and portions subjected to 2-dimensional phosphopeptide mapping by HVE/TLC using pH 1.9 buffer, 30 min at 1 kV for HVE and *n*-butanol/pyridine/glacial acetic acid/water (75:50:15:60) for TLC. Ser^{621} and Ser^{259} peptides were ^{32}P-labeled by AMPK (Ser^{621} peptide) or C-subunit of PKA (Ser^{259} peptide), purified by HPLC, digested with trypsin, and re-isolated by HPLC before use.

3. Results

3.1. NIH 3T3 cells contain a Ser^{621} peptide kinase

Studies of protein serine kinases have demonstrated that specificity for substrate recognition is dependent on the primary amino acid sequence in which the serine acceptor resides. The residues determining specificity usually reside anywhere from P−5 to P+5 (where P represents the phosphorylated serine) [15]. The synthetic peptide SLPKINRSAS^{621}EPSLHRR was used to screen for Ser^{621} kinases in extracts of NIH 3T3 cells. Two peaks of Ser^{621} peptide kinase activity were detected in fractions from MonoQ chromatography of cytosolic supernatants prepared from NIH 3T3 cells (Fig. 1). The major Ser^{621} peptide kinase activity eluted during the gradient at ~0.2 M NaCl, while a minor peak eluted at ~0.1 M NaCl. No activity was detectable in fractions from the flow-through (Fig. 1).

Ser^{621} resides in a sequence exactly matching the recognition motif of AMPK [16]. AMPK is a heterotrimeric αβγ enzyme consisting of catalytic α-subunit and non-catalytic β- and γ-subunits [10,17,18]. AMPK is extremely sensitive to stresses which deplete ATP, and is readily activated during harvesting of cells unless appropriate methods are used [19–21]. Studies of synthetic peptides and of AMPK sites in physiologic substrates indicate a strong preference of AMPK for Ser/Thr with hydrophobic residues (M, V, L, I, or F) in the P−5 and P+4 positions and a single basic residue (R, K, H) at P−4 or P−3 [16]. Furthermore, AMPK elutes from MonoQ at ~0.2 M NaCl [6,17], as did Ser^{621} kinase activity (Fig. 1).

3.2. Immunodepletion with anti-AMPKβ antiserum

Portions of MonoQ fractions were incubated with control sera or antisera to the rat AMPKβ subunit [10], together with protein A-agarose beads, and tested for depletion of Ser^{621} peptide kinase activity from the supernatants (Fig. 2). Immunoprecipitation with anti-AMPKβ antiserum, but not with pre-immune serum, depleted the major peak of Ser^{621} peptide kinase activity (eluting at 0.2 M NaCl) from the supernatant. The activity was recovered in the pellet. Similarly, when anti-AMPKβ immunoprecipitates were analyzed by Western blotting with the same antibody, a 38 kDa immunoreactive AMPKβ band was found only in the fractions corresponding to the major peak (data not shown). These experiments identify the major peak of Ser^{621} kinase activity as AMPK. The minor peak of Ser^{621} peptide kinase activity (eluting at ~0.1 M NaCl) was not depleted by anti-AMPKβ serum and was inhibited by Walsh PKI peptide [22] (Fig. 2), demonstrating that it corresponded to the C-subunit of PKA.

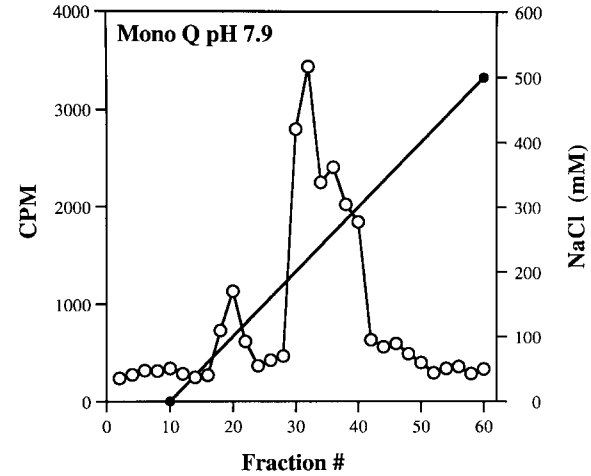


Fig. 1. Characterization of Ser^{621} peptide kinase activity by MonoQ chromatography. Cytosolic supernatants were prepared from NIH 3T3 cells, fractionated by MonoQ HR5/5 chromatography (pH 7.9 at 4°C), and fractions obtained were assayed for Ser^{621} peptide kinase activity.

3.3. AMPK phosphorylates GST-22W Raf-1 exclusively on Ser^{621}

AMPK phosphorylated bacterial GST-22W Raf-1 to a stoichiometry of ~0.2 mol/mol exclusively on serine (data not shown). All of the ^{32}P incorporated appeared in a single [^{32}P]phosphopeptide after tryptic digestion and HPLC (Fig. 3A). This phosphopeptide co-migrated with a synthetic Raf-1 tryptic [^{32}P]phosphopeptide (SAS^{621}EPSLHR), generated by digestion of an NH_2-terminally extended peptide that had been phosphorylated in vitro by AMPK (Fig. 3A). The V_{max} of phosphorylation of the Ser^{621} peptide was 90% of that using the SAMS peptide, while the K_m was somewhat higher (100 vs. 30 μM) (data not shown). Release of ^{32}P from the Raf-1 22W phosphopeptide occurred in cycle 3 during Edman degradation (Fig. 3B), as did release from synthetic peptide (data not shown). These results prove that AMPK phosphorylates GST-22W Raf-1 in vitro exclusively on Ser^{621}.

3.4. Phosphopeptide mapping of K375M Raf-1

To determine whether AMPK also phosphorylates a more native Raf-1 protein on Ser^{621}, we partially purified kinase-defective K375M Raf-1 from *Sf9* cells. Prior to phosphoryla-

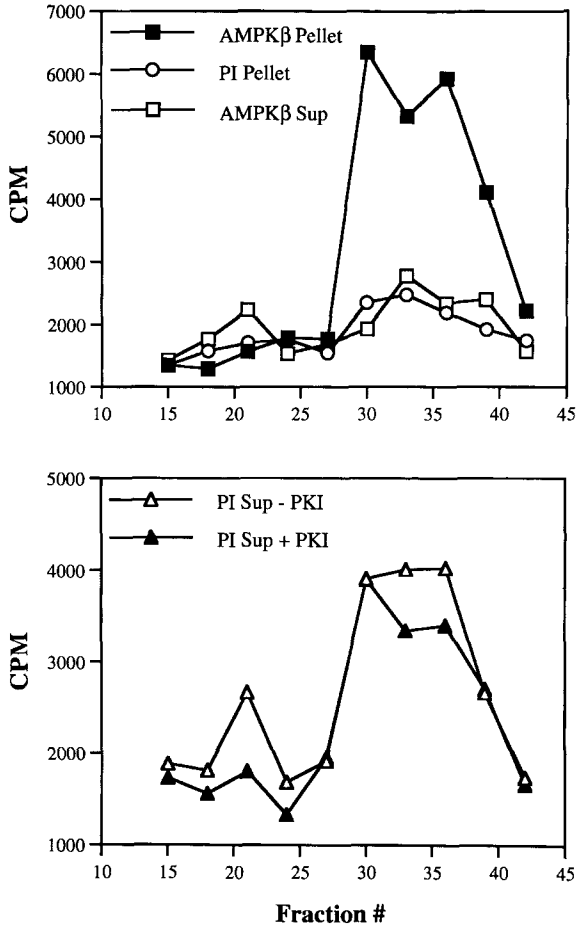


Fig. 2. Immunodepletion of peptide kinase activity from MonoQ fractions. NIH 3T3 cytosols were fractionated over MonoQ as in Fig. 1. Top panel: Selected fractions were mixed with protein A and anti-β-AMPK or pre-immune (PI) serum and the pellet or supernatant (sup) assayed for Ser^{621} peptide kinase activity. Bottom panel: Walsh inhibitor (PKI, 10 μM final) was added to the pre-immune supernatant prior to p81 assay.

tion, the K375M preparation was treated with FSBA to inactivate endogenous kinases, and then treated with PP2A to increase availability of phospho-acceptor sites. In the absence of exogenous kinase, there was no detectable phosphorylation of K375M Raf (data not shown). AMPK generated two tryptic [^{32}P]phosphopeptides in K375M Fig. 4. These phosphopeptides co-migrated with synthetic marker peptides for Ser^{621} and Ser^{259}. Thus, Ser^{621} in insect cell K375M is also an in vitro substrate for AMPK. Ser^{259} has hydrophobic residue at P+4 and the required basic residue at P−3, but lacks hydrophobic residue in the P−5 position [5]. On this basis, Ser^{259} would be predicted to be less favorable as an AMPK site than Ser^{621}. The preparation of AMPK was tested for contaminating PKA by incubation of the kinase with the PKA substrate peptide, Kemptide [23]. Under standard assay conditions, there was no detectable phosphorylation of Kemptide (30 μM final). Similarly, the phosphorylation of SAMS peptide was unaffected by the inclusion of 10 μM PKI (data not shown).

4. Discussion

We identified AMPK as the major Ser^{621} kinase in cytosolic supernatants from NIH 3T3 cells. We strongly suspect that AMPK is a Ser^{621} kinase in vivo. Ser^{621} is known to be highly phosphorylated in Raf-1 isolated from insect and mammalian cells. The sequence surrounding Ser^{621} conforms to the AMPK consensus, and the K_m and V_{max} obtained for the Ser^{621} peptide in vitro are comparable to those for the SAMS peptide, which derives from a known physiologic AMPK phosphorylation site in acetyl-CoA carboxylase. AMPK exclusively phosphorylates Ser^{621} in Raf-1 22W, and phosphorylates Ser^{621} and Ser^{259} in full-length Raf-1. The major peak of Ser^{621} peptide kinase activity detectable in lysates from NIH 3T3 cells is unequivocally AMPK.

The role of phosphorylated Ser^{621} in Raf-1 is unclear. Raf-1 Ser^{621}Ala mutants of Raf-1 expressed in *Sf9* or human T cells are inactive under basal conditions, and cannot be stimulated by co-expression with Ras and Src^{Y527F} [5] or treatment with phorbol esters [10]. These data suggest that phosphorylation of Ser^{621} is required for activity, however, effects of Ser^{621}Ala mutation on folding or thermal lability cannot be excluded. Recently, Mischak et al. [24] reported that incubation of GST-GNXRaf-1 (lacking the NH_2-terminal regulatory domain) with ATP/Mg resulted in loss of activity, concomitant with phosphorylation of Ser^{621}. The kinase responsible for this phosphorylation is likely to be a contaminating or co-purifying activity. The catalytic subunit of PKA also phosphorylated Ser^{621} in vitro, while its overexpression in intact cells resulted in increased phosphorylation of Ser^{621} and decreased activity of Raf-1. These results suggest that phosphorylation

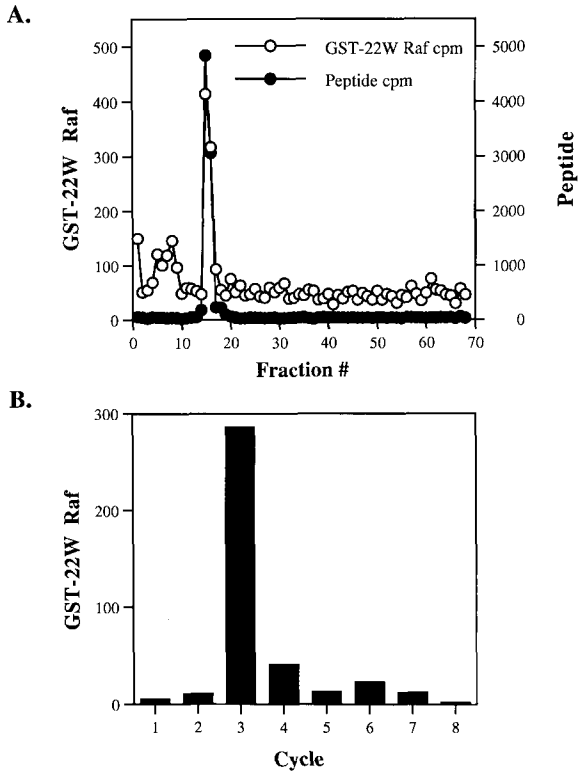


Fig. 3. Tryptic phosphopeptide analysis by HPLC and Edman degradation of AMPK-phosphorylated bacterial GST 22W Raf-1. A: HPLC analysis of in vitro phosphorylated, trypsinized GST 22W Raf or synthetic peptide. B: Edman degradation of tryptic peptide SASEPSLHR with counts released exclusively at the third position, corresponding to Ser^{621}. Data obtained for the synthetic peptide was identical (not shown).

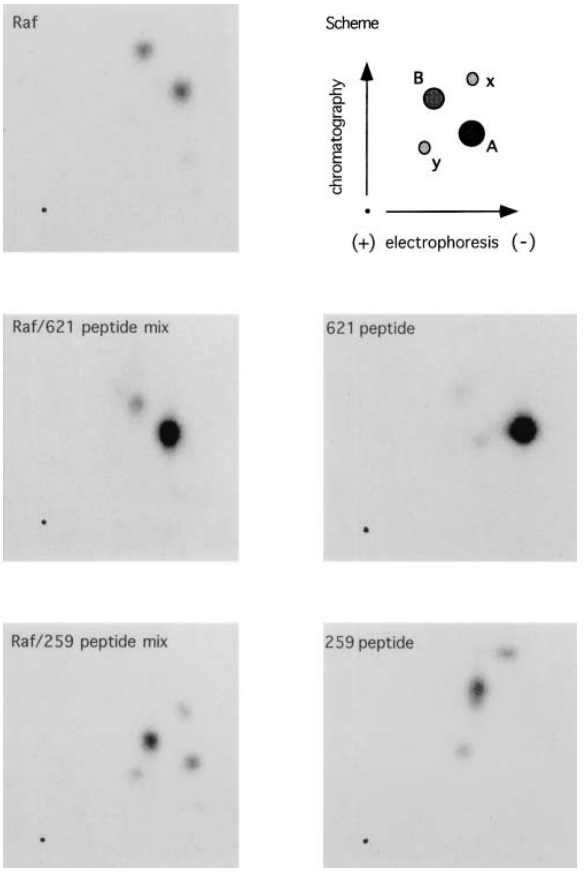


Fig. 4. Phosphopeptide mapping of insect cell K375M Raf. Full-length Raf-1 (K375M) (Raf) was labeled in vitro with AMPK, digested with trypsin and resolved by HVE/TLC. Peptide A in the scheme represents phospho-ser^{621}-containing tryptic peptide, Peptide B represents phospho-ser^{259}-containing peptide, and *x* and *y* are minor peptides produced only when the Ser^{259}-containing synthetic peptide is phosphorylated with PKA.

of Ser^{621} inactivates Raf-1, but do not prove that PKA is responsible for this in vivo. The Ser^{621} site contains only a single arginine at the P−3 position, whereas two adjacent arginines at P−2 and P−3 are normally required for efficient phosphorylation by PKA. On the other hand, Ser^{621} is a perfect match to the recognition motif for AMPK [16], and the Ser^{621} peptide has kinetic parameters comparable with those for the SAMS peptide. To date our own attempts to demonstrate effects of Ser^{621} phosphorylation on Raf-1 activity have been inconclusive. There are several potential explanations for this: for example, both Ser^{259} and Ser^{621} are 14-3-3 protein-binding sites [25,26], and in the case of other enzymes, the presence of 14-3-3 proteins has been shown to be necessary for phosphorylation to have an effect on enzyme activity [27] (and references therein).

A test of the hypothesis that AMPK phosphorylates Ser^{621} in intact cells is now possible by incubation of cells with 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) to activate AMPK [28]. Initial results show that treatment of ^{32}P-labeled CHO cells expressing FLAG-Raf-1 for 10 min with 500 μM AICAR caused a 30–40% increase in Ser^{621} phosphorylation (A. Sprenkle, unpublished). It is worth noting that two physiological substrates for AMPK (acetyl-CoA carboxylase and HMG-CoA reductase) are highly phosphorylated in rat liver under basal conditions [21,29], and though there is 80% inactivation of HMG-CoA reductase under severe stress conditions, [20] phosphorylation increases only 2.5-fold. The phosphorylation of key substrates for AMPK may be poised at this high basal level so that even a mild stress produces a large effect on activity, and thus the 'constitutive' nature of the Ser^{621} phosphorylation of Raf-1 may paradoxically indicate its importance in the regulation of Raf activity.

Acknowledgements: This study was supported by the Howard Hughes Medical Institute (T.W.S.) and by a Programme Grant (to D.G.H.) from the Wellcome Trust. We thank Martin McMahon, Richard Burack and Celeste Smith for reading the manuscript, and Corky Harrison for assistance.

References

[1] Marais, R. and Marshall, C.J. (1996) Cancer Surveys 27, 101–125.
[2] Morrison, D.K. (1995) Mol. Reprod. Dev. 42, 507–514.
[3] Dent, P., Jelinek, T., Morrison, D.K., Weber, M.J. and Sturgill, T.W. (1995) Science 268, 1902–1906.
[4] Marais, R., Light, Y., Paterson, H.F. and Marshall, C.J. (1995) EMBO J. 14, 3136–3145.
[5] Morrison, D.K., Heidecker, G., Rapp, U.R. and Copeland, T.D. (1993) J. Biol. Chem. 268, 17309–17316.
[6] Carling, D., Clarke, P.R., Zammit, V.A. and Hardie, D.G. (1989) Eur. J. Biochem. 186, 129–136.
[7] Sugden, P.H., Holladay, L.A., Reimann, E.M. and Corbin, J.D. (1976) Biochem. J. 159, 409–422.
[8] Stanton, V.P., Jr., Nichols, D.W., Laudano, A.P. and Cooper, G.M. (1989) Mol. Cell. Biol. 9, 639–647.
[9] Dent, P., Reardon, D.B., Morrison, D.K. and Sturgill, T.W. (1995) Mol. Cell. Biol. 15, 4125–4135.
[10] Woods, A., Cheung, P.C., Smith, F.C., Davison, M.D., Scott, J., Beri, R.K. and Carling, D. (1996) J. Biol. Chem. 271, 10282–10290.
[11] Davies, S.P., Sim, A.T. and Hardie, D.G. (1990) Eur. J. Biochem. 187, 183–190.
[12] Stokoe, D., Campbell, D.G., Nakielny, S., Hidaka, H., Leevers, S.J., Marshall, C. and Cohen, P. (1992) EMBO J. 11, 3985–3994.
[13] Dent, P., Chow, Y.H., Wu, J., Morrison, D.K., Jove, R. and Sturgill, T.W. (1994) Biochem. J. 303, 105–112.
[14] Aebersold, R. (1989) A Practical Guide to Protein and Peptide Purification for Microsequencing. (Matsudaira, P.T. ed.), pp. 71–88. Academic Press, San Diego, CA.
[15] Hardie, D.G. and Hanks, S. (1995) The Protein Kinase Factsbook. (Hardie, D.G. and Hanks, S. eds.), Vol. 1, pp. 1–418. Academic Press, San Diego, CA.
[16] Dale, S., Wilson, W.A., Edelman, A.M. and Hardie, D.G. (1995) FEBS Lett. 361, 191–195.
[17] Davies, S.P., Hawley, S.A., Woods, A., Carling, D., Haystead, T.A. and Hardie, D.G. (1994) Eur. J. Biochem. 223, 351–357.
[18] Dyck, J.R.B., Gao, G., Widmer, J., Stapleton, D., Fernandez, C.S., Kemp, B.E. and Witters, L.A. (1996) J. Biol. Chem. 271, 17798–17803.
[19] Hardie, D.G., Carling, D. and Halford, N. (1994) Sem. Cell Biol. 5, 409–416.
[20] Corton, J.M., Gillespie, J.G. and Hardie, D.G. (1994) Curr. Biol. 4, 315–324.
[21] Davies, S.P., Carling, D., Munday, M.R. and Hardie, D.G. (1992) Eur. J. Biochem. 203, 615–623.
[22] Cheng, H.C., Kemp, B.E., Pearson, R.B., Smith, A.J., Misconi, L., Van Patten, S.M. and Walsh, D.A. (1986) J. Biol. Chem. 261, 989–992.
[23] Reed, J., Kinzel, V., Kemp, B.E., Cheng, H.C. and Walsh, D.A. (1985) Biochemistry 24, 2967–2973.
[24] Mischak, H., Seitz, T., Janosch, P., Eulitz, M., Steen, H., Schellerer, M., Philipp, A. and Kolch, W. (1996) Mol. Cell. Biol. 16, 5409–5418.
[25] Muslin, A.J., Tanner, J.W., Allen, P.M. and Shaw, A.S. (1996) Cell 84, 889–897.

[26] Michaud, N.R., Fabian, J.R., Mathes, K.D. and Morrison, D.K. (1995) Mol. Cell. Biol. 15, 3390–3397.
[27] Moorhead, G., Douglas, P., Morrice, N., Scarabel, M., Aitken, A. and Mackintosh, C. (1996) Curr. Biol. 6, 1104–1113.
[28] Corton, J.M., Gillespie, J.G., Hawley, S.A. and Hardie, D.G. (1995) Eur. J. Biochem. 229, 558–565.
[29] Easom, R.A. and Zammit, V.A. (1984) Biochem. J. 220, 739–745.