Volume 317, number 1,2, 12–16 FEBS 12033 February 1993

Functional expression of a MAP kinase kinase in COS cells and recognition by an anti-STE7/byr1 antibody

Clare M.M. Haystead[a], Jie Wu[b], Peter Gregory[a], Thomas W. Sturgill[b] and Timothy A.J. Haystead[a]

Departments of [a]Pharmacology and [b]Internal Medicine University of Virginia, Charlottesville, VA 22908, USA

Received 1 December 1992; revised version received 11 December 1992

Mitogen-activated protein (MAP) kinases $p42^{mapk}$ and $p44^{mapk}$ are activated by dual tyrosine and threonine phosphorylation in vivo. Both MAPKs are phosphorylated and activated in vitro by an activator recently identified as a protein-tyrosine/threonine kinase. We have isolated a putative cDNA for a MAP kinase kinase (MAPKK) and determined its structure [Proc. Natl. Acad. Sci. USA, in press]. The protein encoded by this cDNA shares sequence homology with two yeast protein kinases byr1 and STE7. We now report that stimulation with serum of COS cells expressing this protein amplifies MAPK activator activity markedly. The increased activity co-migrates during chromatography with the expressed 45 kDa protein, recognized by an anti-STE7/byr1 antibody, and is abrogated by treatment with phosphatase 2A. Thus, this cDNA encodes a functional MAPKK. The anti-STE7/byr1 antibody also recognized a 46 kDa COS cell protein that was resolved from the expressed MAPKK by anion-exchange chromatography. This immunoreactive protein co-eluted with endogenous MAPKK activity, suggesting identification of the immunoreactive band as monkey MAPKK.

1. INTRODUCTION

MAP kinases comprise a family of protein serine/threonine kinases which are activated by tyrosine and threonine phosphorylation in response to extracellular stimuli (reviewed in [1]). MAP kinases participate in protein kinase cascades thought to be important for transducing signals from these stimuli. In mammalian cells, three MAP kinases have been characterized, $p42^{mapk}$ [2], $p44^{mapk}$ [3] and p55 MAP kinase [4]. $p42^{mapk}$ and $p44^{mapk}$ are activated in vitro by an activator discovered by Ahn et al. [5] and recently identified as a protein-tyrosine/threonine kinase, a 'MAP kinase kinase'. The enzyme(s) responsible for activating p55 MAP kinase are unknown.

We have isolated and sequenced a cDNA that encodes a novel protein of 393 amino acids (43.5 kDa, predicted mass) with sequence similarity to the yeast protein kinases byr1 and STE7 in the mating pathways of fission and budding yeast, respectively [6,7]. This cDNA was isolated from a rat kidney library using protein sequence data we obtained for p42 MAP kinase kinase isolated from rabbit skeletal muscle [8]. To determine whether the isolated cDNA encodes a functional MAPKK, we have expressed the encoded protein in COS cells and characterized its function.

Correspondence address. T.A.J. Haystead, Department of Pharmacology, Box 448, University of Virginia Health Sciences Center, Charlottesville, VA 22908, USA. Fax: (1) (804) 982 3878.

Abbreviations: MAP, mitogen-activated protein; MAPK, MAP kinase; MAPKK, MAP kinase kinase; $p42^{mapk}$, 42 kDa MAPK; $p44^{mapk}$, 44 kDa MAPK; PP-2A, protein phosphatase 2A; PTPase, protein tyrosine phosphatase; KLH, keyhole limpet hemocyanin; MBP, myelin basic protein.

2. MATERIALS AND METHODS

2.1. *Materials*

Plasmid pCDM8 was purchased from Invitrogen (San Diego, CA). Goat anti-rabbit alkaline phosphatase was obtained from Promega (Madison, WI). Recombinant $p42^{mapk}$ was purified from *E. Coli* strain BL21(DE3)[pET-MK] as described previously [9]. The catalytic subunit of PP-2A and okadaic acid were purified to homogeneity from the sea sponge *Halichondria**. Affinity purified Anti-STE7/byr1 antibody was a generous gift from Dr Steven L. Pelech (Kintech Biotechnology Corp., BC, Canada). Dr. Pelech prepared the antibody with the following protocol. Peptide STE-VIII (FVGTSTYMSPERIC) was synthesized based upon a sequence (underlined) which is completely conserved in the catalytic subdomains VIII of *Saccharomyces cerevisiae* STE7 and *Schizossacharomyces pombe* byr1 [10]. The peptide was coupled to KLH via a cysteine residue added to the C terminus and used to immunize New Zealand white rabbits. Subsequently, STE7/ byr1 antipeptide antibodies were purified from rabbit antisera over a STE7-VIII peptide-agarose affinity column. MAPKK cDNA (GenBank accession no. L04485) was isolated and characterized as described [6].

2.2. *Expression of rat kidney MAPKK cDNA in COS cells*

MAPKK cDNA was excised from plasmid pK28 [6] by digestion with *Hin*dIII and *Xba*I and cloned into pCDM8 generating pCH1. Cos-1 cells were transfected with pCH1 or pCDM8 (control) by the DEAE-dextran method [11], or left untreated. Conditions for expres-

* T.A.J. Haystead, unpublished work.

 Published by Elsevier Science Publishers B.V.

sion (48 h) and serum-deprivation (18 h) were essentially as described [12]. Cells were stimulated with serum for the indicated times, immediately rinsed with 20 ml of chilled phosphate-buffered saline, and snap-frozen by floating the culture plates on an ethanol/dry ice bath. The plates were stored at − 20°C.

2.3. *Assays and chromatography*

Plates were thawed on ice in the presence of 1.0 ml of homogenization buffer containing 50 mM β-glycerophosphate, 1.5 mM EGTA, 1 mM dithiothreitol, 1 mM PMSF and 1 mM benzamidine, 0.15 mM sodium orthovanadate. The cells were briefly sonicated (10 s) on ice and centrifuged (14 000 × *g*) for 15 min at 4°C. Portions of the supernatant (normalized for protein) were assayed for ability to activate $p42^{mapk}$ as described [9]. Extracts from pCDM8 and pCH1 transfected COS cells (± serum treatment) were characterized for MAPKK and MAPK by anion-exchange chromatography as described [9,13]. Conditions for protein phosphatase treatments were also as described previously [13].

3. RESULTS AND DISCUSSION

Transient expression of the protein was demonstrated by Western blotting using an anti-STE7/byr1 peptide antibody (Fig. 1). The rationale for use of the antibody was based on functional and structural homologies between MAPKK and STE7/byr1. STE7 and byr1 have been identified as essential proteins in the signal transduction pathways for response to pheromones in *S. cerevisiae* and *S. pombe*, respectively [14,15]. STE7 and byr1 are thought to phosphorylate and activate yeast homologs of MAP kinase in each yeast [16]. Comparison with protein sequences in the data base revealed that the encoded protein was most similar to byr1 (45% identity) [6]. The fourth -best FASTA score was obtained for comparison to STE7 [6]. The anti-STE7/byr1 antibody was based on a sequence of twelve amino acids (FVGTSTYMSPERI) that are conserved in domain VIII of protein kinases STE7 and byr1. Ten of twelve amino acids of the corresponding sequence from the cDNA, FVGTRSYMSPERL, are also identical; non-identical residues (underlined) are separated by seven residues. FVGT and SYMSPERL are each of sufficient length to form an epitope. For these reasons, we evaluated expression of protein with this antibody.

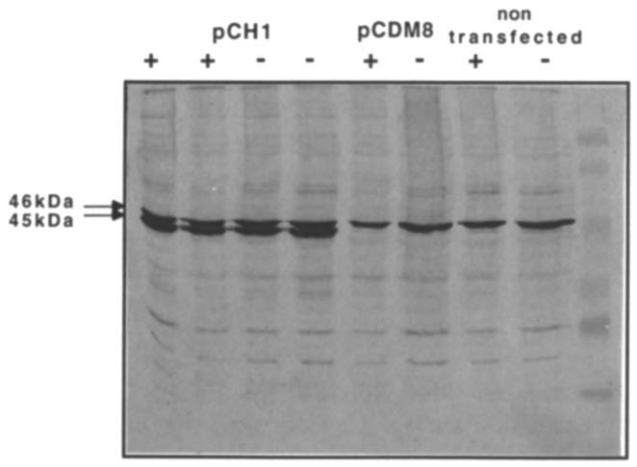


Fig. 1. Western blotting with STE7/byr1 antibody detects the expressed protein encoded by pCH1 and an endogenous COS cell protein. Single dishes of COS cells transfected with pCH1, pCDM8 (control), or non- transfected cells were treated with (+) or without (−) 10% fetal calf serum for 5 min. The cells were washed with ice cold phosphate-buffered saline and lysed in 1.0 ml homogenization buffer. Extracts were prepared and corrected for protein. Samples (20μl, 20 μg total protein) from each condition shown were boiled in SDS sample buffer and characterized by SDS-PAGE and Western blotting with 1μg/ml of STE7/byr1 antibody. The antibody was detected with anti-rabbit alkaline phosphatase-linked goat antibody. Each lane shown for pCH1 and pCDM8 represent a separate transfection of COS cells.

The antibody detected a 45 kDa band in COS cells transfected with pCH1 but not in control cells. The apparent mass of this band is nearly identical to the predicted molecular mass of 43.5 kDa. These data establish that the antibody specifically recognizes the encoded protein. The antibody also detected an additional band of 46 kDa. Recognition of both the endogenous 46 kDa protein and the 45 kDa band appear to be specific because Western blotting with non-immune serum did not show cross reactivity with either band. Similarly, peptide competition studies with the STE7-VIII peptide, confirmed that the STE7/byr1 antibody was specific for these proteins (data not shown). Thus the immunoreactive COS cell protein may be a related protein.

To more fully characterize MAPKK activity in pCH1 and pCDM8 transfected cells, extract supernatants were fractionated by anion-exchange chromatography and assayed for MBP kinase activity in the presence and absence of recombinant $p42^{mapk}$ (Fig. 2). Fractionation of extracts from serum-treated pCH1 cells resolved a minor (fraction 5) and a major peak (fraction 7) of MAPKK activity (Fig. 2A). Neither of these activities were detectable in pCH1-transfected COS cells that were not stimulated with serum, indicating that MAPKK protein expressed from pCH1, like endogenous COS cell MAPKK, was not constitutively active. Fractionation of extracts from serum treated pCDM8-transfected COS cells revealed a small activation of endogenous COS cell MAPKK (Fig. 2B). No stimulation of MAPKK or MAPK was detected in column fractions from non-serum treated pCDM8 cells. When column fractions were assayed for MBP kinase activity, two serum stimulated peaks were resolved eluting at 125 mM NaCl and 150 mM NaCl in both pCDM8-or pCH1-transfected cells. These corresponded respectively to $p42^{mapk}$ and $p44^{mapk}$, as determined by cross reactivity with an antipeptide antibody raised to the amino acid sequence RRITVEEALAHPYLEQYYDPTDE representing (residues 307–327) a conserved sequence in both MAPK isoforms [2] (data not shown). Interestingly, $p44^{mapk}$ appeared to be more significantly activated (4- to 5-fold) relative to $p42^{mapk}$ (2-fold) in pCH1 transfected cells (compare Fig. 2A with 2B). These results suggest that distinct cellular isoforms of MAPKK exist which show a specificifity towards either $p42^{mapk}$ or $p44^{mapk}$.

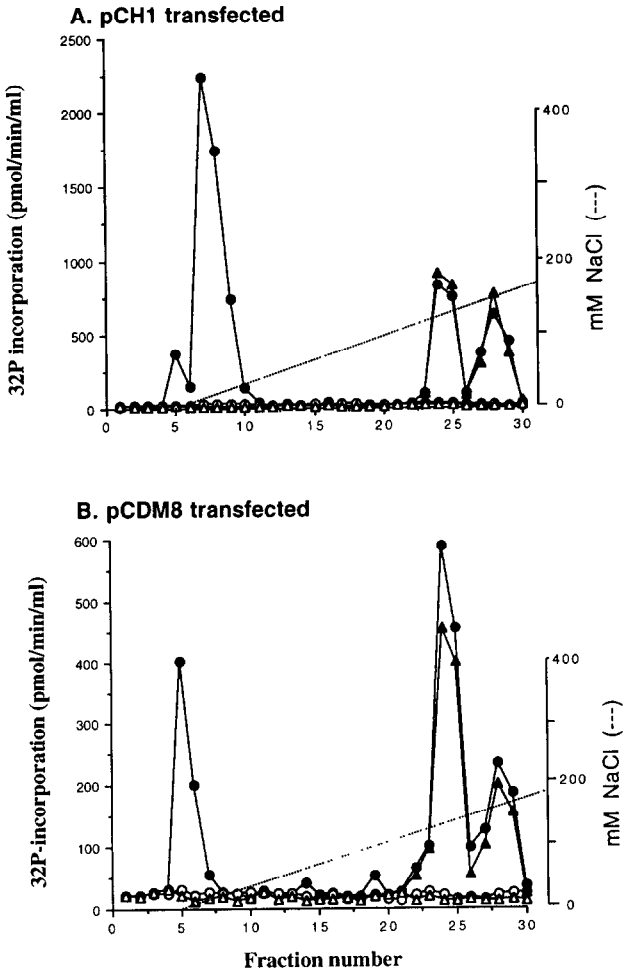


Fig. 2. Anion-exchange chromatography of pCH1 and pCDM8 transfected COS cell extracts. Extracts prepared from pCH1- (A) or pCDM8-transfected (B) COS cells treated with (closed symbols) or without serum (open symbols) for 5 min were applied to a Waters AP-1 anion-exchange column (0.8 mm × 10 cm). The column was developed at a flow rate of 0.5 ml per minute with a 50 ml linear salt gradient (0 to 400 mM NaCl) in 50 mM β-glycerophosphate, pH 7.31 4°C/0.15 mM sodium orthovanidate/1.5 mM EGTA/1 mM dithiothreitol. Fractions were assayed for MBP kinase activity in the presence (○) and absence (△) of recombinant $p42^{mapk}$ as described in Methods. Only the first 30 fractions are shown; no significant activity was detected later in the profile.

The data shown in Fig. 2 demonstrate that pCH1-transfected cells contain two resolvable peaks of MAPKK. The second peak of activity is derived from the MAPKK expressed from pCH1 because this peak appears only in pCH1-transfected cells. The first peak of activity is the endogenous COS cell MAPKK. Since two proteins in pCH1-transfected cells were immunoreactive with anti-STE7/byr1 antibody, the data suggest that the immunoreactive proteins (Fig. 1) corresponded to two forms of MAPKK. This was confirmed by Western blotting of column fractions from each of the conditions with the STE7/byr1 antibody. Fig. 3 shows that the major peak of MAPKK activity appearing in serum-stimulated pCH1-transfected cells (fraction 7, Fig. 2A) co-elutes with the 45kDa protein expressed from pCH1 in Fig. 1. The peak of endogenous MAPKK activity in pCDM8-transfected cells co-elutes with the 46 kDa protein detected in both pCH1- and pCDM8-transfected cells (Fig. 1). These data show that the 45 kDa band is derived from the rat kidney MAPKK cDNA. The data also demonstrate conclusively that pCH1 cells express a fully functional MAPKK which is utilized the same serum-stimulated pathways that bring about activation of endogenous MAPKK. Furthermore, the amplified activation of $p42^{mapk}$ and $p44^{mapk}$ in pCH1-transfected cells suggests that the expressed MAPKK functions normally to activate both endogenous COS cell MAPKs.

To further investigate pCH1 MAPKK activation the most active fractions from anion-exchange were pooled and treated with purified protein phosphatases (Fig. 4). As with MAPKK isolated from other sources [17], pCH1 expressed MAPKK could be completely inactivated by PP-2A. The enzyme showed some susceptibility to PP-1, but was insensitive to PTPase 1b or CD45 (data not shown). Furthermore treatment of pCH1 COS cells with micromolar concentrations of okadaic acid resulted in a profound activation of the expressed rat kidney MAPKK and endogenous COS cell MAPKK*. This data indicates that pCH1 MAPKK was activated by serine/threonine phosphorylation in response to serum and that the enzyme showed the same susceptibility to dephosphorylation and inactivation by PP-2A as wild type MAPKK.

The finding that the endogenous monkey MAPKK was separable from the expressed rat MAPKK from pCH1 and apparently recognized by the anti-STE7/byr1 was unexpected. This may represent a species difference. However, heterogeneity of MAPKK activity has been detected during anion-exchange chromatography at early steps of purification of MAPKK from rabbit skeletal muscle [8]. In addition, two SDS-PAGE bands containing MAPKK activity were purified from rabbit skeletal muscle [8] and A431 cells [18].

This heterogeneity could be explained by differences in phosphorylation or proteolytic cleavage of a single protein or alternatively, by distinct isoforms. Distinct isoforms could arise from distinct mRNAs. Northern analysis with a 1.6 kb fragment of MAPKK cDNA revealed two transcripts (2.7 and 1.7 kb) in adult rat tissues and an additional 2.3 kb transcript in neonatal tongue, each sufficiently large to enclose MAPKK. These mRNAs appear to be derived from a single gene. We do not know as yet whether these mRNAs encode

* C.M.M. Haystead and T.A.J. Haystead, unpublished work.

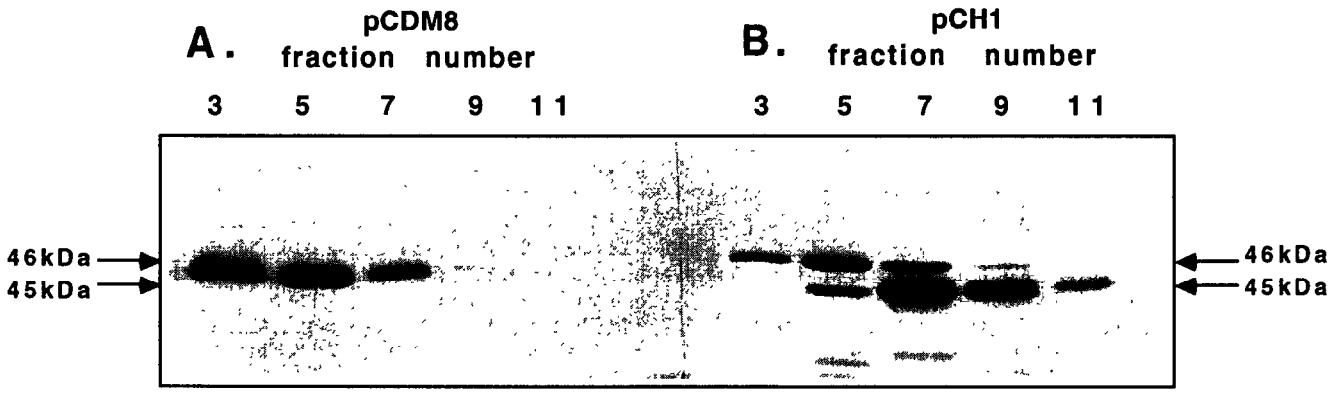
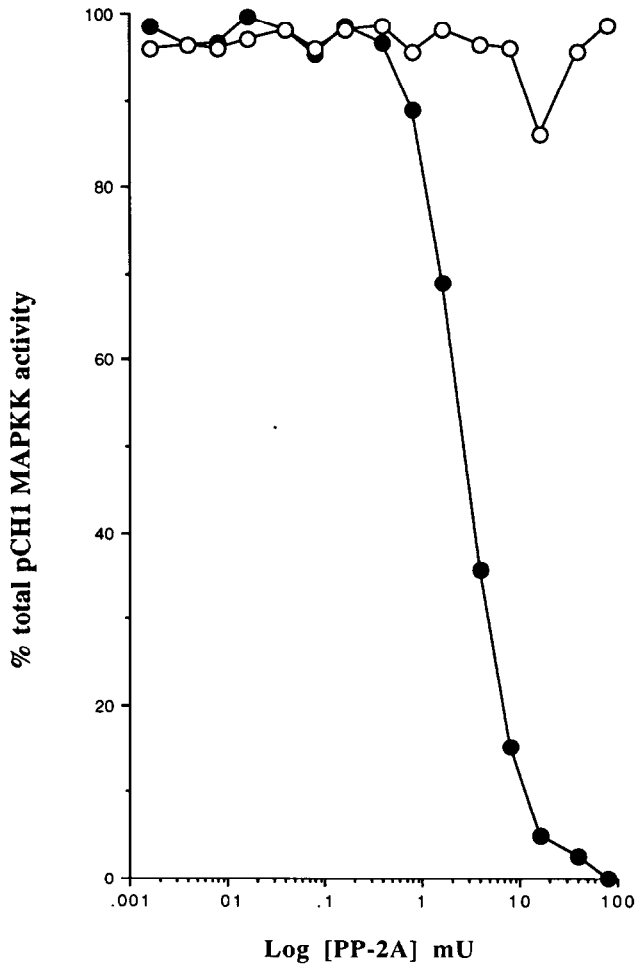


Fig. 3. Western blotting of anion-exchange column fractions with STE7/byr1 peptide antibody. Column fractions from pCH1 and pCDM8 serum stimulated COS cell extracts were probed for MAPKK with the STE7/byr1 as in Fig. 1.

distinct proteins. Additional studies, including characterization of additional MAPKK cDNAs, will be necessary to determine the numbers of MAPKKs and the inter-species variation between any forms identified.

Recently, v-raf [19] and activated c-raf-1 [20] were shown to activate MAPKK in vitro. The data obtained suggest that c-raf-1 may be an activator of MAPKK in vivo, but the number and identity of intracellular MAPKK-kinase(s) remains to be established. The demonstration herein that the isolated cDNA encodes a functional MAPKK will greatly expand approaches available to enumerate and identify these enzyme(s).



Acknowledgements: C.M.M.H. and J.W. contributed equally to this work. We thank Dr. Paul Dent for invaluable discussions. We also express our gratitude to Dr. Steven Pelech of Kintech Biotechnology Corp (Vancouver, BC) for the generous gift of the STE7/byr1 anti-peptide antibody. Support for this work was provided to T.A.J.H. by the Markey Center of the University of Virginia and the American Cancer Society to T.A.J.H. and T.W.S. (BE-69D), National Institutes of Health to T.W.S. (DK41077). J.W. is a postdoctoral fellow of the Juvenile Diabetes Foundation.

REFERENCES

1 Pelech, S.L. and Sanghera, J.S. (1992) Science 257, 1355–1356.
2 Boulton, T.G., Yanacopoulos, G.D., Slaughter, C., Moomaw, C., Hsu, J. and Cobb, M. (1990) Science 249, 64–67.
3 Her, J.H., Wu, J., Rall, T.B., Sturgill, T.W. and Weber, M.J. (1992) Nucleic Acids Res. 19, 3743.
4 Kyriakis, J.M., Brautigan, D.L., Ingerbritsen, T.S. and Avruch, J. (1991) J. Biol. Chem. 266, 10043–10046.

←

Fig. 4. pCH1 expressed serum-stimulated MAPKK is inactivated by protein phosphatase 2A. The most active column fractions containing the expressed rat kidney MAPKK (fractions 7 and 8, Fig. 2A) were pooled after anion-exchange and treated for 20 min at 30°C with the indicated concentrations of purified catalytic subunit of PP-2A in the presence (open symbols) or absence (filled symbols) of 10 μM okadaic acid. Phosphatase activity was terminated by the addition of 40 μM okadaic acid and the reactions assayed for MAPKK by activation of $p42^{mapk}$ as described in section 2. Similar results were obtained with column fractions containing endogenous COS cell MAPKK (data not shown). Results shown are from a single experiment, although this was repeated on a separate occasion.

5 Ahn, N.G., Segar, R., Bratlien, R.L., Diltz, C.D., Tonks, N.K. and Krebs, E.G. (1991) J. Biol. Chem. 266, 4220–4227.
6 Wu, J., Harrison, J.K., Vincent, L.A., Haystead, C., Haystead, T.A.J., Michel, H., Hunt, D.F., Lynch, K.R. and Sturgill, T.W., Proc. Natl. Acad. Sci. USA, in press.
7 Crews, C.M., Alessandrini, A. and Erikson, R.L. (1992) Science, 258, 478–480.
8 Wu, J., Michel, H., Rossomando, A., Haystead, T.A.J., Shabanowitz, J., Hunt, D.F. and Sturgill, T.W. (1992) Biochem. J. 285, 701–705.
9 Haystead, T.A.J., Dent, P., Wu, J., Haystead, C.M.M. and Sturgill, T.W. (1992) FEBS Lett. 306, 17–22.
10 Hanks, S. and Quinn, A. (1991) Methods Enzymol. 200, 38–62.
11 Cullen, B.R. (1987) Methods Enzymol. 152, 684–704.
12 L'Allemain, G., Her, J.-H., Del Vecchio, R.L. and Weber, M.J. (1991) FEBS Lett. 292, 191–195.
13 Haystead, T.A.J., Weiel, J.E., Litchfield, D.W., Tsukitani, Y., Fischer, E.H. and Krebs, E.G. (1990) J. Biol. Chem. 265, 16571–16580.
14 Elion, E.A., Grisafi, P.L. and Fink, G.R. (1990) Cell 60, 649–664.
15 Courchesne, W.E., Kunisawa, R. and Thorner, J. (1992) Cell 58, 1107–1119.
16 Garner, A., Nasmyth, K. and Ammerer, G. (1992) Genes Dev. 6, 1280–1292.
17 Gomez, N. and Cohen, P. (1991) Nature 353, 170–175
18 Segar, R., Ahn, N.G. , Posada, J., Munar, E.S., Jensen, A.M., Cooper, J.A., Cobb, M.H. and Krebs, E.G. (1992) J.Biol. Chem. 267, 14373–14381.
19 Dent, P., Haser, W., Haystead, T.A.J., Vincent, L.A., Roberts, T.M. and Sturgill, T.W. (1992) Science 257, 1404–1407.
20 Kyriakis, J.M., App, H., Zhang, X., Banerjee, P., Brautigan, D.L., Rapp, U.R. and Avruch, J. (1992) Nature 358, 417–421.