BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **227,** 20–26 (1996)
ARTICLE NO. 1461

Inhibition of Raf/MAPK Signaling in *Xenopus* Oocyte Extracts by Raf-1-Specific Peptides

Gerald Radziwill, Ulrike Steinhusen,[1] Alastair Aitken,* and Karin Moelling[2]

*Institut für Medizinische Virologie, Universität Zürich, Gloriastrasse 30, CH-8028 Zürich, Switzerland; and *Laboratory of Protein Structure, National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, United Kingdom*

Received August 11, 1996

Raf-1 is an upstream element of the mitogen-activated protein kinase (MAPK) pathway which leads to cell proliferation and differentiation. In this study Raf-1 derived peptides comprising the conserved amino acid residues Arg89 and Ser259, involved in binding of activated Ras and 14-3-3 proteins, respectively, were shown to interfere with MAPK activation in extracts from immature *Xenopus* oocytes. Lipids prepared from oocyte extracts can stimulate MAPK in a Ras- and protein kinase C-independent manner. This lipid-induced MAPK activation is blocked by a Raf-1 derived peptide comprising Ser259. © 1996 Academic Press, Inc.

Raf-1 is a cytoplasmic serine/threonine-specific protein kinase (1) and functions as an important mediator in different signaling pathways involved in cell growth and differentiation (2). The members of the Raf kinase family contain three conserved regions (CR) (Figure 1A). CR1 and CR2 are part of the amino terminal regulatory domain of Raf-1 and contain a cysteine-rich zinc-finger motif similar to members of the protein kinase C (PKC) family and a serine/threonine-rich region including one of the major phosphorylation sites *in vivo*, Ser259, respectively. CR3, located at the carboxy terminus, represents the catalytic domain.

One of the best characterized pathways involved the stimulation of the receptor-tyrosine kinases leading to activation of Ras. The active GTP-bound form of Ras directly interacts with Raf-1 and recruits it to the plasma membrane for further activation (2). Raf-1 in turn activates the kinase MEK (MAPK/ERK kinase) which phosphorylates extracellular-regulated kinases (ERKs), members of the family of mitogen-activated protein kinases (MAPKs) (3). The activated ERKs can phosphorylate cytoplasmic substrates such as the ribosomal S6-kinase and the cytosolic phospholipase A_2 as well as nuclear proteins such as the transcription factors c-Myc, Elk-1and c-Jun (3). Recently we described the direct association of c-Jun with c-Mil, the avian homologue of Raf-1, suggesting a bypass of the Raf-1/MEK/ERK pathway which also results in phosphorylation of c-Jun (4).

In vivo Raf-1 can not only associate with activated Ras and MEK but also with an unknown Raf-1 phosphorylating kinase (5), the heat shock protein hsp90 (6), a phosphoprotein of 34 kDa (7) and with 14-3-3 proteins (8). 14-3-3 proteins are a family of highly conserved proteins involved in several biological processes including the regulation of Raf-1 activity (9). Recently we showed that 14-3-3 proteins can bind to the amino terminus as well as to the carboxy terminus of Raf-1 and that activated Ras can displace the 14-3-3 isoforms ζ from the amino terminus of Raf-1 indicating a role of 14-3-3 proteins in regulating the Raf-1 activity (10).

The Ras/Raf-1/MEK/ERK signaling pathway has been described in various vertebrate cell lines

[1] Present address: Max-Delbrück-Center für molekulare Medizin, Robert Rössle-Str. 10, D-13122 Berlin-Buch.
[2] Corresponding author. Fax: +41 1 257 6967.
Abbreviations: MAPK, mitogen-activated protein kinase; GST, glutathione S-transferase; PKC, protein kinase C.

0006-291X/96 $18.00

and also in *Drosophila melanogaster, Caenorhabditis elegans* and *Xenopus laevis* (2). It has been shown that treatment of immature *Xenopus* oocytes with progesterone or insulin activates the 42 kDa *Xenopus* MAPK in a Raf-1 dependent manner (11, 12). Microinjection of RNA of either Ras, active Raf-1 mutants or 14-3-3 isoforms (β or ζ) results in oocyte maturation and MAPK activation (11, 13). Moreover, in cell-free extracts prepared from immature *Xenopus* oocytes, activated Ras can enhance endogenous Raf-1 activity leading to stimulation of the 45 kDa *Xenopus* MAPK kinase followed by activation of 42 kDa MAPK (14).

In this study we used peptides derived from regions of Raf-1 that have been shown to be critical for binding of activated Ras and 14-3-3, to dissect Raf-1 mediated MAPK activating pathways. In cell-free extracts from immature *Xenopus* oocytes 14-3-3 -induced MAPK activation is independent of the Ras-induced pathway. Ras-triggered MAPK activation depends on direct binding of Ras to Raf-1 via Arg89 but also interferes with the 14-3-3 -induced MAPK pathway via Ser259. Moreover, lipids extracted from immature oocytes can activate MAPK in a Ras- and PKC-independent manner. This lipid-induced MAPK activation is inhibited by the Raf-1 derived peptide comprising Ser259.

MATERIALS AND METHODS

Materials. pGEX (Pharmacia) derived plasmids expressing the amino terminal (NT) residues 1-331 of the human Raf-1 as glutathionine-S-transferase (GST) fusion proteins, GST-RafNT, and the GST-Raf mutant proteins GST-RafNT (R89L), GST-RafNT (C168S), GST-RafNT (S259A) and GST-RafNT (ΔCys), have been published (15, 16). The recombinant Ras mutant protein, Ha-Ras (G12V), was a generous gift of A. Wittinghofer (17). 14-3-3 proteins partially purified from sheep brain and recombinant 14-3-3 ζ were prepared as described (18). Peptides were synthesized by Birsner & Grob Inc. (Denzlingen, FRG) with sequences as follows: peptide A corresponding to the Raf-1 residues 77-101 (SLHDCLMKALKVRGLQPECCAV TRL); mutant peptide A, same as peptide A except for exchange of residue Arg 89 to Leu; peptide B, Raf-1 residues 252-275 (SQRQRSTSTPNVHMVSTTLPVDSR); mutant peptide B, same as peptide B except for a single amino acid exchange of residue Ser 259 to Ala. PKCζ peptide inhibitor corresponds to the pseudosubstrate site on PKCζ (RRGARRWRK) (19). PKCα inhibitory peptide (RKGALRQKW) was purchased from Boehringer Mannheim. Antiserum to *Xenopus* p42 MAPK was raised in rabbits against a peptide comprising the residues 336-348 (MELDDLPKETLKE) conjugated to keyhole limpet haemocyanin. Female frogs (*Xenopus laevis*) were purchased from H. Kaehler (Hamburg, FRG).

In vitro binding assays. The assay has been described in detail (16). For each assay 3 μg GST-RafNT fusion protein bound to GSH-agarose beads (Sigma) were incubated with 1μg recombinant mutant Ha-Ras (G12V) loaded with GTPγS (Boehringer Mannheim) or 2 μg partially purified 14-3-3 proteins in 300 ml NETN-buffer (0.1 M NaCl, 1 mM EDTA, 1 mM DTT, 0.5 % NP40, 10 mM PMSF, 20 KIU/ml Trasylol (Bayer), 20 mM Tris-HCl, pH 8.0) for 2h at 4°C. The beads were washed and analyzed by 15% SDS-PAGE followed by immunoblotting using monoclonal pan-Ras antibody (dilution 1:2000) or anti-pan-14-3-3 antiserum (dilution 1:4000) and enhanced chemiluminescence (ECL, Amersham).

Preparation of cell-free extracts and lipids from Xenopus oocytes. Cell-free extracts were prepared from immature *Xenopus* oocytes essentially as described by Shibuya et al. (20). The lipid cap obtained from preparations of *Xenopus* oocytes extracts was diluted with five volumes chloroform:methanol (2:1, vol/vol), mixed thoroughly and centrifuged to separate the two phases. The chloroform phase containing the extracted lipids was evaporated in a speed-vac centrifuge. The concentrated lipid solution was stored at −80°C.

Immunoblot analysis of MAPK. Samples for MAPK immunoblots were prepared by mixing 10 μl of oocyte extract, 0.5 μl ATP-regenerating system (25 mM ATP, 25 mM $MgCl_2$, 0.25 M creatine phosphate, 1.25 mg/ml creatine kinase (Boehringer Mannheim), 4000 KIU/ml Trasylol, 25 mg/ml leupeptin in EB), 4.5 μl EB or other additives. For analysis of MAPK-activation the extract was incubated with 1 μg activated GTPgS- loaded Ha-Ras (G12V) (16), 2 μg recombinant 14-3-3ζ or 1 ml of extracted lipids for 4h at 20°C. The samples were analyzed by 10% SDS-PAGE followed by immunoblotting using antiserum to MAPK (dilution 1:2000) and ECL. To analyse the effect of the peptides on MAPK stimulation activated Ras and 14-3-3ζ were preincubated with an one hundred molar excess of the indicated peptides for 30 min at 4°C. To analyze the effects on the lipid-induced MAPK activation the peptides were added to the oocyte extract just prior to addition of the lipids.

RESULTS AND DISCUSSION

Two modulators of Raf-1 activity which directly bind to the protein are the active GTP-bound form of the mutant Ras(G12V) protein and 14-3-3 proteins (2, 9). By using the amino

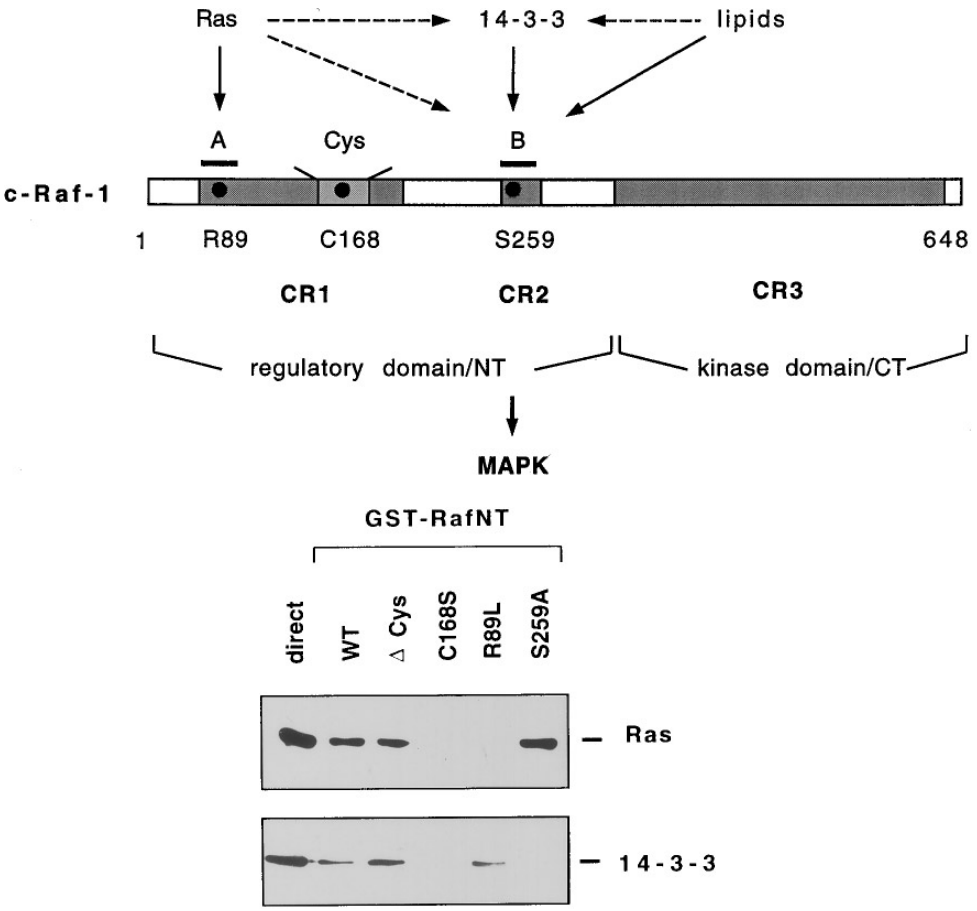

FIG. 1. (A) Schematic representation of the protein kinase Raf-1. The amino terminally (NT) located regulatory domain of Raf-1 contains the conserved regions CR1 (residues 62–196), including Arg 89 (R89) and the cysteine-rich region (Cys, residues 152–184), and CR2 (residues 255–268), a Ser/Thr-rich region including Ser 259 (S259). The conserved region CR3 (residues 331–625) corresponds to the catalytic domain at the carboxy terminus (CT). The bars represent peptide A (residues 77–101) and peptide B (residues 252–275). The top summarizes the results of this study. The dotted arrows indicate that the interactions may be indirect. (B) Recombinant GST-RafNT and various mutant proteins, as indicated and described in Materials and Methods, were immobilized on GSH-agarose beads and incubated with recombinant GTPγS-loaded Ras or 14-3-3 proteins partially purified from sheep brain. After washing, the GSH-agarose beads were analyzed by SDS–PAGE followed by immunoblotting with Ras-specific antibody (upper panel) or pan-14-3-3 antiserum (lower panel) and developed with ECL.

terminus (NT) of Raf-1 fused to glutathionine S-transferase (GST-RafNT) and mutant GST-RafNT proteins for *in vitro* binding studies we can dissect the regions on Raf-1 important for binding of activated Ras and 14-3-3 proteins (Figure 1B). GST-RafNT can bind activated Ras as well as 14-3-3 proteins. GST-RafNT(R89L) can no longer bind to activated Ras but can still bind 14-3-3 proteins. In contrast, GST-RafNT(S259A) can bind to activated Ras but not to 14-3-3 proteins. A deletion of residues 152-184 comprising the cysteine-rich region (GST-RafNTΔCys) does not interfere with binding of activated Ras and Raf-1 *in vitro* (Figure 1B). However, a single amino acid exchange of Cys168 to Ser in the amino terminus of Raf-1, GST-RafNT(C168S), prevents binding of both proteins suggesting an overall conformational change rather than a direct involvement of Cys168 in binding to activated Ras or 14-3-3. These data indicate that the regions on Raf-1 which are involved in binding of activated Ras

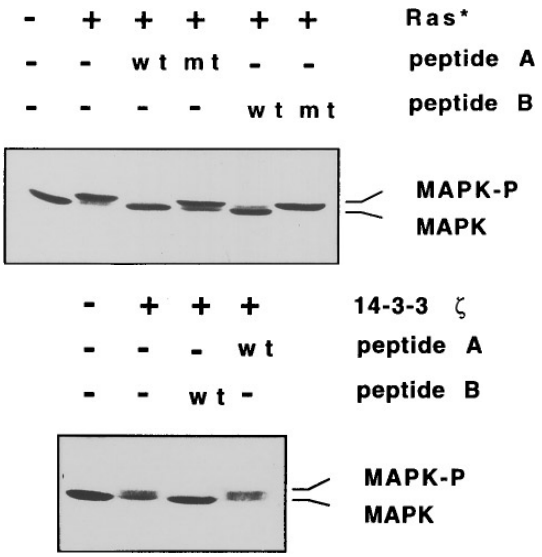

FIG. 2. Raf-1 derived peptides inhibit MAPK activation in oocyte extracts. (A) Extracts of immature oocytes were incubated with recombinant activated GTP-loaded mutant Ras(G12V), Ras*, for four hours at 20°C. Activated Ras protein was preincubated for 30 min at 4°C with peptide A, corresponding to the residues 77–101 of Raf-1 (peptide A, wt); the mutant peptide A, containing a single amino acid exchange of Arg 89 to Leu (peptide A, mt); peptide B corresponding to residues 252–275 of Raf-1 (peptide B, wt); or the mutant peptide B carrying a single amino acid exchange of Ser 259 to Ala (peptide B, mt). The extracts were analyzed by SDS–PAGE and immunoblotting with antiserum to MAPK. The hyperphosphorylated activated MAPK (MAPK-P) is detectable by its reduced electrophoretic mobility compared to the inactive form. (B) Effects on MAPK activation were also studied with recombinant 14-3-3ζ, in the presence or absence of the Raf-derived peptides (A and B, wt) and analyzed as described in (A).

and 14-3-3 proteins are separated by the cysteine-rich region. Recently two binding sequences on Raf-1 for Ras have been identified, one comprising residues 51-131 and the other one residues 139-186, the region around the zinc-finger (21). We have shown that a peptide corresponding to residues 77-101, which is part of the first binding site and contains the highly conserved residue Arg89, can prevent binding of activated Ras to Raf-1 *in vitro* (16). Amino terminal fragments of Raf-1 containing in addition to the first binding site the residues 132-149, a portion of the second binding site, displayed increased affinity for binding of active Ras (22, 23). Whether the adjacent sequences up to 186 are also essential for Raf-1/Ras binding is unclear. Since these sequences are deleted in the mutant GST-RafNTΔCys our results indicate that the cysteine-rich region (residues 152-184) is not directly involved in binding of activated Ras and also not of 14-3-3 proteins to Raf-1 *in vitro.*

Based on these results we asked the question whether it was possible to use Raf-1 derived peptides corresponding to the proposed binding sites of active Ras and 14-3-3 proteins to interrupt the Raf-1/MAPK signaling pathway. Two Raf-1 derived peptides, peptide A corresponding to residues 77-101 and peptide B corresponding to residues 252-275 along with the respective mutant derivates R89L and S259A were synthesized (see Figure 1A). We used cell-free extracts of immature *Xenopus* oocytes to investigate signal transduction pathways leading to activation of MAPK. The activation status of the MAPK was monitored by immunoblotting with antiserum to MAPK. The active hyperphosphorylated form of the MAPK shows reduction of electrophoretic mobility compared to the inactive form (20).

Incubation of the oocyte extract in the presence of activated Ras (Ras*) resulted in activation of MAPK (Figure 2A). In order to test the effect of the Raf-1 derived peptides, the activated

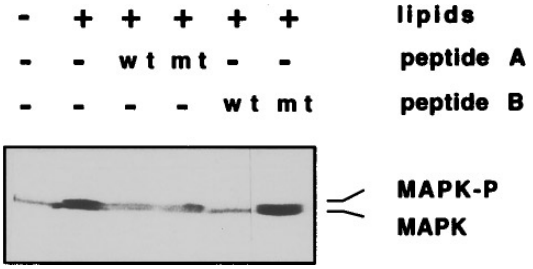

FIG. 3. Lipid-induced MAPK activation is inhibited by the Raf-1-derived peptide B. Extracts of immature oocytes were incubated with lipids extracted from the oocyte membrane fraction and effects of wild-type (wt) and mutant (mt) peptides A and B on lipid-induced MAPK activation were analyzed as described for Fig. 2A.

Ras was preincubated with the peptides before the mixture was added to the oocyte extract for further incubation. Peptide A (wt) and peptide B (wt) significantly inhibited the MAPK activation induced by activated Ras. In contrast, the mutant peptides A (mt) and B (mt) had no influence on Ras-triggered MAPK activation. We then studied one member of the 14-3-3 protein family,14-3-3ζ, which has been shown to stimulate Raf-1 activity (13). As expecteed, addition of 14-3-3ζ to the oocyte extract led to activation of MAPK (Figure 2B). This 14-3-3ζ-induced MAPK activation was blocked by peptide B but not by peptide A, suggesting that signaling involves the conserved region CR2 of Raf-1.

In summary, 14-3-3 -induced stimulation of MAPK was only inhibited with peptide B comprising the sequence which interferes with binding of 14-3-3 (Figure 1B) (10), but not with peptide A indicating that this effect is independent of Ras or functions downstream of Ras. The Ras-induced MAPK activation was not only inhibited by peptide A which prevents the direct binding of Ras to Raf-1 (16), but also by peptide B. This was unexpected since peptide B does not directly influence the association of Raf-1 and activated Ras. In the yeast system a similar finding was made in that the 14-3-3 homologue BMH1 was required for the Ras-induced activation of Raf-1 (24). Therefore it appears that in certain systems the Ras pathway depends also on steps involved in 14-3-3 -induced MAPK activation.

Most recently, while this work was in progress it has been shown by Muslin et al. (25) that Raf-1 binds to 14-3-3 via its motif RSTpSTP (residues 256-261) in a sequence and phosphoserine dependent manner whereby Ser259 was phosphorylated. A second similar motif can be found at the carboxy terminus of the human Raf-1 (RSApSEP, residues 616-623). Ser621 is however not conserved in the Raf-1 homologue of *Xenopus laevis*, Xe-Raf (RSALEP) (26). Because the peptide B mutant derivate S259A did not interfere with MAPK activation the position of this serine residue appears to be important. Therefore the peptide B used here is expected to mimick the amino terminal binding site for 14-3-3. Taken together our data clearly show that the sequence of peptide B containing the conserved region CR2 of Raf-1 is important for the regulation of the Raf-1 activity by Ras and 14-3-3 as summarized schematically in Figure 1A.

During these studies we noticed that in oocyte extracts the MAPK could also be activated by supplementing the oocyte extract with an aliquot of the membrane fraction which had been removed during the oocyte extract preparation (data not shown). To address the question whether the lipid components of the membrane fraction were responsible for the MAPK activation, the lipids were extracted from the membrane fraction with chloroform-methanol and added to an oocyte extract. This lipid extract was indeed sufficient to activate MAPK (Figure 3). The lipid-induced MAPK activation could be inhibited with peptide B but not with peptide A or either of the two mutant peptides (Figure 3). In principle, the lipid effect could

have been mediated through members of the protein kinase C (PKC) family since it has been shown that PKCα can phosphorylate and activate Raf-1 (27). The lipid-induced MAPK activation was however not influenced by two PKC inhibitory peptides specific for the PKC isoforms α, β, γ or ζ, respectively (data not shown), indicating that these members of the PKC family were not involved in this lipid-induced activation of the MAPK. Thus, Raf-1 itself appears to be activated by the lipids. It has been shown that the cysteine-rich region of Raf-1 which is very similar to the zink-finger motif of PKC can bind phosphatidylserine (21). Force et al. (28) tested various commercially available lipids including phosphatidylserine and showed that these were not sufficient to modulate Raf-1 kinase activity *in vitro*. Recently it has been shown that *in vitro* Raf-1 kinase activity can be stimulated in a Ras- dependent manner by the addition of plasma membranes prepared from cells transformed with v-Ras or v-Src (29). Further modulation of Raf-1 kinase activity was possible by addition of lipids extracted from membranes of unstimulated cells (30). This lipid-induced activation was not Ras-dependent and was not blocked by an amino terminal Raf-1 fragment comprising residues 1-257. This Raf-1 fragment does not contain the whole sequence of peptide B (residues 252-275) which we demonstrate here to inhibit the lipid-induced MAPK activation in oocyte extracts. Thus the conserved Ser259 appears be important for the lipid effect. Furthermore the here described lipid activation is independent of Ras and PKC.

The data presented here do not show whether lipids bind to Raf-1 directly or act through 14-3-3. Furthermore, lipids may also regulate other kinase(s) phosphorylating Raf-1, one of which we have described to bind to the amino terminus of Raf-1 and to phosphorylate it at Ser259 (5). It will be of interest to characterize the role of lipids involved in the stimulation of the Raf-1/MAPK pathway and to analyze in detail the effects of lipids on regulation of Raf-1 activity.

ACKNOWLEDGMENTS

The authors thank Dr. A. Wittinghofer for Ha-Ras(G12V) protein, Y. Soneji for 14-3-3ζ, Dr. H. Martin for 14-3-3 antiserum and Dr. J. Lovric′ and C. Rommel for critical reading of the manuscript. Gratefully acknowledged is the excellent technical assistance of A. Rabien and I. Benz. This work was supported by the Schweizer Nationalfond, the Schweizer Krebsliga and the Krebsliga des Kantons Zürich.

REFERENCES

1. Moelling, K., Heimann, B., Beimling, P., Rapp, U. R., and Sander, T. (1984) *Nature* **312,** 558–661.
2. Daum, G., Eisenmann-Tappe, I., Fries, H., Troppmair, J., and Rapp, U. R. (1994) *TIBS* **19,** 474–479.
3. Cano, E., and Mahadevan, L. C. (1995) *TIBS* **20,** 117–122.
4. Radziwill, G., Niehof, M., Rommel, C., and Moelling, K. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 1421–1425.
5. Beimling, P., Niehof, M., Radziwill, G., and Moelling, K. (1994) *Biochem. & Biophys. Res. Commun.* **204,** 841–848.
6. Lovric′, J., Bischof, O., and Moelling, K. (1994) *FEBS Let.* **343,** 15–21.
7. Lovric′, J., Radziwill, G., and Moelling, K. (1996) *Oncogene* **12,** 1145–1151.
8. Morrison, D. (1994) *Science* **266,** 56–57.
9. Aitken, A. (1995) *TIBS* **20,** 95–97.
10. Rommel, C., Radziwill, G., Lovric′, J., Noeldeke, J., Heinicke, T., Jones, D., Aitken, A., and Moelling, K. (1996) *Oncogene* **12,** 609–619.
11. Fabian, J. R., Morrison, D. K., and Daar, I. O. (1993) *J. Cell Biol.* **122,** 645–652.
12. Muslin, A. J., MacNicol, A. M., and Williams, L. T. (1993) *Mol. Cell. Biol.* **13,** 4197–4202.
13. Fantl, W. J., Muslin, A. J., Kikuchi, A., Martin, J. A., MacNicol, A. M., Gross, R. W., and Williams, L. T. (1994) *Nature* **371,** 612–614.
14. Fukuda, M., Gotoh, Y., Kosako, H., Hattori, S., and Nishida, E. (1994) *J. Biol. Chem.* **269,** 33097–33101.
15. Niehof, M., Beimling, P., and Moelling, K. (1994) *Biochem. Biophys. Res. Commun.* **203,** 1560–1566.
16. Niehof, M., Radziwill, G., Klauser, S., and Moelling, K. (1994) *Biochem. Biophys. Res. Commun.* **206,** 46–50.
17. John, J., Frech, M., and Wittinghofer, A. (1988) *J. Biol. Chem.* **263,** 11792–11799.
18. Robinson, K., Jones, D., Patel, Y., Martin, H., Madrazo, J., Martin, S., Howell, S., Elmore, M., Finnen, M. J., and Aitken, A. (1994) *Biochem J.* **299,** 853–861.

19. Berra, E., Diaz-Meco, M. T., Dominguez, I., Municio, M. M., Sanz, L., Lozano, J., Chapkin, R. S., and Moscat, J. (1993) *Cell* **74,** 555–563.
20. Shibuya, E. K., Polverino, A. J., Chang, E., Wigler, M., and Ruderman, J. V. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 9831–9835.
21. Brtva, T. R., Drugan, J. K., Ghosh, S., Terrell, R. S., Campbell-Burk, S., Bell, R. M., and Der, C. J. (1995) *J. Biol. Chem.* **270,** 9809–9812.
22. Chuang, E., Barnard, D., Hettich, L., Zhang, X., Avruch, J., and Marshall, M. S. (1994) *Mol. Cell. Biol.* **14,** 5318–5325.
23. Ghosh, S., Xie, W. Q., Quest, A. F. G., Mabrouk, G. M., Sturm, J. C., and Bell, R. M. (1994) *J. Biol. Chem.* **269,** 10000–10007.
24. Irie, K., Gotoh, Y., Yashar, B. M., Errede, B., Nishida, E., and Matsumoto, K. (1994) *Science* **265,** 1716–1719.
25. Muslin, A. J., Tanner, J. W., Allen, P. M., and Shaw, A. S. (1996) *Cell* **84,** 889–897.
26. Le Guellec, R., Le Guellec, K., Paris, J., and Philipe, M. (1988) *Nucleic Acids Res.* **21,** 10357.
27. Kolch, W., Heidecker, G., Kochs, G., Hummel, R., Vahidi, H., Mischak, H., Finkenzeller, G., Marmé, D., and Rapp, U. R. (1993) *Nature* **364,** 249–252.
28. Force, T., Bonventre, J. V., Heidecker, G., Rapp, U., Avruch, J., and Kyriakis, J. M. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 1270–1274.
29. Dent, P., and Sturgill, T. W. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 9544–9548.
30. Dent, P., Reardon, D. B., Morrison, D. K., and Sturgill, T. W. (1995) *Mol. Cell. Biol.* **15,** 4125–4135.