FEBS Letters 394 (1996) 34–38 FEBS 17533

Activation-independent nuclear translocation of mitogen activated protein kinase ERK1 mediated by thiol-modifying chemicals

Ruedi Meili, Kurt Ballmer-Hofer*

Friedrich Miescher Institute, P.O. Box 2543, CH-4002 Basel, Switzerland

Received 2 August 1996

Abstract The extracellular signal-regulated kinases ERK1 and ERK2 are key mediators of mitogenic signals in most cell types. In fibroblasts, sustained activation and nuclear translocation are mandatory for S-phase induction. The events leading to activation of these kinases are well understood, whereas little is known about the mechanism of their translocation. Using indirect immunofluorescence and biochemical analysis we show that ERK1 can translocate to the nucleus in the absence of activation and phosphorylation by upstream kinases when cells are treated with thiol-modifying chemicals. We propose that these chemicals inactivate a protein contributing to the cytoplasmic localization of ERK1.

Key words: Extracellular signal-regulated kinase; Mitogen-activated protein kinase; Localization; Phenylarsine oxide; Microtubule

1. Introduction

An important part of the cellular response to a changing environment is adjusting the levels of gene products to new conditions. To initiate these adaptations, signals have to be relayed from the plasma membrane to the nucleus.

A kinase early implicated in the transduction of these signals [1] was later dubbed extracellular signal-regulated kinase (ERK). Two closely related isoforms, ERK1 and ERK2 are found in mammalian cells which seem to be functionally equivalent in most aspects. Following activation, translocation of ERK from the cytoplasm to the nucleus has been observed in many cell systems [2–6].

The extent of translocation seems to determine the response of a cell to the signalling event. In fibroblasts, only stimuli which lead to detectable nuclear localization of ERK are mitogenic while in PC12 rat pheocytochroma cells mitogenesis can occur without apparent translocation of ERK to the nucleus. Instead, a correlation exists between differentiation of PC12 cells and detectable nuclear localization of ERK (reviewed in [7]).

ERK becomes activated by a chain of interacting proteins. At the plasma membrane, receptor activation results in the conversion of the small GTP binding protein Ras from the GDP-bound, inactive to the GTP-bound, active form via intermediary proteins. The signal transducing proteins involved vary depending on the external stimuli and the receptors engaged (reviewed in [8]).

Activated Ras recruits the serine/threonine kinase Raf to the plasma membrane [9,10] where Raf is fully activated by additional phosphorylation [11]. Raf then phosphorylates and activates MEK (for MAPK/ERK kinase). MEK is a dual specificity kinase which in turn activates ERK by phosphorylation of a threonine and a tyrosine residue. The three kinases Raf, MEK and ERK together form a mitogen-activated protein kinase (MAPK) cascade (reviewed in [12]). The mechanism responsible for nuclear translocation of ERK is unknown.

Here we describe that several thiol-modifying chemicals induce nuclear translocation of ERK in the absence of its activation. This implies that ERK activation and translocation are separable events.

2. Materials and methods

2.1. Materials

Diamide, phenylarsine oxide, myelin basic protein and sodium orthovanadate were obtained from Sigma. *N*-Ethylmaleimide (NEM), aprotinin, leupeptin, and nocodazole was purchased from Boehringer Mannheim. [γ-^{32}P]ATP was from ICN. β-Glycerophosphate and protein A-Sepharose CL-4B were obtained from Pharmacia Biotech Inc. 4′,6-Diamidine-2′-phenylindole dihydrochloride (DAPI) for DNA staining was obtained from Serva. Taxol was purchased from Calbiochem. Recombinant B-chain homodimer platelet-derived growth factor was from Saxon Biochemicals GmbH. Secondary antibodies for Western blotting and immunofluorescence were purchased from Southern Biotechnology Associates.

Vanadyl hydroperoxide was prepared [13] by mixing equivalent concentrations (10 mM) of orthovanadate and hydrogen peroxide followed by a 15 min incubation at room temperature. To destroy unreacted hydrogen peroxide, catalase (200 μg/ml) was added.

2.2. Tissue culture

REF52 cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (GIBCO BRL). Growth arrest was achieved by culturing the cells in DMEM containing 0.1% FCS for 48 h.

2.3. Immunofluorescence

Subcellular localization of ERK1 in REF 52 cells was determined as described in [14] by indirect immunofluorescence using a polyclonal rabbit antibody raised against a C-terminal peptide of rat ERK1 [15]. The antibody was purchased from Upstate Biotechnology Inc. (UBI). Phosphotyrosine was detected using the mouse monoclonal anti phosphotyrosine antibody 4G10 [16]. Nuclei were stained with the DNA binding dye DAPI (0.1 μg/ml).

2.4. Microinjection

REF52 cells were microinjected with the plasmid pCMV NΔRaf as described earlier [14]. This construct directs expression of a c-Raf1 mutant lacking the kinase domain which acts on signalling in a dominant negative fashion [17] under the control of a cytomegalo virus early promoter. Expression of NΔRaf protein was detected by indirect immunofluorescence using a mouse monoclonal anti-c-Raf1 antibody purchased from UBI.

2.5. MAPkinase activity assay

ERK1 activity was measured in an immune complex kinase assay

*Corresponding author. Fax: +41 61 6973976.
E-mail: meiliru1@fmi.ch

PII S0014-5793(96)00927-1

using a rabbit polyclonal antibody specific for ERK1 (F15P) and myelin basic protein as a substrate as described previously [18].

2.6. Western analysis

24 μl of lysates prepared in the same way as for ERK activity assays were added to 6 μl of 5× concentrated SDS sample buffer, separated on 12.5% SDS PAGE and transferred to a PVDF membrane. The membranes were immunodecorated following standard Western blotting protocols: for ERK1 with the anti-ERK1 CT antibody (UBI), for phosphotyrosine with the monoclonal antibody 4G10 and for MEK1 with an antibody raised against a N-terminal peptide [19]. An alkaline phosphatase-linked secondary antibody (Southern Biotechnology Associates) was used. The membranes were developed using a colorigenic substrate combination (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium, Boehringer Mannheim).

3. Results

3.1. Thiol-reactive chemicals induce relocalization of ERK1 to the nucleus

Different chemicals were tested for their effect on the subcellular localization of ERK in a rat-embryo-derived fibroblastic cell line, REF52 using indirect immunofluorescence. In growth-arrested cells (Fig. 1, panel A1) the fluorescence intensity in the nucleus was lower than in the adjacent cytoplasm. As described previously [2,20], stimulation by mitogens for 10 min (Fig. 1, panels A3 and A4) increased nuclear fluorescence to a level equal to or higher than that of the surrounding cytoplasm, indicating that ERK1 accumulated in the nucleus in response to mitogen stimulation.

A 10-min treatment with membrane-permeable thiol-reactive compounds like phenylarsine oxide (PAO, 40 μM) (Fig. 1, panel A2), *N*-ethylmaleimide (NEM, 1 mM) (data not shown) or 250 μM diamide (data not shown) resulted in a nuclear localization of ERK1 which was indistinguishable from that induced by mitogens. The membrane impermeable sodium arsineoxide, which has a reactivity towards thiols like PAO, did not affect subcellular localization of ERK1 at concentrations of up to 1 mM (data not shown). This suggests that modifications of intracellular thiol groups mediate the effect of PAO on ERK1 localization.

3.2. Thiol-reactive reagents neither activate nor promote phosphorylation of ERK

Biochemical analysis was used to determine the effect of PAO on the activity and phosphorylation state of ERK1.

An anti-phosphotyrosine Western blot (Fig. 2A) of whole cell lysates demonstrated that PAO treatment (lane 2) did not change tyrosine phosphorylation in comparison to growth-arrested cells (lane 1). Mitogen stimulation on the other hand led to an increase in the phosphorylation of discrete bands: PDGF (lane 3) promoted phosphorylation of the 180 kDa PDGF receptor and two other bands, which were identified as the phosphorylated forms of ERK1 and ERK2 by anti-ERK1 Western blotting (Fig. 2B). ERK1 and ERK2 were phosphorylated following serum stimulation as well (lane 4).

When ERK1 or ERK2 are phosphorylated at either one of their two regulatory sites, reduced mobility in SDS PAGE is observed shifting the protein band to a higher apparent molecular mass [21]. An anti-ERK1 Western blot (Fig. 2B) showed that PAO did not promote this shift (lane 2), indicating that no phosphorylation occurred while the expected shift was present following mitogen stimulation (Fig. 2B, lanes 3 and 4). Cross-reactivity of the anti-ERK1 antibody with Rat ERK2 showed that changes of ERK2 mobility in response to the applied stimuli paralleled those observed for ERK1. Satisfactory detection of ERK2 required loading of 10 times more lysate (data not shown).

The absence of a shift in apparent molecular mass following PAO treatment indicated that ERK1 was not activated by PAO. To confirm this, immune complex kinase assay of ERK1 with myelin basic protein as a substrate was performed. ERK1 isolated from PAO-treated cells was not activated (Fig. 2C, lane 2) when compared to ERK1 isolated from growth-arrested cells (Fig. 2C, lane 1), while ERK1 isolated from PDGF-stimulated cells showed a 5-fold activation (Fig. 2C, lane 3).

3.3. Induction of nuclear localization of ERK by PAO is not mediated by the MAPK cascade

Since mitogen-induced nuclear localization of ERK correlates with the activation of the MAPK cascade, we investigated the role of Raf and MEK in PAO-induced nuclear localization. An expression vector coding for NΔRaf, a mutant known to block mitogenic signalling in a dominant negative fashion [17], was microinjected into serum-starved cells. After further incubation for 6 h to allow for expression of the protein, cells were either stimulated by serum (Fig. 3A, left column) or treated with PAO (Fig. 3A, right column). NΔRaf expression was confirmed by staining the injected cells with an anti-c-Raf1 antibody (Fig. 3A, lower row). Expression of the dominant negative mutant of Raf1 blocked serum-induced nuclear localization of ERK1 but did not prevent nuclear localization induced by PAO (Fig. 3A, upper row). This indicates that the mechanism of PAO action is independent of Raf1 activity while nuclear localization in response to mitogen stimulation requires Raf1. Since ERKs were not phosphorylated in response to PAO, a contribution of MEK kinase activity to its effect seemed unlikely as well. To confirm this assumption a Western blot of cell lysates was decorated with an anti-MEK1 antibody. As shown in Fig. 3B (lane 2) MEK1 is not shifted in lysates of PAO-treated cells compared to those of growth-arrested control cells (Fig. 3B, lane 1). A mobility shift of MEK1 like that seen in lysates of mitogen-stimulated cells (Fig. 3B, lanes 3 and 4) has been shown earlier to correlate with MEK1 activity [22]. Therefore we concluded that MEK1 is not activated in response to PAO and MEK1 kinase activity is not required for PAO-induced nuclear localization of ERK1.

3.4. PAO, in contrast to vanadyl hydroperoxide, does not act via general inhibition of tyrosine phosphatases

Although PAO has been reported to act as a tyrosine phosphatase inhibitor [23–25], no general increase in tyrosine phosphorylation (Fig. 2A, lane 2) and no increase of tyrosine phosphorylation of specific proteins was detected. To assess whether nuclear localization of ERK1 could still be a consequence of PAO acting as a tyrosine phosphatase inhibitor, another tyrosine phosphatase inhibitor, vanadyl hydroperoxide, was used as an additional control. It is acting similar to orthovanadate [26] but it is more potent [27]. Treating cells for 10 min with 100 μM vanadyl hydroperoxide promoted nuclear localization of ERK1 comparable to treatment with PAO or stimulation with mitogens (Fig. 1, panel A5). Judged by immunofluorescence (Fig. 1, panel B5) and Western blotting (Fig. 2A, lane 5), vanadyl hydroperoxide treatment led to

Figure 1

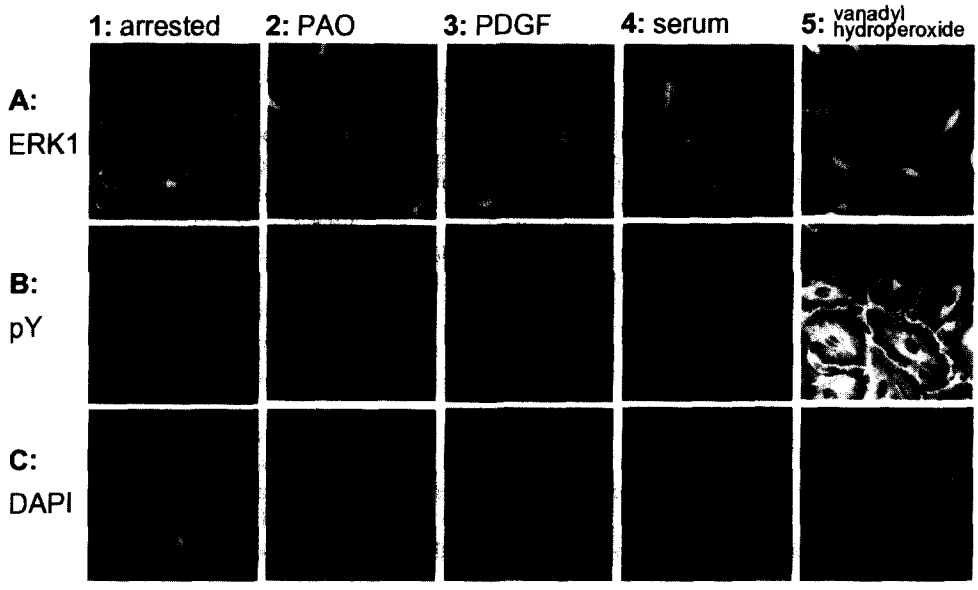

Figure 2

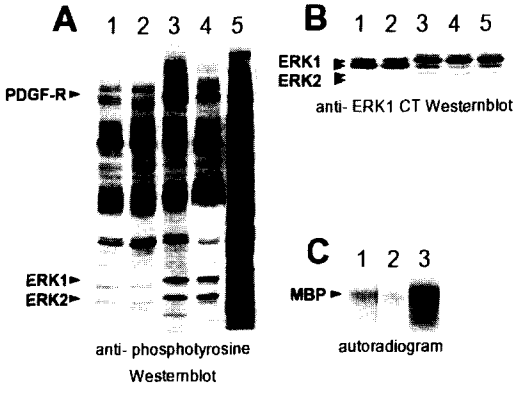

Figure 3

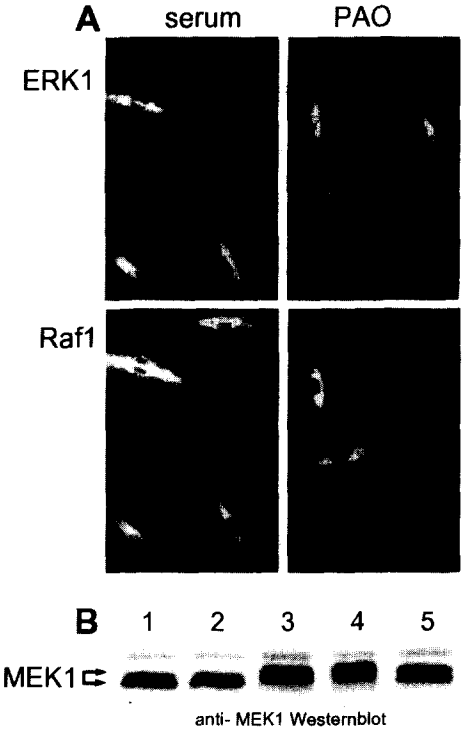

Figure 4

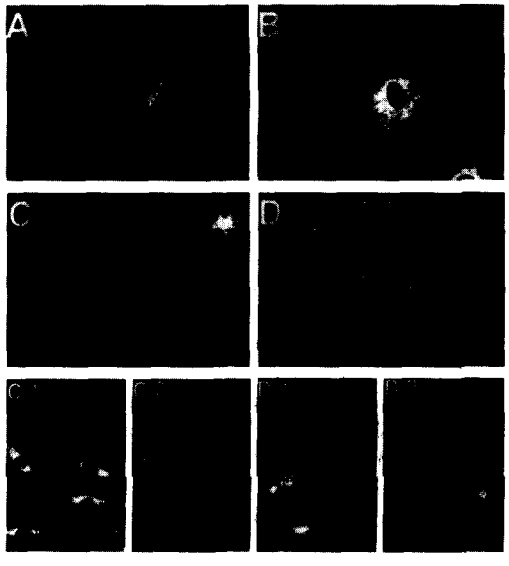

Fig. 1. Localization of ERK1, phosphotyrosine (pY) and DNA (DAPI) (top to bottom) by immunofluorescent staining of REF 52 cells (magnification 125×). Column 1 shows growth-arrested cells, the cells depicted in the other columns were treated for 10 min at 37°C as follows: 2: 40 μM PAO, 3: 30 ng/ml PDGF BB, 4: 20% FCS, 5: 100 μM vanadyl hydroperoxide.

Fig. 2. Tyrosine phosphorylation and ERK1 activity. The cells analyzed in lanes 1–5 were treated as described for columns 1–5 of Fig. 1. The autoradiogramm in panel C shows the result of a MAPkinase activity assay for ERK1 activity.

Fig. 3. A: Localization of ERK1 (top) and check for NΔRaf expression (bottom) in REF 52 cells microinjected with an expression plasmid coding for NΔRaf by immunofluorescent staining (magnification 125×). B: MEK1 phosphorylation detected as reduced mobility in SDS PAGE by Western blotting. Treatment of cells used for lysates in lanes 1–5 was as described for columns 1–5 in Fig. 1.

Fig. 4. In panels A–D (magnification 200×) the presence or absence of microtubules was determined by indirect immunofluorescence with the anti-tubulin antibody IA2 (T.E. Kreis, Geneva). The cells in panels C.1 and D.1 were stained against ERK1 and panels C.2 and D.2 show the same fields stained with the DNA binding dye DAPI (magnification 125×). All cells were growth-arrested like for panel A, cells for panels C, C.1 and C.2 were treated additionally with 8 μM nocodazole for 30 min at 37°C. The cells for panel B were treated for 10 min with 40 μM PAO and the cells for panels D, D.1 and D.2 were first incubated with 10 μM taxol for 30 min and then treated with 40 μM PAO for 10 min. ←

a significant increase in total tyrosine phosphorylation as reported previously [13]. As a consequence of such phosphorylation, ERK1 and ERK2 had a reduced mobility in SDS PAGE (Fig. 2B, lane 5). Gel retardation and activation of ERK has also been reported in response to treatment with the less potent orthovanadate [28]. REF52 cells, however, showed no increase in phosphotyrosine content when treated for 10 min with 1 mM orthovanadate or 1 mM hydrogen peroxide (data not shown).

Although MEK1 mobility shift is due to serine phosphorylation alone, MEK1 mobility was reduced in response to vanadyl hydroperoxide (Fig. 3B, lane 5). This indicates that inhibition of tyrosine phosphatases maintains at least one signal transduction pathway which can lead to MEK1 phosphorylation in an activated state.

Taken together, PAO acts in a different way than a general tyrosine phosphatase inhibitor, although involvement of a specific phosphatase cannot be excluded at this point.

3.5. Depolymerization of microtubules by thiol-reactive compounds is not the cause for ERK1 translocation

While stimulation by mitogens did not affect microtubules (data not shown), PAO treatment was associated with microtubule depolymerization (Fig. 4B). It is known that thiol-reactive compounds can inhibit microtubule polymerization in vitro by directly modifying tubulin at cysteins [29]. The actin cytoskeleton on the other hand was affected neither by PAO nor by mitogens (data not shown).

Depolymerization of microtubules induced by nocodazole (Fig. 4C) demonstrated that disruption of microtubules alone did not alter ERK1 localization in growth-arrested cells (Fig. 4C.1). By stabilizing microtubules with taxol to prevent depolymerization by PAO (Fig. 4D), it was possible to show that depolymerization of the microtubule network was not required for relocalization of ERK1 to the nucleus by PAO (Fig. 4D.1).

4. Discussion

Our experiments show that ERK1 can be induced to translocate to the nucleus in the absence of both activation and the upstream signals usually required for activation. Nuclear translocation in response to thiol-modifying chemicals was indistinguishable from that following mitogenic stimulation of fibroblasts when studied by immunofluorescence.

NEM, diamide and PAO, the three thiol-reactive chemicals used in this study, are structurally very different. Therefore, it can be assumed that their shared reactivity towards thiols alone is causing ERK1 translocation. Diamide [30] and NEM react with mono- and dithiols while PAO preferentially bridges covalently between two vicinal or adjacent thiol groups [31] found in protein motifs like -CXXC-. Since PAO is as effective as the other two chemicals in inducing translocation, it is likely that a reaction with a protein containing such a dithiol is involved.

ERKs neither contain a nuclear localization signal [32] nor one of the recently described nuclear export signals [33] which could determine their localization. Since they are small enough to enter the nucleus by passive diffusion across nuclear pores, a mechanism to prevent nuclear localization in unstimulated cells has to be proposed. Such a mechanism could be based on one of the two following principles: (1) a cytoplasmic anchor retaining ERK1 in the cytoplasm or (2) active export clearing ERK1 from the nucleus. The available data do not allow discrimination between these two possibilities.

To allow translocation in stimulated cells, the localization machinery has to be inactivated. We have demonstrated that the kinase activity of Raf is required for mediating this event in response to serum. Raf could inactivate components of the putative localization machinery by direct phosphorylation or indirectly via activation of MEK or ERK.

Since PAO, in contrast to vanadyl hydroperoxide, did not activate the MAPK cascade, it can be excluded that it works by simply mimicking mitogenic stimulation. We suggest therefore that direct modification of proteins regulating ERK localization is the most likely mechanism by which thiol-modifying reagents induce ERK1 translocation.

No potential target involved in an active nuclear export mechanism for ERK1 is known. However, a candidate component for a cytoplasmic retention mechanism is found in the yeast mating pheromone pathway, the STE5 gene product. The STE5 protein (STE5p) has been shown to bind all members of the particular yeast MAPK cascade transmitting the pheromone signal [34]. It thus tethers the components together and may thereby localize the yeast homologue of ERK to the cytoplasm. The general relevance of STE5p as a candidate cytoplasmic anchor is unclear since no homologue has been found so far in other organisms.

Alternatively cytoplasmic anchoring of ERK could be achieved by direct binding of the kinases of the MAPK cascade to each other. Supporting such a concept is the fact that Ras, Raf and MEK can form a stable complex [19]. In addi-

tion, interaction between a member of the MEK family, MEK5 and the corresponding member of the ERK family, ERK5, has been demonstrated using the two hybrid system [35]. Although attractive conceptually since such a complex comprising the whole cascade could explain the localization of ERK and link its regulation to changes in protein–protein affinities upon mitogen stimulation, its existence and relevance for localization awaits experimental demonstration.

Microtubule association has been reported for a subpopulation of ERK which remains in the cytoplasm after mitogen stimulation of fibroblasts [36]. A similar interaction with cytoskeletal proteins might contribute to the localization of the subpopulation of ERK susceptible to translocation. Our observation that nocodazole had no influence on ERK1 localization cannot rule out this possibility although it makes an involvement of microtubules unlikely.

Allowing the separation of activation and translocation of ERK, the thiol-modifying chemicals used in this study are tools that can be used to define in more detail the conditions for nuclear translocation of ERK1. Having the most limited reactivity towards thiol groups while still fully active in inducing translocation, PAO could serve to chemically modify and subsequently isolate proteins involved in ERK localization.

Acknowledgements: We would like to thank our colleagues Claudia Wiedemann, Theo Schäfer, Markus Wartmann, Roger Beerli, Marc Urich and Anja Messerschmitt for helpful discussions and careful proofreading of the manuscript.

References

[1] Ray, L.B. and Sturgill, T.W. (1987) Proc. Natl. Acad. Sci. USA 84, 1502–1506.
[2] Chen, R.H., Sarnecki, C. and Blenis, J. (1992) Mol. Cell. Biol. 12, 915–927.
[3] Gonzalez, F.A., Seth, A., Raden, D.L., Bowman, D.S., Fay, F.S. and Davis, R.J. (1993) J. Cell Biol. 122, 1089–1101.
[4] Nguyen, T.T., Scimeca, J.C., Filloux, C., Peraldi, P., Carpentier, J.L. and Van Obberghen, E. (1993) J. Biol. Chem. 268, 9803–9810.
[5] Traverse, S., Gomez, N., Paterson, H., Marshall, C. and Cohen, P. (1992) Biochem. J. 288, 351–355.
[6] Lenormand, P., Sardet, C., Pagès, G., L'Allemain, G., Brunet, A. and Pouysségur, J. (1993) J. Cell Biol. 122, 1079–1088.
[7] Marshall, C.J. (1995) Cell 80, 179–185.
[8] Van der Geer, P. and Hunter, T. (1994) Ann. Rev. Cell Biol. 10, 251–337.
[9] Warne, P.H., Viciana, P.R. and Downward, J. (1993) Nature 364, 352–355.
[10] Zhang, X.F., Settleman, J., Kyriakis, J.M., et al. (1993) Nature 364, 308–313.
[11] Marais, R., Light, Y., Paterson, H.F. and Marshall, C.J. (1995) Embo J. 14, 3136–3145.
[12] Seger, R. and Krebs, E.G. (1996) FASEB J. 9, 726–735.
[13] Trudel, S., Paquet, M.R. and Grinstein, S. (1991) Biochem. J. 276, 611–619.
[14] Messerschmitt, A., Disela, C., Dilworth, S., Marti, A.G. and Ballmer-Hofer, K. (1996) J. Gen. Virol. 77, 17–26.
[15] Sanghera, J.S., Peter, M., Nigg, E.A. and Pelech, S.L. (1992) Mol. Biol. Cell 3, 775–787.
[16] Druker, B.J., Mamon, H.J. and Roberts, T.M. (1989) New Engl. J. Med. 321, 1383–1391.
[17] Schaap, D., Van der Wal, J., Howe, L.R., Marshall, C.J. and Van Blitterswijk, W.J. (1993) J. Biol. Chem. 268, 20232–20236.
[18] Marte, B.M., Graus Porta, D., Jeschke, M., Fabbro, D., Hynes, N.E. and Taverna, D. (1995) Oncogene 10, 167–175.
[19] Wartmann, M. and Davis, R.J. (1994) J. Biol. Chem. 269, 6695–6701.
[20] Urich, M., El Shemerly, M.Y., Besser, D., Nagamine, Y. and Ballmer-Hofer, K. (1995) J. Biol. Chem. 270, 29286–29292.
[21] Posada, J. and Cooper, J.A. (1992) Science 255, 212–215.
[22] Grinstein, S., Butler, J.R., Furuya, W., L'Allemain, G. and Downey, G.P. (1994) J. Biol. Chem. 269, 19313–19320.
[23] Levenson, R.M. and Blackshear, P.J. (1989) J. Biol. Chem. 264, 19984–19993.
[24] Garcia Morales, P., Minami, Y., Luong, E., Klausner, R.D. and Samelson, L.E. (1990) Proc. Natl. Acad. Sci. USA 87, 9255–9259.
[25] Liao, K., Hoffman, R.D. and Lane, M.D. (1991) J. Biol. Chem. 266, 6544–6553.
[26] Swarup, G., Cohen, S. and Garbers, D.L. (1982) Biochem. Biophys. Res. Commun. 107, 1104–1109.
[27] Fantus, I.G., Kadota, S., Deragon, G., Foster, B. and Posner, B.I. (1989) Biochemistry 28, 8864–8871.
[28] D'Onofrio, F., Le, M.Q., Chiasson, J.L. and Srivastava, A.K. (1994) FEBS Lett. 340, 269–275.
[29] Mellon, M.G. and Rebhun, L.I. (1976) J. Cell Biol. 70, 226–238.
[30] Kosower, N.S., Kosower, E.M. and Wertheim, B. (1969) Biochem. Biophys. Res. Commun. 37, 593–596.
[31] Webb, J.L. (1966) in: Enzymes and metabolic inhibitors, Vol. 3, pp. 595–793, Academic Press, New York.
[32] Dingwall, C. and Laskey, R.A. (1991) Trends Biochem. Sci. 16, 478–481.
[33] Wen, W., Meinkoth, J.L., Tsien, R.Y. and Taylor, S.S. (1995) Cell 82, 463–473.
[34] Choi, K.Y., Satterberg, B., Lyons, D.M. and Elion, E.A. (1994) Cell 78, 499–512.
[35] Zhou, G., Bao, Z.Q. and Dixon, J.E. (1995) J. Biol. Chem. 2705, 12665–12669.
[36] Reszka, A.A., Seger, R., Diltz, C.D., Krebs, E.G. and Fischer, E.H. (1995) Proc. Natl. Acad. Sci. USA 92, 8881–8885.