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MAP kinase activation during heat shock in quiescent and exponentially growing mammalian cells

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In numerous cases of signal transduction, the mitogen-activated protein kinases (MAP kinases) or extracellular regulated kinases (ERKs) are found to be activated by phosphorylations which result in electrophoretic mobility changes. Activities of MAP kinases in cytosolic extracts can also be monitored by the capacity of such extracts to phosphorylate myelin basic protein. These two assays were used to demonstrate that MAP kinases were rapidly activated during heat shock of both quiescent and exponentially growing mammalian (hamster, rat, mouse and human) cells. Thus, the MAP kinase cascade is likely to also ensure heat-shock signal transduction and contribute to the regulation of the complex array of metabolic changes designated as the heat-shock response.

MAP kinase; Heat shock

1. INTRODUCTION

The metabolism of cells is markedly altered by heat-shock stress. Numerous cellular functions related to gene expression are impaired, such as protein translation, RNA splicing, 'normal' gene transcription [1,2]. Meanwhile, the transcription of heat-shock genes is activated or enhanced.

The rapid changes in the translational efficiency are likely to be controlled, in part, by phosphorylation or dephosphorylation of regulatory proteins [3]. Indeed, the heat-shock enhanced phosphorylation of the eukaryotic translation initiation factor 2 (eIF-2) [4] and dephosphorylation of initiation factor 4F (eIF-4F) [5], as well as that of ribosomal protein S6 [6–9] may contribute to the general shut-off of protein synthesis and to the selective translation of heat-shock mRNAs. Heat-shock induced phosphorylation of the largest subunit of RNA polymerase II [10] and of heat-shock factors may contribute to the transcriptional activation of heat-shock genes [11–13].

These changes are likely to result from the activation of specific protein kinases and protein phosphatases. For example, an eIF2-kinase, related to the heme-regulated protein kinase (HRI), is activated by heat-shock in rabbit reticulocyte lysates [14] and HeLa cells [15]. Activity of this kinase may be regulated by changes in the phosphorylation state and binding of heat-shock proteins to the catalytic subunit [16,17].

A possible involvement of MAP kinases, the mitogen-activated protein kinases, in the heat-shock response of quiescent cells has been suggested recently by the observation that the MAP kinase-activated protein kinase 2 (MAPKAP kinase 2) was a major heat-shock-activated enzyme responsible for the phosphorylation of the small 25–28 kDa mammalian heat-shock protein (HSP 27) in quiescent cells [18]. MAP kinases are also designated as extracellular signal-regulated kinases (ERKs) and belong to a family of Ser/Thr protein kinases that can be rapidly activated by a wide variety of stimuli, such as the addition of serum to quiescent cells [19–21]. It had recently been mentioned that MAP kinases were activated during heat-shock in quiescent mouse fibroblasts [22]. The $pp90^{rsk}$ S6 kinase is known to be activated in various cases of extracellular-signalled cell stimulation through phosphorylation by MAP kinases and is often designated as MAP kinase activated protein kinase 1 (MAPKAP kinase 1) [23]. In contrast with extensive observations reported for growing plant and animal cells [6–9], the ribosomal protein S6 was found to be increasingly phosphorylated in heat-shocked quiescent mammalian cells by both the $pp90^{rsk}$ and $pp70^{S6K}$ protein kinases [24]. Since the heat-shock response is not dependent on cell growth, we have been examining the activation of MAP kinases in exponentially growing cells submitted to a heat-shock stress. Heat-shock treatment was found to activate MAP kinases in several species of mammalian cells independently of cell growth.

2. MATERIALS AND METHODS

Chinese hamster lung fibroblast line CCL39, mouse Swiss 3T3 fibroblasts and human HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, NY) supplemented

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with 10% fetal calf serum. Rat hepatoma cells, cell line H56 [25] were kindly provided by Dr Aniko Venetianer and cultured in F12 medium (Gibco) supplemented with 10% fetal calf serum. Cells were made quiescent by incubating confluent cultures for 24 h in serum-free medium. Heat shocks were performed by immersion of the culture tubes in a water-bath.

2.1. *Protein kinase assay*

After serum or heat-shock treatment, cells (3×10^5 per tube) were washed in ice-cold buffer A (sodium glycerophosphate (pH 7.3) 20 mM, magnesium chloride 5 mM, ethylene glycol tetraacetic acid 1 mM, sodium vanadate 1 mM, phenylmethyl sulfonyl fluoride 0.2 mM, glycerol 10% and β-mercaptoethanol 1 mM) and lysed on ice with 100 ml buffer A containing 1% Nonidet P40. Cytosolic extracts were cleared by centrifugation for 5 min at $10,000 \times g$ and stored at −80°C. Phosphorylation of myelin basic protein (MBP) was performed by incubating 2 ml of cytosolic extracts at 30°C with 20 ml of buffer A containing [^{32}P]-γ-ATP (0.05 mCi/ml, 0.1 mM) and MBP (0.4 mg/ml) for 10 min. The reaction was stopped by the addition of 20 ml of 2 × Laemmli buffer (Tris-HCl (pH 6.8) 120 mM, sodium dodecyl sulfate 4%, glycerol 20%, β-mercaptoethanol 2%, and Bromophenol blue 0.002%). After electrophoresis in 15% polyacrylamide gels, the gels were autoradiographed. MBP was the major phosphorylated band and migrated with an apparent molecular weight of 22 kDa.

2.2. *Immunoprecipitation*

Cytosolic extracts were prepared from 5×10^6 NIH 3T3 cells lysed in 300 ml of buffer A. These extracts were preincubated first with protein A–Sepharose-CL4B, then 5 ml anti-ERK1 polyclonal antibody (Santa Cruz Biotechnology) was added to 50 ml of supernatant. After 1 h at 4°C, the antibody was adsorbed on 20 ml of protein A–Sepharose beads. After 2 h of incubation at 4°C, the beads were washed in buffer A and the protein kinase assay was performed as described above using 5 ml of beads.

2.3. *Western blot analysis*

Immediately after heat shock, cells were washed once with cold phosphate-buffered saline and lysed in Laemmli buffer. The lysates were heated for 10 min at 90°C and electrophoresed in 10% polyacrylamide gels. Proteins in the gel were then electrotransferred to a sheet of nitrocellulose (0.2 mm; Schleicher and Schuell, Dassel, Germany). The blots were blocked in TBS (Tris-HCl (pH 7.6) 20 mM, NaCl 137 mM, and Tween-20 0.2%) containing 5% non-fat dry milk, and then incubated with a rabbit anti-MAP kinase antiserum (1:2,000) kindly provided by Dr Jacques Pouysségur. This antiserum recognized both p42^{mapk} and p44^{mapk} [26]. After washing in TBS, the blots were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:7,500, Promega) in TBS for 1 h. Immunoreactivity was determined using the ECL chemiluminescence reaction (Amersham, UK).

3. RESULTS

3.1. *Transient MAP kinase activation following addition of serum to quiescent Chinese hamster lung fibroblasts*

The activation of MAP kinases by addition of serum to quiescent CCL39 Chinese hamster lung fibroblasts is well described ([27] and references therein). Myelin basic protein (MBP) is considered to be a typical specific substrate for in vitro assay of MAP kinase activity. Indeed, we found a strong increase in MBP kinase activity in cytosolic extracts within 5 min of serum addition (Fig. 1A). This activation decreased after 30 min of serum stimulation. When total lysates from quiescent CCL39 cells were probed by Western blot with anti-MAP kinases antiserum, two protein bands were detected with molecular weights between 40 and 45 kDa (Fig. 1B). The upper band (p44) was preferentially recognized by the anti-ERK1 polyclonal antibody (Santa Cruz Biotechnology), whereas the lower band (p42) was detected with an anti-p42^{mapk} monoclonal antibody (Zymed) (data not shown). Within less than 5 min of serum addition, two new bands of lower electrophoretic mobilities p42* and p44* were observed; meanwhile the

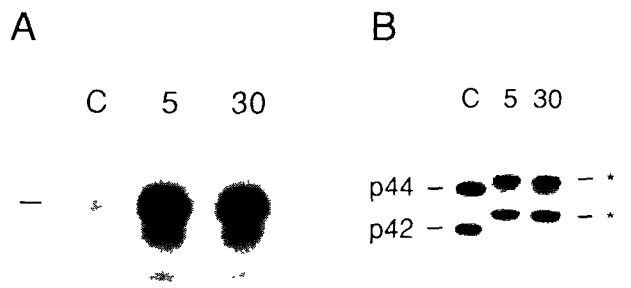

Fig. 1. MAP kinases activation by serum in quiescent CCL39 cells. (A) In vitro phosphorylation of MBP by cytosolic extracts from quiescent cells without stimulation (control, C) or which had been stimulated for 5 min (5) or 30 min (30) with 20% fetal calf serum. (B) Total cell lysates were analyzed by Western blot using a rabbit anti-MAP kinase antiserum. *Refers to the phosphorylated p42^{mapk} and p44^{mapk}.

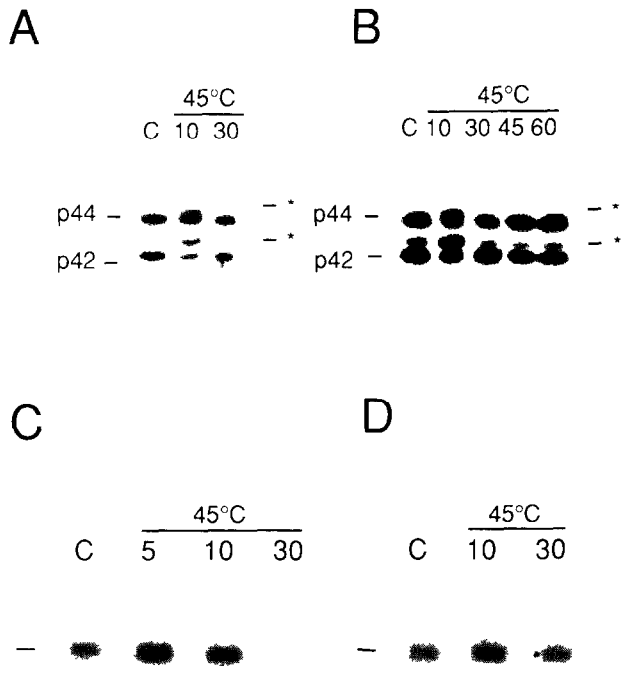

Fig. 2. Activation of MAP kinases by heat shock in CCL39 cells. Quiescent (A,C) and exponentially growing CCL39 (B,D) cells were submitted to a 45°C heat shock for 5–60 min, or were unstimulated (lanes C). Total cell lysates were analyzed by Western blot using an anti-MAP kinase rabbit serum (A,B) and cytosolic extracts were assayed for MBP phosphorylation (C,D).

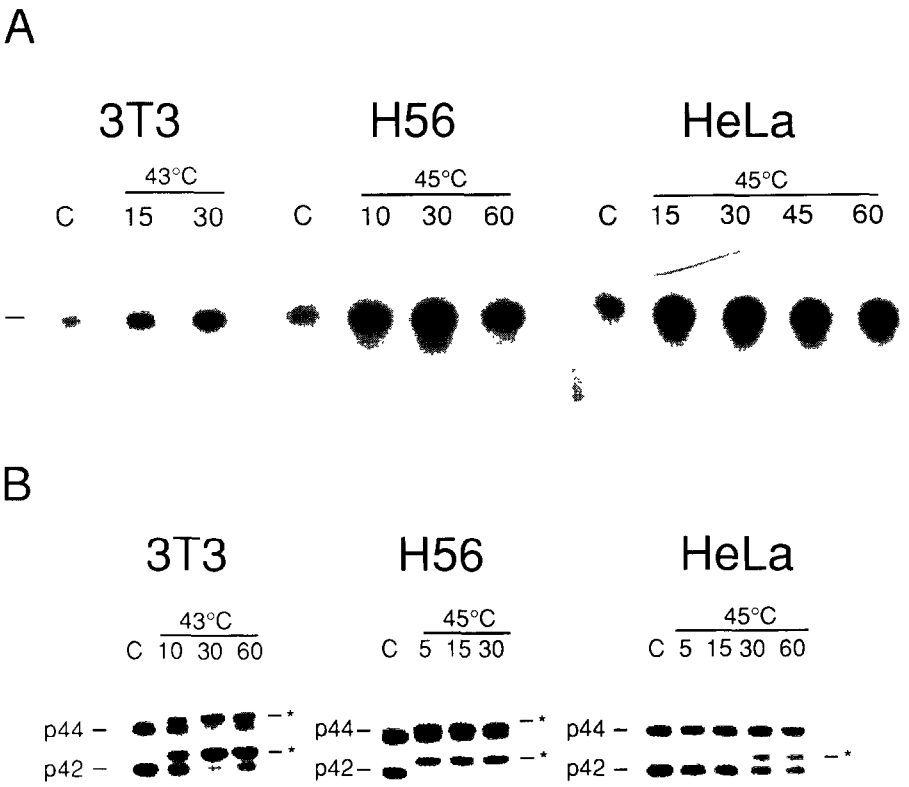

Fig. 3. Activation of MAP kinases by heat shock in various cell species. Exponentially growing mouse NIH 3T3 fibroblasts, rat H56 hepatoma cells and human HeLa cells were submitted to heat shocks at 43°C or 45°C for from 5 to 60 min, or were unstimulated (lanes C). 43°C with mouse cells was used instead of 45°C because mouse cells are more heat-sensitive than the other cells used in this study – after 1 h at 43°C mouse cells retained 80% cloning efficiency like HeLa cells after 1 h at 45°C. Cytosolic extracts were assayed for MBP phosphorylation (A), and total cell lysates were analyzed by Western blot using anti-MAP kinase antiserum (B).

p42 and p44 bands disappeared. These bands should correspond to the activated $p42^{mapk}$ and $p44^{mapk}$ proteins phosphorylated on a tyrosine and on a threonine by a MAPK kinase [19,28].

These two assays were used next to analyze MAP kinase activation during heat-shock stress.

3.2. *Transient MAP kinase activation during heat shock of Chinese hamster lung fibroblasts*

First, we analyzed the effect of heat shock on quiescent CCL39 cells. A small but reproducible increase in MBP kinase activity was observed in cytosolic extracts from quiescent CCL39 cells submitted to a 45°C heat shock. This increase was transient: it was clear after 5 min of stress and decreased thereafter (Fig. 2C). A transient increase in the p42* and p44* band intensities was observed within 10 min of stress on Western blots of the total lysates (Fig. 2A). This increase correlated in time with the MBP kinase activity.

Since quiescent cells might be prepared to respond to external stimuli, exponentially growing cells (kept in the presence of the serum growth factors) were next analyzed. Again, a small and transient but reproducible increase in MBP kinase activity was observed in the cytosolic extracts from heat-shocked cells (Fig. 2D). CCL39 total cell lysates were then analyzed by Western blot and a small amount of p42* and p44* was already detected in control cell lysates, however, the intensity of the p42* and p44* bands increased and peaked in total lysates from cells incubated for 10 min at 45°C (Fig. 2B). Since 45°C is a moderately high heat-shock temperature, these experiments were performed at lower temperature and a low but reliable MAP kinase activation was detectable after heat-shock at milder temperatures such as 42°C (data not shown).

Thus, both $p42^{mapk}$ and $p44^{mapk}$ kinases were transiently activated upon heat-shock in either quiescent or exponentially growing CCL39 hamster cells.

3.3. *Activation by heat shock of MAP kinases in various mammalian cell lines*

The heat-shock response is ubiquitous among all organisms from bacteria to man. Therefore to determine whether the activation of MAP kinase by stress was a general phenomenon, we have analyzed the behaviour of exponentially growing mouse, rat and human cells submitted to heat shock. As shown in Fig. 3A, in mouse NIH 3T3 fibroblasts, rat H56 hepatoma cells, and

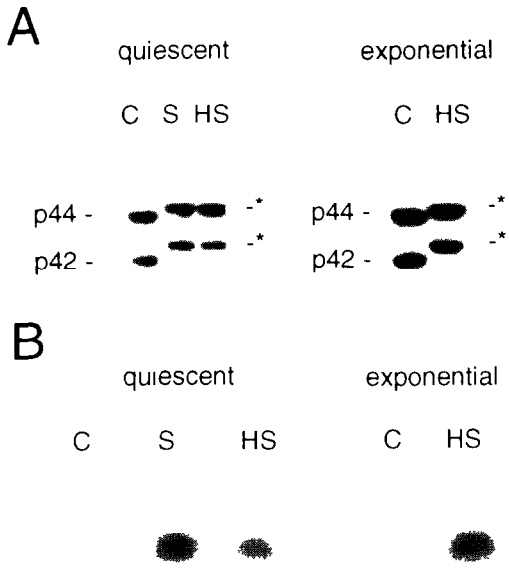

Fig. 4. Activation of MAP kinase in quiescent and exponentially growing NIH 3T3 fibroblasts. Quiescent cells, control (C), 15 min after addition of 20% serum (S) or heat shocked for 30 min at 43°C (HS). Exponentially growing cells: control (C) or heat shocked for 30 min at 43°C (HS) (A) Cytosolic extracts were analyzed by Western blot using anti-ERK1 antibody. (B) Immune complex kinase assays were performed using myelin basic protein as substrate

human HeLa cells, strong increases in MBP kinase activities were observed after heat shock. In rat H45 cells, the conversion of the p42 and p44 MAP kinases to the p42* and p44* forms was almost complete after 30 min at 45°C (Fig. 3B). In mouse 3T3 cells, most of the MAP kinases were converted to the p42* and p44* forms after 30 min at 43°C, but after 60 min at 43°C the signals corresponding to the p42 and p44 forms increased again. In unstressed exponentially growing human HeLa cells, the p44 band was surrounded by two minor bands of approximately 43 and 45 kDa, which were distinct from p42* and p44*. During stress at 45°C, a strong increase of the p42* intensity was observed while the p43 band disappeared, however, no increase of a putative p44* could be seen. Like in cells from other species, the magnitude of MAP kinase activation in human HeLa cells was dependent on the stress temperature, and p42 activation could be clearly detected after mild stress such as 30 min at 42°C (data not shown).

Thus, heat-shock activation of MAP kinases was likely to occur in cells from different mammalian species.

3.4. *Increased MBP kinase activity in immune complexes formed with anti-ERK1 antibody in lysates from heat shocked cells*

As a control for MAP kinase activation, an immune complex assay was performed using an anti-ERK1 antibody suitable for immunoprecipitation. In NIH 3T3 cells, the anti-ERK1 antibody recognized both the p42 and p44 MAP kinases on Western blots (Fig. 4A). A complete shift-up of the p42 and p44 forms into the p42* and p44* forms was detected following either addition of serum or heat shock. The same anti-ERK1 antibody was added to cytosolic extracts, and adsorbed on protein A-agarose beads. The beads retained some MBP kinase activity which was strongly enhanced by addition of serum to quiescent cells and by heat shock of both quiescent and exponentially growing cells (Fig. 4B).

This immune complex kinase assay confirmed that the p42 and p44 MAP kinase electrophoretic shift-up and the increased MBP kinase activity in cytosolic extracts are reliable indications for MAP kinases activation during heat shock.

4. DISCUSSION

We report here the activation of p42 kDa and p44 kDa MAP kinases in quiescent as well as in exponentially growing cells during heat-shock stress. The lack of p44 activation in HeLa cells is surprising since both p42 and p44 were expected to be substrates for MAP kinase kinase [20,29]. The mechanism of MAP kinase activation during stress remains, however, to be established; a MAP kinase kinase distinct from the growth factor-activated one might be involved. It should be noted that heat shock is accompanied by typical second messenger signalling, such as increased intracellular Ca^{2+} levels and inositol triphosphate release [30,31]. The expression of a protein–tyrosine phosphatase has recently been reported to be induced after stress; since MAP kinases are activated by tyrosine phosphorylation, this phosphatase might be involved in MAP kinase deactivation [32].

Among the numerous substrates which have been proposed for MAP kinases, there are two protein kinases which concern stress. Thus, in serum-deprived cells, the MAP kinase activated protein kinase 1 (MAPKAP kinase 1) has been shown to be heat activated and to result in increased S6 protein phosphorylation [24]. Such an observation contrasted with previous reports of S6 protein dephosphorylation upon heat shock in exponentially growing cells [6–8], and further experiments are required to examine the S6 kinase activities in growing cells. The second characterized MAP kinase-activated protein kinase is MAPKAP kinase 2. The 25–28 kDa small heat-shock protein (HSP 27) has been shown to be an excellent substrate for MAPKAP kinase 2 [18]. Thus, activation of MAP kinase may account for the heat-shock-induced phosphorylation of HSP 27 observed in exponentially growing cells [33–36]. It is noteworthy that HSP 27 has been found to be phosphorylated in most cases of signal transduction where it had been looked for, provided the protein was present.

In previous work, we reported that heat shock activated a protein kinase which phosphorylated the heptapeptide consensus sequence present in the carboxy-terminal domain of the large subunit of RNA polymerase II [37]. This heptapeptide repeat (Tyr-Ser-Pro-Tyr-Ser-Pro-Ser) had been shown to be a good substrate for MAP kinases [38]. Since the largest subunit of RNA polymerase II became highly phosphorylated during heat shock in HeLa cells ([10] and article in preparation), experiments are in progress to determine whether MAP kinases might be involved in this process.

Since MAP kinases are thought to play an essential and pleiotropic role in cell signal transduction, they are also likely to contribute significantly to the heat-shock response.

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REFERENCES

[1] Morimoto, R.I., Tissières, A. and Georgopoulos, C. (1990) Stress Proteins in Biology and Medicine, Cold Spring Harbor Laboratory Press, New York.
[2] Nover, L. (1991) Heat Shock Response, CRC Press, Boca Raton
[3] Hershey, J.W.B. (1989) J. Biol. Chem. 264, 20823–20826.
[4] Duncan, R.F. and Hershey, J.W B. (1989) J. Cell Physiol. 109, 1467–1481.
[5] Lamphear, B J and Panniers, R. (1991) J. Biol. Chem. 266, 2789–2794.
[6] Scharf, K.D. and Nover, L. (1982) Cell 30, 427–437.
[7] Glover, C.V.C. (1982) Proc. Natl. Acad. Sci. USA 79, 1781–1785.
[8] Kennedy, I.M., Burdon, R.H. and Leader, D.P. (1984) FEBS Lett. 169, 267–273.
[9] Pekkala, D. and Silver, J.C. (1987) Exp. Cell Res. 168, 325–337.
[10] Dubois, M.-F., Bensaude, O. and Morange, M. (1991) Compt. Rend. Acad. Sci. (Paris) 313, 165–170
[11] Larson, J.S., Schuetz, T.J. and Kingston, R.E (1988) Nature 335, 372–375.
[12] Sorger, P.K. and Pelham, H.R.B. (1988) Cell 54, 855–864
[13] Sarge, K., Murphy, S.P. and Morimoto, R.I. (1993) Mol. Cell. Biol. 13, 1392–1407.
[14] Farrell, P.J., Balkow, K., Hunt, T. and Jackson, R.J (1977) Cell 11, 187–200.
[15] De Benedetti, A. and Baglioni, C. (1986) J. Biol. Chem. 261, 338–342.
[16] Szyska, R., Kramer, G. and Hardesty, B. (1989) Biochemistry 28, 1435–1438.
[17] Matts, R.L., Xu, Z., Pal, J.K. and Chen, J.-J. (1992) J. Biol. Chem. 267, 18160–18167.
[18] Stokoe, D., Engel, K., Campbell, D.G., Cohen, P. and Gaestel, M. (1992) FEBS Lett. 313, 307–313.
[19] Cobb, M., Robbins, D.J. and Boulton, T.G. (1991) Curr. Opin. Cell Biol. 3, 1025–1032.
[20] Pelech, S L. and Sanghera, J.S. (1992) Science 257, 1355–1356.
[21] Thomas, G. (1992) Cell 68, 3–6.
[22] Chung, J., Kuo, C.J., Crabtree, G.R and Blenis, J. (1992) Cell 69.
[23] Erikson, R.L. (1991) J Biol. Chem. 266, 6007–6010.
[24] Jurivich, D.A , Chung, J. and Blenis, J. (1991) J Cell Physiol. 148, 252–259.
[25] Baranyi, M., Hevér-Szabo, A. and Venetianer, A. (1991) Eur. J. Biochem. 200, 707–713.
[26] Wang, Y., Simonson, M.S., Pouysségur, J. and Dunn, M.J. (1992) Biochem. J. 287, 589–594.
[27] Meloche, S., Pagès, G. and Pouysségur, J. (1992) Mol. Biol. Cell 3, 63–71.
[28] Maller, J. (1991) Curr. Biol. 1, 334–335
[29] Gómez, N. and Cohen, P (1991) Nature 353, 170–173.
[30] Calderwood, S.K , Stevenson, M.A. and Hahn, G. (1987) J Cell. Physiol. 130, 369–376.
[31] Stevenson, M.A., Calderwood, S.K. and Hahn, G.M. (1987) Cancer Res. 47, 3712–3717.
[32] Keyse, S.M. and Emslie, E.A. (1992) Nature 359, 644–646.
[33] Kim, Y.-J., Shuman, M., Sette, M. and Przybyla, A. (1984) Mol. Cell. Biol. 4, 468–474.
[34] Arrigo, A.-P. and Welch, W. (1987) J. Biol. Chem. 262, 15359–15369.
[35] Crête, P and Landry, J. (1990) Rad. Res 121, 320–327.
[36] Zhou, M., Lambert, H and Landry, J. (1993) J. Biol. Chem. 268, 35–43.
[37] Legagneux, V., Morange, M. and Bensaude, O. (1990) Eur. J. Biochem. 193, 121–126.
[38] Clark-Lewis, I., Sanghera, J.S. and Pelech, S.L. (1991) J. Biol Chem 266, 15180–15184.