Transforming growth factor-β1 induces activation of Ras, Raf-1, MEK and MAPK in rat hepatic stellate cells

Thomas Reimann[a], Ute Hempel[a,*], Stefan Krautwald[b], Andreas Axmann[a], Roland Scheibe[a], Dagmar Seidel[a], Klaus-Wolfgang Wenzel[a]

[a] *Institute of Physiological Chemistry, Medical Faculty Carl Gustav Carus, Technical University Dresden, Karl-Marx-Straße 3, D-01109 Dresden, Germany*

[b] *Fraunhofer Institute for Toxicology and Aerosol Research, Nikolai-Fuchs-Straße 1, D-30625 Hannover, Germany*

Received 6 December 1996

Abstract The transdifferentiation of hepatic stellate cells into myofibroblast-like cells and the proliferation of the transdifferentiated cells are controlled by TGF-β1. Little is known about the intracellular signal transducers of TGF-β1. In this paper we show that in cultured hepatic stellate cells TGF-β1 induces activation of Ras, Raf-1, MEK and MAPK p42 and p44. The activation of MAPK depends on the activation of MEK. Our data exclude that the observed effects are mediated by a bFGF or PDGF autocrine loop.

Key words: Transforming growth factor-β1; Ras; Raf-1; MEK; MAPK; Hepatic stellate cell; Signal transduction

1. Introduction

Hepatic stellate cells (Ito cells, fat-storing cells) play a central role in the pathogenesis of liver fibrosis. They transdifferentiate into myofibroblast-like cells, which contribute mainly to the excessive collagen synthesis in the liver [1,2]. TGF-β1 is involved in the regulation of transdifferentiation and proliferation as well as in the stimulation of collagen synthesis of the myofibroblast-like cells [3–7]. It has been demonstrated that TGF-β1 in vitro strongly inhibits the proliferation of hepatic stellate cells, but promotes their proliferation in vivo by a bFGF or PDGF autocrine loop [8–10]. The collagen synthesis is stimulated by TGF-β1 at transcriptional level [11,12]. Recently, Davis et al. [13] reported that in rat hepatic stellate cells the protein kinases Raf-1 and MAPK are involved in the regulation of collagen gene transcription. However, it remains to be elucidated whether they also mediate the TGF-β1 induced effects.

Little is known about the intracellular transduction of the TGF-β1 signal and the regulation of its diverse biological effects. The TGF-β1 receptor is a heterodimer of two serine-threonine kinases, the type I and the type II receptor, which is formed upon ligand binding [14,15]. Several isoforms of the type I receptor have been described that may activate different signal transduction pathways inducing different TGF-β1 responses [14–16]. The immunophilin FKBP12 and two variants of the Ras-farnesyltransferase α-subunit have been demonstrated to associate with the type I receptor, but they are not involved directly in signal transduction [17–20]. Several proteins have been found to become activated by the TGF-β receptor kinases, i.e. proteins of the Mad family [16,21,22], the TGF-β activated kinase TAK-1 [23–25] as well as a yet unidentified 78 kDa protein kinase [26].

Studies on transformed intestinal epithelial cells revealed that TGF-β induces activation of the MAPK isoenzyme p44 depending on Ras [27–30]. The activation of Ras and MAPK p44 induced by TGF-β has been observed only in sublines which were growth-inhibited by TGF-β but not in TGF-β-insensitive cells. This indicates that Ras and MAPK not only transduce mitogenic but also growth inhibitory signals [28–30]. However, the TGF-β1 induced effects on the activation of the Ras-MAPK pathway largely depend on the cell type investigated. Yan et al. [31] found that in carcinoma cells TGF-β1 induces growth inhibition and activation of Ras but does not activate MAPK p42 or p44. Contrarily, it has been demonstrated in NIH 3T3 cells that TGF-β1 induces activation of MAPK independently of Ras [32]. In Swiss 3T3 and BALB/c 3T3 fibroblasts TGF-β1 does not induce activation of MAPK [33]. Moreover, in epithelial cells TGF-β1 suppresses the growth factor induced activation of Ras and MAPK [34].

In this study, we demonstrate that in cultured stellate cells isolated from normal rat liver TGF-β1 induces activation of Ras, Raf-1, MEK and MAPK p42 and p44. We show that the activation of MAPK p42 and p44 by TGF-β1 requires the activation of MEK. Our data exclude that these effects are mediated by a bFGF or PDGF autocrine loop.

2. Materials and methods

2.1. Materials

TGF-β1, bFGF and PDGF-BB and the anti-TGF-β1 antibody were purchased from Genzyme (Rüsselsheim, Germany). MBP was obtained from Sigma (Munich, Germany). [γ-^{32}P]ATP (5000 Ci/mmol) was obtained from Amersham Buchler (Braunschweig, Germany). All inhibitors were purchased from Calbiochem (Bad Soden, Germany). Polyclonal anti-MEK antibody and monoclonal anti-c-Raf-1 antibody were provided by Affiniti (Nottingham, UK). The antibodies against bFGF or PDGF-AB were obtained from Biomol (Hamburg, Germany).

2.2. Cell isolation and cultivation

Hepatic stellate cells were prepared from rat according to Schäfer et al. [35]. Male Wistar rats (Charles-River, Sulzfeld, Germany) weighing 400–500 g were used. Hepatic stellate cells were grown in DMEM supplemented with 10% FCS at 37°C under a 5% CO_2 atmosphere.

2.3. Treatment of cells and preparation of cell lysates

Subconfluent cells were growth-arrested by incubation in DMEM

*Corresponding author. Fax: (49) (351) 8832 875.

Abbreviations: bFGF, basic fibroblast growth factor; EGF, epidermal growth factor; EGTA, ethyleneglycol-bis (β-amino-ethyl)-*N*,*N*,*N*′,*N*′-tetraacetic acid; MBP, myelin basic protein; MAPK, mitogen-activated protein kinase; MEK, MAPK or ERK kinase; PDGF, platelet derived growth factor; PMSF, phenylmethylsulfonylfluoride; TGF-β1, transforming growth factor-β1

PII S0014-5793(97)00024-0

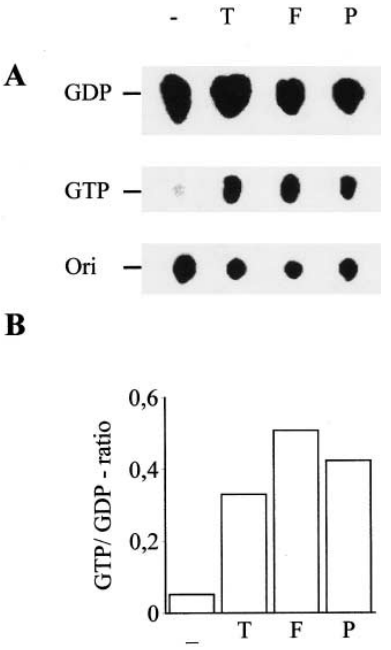

Fig. 1. Activation of Ras by TGF-β1, bFGF and PDGF. Rat hepatic stellate cells were labeled with [^{32}P]ortho-phosphate and stimulated with either TGF-β1 (T), bFGF (F) or PDGF (P) for 10 min. Ras activity was assessed analyzing the Ras-bound nucleotides by thin-layer chromatography (A) and densitometric determination of the GTP/GDP ratio (B).

without FCS 24 h prior to stimulation. The cells were stimulated with either TGF-β1 (5 ng/ml), bFGF (25 ng/ml) or PDGF-BB (10 ng/ml) for time periods indicated. For antagonizing the effects of bFGF or PDGF, the cells were either preincubated with suramine (0.4 mM) for 4 h or the growth factors were preincubated with anti-bFGF antibody or anti-PDGF antibody in 10-fold stoichiometric excess for 1 h prior to their addition to the medium. For inhibition of tyrosine kinases the cells were preincubated with genistein (40 μM) or tyrphostin A9 (5 μM) for 4 h. MEK was blocked by preincubation of the cells with PD98059 (50 μM) for 30 min. After stimulation, the cells were washed with PBS and lysed in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100 (w/v), 0.1 mM Na_3VO_4, 1 mM PMSF and 0.1 mM aprotinin. Cell debris were removed by centrifugation at 15000×*g* for 30 min at 4°C. Protein concentrations were determined by the BioRad protein dye assay.

2.4. Analysis of Ras bound nucleotides

The GTP/GDP-ratio of Ras bound nucleotides was determined as described previously [36]. The chromatograms were analyzed autoradiographically in a PhosphorImager (Molecular Dynamics).

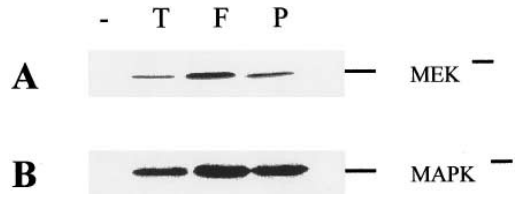

Fig. 2. Activation of Raf-1 and MEK by TGF-β1, bFGF and PDGF. Rat hepatic stellate cells were stimulated with either TGF-β1 (T), bFGF (F) or PDGF (P) for 10 min. The activity of Raf-1 (A) and MEK (B) was assessed by immune complex kinase assay using catalytically compromised MEK-1 and MAPK, respectively, as specific substrates.

2.5. Immune complex kinase assay

The assessment of Raf-1 activity was performed as described previously [37]. For immunoprecipitation, anti-c-Raf-1 monoclonal antibody was used. The kinase reaction was performed in 25 mM PIPES, pH 7.0, 2 mM EGTA, 20 mM $MgCl_2$, 5 mM $MnCl_2$, 2 mM DTT, 0.2 mM Na_3VO_4, 0.2 μM okadaic acid using 300 ng recombinant catalytically compromised MEK-1 as substrate for Raf-1. The assessment of MEK activity was performed according to Lange-Carter et al. [38]. The lysis buffer was supplemented with 10% glycerol (w/v). For immunoprecipitation, anti-MEK-1,2 polyclonal antibody was used. The kinase assays were analyzed autoradiographically in a PhosphorImager (Molecular Dynamics).

2.6. In vitro renaturation assay for MAPK activity

MAPK activity was assessed by an in vitro renaturation assay (in-gel kinase assay) as described previously [37]. After the denaturation-renaturation procedure, the gels were preincubated in 25 mM HEPES, pH 7.4, 2 mM 2-mercaptoethanol, 10 mM $MgCl_2$, 0.1 mM Na_3VO_4, and 0.5 mM EGTA at 30°C for 30 min. The kinase reaction was performed by incubation of the gels in 25 mM HEPES, pH 7.4, 2 mM 2-mercaptoethanol, 10 mM $MgCl_2$, 0.1 mM Na_3VO_4, 0.5 mM EGTA, 25 μM ATP and 250 μCi [γ-^{32}P]ATP.

3. Results and discussion

3.1. TGF-β1 induces activation of Ras

We assessed the activation of Ras in cultured stellate cells isolated from normal rat liver following stimulation with TGF-β1, bFGF or PDGF for 10 min. Densitometric analysis of Ras-bound nucleotides revealed that TGF-β1 induces rapid activation of Ras by approximately 6-fold to about the same extent as observed for bFGF and PDGF (Fig. 1).

3.2. TGF-β1 induces activation of Raf-1 and MEK

Ras is known to be involved in the activation of Raf-1, which in turn activates MEK. We were therefore interested in whether TGF-β1 also activates Raf-1 and MEK. We exam-

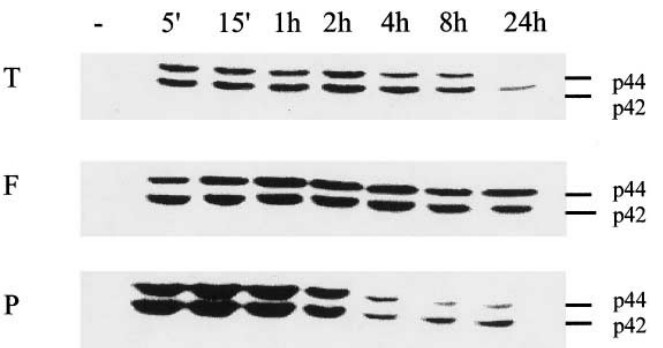

Fig. 3. Activation of MAPK by TGF-β1, bFGF and PDGF. Rat hepatic stellate cells were treated with either TGF-β1 (T), bFGF (F) or PDGF-BB (P) for indicated time periods. MAPK activity was assessed by an in vitro renaturation assay using myelin basic protein as substrate.

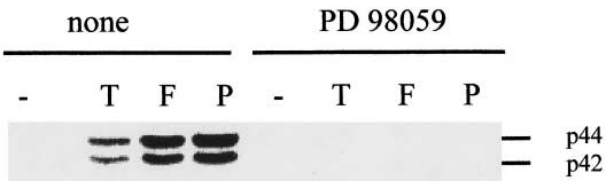

Fig. 4. Inhibition of MAPK activation induced by TGF-β1, bFGF and PDGF. Rat hepatic stellate cells were preincubated with PD 98059 for 30 min and stimulated with either TGF-β1 (T), bFGF (F) or PDGF (P) for 10 min. MAPK activity was assessed by an in vitro renaturation assay.

ined the activity of Raf-1 and MEK by immune complex kinase assays in lysates of hepatic stellate cells which were stimulated with either TGF-β1, bFGF or PDGF. TGF-β1 was found to activate Raf-1 and MEK, but to a lesser extent than their activation by bFGF or PDGF (Fig. 2).

3.3. TGF-β1 induces activation of MAPK p42 and p44

It has been demonstrated in transformed epithelial and NIH3T3 cells that TGF-β1 activates MAPK [29,30,32]. However, in Swiss 3T3 cells or even in epithelial cell lines other groups found no TGF-β1 induced activation of MAPK [31,33]. We examined MAPK activation by an in vitro renaturation assay in lysates of hepatic stellate cells stimulated with TGF-β1, bFGF or PDGF. We found that TGF-β1 as well as bFGF and PDGF induce activation of MAPK p42 and p44. However, as observed for Raf-1 and MEK, the extent of the MAPK activation by TGF-β1 was substantially weaker as compared to its activation by the growth factors bFGF or PDGF (Fig. 3). The time course of MAPK activation demonstrates that MAPK p42 and p44 are already fully activated by TGF-β1, bFGF or PDGF after 5 min and persist up to 8 h in the activated state in presence of TGF-β1.

In order to investigate whether the TGF-β1 induced activation of MAPK depends on MEK, we preincubated the cells with PD 98059, which is described to inhibit MEK [39]. This results in complete suppression of the MAPK activation induced by TGF-β1 as well as by bFGF or PDGF (Fig. 4) and shows that activation of MEK is a prerequisite for activation of MAPK by TGF-β1.

3.4. Activation of MAPK by TGF-β1 occurs independently of autocrine bFGF or PDGF

The activation of MAPK occurs rapidly and hence autocrine growth stimulation is not likely. In order to get further evidence that the activation of MAPK by TGF-β1 is not mediated by an autocrine loop involving bFGF or PDGF, the cells were preincubated with suramine, which is known to inhibit the binding of various growth factors to their cell surface receptors [40]. Suramine did not affect the activation of MAPK induced by TGF-β1 but suppressed completely the activation of MAPK by bFGF or PDGF (Fig. 5A). Further, specific inhibition of bFGF or PDGF by antibodies antagonizing their mitogenic effects did not block the TGF-β1 induced activation of MAPK but diminished substantially the activation of MAPK by bFGF and PDGF (Fig. 5B). Finally, the inhibition of tyrosine kinases with genistein or tyrphostin A9 completely abrogated the activation of MAPK by bFGF or PDGF, but did not affect the TGF-β1 induced activation of MAPK (Fig. 5C). This indicates that the activation of MAPK by TGF-β1 is independent of tyrosine kinases. This observation is somewhat intriguing, since the phosphorylation of tyrosine-340 in Raf-1 has been shown to be important in the activation of Raf-1 via Ras [41]. However, it remains to be elucidated whether the TGF-β1 induced activation of Raf-1 requires Ras. It has been demonstrated that Raf-1 can also be activated independently of Ras and tyrosine kinases by protein kinase C [42].

Thus, in hepatic stellate cells TGF-β1 may activate Raf-1,

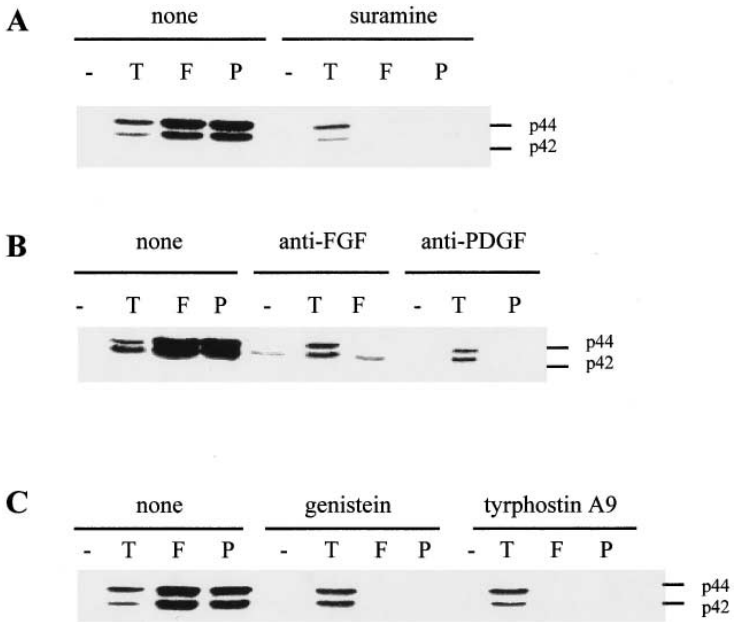

Fig. 5. Effects of suramine, neutralizing antibodies of bFGF and PDGF and inhibitors of tyrosine kinases on TGF-β1 induced activation of MAPK. A: Rat hepatic stellate cells were preincubated with suramine for 4 h and then stimulated with either TGF-β1 (T), bFGF (F) or PDGF (P), B: TGF-β1 (T), bFGF (F) and PDGF (P) were preincubated for 1 h with neutralizing antibodies in 10-fold stoichiometric excess prior to their addition to the medium. C: Rat hepatic stellate cells were pretreated with either genistein or tyrphostin A 9 for 4 h and then stimulated with either TGF-β1 (T), bFGF (F) or PDGF (P). MAPK activity was assessed by an in vitro renaturation assay.

MEK and MAPK not exclusively via Ras or may even activate Raf-1 and Ras independently from each other. Moreover, it remains to be established whether in hepatic stellate cells MEK and MAPK are activated via Raf-1 or via other pathways. It has been shown that in hepatic stellate cells Raf-1 and MAPK differentially regulate collagen gene expression [13].

The activation of different pathways could also reflect the different effects of TGF-β1 on cell growth, differentiation and transcriptional activation. Although it has been demonstrated that Ras and MAPK mediate at least in part the TGF-β-induced growth inhibition in transformed epithelial cells [30], it has to be clarified whether in hepatic stellate cells Ras, Raf-1, MEK and MAPK might also be involved in the stimulation of collagen gene expression by TGF-β1.

Acknowledgements: We are grateful to Dr. C. Marshall and Dr. G.L. Johnson for providing the clones expressing catalytically compromised MAPK and MEK-1. The authors thank C. Wahren and C. Kupke for their technical assistance.

References

[1] Pinzani, M. (1995) J. Hepatol. 22, 65–70.
[2] Gressner, A.M. and Bachem, M.G. (1995) Digestion 56, 335–346.
[3] Rosenbaum, J. and Blazejewski, S. (1995) J. Hepatol. 22, 65–70.
[4] Gressner, A.M. (1995) J. Hepatol. 22, 28–36.
[5] Pinzani, M. (1995) Pharmacol. Ther. 66, 387–412.
[6] Pinzani, M., Gentilini, A., Galigiuri, A., DeFranco, R., Pellegrini, G., Milani, S., Marra, F. and Gentilini, P. (1995) Hepatology 21, 232–239.
[7] Casini, A., Pinzani, M., Milani, S., Grappone, C., Galli, G., Jezequel, A.M., Schuppan, D., Rotella, C.M. and Surrenti, C. (1993) Gastroenterology 105, 245–253.
[8] Battegay, E.J., Raines, E.W., Seifert, R.A., Bowen-Pope, D.F. and Ross, R. (1990) Cell 63, 515–524.
[9] Win, K.M., Charlotte, F., Mallat, A., Cherqui, D., Martin, N., Mavier, P., Preaux, A.-M., Dhumeaux, D. and Rosenbaum, J. (1993) Hepatology 18, 137–145.
[10] Rosenbaum, J., Blazejewski, S., Preaux, A.-M., Mallat, A., Dhumeaux, D. and Movier, P. (1995) Gastroenterology 109, 1986–1993.
[11] Ritzenthaler, J.D., Goldstein, R.H., Fine, A., Lichtler, A., Rowe, D.W. and Smith, B.D. (1991) Biochem. J. 280, 157–162.
[12] Ritzenthaler, J.D., Goldstein, R.H., Fine, A. and Smith, B.D. (1993) J. Biol. Chem. 268, 13625–13631.
[13] Davis, B.H., Chen, A. and Beno, D.W.A. (1996) J. Biol. Chem. 271, 11039–11042.
[14] Attisano, L., Carcamo, J., Ventura, F., Weis, F.M.B., Massague, J. and Wrana, J.L. (1993) Cell 75, 671–680.
[15] Massague, J. (1996) Cell 85, 947–950.
[16] Carcamo, J., Weis, F.M.B., Ventura, F., Wieser, R., Wrana, J.L., Attisano, L. and Massague, J. (1994) Mol. Cell. Biol. 14, 3810–3821.
[17] Wang, T., Li, B., Danielson, P.P., Shah, P.C., Rockwell, S., Lechleider, R.J., Martin, J., Manganaro, T. and Donahoe, P.D. (1996) Cell 86, 435–444.
[18] Okadone, T., Oeda, E., Satoh, M., Ichijo, H., Moses, H.L., Miyazono, K. and Kawabata, M. (1996) J. Biol. Chem. 271, 21687–21690.
[19] Wang, T., Danielson, P.D., Li, B., Shah, P.C., Kim, S.D. and Donahoe, P.K. (1996) Science 271, 1120–1122.
[20] Kawabata, M., Imamura, T., Miyazono, K., Engel, M.E. and Moses, H.L. (1995) J. Biol. Chem. 270, 29628–29631.
[21] Ventura, F., Liu, F., Doody, J. and Massague, J. (1996) J. Biol. Chem. 271, 13931–13934.
[22] Graff, J.M., Bansal, A. and Melton, D.A. (1996) Cell 85, 479–487.
[23] Hoodless, P., Haerry, T., Abdollah, S., Stapleton, M., O'Connor, M.B., Attisano, L. and Wrana, G. (1996) Cell 85, 489–500.
[24] Yamaguchi, K., Shirikabe, T., Shibuya, H., Irie, K., Oishi, I., Ueno, N., Taniguchi, T., Nishida, E. and Matsumoto, K. (1995) Science 270, 2008–2011.
[25] Shibuya, H., Yamaguchi, K., Shirakabe, K., Tonegawa, A., Gotoh, Y., Ueno, N., Irie, K., Nishida, E. and Matsumoto, K. (1996) Science 272, 1179–1182.
[26] Atfi, A., Lepage, K., Allard, P., Chapdelaine, A. and Chevalier, S. (1995) Proc. Natl. Acad. Sci. USA 92, 12110–12114.
[27] Mulder, K.M., Zhong, Q., Choi, H.G., Humphrey, L.E. and Brattain, M.G. (1990) Cancer Res. 50, 7581–7586.
[28] Mulder, K.M. and Morris, S.L. (1992) J. Biol. Chem. 267, 5029–5031.
[29] Hartsough, M. and Mulder, K.M. (1995) J. Biol. Chem. 270, 7117–7124.
[30] Hartsough, M.T., Frey, A.S., Zipfel, P.A., Buard, A., Cook, S.J., McCormick, F. and Mulder, K.M. (1996) J. Biol. Chem. 271, 22368–22375.
[31] Yan, Z., Winaver, S. and Friedman, E. (1994) J. Biol. Chem. 269, 13231–13237.
[32] Mucsi, I., Skorecki, K.L. and Goldberg, H.J. (1996) J. Biol. Chem. 12, 16567–16572.
[33] Xu, R.H., Dong, Z.G., Maeno, M., Kim, J., Suzuki, A., Ueno, N., Sredni, D., Colburn, N.H. and Kung, H.F. (1996) Proc. Natl. Acad. Sci. USA 93, 834–838.
[34] Howe, P.H., Dobrowolski, S.D., Reddy, K.R. and Stacey, D.W. (1993) J. Biol. Chem. 268, 21448–21452.
[35] Schäfer, S., Yerbe, O. and Greßner, A. (1987) Hepatology 7, 680–687.
[36] Reimann, T., Büscher, D., Krautwald, S., Lohmann-Matthes, M.-L. and Baccarini, M. (1994) J. Immunol. 153, 5740–5749.
[37] Büscher, D., Hipskind, R.A., Krautwald, S., Reimann, T. and Baccarini, M. (1995) Mol. Cell. Biol. 15, 466–475.
[38] Lange-Carter, C.A., Pleiman, C.M., Gardner, A.M. and Blumer, K.J. (1993) Science 260, 315–319.
[39] Dudley, D.T., Pang, L., Decker, S.J., Bridges, A.J. and Saltiel, A.R. (1995) Proc. Natl. Acad. Sci. USA 92, 7686–7689.
[40] Valgeirsdottir, S., Eriksson, A., Nister, M., Heldin, C.H., Westermark, B. and Claesson-Welsh, L. (1995) J. Biol. Chem. 270, 10161–10170.
[41] Dent, P., Reardon, D.B., Morrison, D.K. and Sturgill, T.W. (1995) Mol. Cell. Biol. 15, 4125–4135.
[42] Kolch, W., Heidecker, G., Kochs, G., Hummel, R., Vahidi, H., Mischak, H., Finkenzeller, G., Marme, D. and Rapp, U.R. (1994) Nature 364, 249–252.