FEBS Letters 350 (1994) 113–116

FEBS 14358

Stimulation of tyrosine- and serine-phosphorylation of focal adhesion kinase in mouse 3T3 cells by fibronectin and fibroblast growth factor

Mika Hatai[a,*], Hidetaka Hashi[b], Akira Mogi[a], Hisae Soga[a], Jun Yokota[a], Yoshihito Yaoi[a,*]

[a]*Biology Division, National Cancer Center Research Institute, Tsukiji 5-1-1, Chuo-ku, Tokyo 104, Japan*
[b]*Biotechnology Research Laboratories, Takara Shuzo Co., Seta 3-4-1 Ohtsu, Shiga 520-21, Japan*

Received 28 June 1994

Abstract

Phosphorylation of both tyrosine and serine residues of focal adhesion kinase (FAK) was stimulated by the adhesion of BALB/c mouse 3T3 cells to fibronectin, but phosphorylation of threonine was not detectable. Acidic and basic fibroblast growth factors also stimulated the phosphorylation of serine and tyrosine of FAK in cells adhered to poly-L-lysine, but epidermal growth factor and platelet-derived growth factor did not. A fusion protein of fibronectin and basic fibroblast growth factor effectively induced the phosphorylation of FAK. Phosphorylation of FAK in the rat myoblast L-6 cell line, which lacks fibroblast growth factor receptors, was not stimulated by fibroblast growth factors, suggesting that the interaction of fibroblast growth factors with their receptors might cause the phosphorylation of FAK.

Key words: Focal adhesion kinase; Fibronectin; Fibroblast growth factor; Phosphorylation

1. Introduction

Focal adhesion kinase (FAK, pp125^{FAK}) is a member of the family of protein tyrosine kinases which localizes to focal adhesion plaques in cells [1]. Phosphorylation of tyrosine residues of FAK is stimulated in cells transformed by pp60$^{v\text{-}src}$ [2], attachment of cells to fibronectin or other cell-adhesive proteins [1–5], clustering of integrins [6,7], by activation of platelets [8], and the binding of immunoglobulin E [9] or mitogenic neuropeptides to cells [10,11]. It is also stimulated by the activation of protein kinase C [11,12]. Thus, FAK is thought to play a crucial role in the adhesion, migration, and proliferation of normal and transformed cells.

Kimizuka et al. [13,14] prepared several recombinant fibronectin fragments that contained the cell-binding sequence Arg-Gly-Asp-Ser. One of them, termed peptide C-274, had three type III homology repeats of human fibronectin in the molecule, and showed cell-adhesive activity. We constructed a fusion protein of C-274 with human basic fibroblast growth factor (bFGF), in which bFGF was linked covalently to the C-terminal end of C-274 [15]. The fusion protein, termed FN-FGF, had cell-adhesive activity towards BALB/c 3T3 cells, and stimulated the in vitro growth of human umbilical vein endothelial cells, as well as the in vivo angiogenesis on chorioallantoic membranes of developing chick embryos [15]. We then examined the effects of FN-FGF on the phosphorylation of cellular proteins and found that it stimulated the phosphorylation of a 120-kDa protein of 3T3 cells, which turned out to be FAK. These findings led us to investigate the effects of FGF on the phosphorylation of FAK. Here we report that the phosphorylation of both tyrosine and serine residues of FAK in BALB/c mouse 3T3 cells is stimulated by fibronectin and FGF.

2. Materials and methods

2.1. Materials

The following materials were used: cell-adhesive fragment of fibronectin (C-274; Takara Shuzo Co.), poly-L-lysine (Sigma), monoclonal antibodies against phosphotyrosine (PY69; ICN) and against chick FAK (UBI), Dulbecco's modification of Eagle's minimum essential medium (D-MEM; Nissui Pharmaceutical Co.). Bovine pituitary aFGF, bovine brain bFGF, bovine pituitary epidermal growth factor (EGF), human platelet-derived growth factor (PDGF) were purchased from Takara Shuzo Co. Fibronectin-bFGF fusion protein (FN-FGF) was purified by heparin affinity chromatography from extracts from *E. coli* strain JM109 that carried the expression vector for the fusion protein [15].

2.2. Immunoblot analysis

BALB/c mouse 3T3/K3 cells and rat myoblast L6 cells were cultivated in D-MEM containing either 10% calf serum or 10% fetal bovine serum. Sub-confluent cells were dispersed by trypsin treatment and suspended in D-MEM containing 0.5 mg/ml soybean trypsin inhibitor (Sigma). After being washed twice with D-MEM, 2×10^6 cells suspended in 5 ml of serum-free D-MEM were put in Corning tissue culture dishes (10 cm) that had previously been coated at room temperature for 2 h with 1 μM each of human plasma fibronectin, fibronectin fragment C-274, fusion protein FN-FGF, or with 1 mg/ml poly-L-lysine. After incubating the cells at 37°C for 1–6 h, attached cells were washed with phosphate-buffered saline (PBS) containing 100 μM sodium orthovanadate, and lysed with 0.5 ml lysis buffer; 10 mM Tris-HCl, pH 7.4, containing 50 mM NaCl, 5 mM EDTA, 50 mM NaF, 30 mM sodium pyrophosphate, 100 μM sodium orthovanadate, 1% Triton X-100, and 1 mM phenylmethane sulfonyl fluoride (PMSF). Equal

*Corresponding author. Fax: (81) (3) 3542 0807.

Abbreviations: aFGF, acidic fibroblast growth factor; bFGF, basic fibroblast growth factor; C-274, recombinant fibronectin fragment having cell-binding domain; EGF, epidermal growth factor; FAK, focal adhesion kinase; FN-FGF, fibronectin–basic fibroblast growth factor fusion protein; PDGF, platelet-derived growth factor.

SSDI 0014-5793(94)00745-4

amounts of proteins (5–10 μg) were separated by SDS-PAGE in Laemmli's buffer system [16]. Immunoblot analysis was carried out using an Amersham ECL immunoblot detection system with anti-phosphotyrosine or anti-FAK monoclonal antibodies.

2.3. Chemical cross-linking of FGF and fusion protein to cell surface receptors

Purified fusion protein FN-FGF (1 mg) was labeled with 2 mCi of ^{125}I-labelled Na by a radioiodination kit using the lactoperoxidase method (New England Nuclear), and the labeled protein was purified by a heparin–Sepharose column. The specific radioactivity of the labeled FN-FGF was 5.76×10^6 cpm/nmol. Confluent 3T3 cells grown in 10-cm dishes were washed twice with binding buffer (D-MEM and 0.1% gelatin in 50 mM HEPES, pH 7.5), and incubated with 0.4 nmol of [^{125}I]FN-FGF or 0.001 nmol of [^{125}I]bFGF (2.02×10^9 cpm/nmol; Amersham) in the binding buffer at 4°C for 90 min. After being washed with the binding buffer, the cells were further incubated at 4°C for 20 min in PBS containing 0.3 mM disuccinimidyl suberate (Pierce). The cells were collected and lysed in 100 μl of lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1.5 mM $MgCl_2$, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 0.1 mM PMSF). Proteins were separated by SDS-PAGE in 4–20% gels, and detected by autoradiography using the Fujix Bio-Imaging Analyzer BAS2000 (Fuji Photo Film Co.).

2.4. Analysis of phosphoamino acids

Cells were inoculated in phosphate-free D-MEM (Gibco) containing 1 mCi/ml ortho[^{32}P]phosphate (NEN) in 10-cm culture dishes coated with fibronectin, C-274, or FN-FGF, or poly-L-lysine as described above. To the cultures in poly-L-lysine-coated dishes, aFGF, bFGF, EGF, or PDGF were added, and the cells were incubated at 37°C for 2 h. Cells were lysed and FAK was precipitated with anti-FAK monoclonal antibodies. The immunoprecipitate was separated by SDS-PAGE in 6% gels, and the region of the gel corresponding to FAK was excised. Proteins were extracted from the gel, hydrolysed with 6 N HCl, and phosphoamino acids were separated by one-dimensional thin-layer electrophoresis in 5% acetic acid, 0.5% pyridine (pH 3.5) [17], and detected by autoradiography.

3. Results

BALB/c mouse 3T3 cells were inoculated in dishes coated with fibronectin, recombinant fibronectin fragment C-274, or fibronectin-bFGF fusion protein FN-FGF, and the tyrosine-phosphorylation of cellular proteins was examined by immunoblot analysis using anti-phosphotyrosine monoclonal antibodies (Fig. 1, left panel). After 1 h of incubation, a 120-kDa protein was strongly phosphorylated on all substrata, but C-274 was less effective than fibronectin or FN-FGF in stimulating the phosphorylation of this protein. After 4–6 h, the level of tyrosine phosphorylation of the 120-kDa protein seemed to be higher in the cells that adhered to the FN-FGF substratum than to those that adhered to fibronectin. Immunoblot analysis using anti-FAK antibodies showed that the amount of FAK at 6 h on these substrata was similar (Fig. 1, right panel). The 120-kDa phosphorylated protein was precipitated with anti-FAK (see below) or anti-phosphotyrosine (data not shown) antibodies, so at least a part of the 120-kDa protein(s) was FAK.

Mouse 3T3 cells were inoculated in dishes coated with fibronectin or poly-L-lysine, and aFGF, bFGF, EGF, or PDGF was added to the cells on the poly-L-lysine substrata at the same time. Tyrosine phosphorylation of the 120-kDa protein was very small in the cells on the poly-L-lysine substratum, but it was stimulated by the addition of aFGF or bFGF to the cultures (Fig. 2, left panel). In contrast, EGF or PDGF did not stimulate the tyrosine phosphorylation of FAK. In a separate set of experiments, the cells were labeled with ortho[^{32}P]phosphate for 2 h, and FAK was immunoprecipitated with anti-FAK antibodies, followed by SDS-PAGE and autoradiography. Phosphorylation of FAK was stimulated by aFGF or bFGF, but not significantly by EGF or PDGF (Fig. 2, right panel).

Rat myoblast cell line L6 lacks FGF receptors [18]. These cells were inoculated onto various adhesion substrata, and the tyrosine phosphorylation of FAK was

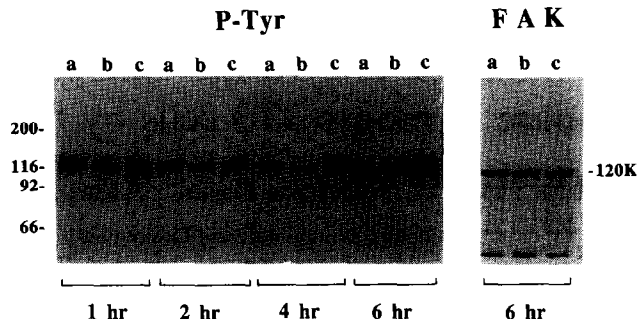

Fig. 1. Tyrosine phosphorylation of the proteins in BALB/c mouse 3T3 cells on different adhesion substrata. Cells were inoculated into dishes previously coated with fibronectin (lanes a), C-274 (lanes b), and FN-FGF (lanes c). After incubation of the cells at 37°C for the indicated periods, lysates of the attached cells were prepared and separated by 6% SDS-PAGE, followed by immunoblot analysis using anti-phosphotyrosine (P-Tyr, left panel) and anti-FAK (FAK, right panel) antibodies.

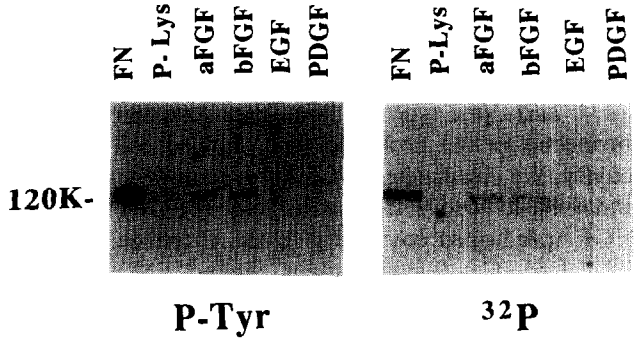

Fig. 2. Effects of growth factors on the phosphorylation of FAK. BALB/c 3T3 cells were inoculated in 10-cm culture dishes coated with 1 μM fibronectin (FN) or 1 mg/ml poly-L-lysine (P-Lys). To cultures on poly-lysine substrata, 10 ng/ml aFGF, 10 ng/ml bFGF, 50 ng/ml EGF, or 5 ng/ml PDGF were added at the time of the inoculation of cells, and the cells were incubated at 37°C for 2 h. (Left panel) Lysates of attached cells were separated by SDS-PAGE in 6% gels, followed by immunoblot analysis using anti-phosphotyrosine antibodies. (Right panel) Cells were labeled with ortho[^{32}P]phosphate at 37°C for 2 h after inoculation. FAK was precipitated with anti-FAK antibodies, and the immunoprecipitate was separated by SDS-PAGE, followed by autoradiography.

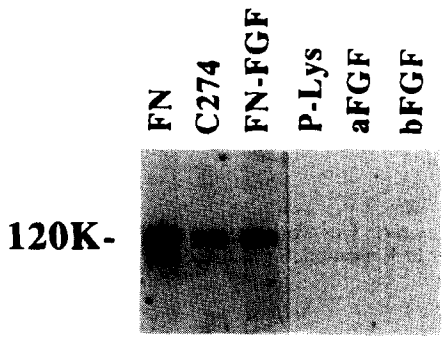

Fig. 3. Phosphorylation of FAK in rat myoblast L-6 cells. The cells were inoculated into dishes coated with fibronectin (FN), C-274, FN-FGF, or poly-L-lysine (P-Lys). aFGF or bFGF (10 ng/ml each) was added to the cultures on poly-lysine substrata, and the cells were incubated at 37°C for 2 h. Total cell lysate was analysed by immunoblot using anti-phosphotyrosine antibodies.

examined. Phosphorylation of FAK was stimulated in the cells adhering to fibronectin, C-274, or FN-FGF, but aFGF and bFGF did not stimulate the tyrosine phosphorylation of FAK in L6 cells adhered to the poly-L-lysine substrata (Fig. 3).

Fig. 4 shows the results of chemical cross-linking studies using [^{125}I]bFGF and [^{125}I]FN-FGF. Cross-linking of bFGF to 3T3 cells gave a broad band on SDS-PAGE at around 150 kDa (Fig. 4, lane a), which is likely to be a cross-linked product of bFGF (17 kDa) and its receptors (130–150 kDa). No cross-linked product was observed in rat L6 cells (Fig. 4, lane b). We confirmed that the major FGF receptor of 3T3 cells was *flg* (150 kDa), so the calculated molecular weight of the cross-linked product of bFGF with the receptor is 167 kDa. The discrepancy in the molecular weight was due to the faster mobility of the cross-linked products in the present SDS-PAGE system (unpublished observation). The 33-kDa component, found in both 3T3 and L6 cells, seemed to be a dimer of bFGF.

Following incubation of [^{125}I]FN-FGF with 3T3 cells, 100-, 120- and 180-kDa components were detected (Fig. 4, lane d). The 180-kDa component corresponded to the cross-linked product of FN-FGF (48 kDa) with FGF receptors. At least a part of the 100- to 120-kDa components seemed to be the cross-linked product of FN-FGF with integrin subunits. These results confirmed that FN-FGF could bind to the FGF receptors on 3T3 cells, and that rat L6 cells lacked FGF receptors.

For the analysis of phosphorylated amino acids, 3T3 cells were inoculated on fibronectin or poly-L-lysine, and aFGF or bFGF was added to the cultures on the poly-L-lysine substrata. Following incubation of the cells in the presence of ortho[^{32}P]phosphate for 2 h, FAK was immunoprecipitated from the cell lysate, purified by SDS-PAGE, hydrolysed, and the phosphoamino acids were separated by thin-layer electrophoresis (Fig. 5). Phosphoserine, in addition to phosphotyrosine, was detected in the hydrolysate of FAK of the cells adhered to fibronectin (lane B). aFGF and bFGF stimulated the phosphorylation of serine of FAK in the cells adhered to poly-L-lysine, and tyrosine phosphorylation was also stimulated (lanes C and D), in accordance with the results of the immunoblot analyses (Fig. 3). Fibronectin and FGF did not cause detectable threonine phosphorylation of FAK.

4. Discussion

Hanks et al. [4] showed that bacterially expressed FAK was auto-phosphorylated only on tyrosine in vitro. However, Schaller et al. [19] reported that a truncated, non-catalytic form of FAK (FRNK) is present in cultured chick embryo cells, and suggested that serine and/or threonine residues of FRNK might be phosphorylated in vivo. Our results unequivocally demonstrated that tyrosine and serine, but not threonine, residues of FAK are phosphorylated in BALB/c mouse 3T3 cells. The phosphorylation of serine might be a prerequisite for the tyrosine phosphorylation of FAK, since activation of protein kinase C results in the stimulation of the latter [11,12]. However, Vuori and Ruoslahti [12] reported that FAK was not directly phosphorylated by protein kinase C. Another type of serine/threonine kinase seems to be responsible for the serine phosphorylation of FAK.

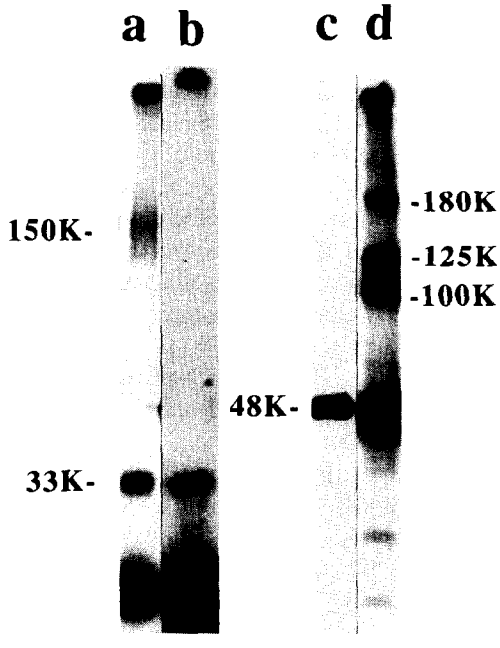

Fig. 4. Chemical cross-linking of bFGF and FN-FGF to cell surface receptors. ^{125}I-labeled bFGF or FN-FGF was cross-linked to mouse 3T3 cells or rat myoblast L6 cells. The cell lysate was separated by SDS-PAGE, followed by autoradiography. Lanes a and b, [^{125}I]bFGF cross-linked to 3T3 cells (a) and L6 cells (b); lane c, purified [^{125}I]FN-FGF; lane d, [^{125}I]FN-FGF cross-linked to 3T3 cells.

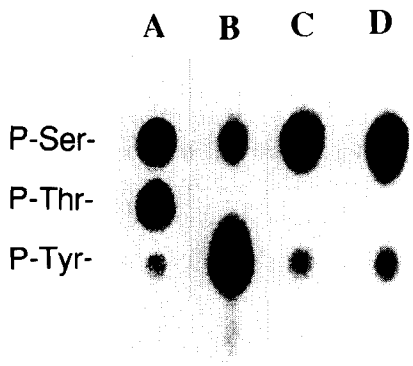

Fig. 5. Analysis of phosphoamino acids of FAK. 3T3 cells were inoculated into dishes coated with fibronectin (lane B), or poly-L-lysine (lanes C and D) in the presence of 1 mCi/ml ortho[^{32}P]phosphate. 10 ng/ml each of aFGF (lane C) or bFGF (lane D) was added to the cells on poly-lysine substrata and the cells were incubated at 37°C for 2 h. FAK was immunoprecipitated from the lysate with anti-FAK antibodies, separated by SDS-PAGE, and hydrolysed. Phosphoamino acids were analysed by one-dimensional thin-layer electrophoresis. Lane A, standard phosphoamino acids detected by ninhydrin; lanes B–D, autoradiography.

Acidic and basic FGF also stimulated the phosphorylation of tyrosine and serine of FAK in 3T3 cells cultured on poly-L-lysine, on which the cells did not spread and focal adhesion plaques were not formed. Phosphorylation of FAK in a rat cell line, L-6, which lacks FGF receptors, was not stimulated by FGF. Chemical cross-linking experiments showed that the fusion protein FN-FGF bound to the FGF receptors on 3T3 cells. Taken together, these data suggested that FGF receptors were necessary for the FGF-induced phosphorylation of FAK. FN-FGF seems to have the phosphorylation-stimulating activity of both fibronectin and FGF. Thus, FGF seems to be a new family of mitogenic polypeptides that can act as an extracellular stimulus leading to FAK phosphorylation. FGF receptors have tyrosine kinase activity [20–22], but it remains to be investigated whether the tyrosine residues of FAK are directly phosphorylated by FGF receptor kinases. The present results do not rule out the possibility that some common mechanisms are involved in the fibronectin and FGF-induced phosphorylation pathways of FAK. Which properties of Ser/Thr kinases cause the FGF-induced serine-phosphorylation of FAK are still unknown.

Rankin and Rozengurt [23] reported that PDGF stimulated tyrosine phosphorylation of FAK in Swiss mouse 3T3 cells. However, our results showed that EGF and PDGF were less effective in stimulating the phosphorylation of FAK in BALB/c 3T3 cells. This discrepancy might be due to the differences in the experimental conditions used in their and our studies.

FGF displays a broad spectrum of biological activity on many types of cells in vitro, including stimulation of cell proliferation and cell migration, and induction of plasminogen activator and collagenase [24]. In vivo, FGF stimulates angiogenesis [24], neuronal differentiation [18], wound healing [25–27], and induces the mesoderm formation in early amphibian embryos [28,29]. At least a part of these multiple functions of FGF may be related to its ability to stimulate the phosphorylation of FAK.

Acknowledgements: This work was supported by a Grant-in-Aid from the Ministry of Education, Science and Culture of Japan (05151018).

References

[1] Schaller, M.D., Borgman, C.A., Cobb, B.S., Vines, R.R., Reynolds, A.B. and Parsons, J.T. (1992) Proc. Natl. Acad. Sci. USA 89, 5192–5196.
[2] Guan, J.-L. and Shalloway, D. (1992) Nature 358, 690–692.
[3] Guan, J.-L., Trevithick, E. and Hynes, R.O. (1992) Cell Reg. 2, 951–946.
[4] Hanks, S.K., Calalb, M.B., Harper, M.C. and Patel, S.K. (1992) Proc. Natl. Acad. Sci. USA 89, 8487–8491.
[5] Burridge, K., Turner, C.E. and Romer, L.H. (1992) J. Cell Biol. 119, 893–903.
[6] Kornberg, L., Earp, H.S., Parsons, J.T., Schaller, M. and Juliano, R.L. (1992) J. Biol. Chem. 267, 23439–23442.
[7] Kornberg, L.J., Earp, H.S., Turner, C.E., Prockop, C. and Juliano, R. (1991) Proc. Natl. Acad. Sci. USA 88, 8392–8396.
[8] Lipfert, L., Haimovich, B., Schaller, M.D., Cobb, B.S., Parsons, J.T. and Brugge, J.S. (1992) J. Cell Biol. 119, 905–912.
[9] Hamawy, M.M., Mergenhagen, S.E. and Siraganian, R.P. (1993) J. Biol. Chem. 268, 6851–6854.
[10] Zachary, I., Sinnett-Smith, J. and Rozengurt, E. (1992) J. Biol. Chem. 267, 19031–19034.
[11] Sinnett-Smith, J., Zachary, I., Valverde, A.M. and Rozengurt, E. (1993) J. Biol. Chem. 268, 14361–14268.
[12] Vuori, K. and Ruoslahti, E. (1993) J. Biol. Chem. 268, 21459–21462.
[13] Kimizuka, F., Ohdate, Y., Kawase, Y., Shimojo, T., Taguchi, Y., Hashino, K., Kato, I., Sekiguchi, K. and Titani, K. (1991) J. Biol. Chem. 266, 3045–3051.
[14] Kimizuka, F., Taguchi, Y., Ohdate, Y., Kawase, Y., Shimojo, Hashino, K., Goto, S., Hashi, H., Kato, I., Sekiguchi, K. and Titani, K. (1991) J. Biochem. 110, 284–291.
[15] Hashi, H., Hatai, M., Kimizuka, F., Kato, I. and Yaoi, Y. (1994) Cell Struct. Funct. 19, 37–47.
[16] Laemmli, U.K. (1970) Nature 277, 680–685.
[17] Boyle, W.J., van der Geer, P. and Hunter, T. (1991) Methods Enzymol. 201, 110–149.
[18] Olwin, B.B. and Hauschka, S.D. (1989) J. Cell. Biochem. 39, 443–459.
[19] Schaller, M.D., Borgman, C.A. and Parsons, J.T. (1993) Mol. Cell. Biol. 13, 785–791.
[20] Huang, S.S. and Huang, J.S. (1986) J. Biol. Chem. 261, 9568–9571.
[21] Coughlin, C.R., Barr, P.J., Couses, L.S., Fretto, L.J. and Williams, L.T. (1988) J. Biol. Chem. 263, 988–993.
[22] Friesel, R., Burgess, W.H. and Maciag, T. (1989) Mol. Cell Biol. 9, 1857 1865.
[23] Rankin, S. and Rozengurt, E. (1994) J. Biol. Chem. 269, 704–710.
[24] Rifkin, D.B. and Moscatelli, D. (1989) J. Cell Biol. 109, 1–6.
[25] Folkman, J. and Klagsbrun, M. (1987) Science 235, 442–447.
[26] Lobb, R.R. (1988) Eur. J. Clin. Invest. 18, 321–336.
[27] Tsuboi, R. and Rifkin, D.B. (1990) J. Exp. Med. 172, 245 251.
[28] Slack, J.M.W., Darlington, B.G., Heath, J.K. and Godsave, S.F. (1987) Nature 326, 197–200.
[29] Kimmelman, D. and Kirschner, M. (1987) Cell 51, 869–877.