FEBS Letters 353 (1994) 133–137

FEBS 14637

Serum alleviates the requirement of the granulocyte-macrophage colony-stimulating factor (GM-CSF)-induced Ras activation for proliferation of BaF3 cells

Kazuhiro Sakamaki[a,*], Shin Yonehara[b]

[a]*Pharmaceutical Basic Research Laboratory, JT Inc., Fukuura, Kanazawa-ku, Yokohama 236, Japan*
[b]*Institute for Virus Research, Kyoto University, Shogoin-Kawahara, Sakyo-ku, Kyoto 606-01, Japan*

Received 29 August 1994

Abstract Deletion analysis of the β subunit of the human granulocyte-macrophage colony-stimulating factor (GM-CSF) receptor previously defined two cytoplasmic regions required for distinct signaling. The membrane-proximal region is responsible for induction of c-*myc* and *pim-1*, and is indispensable for GM-CSF-dependent proliferation of mouse BaF3 transfectants. The distal region is required for activation of Ras, Raf-1, MAP kinase and p70 S6 kinase as well as induction of c-*fos* and c-*jun*, but is dispensable for GM-CSF-dependent proliferation of transfectants under normal culture conditions containing serum. Here we show that signals induced by the distal region of the β subunit are also required for proliferation. GM-CSF supported proliferation of BaF3 transfectants expressing the normal β subunit, even in serum-free medium. However, in the absence of serum, GM-CSF did not support proliferation of BaF3 transfectants that have the β deletion mutants lacking the distal region. Serum-induced activation of Ras, phosphorylation of MAP kinase and expression of c-*fos* in parental BaF3 cells and antisense oligonucleotide against c-*raf* blocked DNA synthesis of BaF3 cells. These results indicate that proliferation of BaF3 cells requires signals induced by the proximal as well as the distal region of the β subunit of the GM-CSF receptor, and that serum alleviates the requirement of signals induced by the distal region.

Key words: Ras; Raf-1; Serum-free culture; GM-CSF receptor; BaF3

1. Introduction

The high affinity receptors for granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-3 (IL-3) and interleukin-5 (IL-5) consist of two distinct subunits, α and β [1]. The α subunits are specific for each cytokine and bind their specific ligand with low affinity. The three receptors share the common β subunit (β_c) that does not bind any cytokine by itself but forms a high affinity receptor for the GM-CSF, IL-3 and IL-5 with the respective α subunit. The β_c subunit is not only required for high affinity binding of a ligand, but is also essential for signal transduction. Although none of these subunits has intrinsic kinase activity, binding of these cytokines to their receptors induces rapid tyrosine phosphorylation including the β_c subunit itself [2–7]. Several tyrosine kinases, Lyn, Fyn, Fes, and Jak2 have been implicated in the GM-CSF/IL-3 signaling pathway [8–11]. Presumably, activation of one or some of these tyrosine kinases leads to activation of $p21^{ras}$ (Ras), and serine/threonine kinases, Raf-1 and MAP kinase as well as induction of nuclear protooncogenes such as c-*myc*, c-*fos* and c-*jun* [12–19].

BaF3 is an IL-3-dependent cell line originally described as a pro-B cell line [20] and has been widely used to study the function of cytokine receptors [21–25]. A series of cytoplasmic deletion mutants of the human β_c subunit has been coexpressed with the human GM-CSF receptor α subunit in BaF3 cells and their functions in transfectants have been examined [7,26]. Two cytoplasmic regions of the β_c subunit were found to be important for induction of distinct signals [26]. The membrane-proximal region of about 60 amino acid residues is responsible for induction of c-*myc* and *pim-1* (referred to as the Myc pathway) and is indispensable for proliferation. The membrane-distal region is required for GM-CSF-induced activation of Ras, Raf-1, MAP kinase and p70 S6 kinase (referred to as the Ras pathway). Like the GM-CSF receptor, the deletions of the interleukin-2 (IL-2) receptor β subunit defined two domains in the cytoplasmic region responsible for different signaling [27,28], i.e. the membrane-proximal serine-rich region is responsible for induction of c-*myc* and indispensable for proliferation of BaF3 transfectants, whereas the distal acidic region is required for activation of Ras and induction of c-*fos* and c-*jun*. The acidic region is also responsible for binding Lck, but this region is dispensable for proliferation.

While it is well established that the Ras pathway plays an important role for proliferation, these results suggest that the Ras pathway activated by the distal region of the β_c subunit is dispensable for proliferation of BaF3 cells. However, it should be noted that proliferation of BaF3 transfectants expressing the GM-CSF receptor or the IL-2 receptor was examined in medium containing serum [7,21]. We therefore have examined the possibility that serum supports growth of transfectants bearing the mutant receptor by activating cellular signaling molecules. Signals mediated through the distal region of the β_c subunit were required for GM-CSF-dependent proliferation when transfectants were cultured in the absence of serum. Consistent with this observation, anti-sense oligonucleotide against c-*raf* inhibited proliferation of BaF3 cells. These results indicate that proliferation of BaF3 cells requires the Ras pathway in addition to the Myc pathway that are activated through the GM-CSF receptor.

2. Materials and methods

2.1. Cell lines

A parental IL-3-dependent mouse pro-B cell line, BaF3 [20] and BaF3 transfectants (BaF/$\alpha\beta_c$, BaF/$\alpha\beta_{763}$, BaF/$\alpha\beta_{544}$, BaF/$\alpha\beta_{517}$, and BaF/$\alpha\beta_{455}$) expressing the normal or mutant GM-CSF receptor [7] were maintained in RPMI 1640 medium (IL-3 medium) supplemented with

*Corresponding author. Fax. (81) (45) 786 7692.

0014-5793/94/$7.00 © 1994 Federation of European Biochemical Societies. All rights reserved.
SSDI 0014-5793(94)01024-2

10% fetal bovine serum (FBS) in the presence of 100 U/ml of mouse IL-3 (mIL-3).

2.2. Cell culture in serum-free medium

Serum-free medium was prepared according to Cormier et al. [29]. BaF3 transfectants cultured in medium containing mIL-3 with 10% FBS were washed with serum-free medium twice, and then incubated at 1×10^5 cells/ml in serum-free medium containing mIL-3 (100 U/ml) or human GM-CSF (1 ng/ml) without or with 10% FBS.

2.3. Analysis of Ras-bound GDP and GTP

Activation of Ras was assayed as described previously [12]. In brief, $0.5–1.0 \times 10^7$ cells were collected and washed with phosphate-free RPMI. Cells were resuspended and incubated in phosphate-free RPMI containing 0.1% bovine serum albumin (BSA) (Fraction V, Sigma), 50 μM Na_3VO_4 and [^{32}P]orthophosphate (1.0 mCi/ml, DuPont-New England Nuclear) for 2.5 h. FBS was added to the cells at final 10%. After incubation with FBS for 0–30 min, cells were harvested and lysed in modified RIPA buffer. Ras protein was recovered by immunoprecipitation with anti-Ras monoclonal antibody, Y13-259. Ras-bound nucleotides were separated by thin-layer chromatography and the radioactivity of GDP and GTP was measured.

2.4. Immunoblot and Northern blot analysis

BaF3 cells were factor- and serum-deprived for 2.5 h, stimulated with FBS for 10 min, and lysed in Laemmli's sample buffer. Total cellular proteins were separated on SDS-polyacrylamide gels and transferred to an immobilon PVDF membrane filter (Millipore). After blocking with PBS containing 3% BSA, the membrane was incubated with the primary rabbit antibody against p44 MAP kinase (anti-ERK1) (UBI). Anti-rabbit Ig (Amersham) coupled with horseradish peroxidase and ECL (Amersham) were used to detect protein–primary antibody complex as described previously [26].

For Northern blot analysis, similarly, BaF3 cells were factor- and serum-deprived for 8 h and stimulated with FBS or mIL3 for 15 min. Total cellular RNA from BaF3 cells was prepared as previously described [26]. Ten micrograms of total RNA from each sample was electrophoresed on a 1% agarose-formaldehyde gel and transferred to the Nytran filter (S&S). cDNA probes for c-*fos* and glyceraldehyde 3-phosphate dehydrogenase (G3PDH) (Clontech) were labeled with [α^{32}P]dCTP and hybridized with the membrane as described previously [30].

2.5. [^{3}H]Thymidine and BrdU incorporation and cell viability assay

Antisense (5′-TCCCTGTATGTGCTCCAT-3′) and sense (5′-ATGGAGCACATACAGGGA-3′) oligonucleotides corresponding to murine c-*raf* were prepared as described by Carroll et al. [31]. BaF3 cells (1×10^4) were incubated with fresh IL-3 medium in the absence or presence of oligonucleotides. After 24 h, 1 μCi of [^{3}H]thymidine (^{3}H-TdR) (NEN) was added. Incorporation of ^{3}H-TdR into DNA was measured after 4 h by liquid scintillation counter. At the same time, cell viability was measured by the calorimetric assay after incubation with alarmaBlue (Biosource) for 4 h following a 24 h incubation.

Similarly, after a 24 h incubation, $0.5–1 \times 10^7$ cells were also labeled with 5-bromo-2-deoxyuridine (BrdU) for 30 min. After fixation with 70% ethanol and denaturation of the DNA with HCl, cells were incubated with FITC-conjugated anti-BrdU antibody (Becton) and then analyzed by flow-cytometry with EPICS ELITE (Coulter).

3. Results

3.1. Proliferation of BaF3 transfectants in serum-free medium

Previously we have shown that BaF3 transfectants expressing both α and β_c subunits of the human GM-CSF receptor proliferate in response to human GM-CSF (hGM-CSF) [32]. A series of cytoplasmic deletion mutants of the β_c subunit was constructed and their functions were examined in BaF3 cells [7,26]. Interestingly, the β_{517} as well as β_{544} deletions that do not induce activation of the Ras pathway were still capable of stimulating proliferation. To understand whether the activated Ras pathway is dispensable for cell proliferation, we examined proliferation assay of BaF3 transfectants in the serum-free medium. Serum in culture medium might substitute the requirement of the Ras pathway induced by hGM-CSF. As shown in Fig. 1, all BaF3 transfectants proliferated continuously in response to mIL-3 even in the absence of serum. In contrast, response of these transfectants to hGM-CSF was different under the serum-free condition. BaF/$\alpha\beta_c$ and BaF/$\alpha\beta_{763}$, which activate the Ras pathway in response to hGM-CSF, proliferated in the presence of hGM-CSF. However, BaF/$\alpha\beta_{544}$ and BaF/$\alpha\beta_{517}$ did not proliferate even in the presence of hGM-CSF, although their viability was maintained by hGM-CSF for a few days. The addition of serum stimulated proliferation of BaF/$\alpha\beta_{544}$ and BaF/$\alpha\beta_{517}$ in the presence of hGM-CSF.

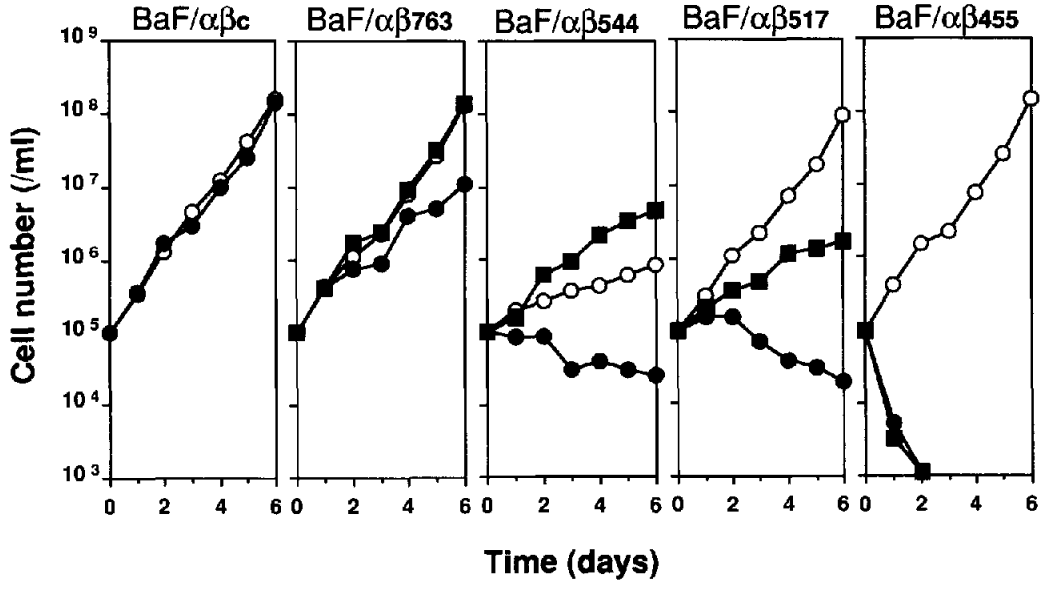

Fig. 1. Cell growth in serum-free medium. Transfectants cultured in medium containing serum were washed with serum-free medium, and then incubated in serum-free medium containing mIL-3 (100 U/ml) (open circle), or hGM-CSF (1 ng/ml) without (closed circle), or with 10% FBS (closed square). Cell growth of transfectants was monitored by counting viable cell number.

BaF/$\alpha\beta_{455}$, which lacks the entire cytoplasmic domain of β_c and was unable to induce any signals, did not proliferate even in the presence of both hGM-CSF and serum. These results indicate that serum supports the growth of BaF/$\alpha\beta_{544}$ and BaF/$\alpha\beta_{517}$ which are unable to activate the Ras pathway, and also suggest that signals induced by the distal region of the β subunit are involved in proliferation of BaF3 transfectants.

3.2. Activation of the Ras pathway by serum

Despite the fact that hGM-CSF failed to activate the Ras pathway in BaF/$\alpha\beta_{544}$ and BaF/$\alpha\beta_{517}$, both transfectants proliferated in response to hGM-CSF in the presence of serum. We therefore examined the possibility that serum induces activation of the Ras pathway in BaF3 cells. BaF3 cells deprived of serum and mIL-3 were stimulated with serum, and the accumulation of GTP-bound Ras (Ras·GTP) was measured (Fig. 2A). Serum increased Ras·GTP approximately 1.5-fold compared with the unstimulated cells. The increase was smaller compared to the stimulation with mIL-3 (5- to 10-fold), but is significant. To further confirm that serum induces activation of the Ras pathway, we examined the phosphorylation of MAP kinase, downstream of Ras. As shown in Fig. 2B, serum as well as mIL-3 clearly induced a mobility shift of MAP kinase, which is known to result from phosphorylation [17]. Similarly, serum induced expression of the c-*fos* mRNA as well (Fig. 2C). These results clearly indicate that serum leads to activation of the Ras pathway in BaF3 cells.

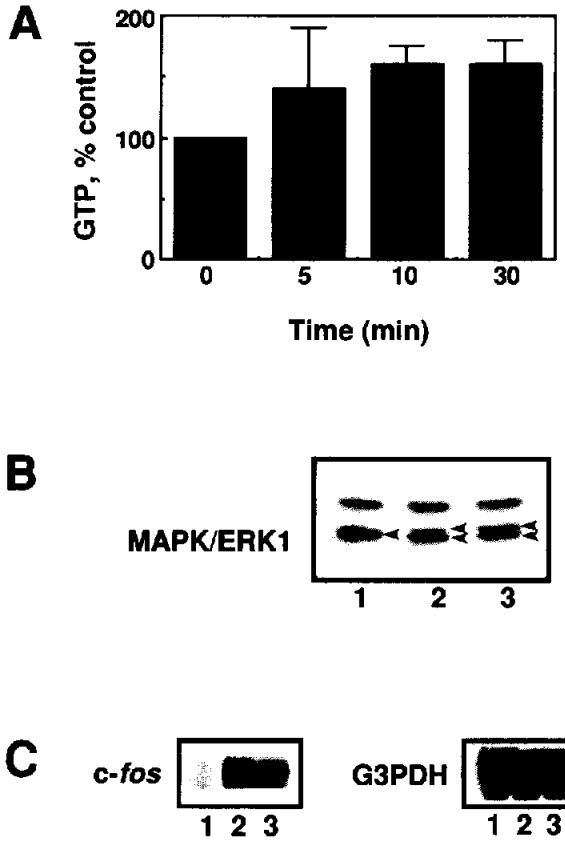

Fig. 2. Activation of molecules on the Ras pathway by serum. (A) Ras activation in BaF3 cells. BaF3 cells were incubated in phosphate-free RPMI medium with [^{32}P]orthophosphate for 2.5 h, stimulated with FBS for 0–30 min at final 10%, and then lysed. Ras protein was immunoprecipitated with anti-Ras antibody, Y13-259 and the radioactivity of GDP and GTP bound to Ras proteins was analyzed as described in section 2. (B) Mobility shift of MAP kinase. mIL-3- and serum-deprived BaF3 cells were incubated without (lane 1) and with 10% FBS (lane 2) or 10 ng/ml mIL-3 (lane 3) for 10 min. Total cellular proteins (1.2×10^5 cells/lane) were separated by SDS-10% PAGE and p44 MAP kinase (ERK1) was identified by immunoblotting using anti-ERK1 antibody. (C) Induction of c-*fos* in BaF3 cells. mIL-3- and serum-deprived BaF3 cells were stimulated with FBS (lane 2) or 10 ng/ml mIL-3 (lane 3) for 15 min. Total RNA was electrophoresed on formaldehyde-1% agarose gel and transferred onto a nylon membrane. The same membrane was used for hybridization with the radiolabeled c-*fos* and G3PDH, respectively.

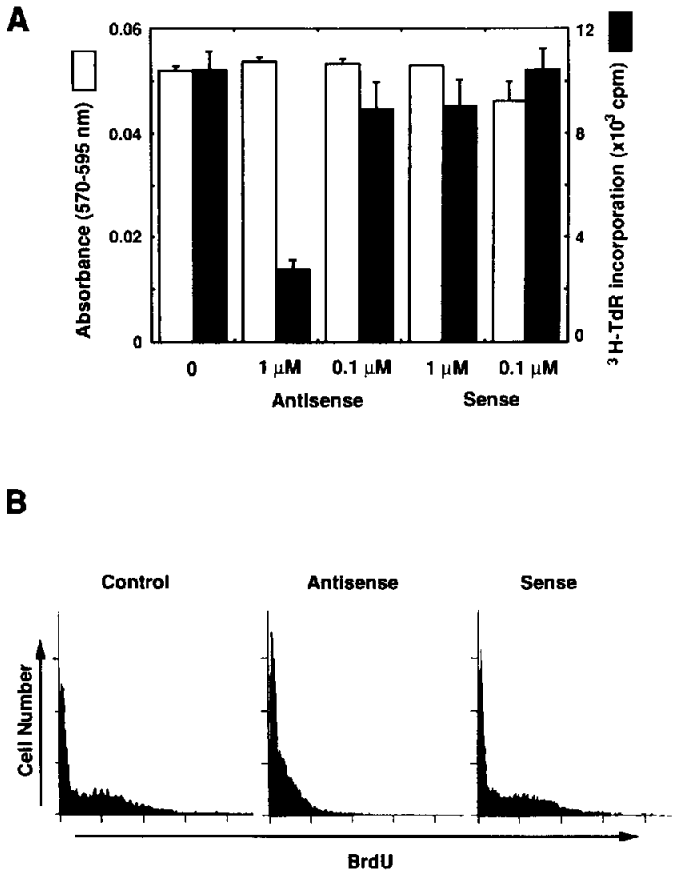

Fig. 3. Effect of c-*raf* antisense oligonucleotide. (A) BaF3 cells were incubated for 24 h in fresh medium containing mIL-3 and 10% FBS without or with antisense (1 and 0.1 μM) or sense (1 and 0.1 μM) oligonucleotides against c-*raf*. For cell viability assay or mitogenic assay, cells were incubated for an additional 4 h alamarBlue (open bar) or ^{3}H-TdR (solid bar), respectively, as described in section 2. (B) BaF3 cells incubated without or with antisense (1 μM) and sense (1 μM) for 24 h were labeled with BrdU for 30 min, stained with FITC-conjugated anti-BrdU antibody, and analyzed by flow-cytometry as described in section 2.

3.3. Inhibition of proliferation by the antisense c-raf oligonucleotide

To investigate the role of the Ras pathway, we focused on Raf-1, which is a downstream molecule of Ras and activates MAP kinase [33,34], and examined the effect of antisense oligonucleotide against c-*raf* to BaF3 cells. Antisense or sense oligonucleotide against c-*raf* was added to culture medium containing mIL-3. After 24 h, cell viability and mitogenic activity of BaF3 cells were measured by alamarBlue and thymidine

uptake (Fig. 3A). Although these oligonucleotides did not affect cell viability of BaF3 cells, DNA synthesis was significantly inhibited by addition of antisense, but not sense, oligonucleotides. We also observed that DNA synthesis of BaF/$\alpha\beta_c$ stimulated by hGM-CSF is inhibited by addition of antisense oligonucleotide (data not shown). Labeling of DNA by BrdU is also decreased by addition of antisense oligonucleotide (Fig. 3B). Further, Myc protein level measured by immunoblotting was not significantly changed by the treatment with antisense- or sense-oligonucleotide against c-*raf* (data not shown). These results indicate that the function of c-*raf* is required for proliferation of BaF3 cells and also suggest that signals on the Ras pathway are important for regulating the G_1 to S phase transition in hematopoietic cells.

4. Discussion

Previously we demonstrated that there are at least two distinct regions in the cytoplasmic domain of the β_c subunit of the GM-CSF receptor: the membrane-proximal region is responsible for induction of c-*myc* and *pim-1* (the Myc pathway) and the distal region is required for activation of Ras, Raf-1, MAP kinase and p70 S6 kinase and for expression of c-*fos* and c-*jun* (the Ras pathway) [26]. Whereas the membrane-proximal region is indispensable for proliferation, the distal region is dispensable for GM-CSF-dependent growth in medium containing serum [7]. Here we demonstrate that serum is required for proliferation of transfectants expressing the truncated GM-CSF receptor which is unable to activate any molecules in the Ras pathway.

Recent studies suggest that Ras, Raf-1, MAP kinase, and c-*fos*/c-*jun* form a signaling pathway referred to as the Ras pathway in this paper [33,34], i.e. activated-Ras activates Raf-1 which activates MAP kinase through MAP kinase kinase. This pathway appears to be common among various growth factors, cytokines and serum, and plays an important role in survival, proliferation and differentiation of cells. The previous report suggested that the Ras pathway is dispensable for proliferation of hematopoietic cells [28]. However, our results provide two lines of evidence that the Ras pathway plays an important role in proliferation of hematopoietic cells driven by IL-3/GM-CSF. First, BaF/$\alpha\beta_{544}$ or BaF/$\alpha\beta_{517}$ lacking the ability to activate the Ras pathway in response to hGM-CSF did not proliferate in serum-free condition (Fig. 1). Second, antisense oligonucleotide against c-*raf* blocked proliferation of BaF3 cells (Fig. 3) and BaF/$\alpha\beta_c$ (data not shown). We have further demonstrated that serum alleviates the requirement of GM-CSF-induced activation of the Ras pathway (Fig. 2). However, it should be noted that serum may activate the Ras pathway not only through Ras, but also other signaling molecules which may directly activate Raf-1, MAP kinase kinase, or MAP kinase. At present it is not known what component of serum activates the Ras pathway in BaF3 cells and BaF3 transfectants.

While Raf-1 is required for proliferation of BaF3 cells (Fig. 3) and IL-3-dependent FDC-P1 cells [31], the activated Raf-1 did not abrogate the IL-3 requirement of FDC-P1 and 32D cells [35]. We have also confirmed that v-*raf* did not abrogate the IL-3 requirement of BaF3 cells (data not shown). Thus the Ras pathway is necessary, but not sufficient for proliferation of hematopoietic cell lines. Expression of c-*myc* is induced by hGM-CSF in BaF/$\alpha\beta_{544}$ and BaF/$\alpha\beta_{517}$, but hGM-CSF did not stimulate proliferation of these cells in serum-free condition, indicating that the signals induced by the membrane proximal region of β_c (the Myc pathway) is also not sufficient for proliferation. Shibuya et al. demonstrated that constitutive expression of c-*myc* was not sufficient for proliferation of BaF3 cells [36]. In combination with v-*raf*, v-*myc* was able to induce factor-independent growth of myeloid cell lines [35]. Thus, like fibroblasts, the Ras pathway and the Myc pathway cooperatively stimulate cell proliferation of hematopoietic cells.

In the previous paper we described that BaF/$\alpha\beta_{517}$ proceeded the G_1 to S phase transition in response to hGM-CSF even in the absence of serum [26]. However, the transition was significantly slower compared to BaF/$\alpha\beta_c$. Furthermore, these cells did not proliferate in the absence of serum (Fig. 1). Although Myc has been implicated in DNA synthesis, controversial results were also reported. Shibuya et al. suggested that Myc plays a role in S to G_2/M transition in BaF3 cells [36]. TGFβ1 is known to arrest cell cycle at G_1 phase in several cell lines by regulating cyclin-dependent kinases [37], and interestingly, this G_1 arrest by TGFβ1 is counteracted by microinjection of oncogenic Ras [38]. Thus, it is possible that the Ras pathway plays an important role in regulation of the transition from G_1 to S phase. To understand the role of the Ras pathway in cytokine-mediated cell proliferation it is necessary to identify the ultimate targets through this pathway.

Acknowledgements: We are grateful to Drs. A. Miyajima, Y. Minami, T. Kinoshita, N. Sato and D. Wylie for helpful discussion and critical reading of the manuscript.

References

[1] Miyajima, A., Mui, A.L.-F., Ogorochi, T. and Sakamaki, K. (1993) Blood 82, 1960–1974.
[2] Morla, A.O., Schreurs, J., Miyajima, A. and Wang, J.Y.J. (1988) J. Mol. Cell. Biol. 8, 2214–2218.
[3] Isfort, R.J. and Ihle, J.N. (1990) Growth Factors 2, 213–220.
[4] Kanakura, Y., Druker, B., Cannistra, S.A., Furukawa, Y., Torimoto, Y. and Griffin, J.D. (1990) Blood 76, 706–715.
[5] Duronio, V., Clarklewis, I., Federsppiel, B., Wieler, J.S. and Schrader, J.W. (1992) J. Biol. Chem. 267, 21856–21863.
[6] Sorensen, P., Mui, A.L.-F. and Krystal, G. (1989) J. Biol. Chem. 264, 19253–19258.
[7] Sakamaki, K., Miyajima, I., Kitamura, T. and Miyajima, A. (1992) EMBO J. 11, 3541–3549.
[8] Torigoe, T., O'Connor, R., Santoli, D. and Reed, J.C. (1992) Blood 80, 617–624.
[9] Minami, Y., Kono, T., Yamada, K., Kobayashi, N., Kawahara, A., Perlmutter, R.M. and Taniguchi, T. (1993) EMBO J. 12, 759–768.
[10] Hanazono, Y., Chiba, S., Sasaki, K., Mano, H., Miyajima, A., Arai, K.-i., Yazaki, Y. and Hirai, H. (1993) EMBO J. 12, 1641–1646.
[11] Silvennoinen, O., Witthuhn, B.A., Quelle, F.W. and Cleveland, J.L. (1993) Proc. Natl. Acad. Sci. USA 90, 8429–8433.
[12] Satoh, T., Nakafuku, M., Miyajima, A. and Kaziro, Y. (1991) Proc. Natl. Acad. Sci. USA 88, 3314–3318.
[13] Duronio, V., Welham, M.J., Abraham, S., Dryden, P. and Schrader, J.W. (1992) Proc. Natl. Acad. Sci. USA 89, 1587–1591.
[14] Carroll, M.P., Clark-Lewis, I., Rapp, U.R. and May, W.S. (1990) J. Biol. Chem. 265, 19812–19817.
[15] Kanakura, Y., Druker, B., Wood, K.W., Mamon, H.J., Okuda, K., Roberts, T.M. and Griffin, J.D. (1991) Blood 77, 243–248.
[16] Miyazawa, K., Hendrie, P.C., Mantel, C., Wood, K., Ashman, L.K. and Broxmeyer, H.E. (1991) Exp. Hematol. 19, 1110–1123.

[17] Welham, M.J., Duronio, V., Sanghera, J.S., Pelech, S.L. and Schrader, J.W. (1993) J. Immunol. 149, 1683–1693.
[18] Okuda, K., Sanghera, J.S., Pelech, S.L., Kanakura, Y., Hallek, M., Griffin, J.D. and Druker, B.J. (1992) Blood 79, 2880–2887.
[19] Conscience, J.F., Verrier, B. and Martin, G. (1986) EMBO J. 5, 317–323.
[20] Palacios, R. and Steinmetz, M. (1985) Cell 41, 727–734.
[21] Hatakeyama, M., Mori, H., Doi, T. and Taniguchi, T. (1989) Cell 59, 837–845.
[22] D'Andrea, A.D., Yoshimura, A., Youssoufian, H., Zon, L.I., Koo, J.-W. and Lodish, H.F. (1991) Mol. Cell. Biol. 11, 1980–1987.
[23] Murakami, M., Narazali, M., Hibi, M., Yawata, H., Yasukawa, K., Hamaguchi, M., Taga, T. and Kishimoto, T. (1991) Proc. Natl. Acad. Sci. USA 88, 11349–11353.
[24] Fukunaga, R., Ishizaka-Ikeda, E., Pan, C.-X., Seto, Y. and Nagata, S. (1991) EMBO J. 10, 2855–2865.
[25] Harada, N., Yang, G., Miyajima, A. and Howard, M. (1992) J. Biol. Chem. 267, 22752–22758.
[26] Sato, N., Sakamaki, K., Terada, N., Arai, K.-i. and Miyajima, A. (1993) EMBO J. 12, 4181–4189.
[27] Taniguchi, T. and Minami, Y. (1993) Cell 73, 5–8.
[28] Satoh, T., Minami, Y., Kono, T., Yamada, K., Kawahara, A., Taniguchi, T. and Kaziro, Y. (1992) J. Biol. Chem. 267, 25423–25427.
[29] Cormier, F., Ponting, I.L., Heyworth, C.M. and Dexter, T.M. (1991) Growth Factors 4, 157–164.
[30] Kitamura, T., Sato, N., Arai, K.-i. and Miyajima, A. (1991) Cell 66, 1165–1174.
[31] Carroll, M.P., Spivak, J.L., McMahon, M., Weich, N., Rapp, U.R. and May, W.S. (1991) J. Biol. Chem. 266, 14964–14969.
[32] Kitamura, T., Hayashida, K., Sakamaki, K., Yokota, T., Arai, K.-i. and Miyajima, A. (1991) Proc. Natl. Acad. Sci. USA 88, 5082–5086.
[33] Crews, C.M. and Erikson, R.L. (1993) Cell 74, 215–217.
[34] Nishida, E. and Gotoh, Y. (1993) Trends. Biochem. Sci. 18, 128.
[35] Rapp, U.R. (1991) Oncogene 6, 495–500.
[36] Shibuya, H., Yoneyama, M., Ninomiya-Tuji, J., Matsumoto, K. and Taniguchi, T. (1992) Cell 70, 57–67.
[37] Ewen, M.E., Sluss, H.K., Whitehouse, L.L. and Livingston, D.M. (1993) Cell 74, 1009–1020.
[38] Howe, P.H., Dobrowolski, S.F., Reddy, K.B. and Stacey, D.W. (1993) J. Biol. Chem. 268, 21448–21452.