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c-*fps/fes* protein-tyrosine kinase is implicated in a signaling pathway triggered by granulocyte-macrophage colony-stimulating factor and interleukin-3

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Granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-3 (IL-3) are hematopoietic growth factors which stimulate the proliferation and differentiation of myeloid progenitor cells. There is a considerable degree of overlap in target cell specificity and the functional effects of GM-CSF and IL-3. GM-CSF and IL-3 induce a nearly identical pattern of protein-tyrosine phosphorylation in certain cell lines, although their receptors have no kinase domains. Furthermore, their receptor complexes share one subunit (designated as β). These observations raise the possibility that GM-CSF and IL-3 have a common signaling pathway. Here we show that both GM-CSF and IL-3 induce tyrosine phosphorylation and kinase activity of the c-*fps/fes* proto-oncogene product (p92$^{c\text{-}fes}$), a non-receptor protein-tyrosine kinase, in a human erythroleukemia cell line, TF-1, which requires GM-CSF or IL-3 for growth. In addition, GM-CSF induces physical association between p92$^{c\text{-}fes}$ and the β chain of the GM-CSF receptor. p92$^{c\text{-}fes}$ is therefore a possible signal transducer of several hematopoietic growth factors including GM-CSF and IL-3 through the common β chain.

Key words: c-*fps/fes* protein-tyrosine kinase/common β chain/granulocyte-macrophage colony-stimulating factor/interleukin-3/signal transduction

Introduction

Granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-3 (IL-3) are potent hematopoietic growth factors which stimulate the proliferation and differentiation of various lineages of hematopoietic cells including multipotent stem cells and granulocyte- and macrophage-progenitor cells (Curtis *et al.*, 1991; Gasson, 1991). The high-affinity receptors for GM-CSF and IL-3 are composed of two subunits designated as α and β (Hayashida *et al.*, 1990; Kitamura *et al.*, 1991). Although the α chains are specific for each factor (Gearing *et al.*, 1989; Kitamura *et al.*, 1991), the β chain is shared by the two receptors (Kitamura *et al.*, 1991). It is now assumed that it is the β chain that is essential for transducing GM-CSF and IL-3 growth signals (Lopez *et al.*, 1992; Sakamaki *et al.*, 1992).

The α chains of the GM-CSF and IL-3 receptors and the common β chain are all members of the cytokine receptor superfamily which have no homology with known enzymes involved in receptor-mediated signal transduction such as protein-tyrosine kinases, protein-serine/threonine kinases or GTPase activating proteins (Bazan, 1989; D'Andrea *et al.*, 1989). The molecular events that are activated following the binding of GM-CSF and IL-3 to cell surface receptors are still unknown. However, it has been shown that activation of cytoplasmic protein-tyrosine kinases is crucial in transmitting intracellular signals through cell surface receptors that contain no kinase domains. For instance, CD4/CD8 (Rudd *et al.*, 1988; Veillette *et al.*, 1988), T-cell antigen receptor (Samelson *et al.*, 1990) and B-cell surface immunoglobulin M (Yamanashi *et al.*, 1990) are associated with the *src*-family protein-tyrosine kinases, p56^{lck}, p59^{fyn} and p56^{lyn}, respectively, and these kinases are considered to be implicated in their signal transduction. Notably, the interleukin-2 (IL-2) receptor β chain, a member of the cytokine receptor superfamily, is associated with p56^{lck} (Hatakeyama *et al.*, 1991). GM-CSF and IL-3 also induce rapid tyrosine-phosphorylation of cellular proteins (Koyasu *et al.*, 1987; Isfort *et al.*, 1988; Morla *et al.*, 1988; Sorensen *et al.*, 1989; Kanakura *et al.*, 1990; Quelle *et al.*, 1992). Therefore, receptor activation by the ligands should induce tyrosine-kinase activity of unidentified cellular protein(s). It seems reasonable to expect that that some protein-tyrosine kinase(s) may also be associated with the GM-CSF and IL-3 receptors.

A number of non-receptor protein-tyrosine kinases are expressed in hematopoietic cells. Among these, the c-*fps/fes* proto-oncogene product is found to be expressed exclusively in myeloid progenitor cells (Feldman *et al.*, 1985; MacDonald *et al.*, 1985). Unlike the *src*-related protein-tyrosine kinases, c-*fps/fes* encodes a larger product of 92 kDa in mammals and lacks post-translational myristylation at its N-terminus (Roebroek *et al.*, 1985; Alcalay *et al.*, 1990). The biological function of c-*fps/fes* in hematopoietic cells still remains unknown.

Here we show that both GM-CSF and IL-3 induce tyrosine phosphorylation and kinase activity of the c-*fps/fes* product (p92$^{c\text{-}fes}$) in a human erythroleukemia cell line, TF-1. In addition, we demonstrate that GM-CSF induces physical association between p92$^{c\text{-}fes}$ and the β chain of the GM-CSF receptor (GM-CSFRβ).

Results

GM-CSF and IL-3 induce tyrosine phosphorylation of p92 in a time- and dose-dependent manner

TF-1 cells require GM-CSF or IL-3 for growth, and they die within several days when deprived of GM-CSF and IL-3, even in medium supplemented with fetal calf serum

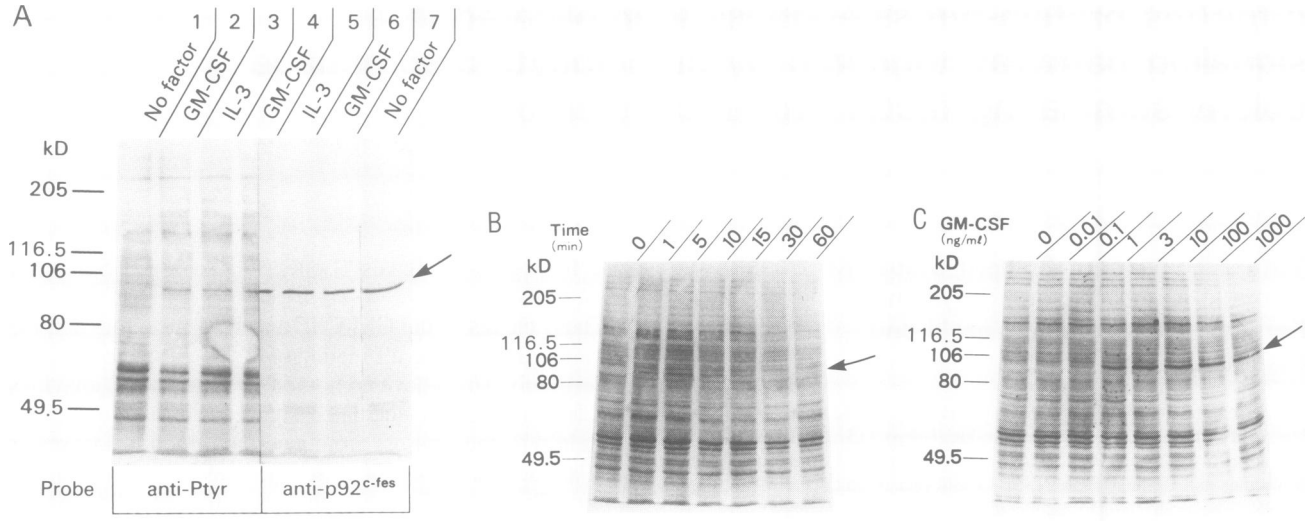

Fig. 1. (**A**) Western blot analysis of phosphotyrosine-containing proteins (lanes 1–4) and p92$^{c\text{-}fes}$ (lanes 4–8) in GM-CSF- or IL-3-treated TF-1 cells. Lane 4 was cut in the middle of the lane; the left half was immunoblotted with anti-Ptyr (PY20) and the right half with anti-p92$^{c\text{-}fes}$ (F115). (**B**) Time dependency of protein-tyrosine phosphorylation in TF-1 cells. Lysates of TF-1 cells treated with 10 ng/ml GM-CSF for the indicated times were analyzed by Western blotting with anti-Ptyr (PY20). (**C**) Dose dependency of protein-tyrosine phosphorylation in TF-1 cells. Lysates of TF-1 cells treated with GM-CSF at the indicated concentrations for 5 min were analyzed by Western blotting with anti-Ptyr (PY20).

(Kitamura *et al.*, 1989). We analyzed tyrosine-phosphorylated proteins in TF-1 cells treated with GM-CSF or IL-3. As shown in Figure 1A, a 92 kDa protein (p92) was tyrosine-phosphorylated upon the addition of GM-CSF and IL-3. p92 was tyrosine-phosphorylated within 1 min of GM-CSF treatment of the cells at 37°C, with maximum levels of phosphorylation attained at 5 min, and dephosphorylated after 30 min (Figure 1B). Tyrosine phosphorylation of p92 was dose-dependent and the phosphorylation occurred at physiological concentrations of GM-CSF (Figure 1C). We obtained similar results when cells were treated with IL-3 (data not shown).

To investigate whether p92 is any of the known tyrosine kinases, we compared the mobility of p92 with that of p92$^{c\text{-}fes}$. On Western blot analysis, the mobility of p92 probed with a mouse monoclonal anti-phosphotyrosine antibody (anti-Ptyr) PY20 was identical to that of p92$^{c\text{-}fes}$ probed with a rat monoclonal anti-p92$^{c\text{-}fes}$ antibody F115 (Veronese *et al.*, 1982) (Figure 1A). Although a distinct 94 kDa protein (p94$^{c\text{-}fer}$), antigenically related to p92$^{c\text{-}fes}$, has been identified in a number of hematopoietic and non-hematopoietic human cells (MacDonald *et al.*, 1985; Feldman *et al.*, 1986), F115 antibody did not recognize p94$^{c\text{-}fer}$ (data not shown).

GM-CSF and IL-3 induce tyrosine phosphorylation and kinase activity of p92$^{c\text{-}fes}$

To determine whether p92 is p92$^{c\text{-}fes}$, we purified p92$^{c\text{-}fes}$ immunologically using the antibody F115 and tested the phosphorylation level of the protein by Western blotting with the antibody PY20. p92$^{c\text{-}fes}$ was induced to be tyrosine-phosphorylated by treatment with GM-CSF or IL-3 (Figure 2), whereas the amount of p92$^{c\text{-}fes}$ was not affected (Figure 1A).

We then evaluated the effect of GM-CSF and IL-3 on the kinase activity of p92$^{c\text{-}fes}$ in TF-1 cells. The factor-starved cells were incubated for 5 min in the presence or absence of GM-CSF or IL-3. The cell lysates were subjected to immunoprecipitation with polyclonal anti-p92$^{c\text{-}fes}$ antibody and the *in vitro* immune-complex kinase assay detected phosphorylation of p92$^{c\text{-}fes}$ induced by GM-CSF or IL-3 (Figure 3).

These results show that GM-CSF and IL-3 induced tyrosine phosphorylation and kinase activity of p92$^{c\text{-}fes}$ in TF-1 cells. As shown in Figure 3, several bands other than that corresponding to p92$^{c\text{-}fes}$ appear to be phosphorylated in response to GM-CSF and IL-3. It is presently unknown whether these bands represent specific proteins associated with p92$^{c\text{-}fes}$ or just non-specific phosphorylated substrates.

Physical association between GM-CSFRβ and p92$^{c\text{-}fes}$

GM-CSF binds to specific cell-surface receptors consisting of two subunits designated α and β (Chiba *et al.*, 1990a; Hayashida *et al.*, 1990). The α chain binds to GM-CSF with low affinity, whereas the β chain does not bind to GM-CSF by itself. The α and β chains together form the high-affinity GM-CSF receptor. It is now assumed that GM-CSFRβ transmits the signal for proliferation (Lopez *et al.*, 1992; Sakamaki *et al.*, 1992). We then looked to ascertain the physical association between GM-CSFRβ and p92$^{c\text{-}fes}$.

Factor-starved TF-1 cells were incubated for 5 min in the presence or absence of GM-CSF or IL-3. The cell lysates were mixed with polyclonal anti-GM-CSFRβ antibody and the immunoprecipitates were immunoblotted with PY20 antibody. The β chain itself was revealed to be tyrosine-phosphorylated, indicating the direct interaction between the β chain and certain protein-tyrosine kinase(s) (Figure 4A). In addition, p92 was co-immunprecipitated with GM-CSFRβ by treatment with GM-CSF and IL-3 (Figure 4A). The immunoprecipitates with anti-GM-CSFRβ were then immunoblotted with F115 antibody (Figure 4B). p92$^{c\text{-}fes}$ was shown to be co-immunoprecipitated with GM-CSFRβ by treatment with GM-CSF, whereas it could not be co-immunoprecipitated with GM-CSFRβ without this treatment. This result suggests that binding of the ligand to the GM-CSF receptor induces physical association between GM-CSFRβ and p92$^{c\text{-}fes}$. The major bands in Figure 4A and B with molecular weights of ~55 kDa represent the immuno-

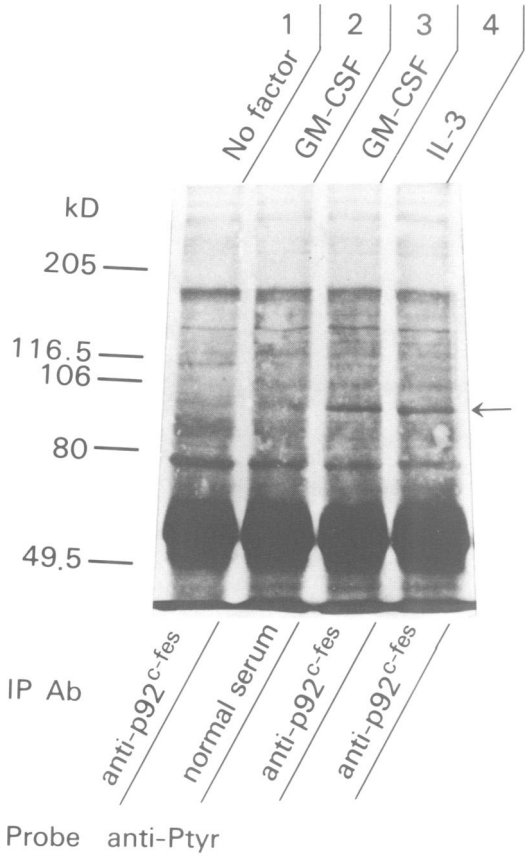

Fig. 2. GM-CSF and IL-3 induce tyrosine phosphorylation of p92$^{c\text{-}fes}$. Lysates of untreated (lane 1), GM-CSF-treated (lanes 2 and 3) and IL-3-treated (lane 4) TF-1 cells were mixed with anti-p92$^{c\text{-}fes}$ (F115) (lanes 1, 3 and 4) or normal serum (lane 2). The immunoprecipitates were collected with protein A−Sepharose, subjected to 7% SDS−PAGE and immunoblotted with anti-Ptyr (PY20).

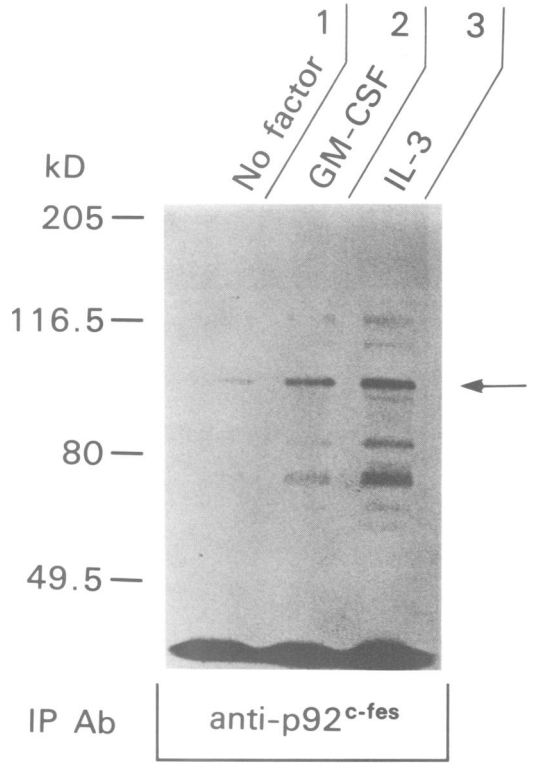

Fig. 3. The effect of GM-CSF and IL-3 on the kinase activity of p92$^{c\text{-}fes}$. Immunoprecipitates with polyclonal anti-p92$^{c\text{-}fes}$ antibody from lysates of untreated (lane 1), GM-CSF-treated (lane 2) and IL-3-treated (lane 3) TF-1 cells were subjected to an *in vitro* kinase assay.

globulin heavy chain detected due to the cross-reaction of secondary alkaline phosphatase-conjugated antibodies.

To analyze conversely whether GM-CSFRβ could be co-immunoprecipitated with anti-p92$^{c\text{-}fes}$ antibody, we cross-linked radiolabeled GM-CSF to its receptor on TF-1 cells in order to label the GM-CSF receptor. If the β chain is associated with p92$^{c\text{-}fes}$, it should be detected on autoradiography when the cross-linked sample is subjected to immunoprecipitation with an anti-p92$^{c\text{-}fes}$ antibody (Figure 5A). TF-1 cells showed two cross-linked proteins at 95 and 150 kDa on autoradiography (Figure 5B, lane 1). Since the molecular weight of human GM-CSF is 15 kDa, the 95 kDa band represents the complex of GM-CSF and the GM-CSF receptor α chain (80 kDa), and the 150 kDa band represents the complex of GM-CSF and GM-CSFRβ (135 kDa) (Chiba *et al.*, 1990b). When the cross-linked sample was subjected to immunoprecipitation with F115 antibody, only the β chain was detected (Figure 5B, lane 4). As a control, when the cross-linked sample was subjected to immunoprecipitation with anti-GM-CSF antibody, both the α and β chains were detected (Figure 5B, lane 3).

From these observations, we conclude that p92$^{c\text{-}fes}$ is physically associated with the β chain of the GM-CSF receptor and that this association is induced by ligand binding. It is likely that p92$^{c\text{-}fes}$ is associated with the β chain by treatment with IL-3 as well, since the two receptors are considered to share the β chain (Kitamura *et al.*, 1991).

Discussion

In this study, we have demonstrated that p92$^{c\text{-}fes}$ is activated by stimulation with GM-CSF and IL-3, and that p92$^{c\text{-}fes}$ becomes associated with GM-CSFRβ upon stimulation with GM-CSF. A number of investigators have noted that GM-CSF and IL-3 induce tyrosine phosphorylation of a protein, or proteins, of ~90−100 kDa (Morla *et al.*, 1988; Sorensen *et al.*, 1989; Kanakura *et al.*, 1990; Quelle *et al.*, 1992). The identity of these phosphotyrosine-containing proteins is a great concern in the field of cytokine research. We have shown that one such protein is p92$^{c\text{-}fes}$. The biological function of the hematopoietic cell-specific c-*fps/fes* protein-tyrosine kinase has been searched for many years. The data in this paper provide a possible clue as to the function of c-*fps/fes* in hematopoietic cells. Similarly, the mechanism by which cytokine receptors transduce signals is an important issue. We have identified the c-*fps/fes* product as a protein-tyrosine kinase implicated in a signaling pathway triggered by GM-CSF and IL-3. However, it should be noted that the c-*fps/fes* product may be only one of the protein-tyrosine kinases involved in the signal transduction of GM-CSF and IL-3, like IL-2 which can regulate the activity of more than one member of protein-tyrosine kinases (p56^{lck} and p56^{lyn}) (Torigoe *et al.*, 1992).

Our results are consistent with previous reports which suggested the possible involvement of p92$^{c\text{-}fes}$ in the signaling pathway in myeloid cells. First, expression of p92$^{c\text{-}fes}$ at relatively high levels is restricted to human hematopoietic cell lineages including granulocyte−macrophage progenitor cells and erythroleukemia cell lines which retain the capacity to differentiate into erythroid or

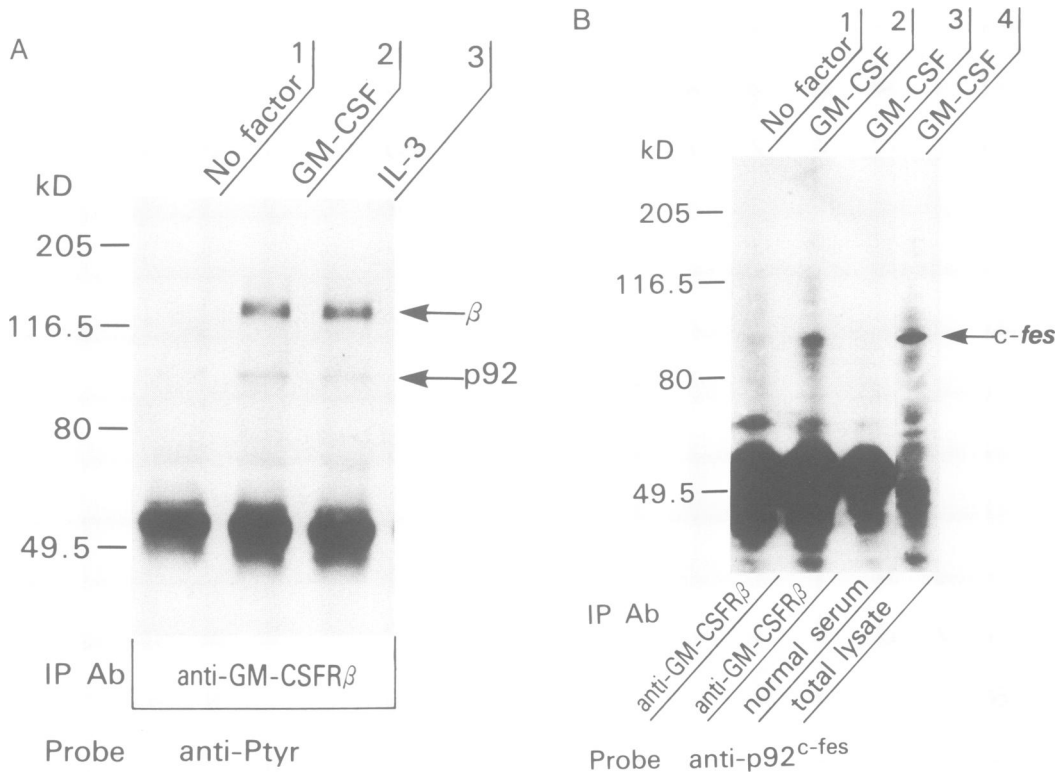

Fig. 4. Co-immunoprecipitation of p92$^{c\text{-}fes}$ with anti-GM-CSFRβ antibody. Cell lysates of TF-1 treated or untreated with GM-CSF or IL-3 were subjected to immunoprecipitation with anti-GM-CSFRβ antibody and the immunoprecipitates were immunoblotted with anti-Ptyr (PY20) (A) and anti-p92$^{c\text{-}fes}$ (F115) (B). (**A**) Lysates of untreated (lane 1), GM-CSF-treated (lane 2) and IL-3-treated (lane 3) TF-1 cells were mixed with a polyclonal anti-GM-CSFRβ antibody. Immunoprecipitates were collected with protein A−Sepharose, subjected to SDS−PAGE and immunoblotted with PY20 antibody. (**B**) Untreated (lane 1) or GM-CSF-treated (lanes 2−4) TF-1 cells were lysed in lysis buffer (20 mM Tris−HCl pH 8.0, 1% digitonin, 50 mM NaF, 500 U/ml aprotinin, 1 mM PMSF, 2 mM EDTA and 1 mM Na_3VO_4) and subjected to immunoprecipitation with a polyclonal anti-GM-CSFRβ antibody. The immune complexes were collected using protein A−Sepharose, and the associated proteins were eluted with RIPA (50 mM Tris pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid and 0.1% SDS), subjected to SDS−PAGE and immunoblotted with F115 antibody. A total lysate of GM-CSF-treated TF-1 cells was also applied (lane 4).

macrophage-like cells (Feldman *et al.*, 1985; MacDonald *et al.*, 1985), and the distribution of the c-*fps/fes* expression is very similar to that of the GM-CSF receptor. Second, chicken myeloid stem cells infected with retroviruses carrying the v-*fps* oncogene do not require exogenous growth factors for *in vitro* differentiation (Carmier and Samarut, 1986). Third, the expression of c-*fps/fes*, its tyrosine kinase activity and the binding of GM-CSF are coordinately increased in human myeloid leukemia HL-60 cells when they are induced by dimethyl sulfoxide to undergo granulocytic differentiation (Smithgall *et al.*, 1988). Finally, human erythroleukemia K562 cells, which do not express the c-*fps/fes* gene and also do not respond to GM-CSF, acquire the ability to undergo myeloid differentiation when transfected with the human c-*fps/fes* gene (Yu *et al.*, 1989).

We have also shown that GM-CSF and IL-3 induce tyrosine phosphorylation of the β chain of their receptors. It has been reported that the IL-2 receptor β chain (Hatakeyama *et al.*, 1991) and the erythropoietin receptor (Dusanter-Fourt *et al.*, 1992) are also tyrosine-phosphorylated by ligand binding. For the IL-2 receptor, the β chain is assumed to be phosphorylated by p56^{lck} which associates with the β chain in its tyrosine-kinase domain (Hatakeyama *et al.*, 1991). Whether the β chain of the GM-CSF and IL-3 receptors is phosphorylated by the associated p92$^{c\text{-}fes}$ is presently unknown.

The c-*fps/fes* proto-oncogene has been captured many times from acutely transforming RNA tumor viruses (Snyder and Theilen, 1969; Gardner *et al.*, 1970; Hanafusa *et al.*, 1980; Lee *et al.*, 1980). All of these viruses involve the N-terminal modification of the c-*fps/fes* product by fusion with viral *gag* sequences (Hampe *et al.*, 1982; Shibuya and Hanafusa, 1982). This fusion with *gag* at the N-terminus results in activation of its enzymatic activity and in unmasking of its oncogenic potential, suggesting that the N-terminal region is a regulatory domain which may interact with certain cellular protein(s) (Foster *et al.*, 1985; Feldman *et al.*, 1987). It is therefore possible that the N-terminal region of p92$^{c\text{-}fes}$ is used for the association with GM-CSFRβ and that p92$^{c\text{-}fes}$ then becomes activated. Further experiments are needed to elucidate the site of interaction.

It has been shown that the receptor for interleukin-5 (IL-5) also shares the same β chain (Tavernier *et al.*, 1991), and, therefore, it is likely that p92$^{c\text{-}fes}$ also becomes associated with the β chain upon stimulation with IL-5 in cells expressing the IL-5 receptor. If this is the case, expression of the α chain of each receptor may determine the specificity of response to a growth factor. Our observations, combined with previous experimental evidence, suggest an involvement of the c-*fps/fes* product in a signaling system for GM-CSF, IL-3 and IL-5 through their receptors, common β chain.

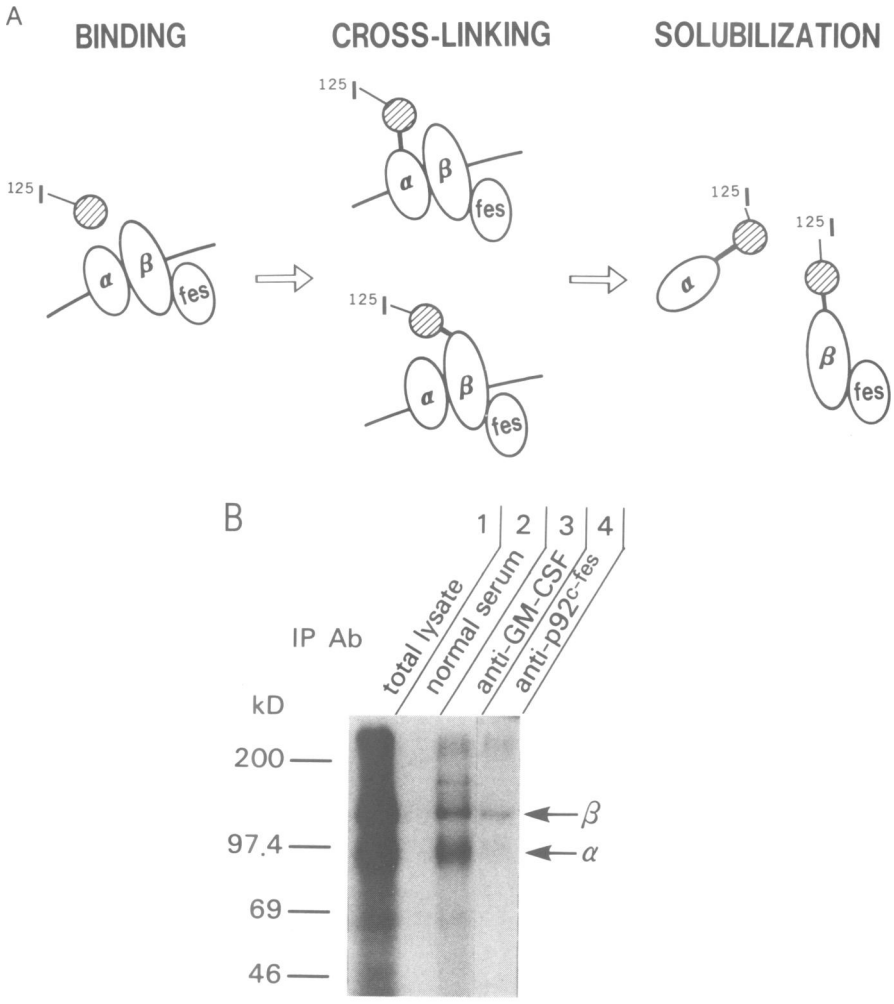

Fig. 5. Co-immunoprecipitation of GM-CSFRβ with anti-p92$^{c\text{-}fes}$ antibody. (**A**) Affinity labeling of a cell surface receptor by ^{125}I-labeled GM-CSF. [^{125}I]GM-CSF is cross-linked to the α or β chain of the GM-CSF receptor. If the β chain is associated with p92$^{c\text{-}fes}$, it can be detected on autoradiography when the cross-linked sample is subjected to immunoprecipitation with an anti-p92$^{c\text{-}fes}$ antibody. (**B**) The β chain was co-immunoprecipitated with anti-p92$^{c\text{-}fes}$ (F115). The cross-linked samples were mixed with normal rabbit serum (lane 2), polyclonal anti-GM-CSF antibody (lane 3) or F115 antibody (lane 4). The immunoprecipitates were collected using protein A−Sepharose and then subjected to SDS−PAGE and autoradiography. A total lysate of the cross-linked sample was also applied (lane 1).

Materials and methods

Cell lines and growth factors

TF-1 cells were maintained in RPMI medium 1640 containing 10% fetal calf serum (FCS) and 5 ng/ml GM-CSF. Recombinant human GM-CSF was supplied by Kirin Brewery Co. Ltd (Tokyo, Japan). Recombinant human IL-3 was provided by Schering Plough Co. Ltd. (Osaka, Japan).

Antibodies

Polyclonal anti-p92$^{c\text{-}fes}$ antibody was prepared from serum of a rabbit immunized against a synthetic peptide which was conjugated with keyhole limpet hemocyanin (KLH) (Pierce). The peptide sequence was LLLQDDRHSTSSSEQEREGG, corresponding to amino acid residues 424−443 of p92$^{c\text{-}fes}$, an upstream region of the SH2 domain (Alcalay *et al.*, 1990).

Polyclonal anti-GM-CSFRβ antibody was prepared from serum of a rabbit immunized against a synthetic peptide which was conjugated with KLH. The peptide sequence was ELPPIEGRSPRSPRNNPVPPE corresponding to amino acid residues 769−789 of KH97, the cytoplasmic domain near the C-terminus (Hayashida *et al.*, 1990).

Polyclonal anti-GM-CSF antibody was prepared from serum of a rabbit immunized against recombinant human GM-CSF (Chiba *et al.*, 1990b).

F115 antibody is a rat monoclonal anti-p92$^{c\text{-}fes}$ antibody (Oncogene Science). F115 antibody recognizes a region within the kinase domain and it blocks the kinase activity (Veronese *et al.*, 1982).

PY20 antibody is a mouse monoclonal anti-phosphotyrosine antibody (ICN).

Preparation of cell lysates

Cells were incubated in RPMI medium 1640 containing 0.1% bovine serum albumin (BSA) without FCS or growth factors for 8−15 h prior to stimulation with growth factors and then resuspended in RPMI medium 1640 containing 100 μM Na_3VO_4. The cells were treated with 10 ng/ml GM-CSF or 10 ng/ml IL-3 for 5 min at 37°C unless otherwise specified and then lysed at 4°C in lysis buffer [20 mM Tris−HCl pH 8.0, 150 mM NaCl, 1% Nonidet P-40 (NP-40), 1 mM phenylmethylsulfonyl fluoride (PMSF), 500 U/ml aprotinin, 2 mM EDTA, 50 mM NaF and 1 mM Na_3VO_4]. Unsolubilized materials were removed by centrifugation for 10 min at 15 000 *g* at 4°C.

Immunoprecipitation

To immunoprecipitate p92$^{c\text{-}fes}$, cell lysates were mixed with a polyclonal anti-p92$^{c\text{-}fes}$ antibody, or F115 antibody (rat IgM) with a secondary rabbit anti-rat IgM (Fc) antibody (Nordic). To immunoprecipitate GM-CSFRβ, cell lysates were mixed with polyclonal anti-GM-CSFRβ antibody. The immune complexes were collected using protein A−Sepharose (Sigma). All of the immunoprecipitates were intensively washed with the lysis buffer before resuspension in Laemmli's sample buffer.

Western blotting

Samples were subjected to 7% SDS−PAGE and electrotransferred onto polyvinylidene difluoride (PVDF) filters (Millipore). Filters were blocked with buffer containing 10 mM Tris−HCl pH 8.0, 150 mM NaCl, 3% BSA and 0.05% Triton X-100. For Western blotting for phosphotyrosine-

containing proteins, filters were incubated with PY20 antibody and then with goat alkaline phosphatase-conjugated anti-mouse IgG (Fc) antibody (Promega). For Western blotting for $p92^{c\text{-}fes}$, filters were sequentially incubated with F115 antibody (rat IgM), rabbit anti-rat IgM (Fc) antibody (Nordic) and an alkaline phosphatase-conjugated goat anti-rabbit IgG (Fc) antibody (Promega). After each incubation, filters were washed four times in the buffer containing 10 mM Tris−HCl pH 8.0, 150 mM NaCl and 0.05% Triton X-100. The color reaction was performed using nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) (Promega).

Kinase assays

Cell lysates were mixed with polyclonal anti-$p92^{c\text{-}fes}$ antibody and immune complexes were collected using protein A−Sepharose and suspended in kinase buffer (25 mM HEPES pH 7.5, 0.1% NP-40 and 3 mM $MnCl_2$). After addition of 10 μCi [γ-^{32}P] ATP, the mixture was incubated for 15 min at room temperature and subjected to 7% SDS−PAGE. Phosphorylated proteins were detected by Fujix BAS 200 Bio-image Analyzer (Fuji Film Co. Ltd).

Radioiodination and cross-linking

Radioiodination of GM-CSF was performed using Bolton−Hunter reagent (ICN) as described previously (Chiba *et al.*, 1990a). The binding characteristics and biological activities of [^{125}I]GM-CSF were fully retained.

Factor-starved TF-1 cells were incubated in binding buffer (α-modified Dulbecco's medium, 50 mM HEPES pH 7.4, 0.1% BSA and 0.02% NaN_3) with 3 nM [^{125}I]GM-CSF for 90 min on ice. After binding, the cells were pelleted down at 1500 *g* and resuspended in the same volume of phosphate-buffered saline; disuccinimidyl suberate (DSS) was immediately added at a final concentration of 400 μM. After a 20 min incubation at 4°C, the reaction was quenched by adding quenching buffer (10 mM Tris pH 7.5, 1 mM EDTA and 150 mM NaCl). The cells were centrifuged at 12 000 *g* for 30 s at 4°C and resuspended in lysis buffer (50 mM HEPES pH 7.4, 1% Triton X-100, 1 mM PMSF, 200 U/ml aprotinin, 1 mM EDTA, 50 mM NaF and 1 mM Na_3VO_4). After a 20 min incubation on ice, the detergent-soluble fractions were separated by centrifugation at 12 000 *g* for 10 min at 4°C.

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