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Differentiation and growth arrest signals are generated through the cytoplasmic region of gp130 that is essential for Stat3 activation

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Interleukin-6 (IL-6) induces growth arrest and macrophage differentiation through its receptor in a murine myeloid leukaemic cell line, M1, although it is largely unknown how the IL-6 receptor generates these signals. By using chimeric receptors consisting of the extracellular domain of growth hormone receptor and the transmembrane and cytoplasmic domain of gp130 with progressive C-terminal truncations, we showed that the membrane-proximal 133, but not 108, amino acids of gp130 could generate the signals for growth arrest, macrophage differentiation, down-regulation of c-*myc* and c-*myb*, induction of *junB* and *IRF1* and Stat3 activation. Mutational analysis of this region showed that the tyrosine residue with the YXXQ motif was critical not only for Stat3 activation but also for growth arrest and differentiation, accompanied by down-regulation of c-*myc* and c-*myb* and immediate early induction of *junB* and *IRF1*. The tight correlation between Stat3 activation and other IL-6 functions was further observed in the context of the full-length cytoplasmic region of gp130. The results suggest that Stat3 plays an essential role in the signals for growth arrest and differentiation.

Keywords: differentiation/gp130/interleukin-6/M1/Stat 3

Introduction

Cytokines play important roles in cellular development, homeostasis and self-defence mechanisms. Cytokines exert pleiotropic functions through their receptors in a variety of cells. The functional redundancy observed in many cytokines could be explained, at least in part, by the sharing of receptor subunits (Miyajima *et al.*, 1993; Hirano *et al.*, 1994; Taniguchi, 1995). However, it is largely unknown how cytokines exert multiple functions through their receptors.

Interleukin-6 (IL-6) is a typical cytokine exerting multiple biological functions through its receptor in a variety of cells (Sehgal *et al.*, 1989; Van Snick, 1990; Hirano, 1992). IL-6 induces the differentiation of B cells to antibody-producing cells (Teranishi *et al.*, 1982; Hirano *et al.*, 1986) and the production of acute-phase proteins in hepatocytes (Andus *et al.*, 1987; Gauldie *et al.*, 1987). IL-6 induces the growth of myelomas and plasmacytomas (Aaden *et al.*, 1985; Nordan and Potter, 1986; Kawano *et al.*, 1988), whereas it inhibits the growth of several carcinoma cells and myeloid leukaemia cells (Chen *et al.*, 1988; Miyaura *et al.*, 1988; Shabo *et al.*, 1988; Oritani *et al.*, 1992). In particular, IL-6 induces both growth arrest and macrophage differentiation in myeloid leukaemia M1 cells (Miyaura *et al.*, 1988; Shabo *et al.*, 1988; Hoffman and Liebermann, 1991a; Lord *et al.*, 1991). The IL-6 receptor (IL-6R) consists of a ligand-binding α-chain and a signal transducer, gp130 (Yamasaki *et al.*, 1988; Taga *et al.*, 1989; Hibi *et al.*, 1990). gp130 is shared among the receptors for leukaemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF), oncostatin M (OSM), IL-11 and cardiotrophin-1 (CT-1), and plays an essential role in generating signals by these members of the IL-6-related cytokine subfamily (Ip *et al.*, 1992; Yin *et al.*, 1993; Hirano *et al.*, 1994; Pennica *et al.*, 1995). Human gp130 has 277 amino acid residues in its cytoplasmic domain and lacks an intrinsic protein kinase domain (Hibi *et al.*, 1990). IL-6 or IL-6-related cytokines, however, have been shown to induce the tyrosine phosphorylation of several proteins including gp130 (Nakajima and Wall, 1991; Boulton *et al.*, 1994; Matsuda *et al.*, 1994). Janus kinases Jak1, Jak2 and Tyk2 have been demonstrated to associate with gp130 constitutively (Matsuda *et al.*, 1994; Stahl *et al.*, 1994), and the binding of IL-6 to its receptor induces the homodimerization of gp130, resulting in phosphorylation of gp130 (Davis *et al.*, 1993; Murakami *et al.*, 1993). Phosphotyrosines and their surrounding sequences in gp130 recruit signal-transducing molecules containing the SH2 domain, such as the signal transducer and activator of transcription 3 (Stat3) and protein tyrosine phosphatase, PTP-1D (Stahl *et al.*, 1995).

Stat3 is a member of the STAT family of transcription factors (Akira *et al.*, 1994; Zhong *et al.*, 1994). Much evidence has been accumulated that shows that STAT family proteins play critical roles in the signal transduction pathways for a variety of cytokine receptors (Darnell *et al.*, 1994; Ihle and Kerr, 1995). Importantly, each cytokine activates different species of the STAT family proteins; IL-6 activates Stat3 and Stat1 (Bonni *et al.*, 1993; Larner *et al.*, 1993; Ruff *et al.*, 1993; Sadowski *et al.*, 1993; Silvennoinen *et al.*, 1993; Fujitani *et al.*, 1994), while interferon α activates Stat1 and Stat2 (Schindler *et al.*, 1992a,b; Darnell *et al.*, 1994). Such specific activation of STAT family proteins by each cytokine may be one of the mechanisms by which a cytokine exerts its specific activity. Although there is evidence that a number of genes are regulated by STAT proteins, including IFNα/β- and IFNγ-inducible genes (Darnell *et al.*, 1994), and IL-6-inducible genes, such as the α_2-macrogloblin, *junB* and *IRF1* genes (Wegenka *et al.*, 1993; Akira *et al.*, 1994; Fujitani *et al.*, 1994; Harroch *et al.*, 1994; Coffer *et al.*, 1995; Nakajima *et al.*,

1995), it remains to be elucidated whether STAT proteins play roles in cell differentiation and cell growth regulation.

To gain insight into the signal transduction mechanisms responsible for terminal differentiation, in this study we examined the functional regions of the cytoplasmic domain of gp130 required for growth arrest and macrophage differentiation in an M1 myeloid leukaemic cell line. This cell line has been shown to be a suitable model for elucidating the molecular mechanisms involved in cytokine-induced cell differentiation accompanied by growth arrest (Kimchi, 1992; Liebermann and Hoffman, 1994). The results of this study showed that Stat3 activation appears to be tightly linked to the induction of growth arrest as well as to macrophage differentiation.

Results

Construction and expression of GHR-gp130 chimeric receptors in M1 cells

We constructed a series of chimeric receptors that consist of the unaltered extracellular domain of growth hormone receptor (GHR) and the unaltered transmembrane domain of gp130 with various truncations and tyrosine mutations of the cytoplasmic domain of gp130 (Figure 1A). We created, by retroviral infection, transformants expressing various chimeric receptors. The expression levels of chimeric receptors were determined by Northern blot analysis and by a GH-binding assay. Neither GHR mRNA nor GH-specific binding activity was detected in the parent M1 cell. We selected at least three clones for each transformant for which the chimeric receptor showed ~1.5–4 fmol binding activity/10^6 cells under the experimental conditions described in Materials and methods (Figure 1B). Scatchard analysis of a representative clone of the GHR-277 transformant showed that it expressed ~20 000 specific GH binding sites/cell with K_d = 180 pM. This suggested that these transformants expressed several hundred-fold more ligand binding sites than the parent M1 cells, which were shown to express ~50 IL-6 binding sites per cell (Taga *et al.*, 1989). We presented the results obtained by a representative clone for each transformant in the following experiments because essentially the same results were obtained among different clones of a given transformant.

Signal transduction through the chimeric receptor carrying the full-length cytoplasmic domain of gp130 in M1 cells

To determine whether the chimeric receptor carrying the full-length cytoplasmic domain of gp130 can transduce growth arrest and differentiation signals, we assessed cell growth, morphological changes and induction of macrophage-associated genes encoding the Fcγ receptor (FcγR) and inducible nitric oxide synthase (iNOS) in these transformants upon stimulation with either IL-6 or GH. In the neo-transformant, IL-6 (but not GH) induced growth arrest in a dose-dependent manner, and caused morphological changes at 4 days following stimulation which included increased cell size, an increased cytoplasm-to-nucleus ratio, and the appearance of many granules, which is characteristic of well-differentiated macrophages (Figure 2A and B). As expected, both GH and IL-6 induced growth arrest and the morphological changes

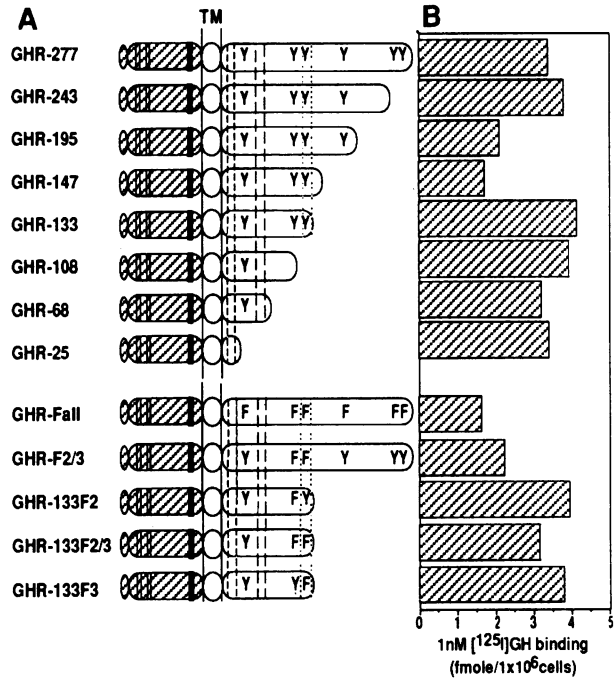

Fig. 1. Structure and expression of the GHR-gp130 chimeric receptors in M1 cells. (**A**) Structures of full-length, truncated and tyrosine-mutated chimeric receptors. Chimeric receptors are composed of the extracellular domain of GHR (hatched boxes), and the transmembrane and cytoplasmic domains of human gp130 (open boxes). The transmembrane domain is indicated as TM. Thick bars indicate the YGEFS sequence in GHR. Vertical dotted lines indicate box 1, box 2 and box 3 from TM, respectively. The chimeric receptors are named according to the last amino acid position from the membrane; residue 1 is the first amino acid of the cytoplasmic domain: full-length cytoplasmic domain (277 amino acids: GHR-277) and receptors truncated at 243 (GHR-243), 195 (GHR-195), 147 (GHR-147), 133 (GHR-133), 108 (GHR-108), 68 (GHR-68) or 25 (GHR-25) amino acids from the membrane. Human gp130 has six tyrosines (amino acid 42, 118, 126, 173, 262 and 274 from the membrane: Y) in its cytoplasmic domain. In the GHR-Fall mutant, all tyrosines in the cytoplasmic domain are replaced by phenylalanines (F). In GHR-F2/3, the second (a.a. 118) and third (a.a. 126) tyrosines are replaced by phenylalanines. In GHR-133F2, GHR-133F3 or GHR-133F2/3, the second and/or third tyrosines in GHR-133 truncation mutants are replaced by phenylalanines, respectively. (**B**) Binding of GH to the chimeric receptors in M1 transformants. Mouse myeloid leukaemia cell line M1 cells were transformed with the chimeric receptor expression vectors using a retroviral vector (Hawley *et al.*, 1994). Transformants were selected by 400 μg/ml of G418. 1×10^6 cells of each transformant were incubated with 1 nmol [^{125}I]human GH (hGH) for 4 h at 4°C and cell-bound [^{125}I]hGH was separated by centrifugation. Radioactivity was determined by a γ-counter. Indicated values are concentrations (fmol) of GH bound to 1×10^6 cells. The values are shown as averages of data obtained from duplicate assays. Variation in the duplicate assays was <10%.

in the transformant expressing the full-length chimeric receptor (GHR-277). Similarly, FcγR and iNOS mRNAs were induced in the neo-transformant by IL-6, and in the M1 transformant expressing GHR-277 by GH (Figure 2C). FcγR was detected 1–3 days after stimulation by IL-6 or GH, and the level of FcγR mRNA peaked 2 days after stimulation. iNOS mRNA was detected at 3 days after stimulation with IL-6 or GH. These results indicate that the chimeric receptor was functional and sufficient for generating signals for growth arrest and macrophage differentiation in M1 cells.

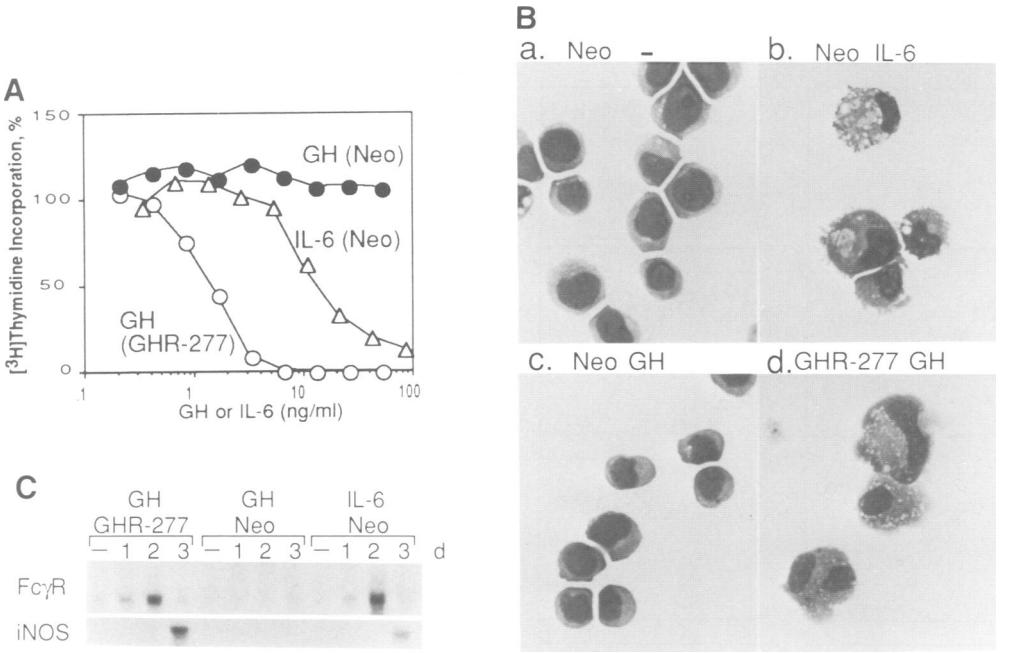

Fig. 2. Induction of macrophage differentiation and growth arrest through the GHR-gp130 chimeric receptors. (**A**) Growth arrest induced by IL-6 or GH. Cells transformed with the control vector (expression vector without inserts: Neo) and cells transformed with the GHR-277 expression vector were cultured in the absence or presence of various indicated concentrations of IL-6 or GH for 4 days, then pulsed for 4 h with 0.5 μCi/well [^{3}H]thymidine. Cells were harvested by an automated cell harvester and incorporated radioactivity was determined using a scintillation counter. [^{3}H]Thymidine incorporation was indicated as ratios (percentages) of radioactivity incorporated by stimulated cells versus radioactivity incorporated by unstimulated cells. (**B**) May–Grunwald–Giemsa (MG) staining of the transformants stimulated by IL-6 or GH. Control transformants (Neo: a, b and c) or GHR-gp130 full-length transformants (GHR-277: d) were cultured in the absence (a) or presence of IL-6 (100 ng/ml; b) or GH (50 ng/ml; c and d) for 4 days, spun down on slide glass using a cytospin (Shandon) and subjected to MG staining. Magnification of the indicated photographs was ×400. (**C**) Expression of the mRNA of FcγR and iNOS. Control transformants (Neo; middle and right panel) and GHR-277 transformants (GHR-277; left panel) were cultured with IL-6 (100 ng/ml; right panel) or GH (50 ng/ml; left and middle panels) for the time periods indicated (d, days; –, unstimulated). 15 μg (in each condition) of total RNA extracted from unstimulated or stimulated cells was analysed by Northern blotting using the cDNAs of FcγR (upper panel) and iNOS (lower panel) as probes and hybridized signals were visualized by autoradiography. The films were exposed for 20 h at –80°C.

Identification of the cytoplasmic region of gp130 which is required for growth arrest and differentiation signals in M1 cells

To identify the cytoplasmic region of gp130 required for growth arrest and differentiation, we tested transformants expressing a series of chimeric receptors with progressive C-terminal truncations. GH could inhibit cell growth in a dose-dependent manner in the transformants expressing truncated forms of the gp130 cytoplasmic domain containing at least 133 amino acid residues (Figure 3A). GH could also induce macrophage-like morphological changes in these transformants (Figure 3B). Further truncation forms of the gp130 subunit (GHR-108, GHR-68 and GHR-25), were unable to generate signals for cell growth arrest and morphological changes (Figure 3A and B). GH induced the expression of iNOS mRNA in the M1 transformant expressing GHR-133 but not in the transformant expressing GHR-108, although the level of iNOS mRNA induced in the transformant of GHR-133 was lower than that in the transformant of GHR-277 (Figure 3C). GH induced the expression of FcγR mRNA in a similar time-course as did IL-6 in the transformant expressing GHR-277 and GHR-133, but not in the transformants expressing GHR-108 or GHR-68, although a weak FcγR expression was still detected in the transformant expressing GHR-108 with GH stimulation (Figure 3C). From these data, we concluded that the region from residues 108–133 in the cytoplasmic domain of gp130 was critical in generating signals for growth arrest and macrophage differentiation, although a membrane-proximal region appeared to be involved in the low-level induction of FcγR. These results also suggest that a C-terminal region distal to amino acid 133 may be required for full induction of iNOS.

Stat3 activation and the regulation of the expression of several IL-6 responsive genes in relation to growth arrest and differentiation

IL-6 induces various immediate early response genes, such as *junB*, *IRF1* and *egr-1*, while it represses c-*myb* and c-*myc* genes in M1 cells (Hoffman and Liebermann, 1991b; Lord *et al.*, 1991). These events have been suggested to be involved in IL-6-induced growth arrest and differentiation in M1 cells (Kimchi, 1992; Liebermann and Hoffman, 1994). Since Stat3 has been shown to be involved in the activation of the IL-6 response element in the *junB* gene promoter (Nakajima *et al.*, 1993, 1995; Fujitani *et al.*, 1994; Coffer *et al.*, 1995) and since the third tyrosine residue at amino acid position 126 in the region between 108 and 133 amino acids, is located in the YXXQ motif, a known docking site for Stat3 (Stahl *et al.*, 1995), we examined whether there is any correlation between Stat3 activation and other IL-6 functions in M1 cells. These functions include growth arrest and terminal

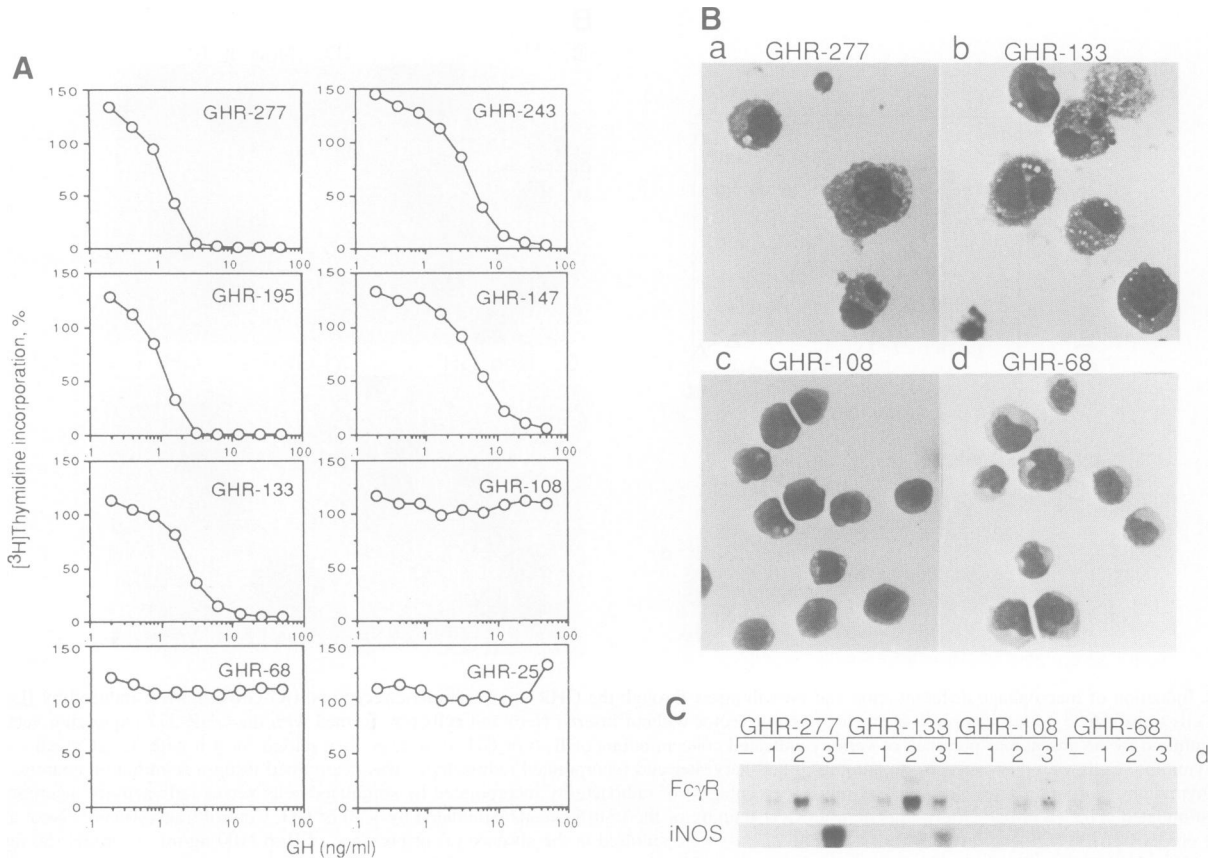

Fig. 3. GH-induced growth arrest and macrophage differentiation through the truncated chimeric receptors. (**A**) Transformants expressing a full-length (GHR-277) or various truncated chimeric receptors (GHR-243, GHR-195, GHR-147, GHR-133, GHR-108, GHR-68, GHR-25) were incubated with various concentrations of GH for 4 days and pulsed with [^{3}H]thymidine for 4 h. Incorporated [^{3}H]thymidine was determined as described in Figure 2A and expressed as a ratio of incorporated radioactivity in stimulated versus unstimulated cells. (**B**) May–Grunwald–Giemsa (MG) staining of transformants stimulated by GH (50 ng/ml). M1 transformants that express full-length (a, GHR-277) or truncated chimeric receptors (b, GHR-133; c, GHR-108; d, GHR-68) were cultured with 50 ng/ml of GH for 4 days, then subjected to cytospin and MG staining. Magnifications were ×400. (**C**) Expression of FcγR and iNOS mRNA. Transformants were either stimulated with 50 ng/ml of GH for the number of days indicated or left unstimulated (–). 15 μg of total RNA extracted from cells in each condition was analysed by Northern blotting using the cDNAs of FcγR and iNOS as probes.

differentiation, induction of *junB*, *IRF1* and *egr-1*, and repression of c-*myb* and c-*myc*.

Figure 4 shows that GH induced the tyrosine phosphorylation of Stat3 in transformants of GHR-277 and GHR-133 but not in the transformants of GHR-108 and GHR-68. This observation is consistent with the results reported by Stahl *et al.* (1995), who demonstrated a requirement of the YXXQ motif for Stat3 activation. IL-6 induced *junB* mRNA expression from 1–24 h after stimulation (Figure 5). The level of *IRF1* mRNA was increased 1 h after IL-6 stimulation but declined thereafter. *egr-1* mRNA was detected 6 h after IL-6 stimulation. GH induced the expression of these genes in the transformants of GHR-277 and GHR-133 in a similar time-course as was observed with IL-6, but did not induce expression in the transformants of GHR-108 or GHR-68. Along with the induction of these genes, IL-6 repressed the expression of c-*myb* and c-*myc*. GH stimulation repressed c-*myb* expression in transformants of GHR-277 and GHR-133, but not in those of GHR-108 or GHR-68. GH induced a transient increase in c-*myc* mRNA at 1 h after stimulation but completely inhibited its expression at 24 h in the transformants of GHR-277 and GHR-133, as did IL-6. However, c-*myc* was not repressed by GH in the transformants of GHR-108 and GHR-68, although a transient increase of c-*myc* was detected. Taken together, these results show that the cytoplasmic region from 108 and 133 amino acids that is critical for growth arrest and macrophage differentiation is also required for generating signals activating Stat3 and for regulating the expression of *junB*, *egr-1*, *IRF1*, c-*myc* and c-*myb* genes in the

Fig. 4. GH-induced tyrosine phosphorylation of Stat3 through the truncated chimeric receptors. Transformants (2×10^7 cells/condition) expressing full-length (GHR-277) or various truncated chimeric receptors (GHR-133, GHR-108, GHR-68) were either stimulated (+) with GH (50 ng/ml) for 15 min or left unstimulated (–). Stat3 was immunoprecipitated from unstimulated or stimulated cells using an anti-Stat3-specific antibody, and probed with either an anti-phosphotyrosine antibody (4G10; upper panel) or an anti-Stat3 antibody (lower panel). The immune complex was visualized by chemiluminescence (Amersham).

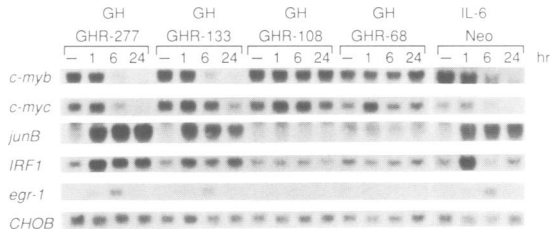

Fig. 5. Induction of the *junB*, *IRF1* and *egr-1* genes and repression of the c-*myb* and c-*myc* genes through the truncated chimeric receptors. Transformants expressing full-length or truncated chimeric receptors were stimulated with IL-6 (100 ng/ml) or GH (50 ng/ml) for 1, 6 or 24 h as indicated (hr). Total RNA was extracted and 15 μg of RNA was analysed by Northern blotting using the cDNAs of *junB*, *IRF1*, *egr-1*, c-*myb*, c-*myc* and *CHOB* as probes.

C-terminal truncated receptors. These results also suggest a correlation between Stat3 activation and other IL-6 functions in M1 cells.

Requirement of tyrosine residues essential for Stat3 activation, for growth arrest and for differentiation signals

We further examined the relationship between Stat3 activation and other IL-6 functions by using chimeric receptors in which various tyrosine residues in gp130 were replaced by phenylalanine. Human gp130 has six tyrosine residues in its cytoplasmic domain, three of which are located in the membrane-proximal region upstream from amino acid 133. Of these three tyrosine residues, only the third, at amino acid position 126, is located in a YXXQ motif. To examine whether these tyrosine residues are essential for the chimeric receptor, GHR-133, to generate the signals for growth arrest and differentiation, we introduced mutations into the second tyrosine residue (GHR-133F2), the third tyrosine residue (GHR-133F3) or both the second and third tyrosine residues (GHR-133F2/3) in the GHR-133 transformant. GHR-133F2 could induce both growth arrest and macrophage differentiation, whereas neither GHR-133F3 nor GHR-133F2/3 could induce these changes (Figure 6). As shown in Figure 7, GHR-133F3 and GHR-133F2/3 did not induce tyrosine phosphorylation of Stat3 either, while GHR-133F2 did. Furthermore, the other tyrosine residues contained in the YXXQ motifs located downstream of amino acid 133 could substitute for the tyrosine residue at amino acid position 126 in GHR-133 in the signal transduction process, since GHR-F2/3—where the second and third tyrosine residues were mutated in the context of the full-length GHR-gp130 chimeric receptor—could induce both growth arrest, differentiation (Figure 6) and Stat3 activation (Figure 7). We confirmed that the tyrosine residues of gp130 have an important role in generating signals for growth arrest, differentiation and Stat3 activation by showing that GHR-Fall, the transformant in which all six tyrosine residues of the full-length GHR-gp130 chimeric receptor were replaced with phenylalanine, induced none of the IL-6 responses except for the induction of FcγR mRNA (Figures 6 and 7). These results suggest that Stat3 plays a critical role in IL-6-induced growth arrest and differentiation. It is likely that a signalling pathway distinct from a Stat-mediated one is operating for FcγR gene activation. This suggestion is also consistent with the FcγR induction seen in the transformants of GHR-108 (Figure 3C).

Correlation of Stat3 activation and the regulation of the expression of junB, IRF1, c-myb and c-myc genes

The repression of the c-*myb* and c-*myc* genes was induced through GHR-133F2 but not through either GHR-133F3 or GHR-133F2/3 (Figure 8). GHR-133F2, but not GHR-133F3 or GHR-133F2/3, induced the expression of *junB* and *IRF1* within 1 h, although GHR-133F3 could induce delayed expression of the *junB* gene at 24 h after stimulation. The results indicated a critical role of the third tyrosine residue at amino acid position 126 in the down-regulation of the c-*myc* and c-*myb* genes and immediate early induction of the *junB* and *IRF1* genes. On the other hand, the transient increase of the c-*myc* gene was not impaired by these mutations, consistent with the fact that GHR-108 did induce a transient increase of the c-*myc* gene. The other tyrosine residues in the YXXQ motifs located downstream from amino acid 133 could substitute for the third tyrosine residue at amino acid position 126 in regulating these genes in the context of the full-length GHR-gp130 chimeric receptor: GHR-F2/3 but not GHR-Fall induced the down-regulation of c-*myb* and c-*myc* and up-regulation of the *junB* and *IRF1* genes (Figure 8). The results showed a correlation between Stat3 activation and the immediate early induction of the *junB* and *IRF1*, and of the repression of c-*myc* and c-*myb*. Only GHR-133F3 induced *egr-1* gene expression, showing that the signals generated through the second tyrosine residue are essential for *egr-1* gene expression. Such a requirement of the second tyrosine residue of gp130 was further shown by the fact that GHR-F2, in which only the second tyrosine residue was mutated in the full-length chimeric receptor, could not induce *egr-1* gene expression (data not shown).

Discussion

It is known that the homodimerization of gp130 is enough to trigger the signals for transient DNA synthesis as well as acute-phase protein synthesis (Ziegler *et al.*, 1993; Baumann *et al.*, 1994), although it remains to be elucidated whether the homodimerization of gp130 can induce the signals for long-term cell growth, growth arrest and differentiation without the involvement of IL-6R α-chain. In this study, we showed that GH induces growth arrest and macrophage differentiation, accompanied by morphological changes and the expression of FcγR and iNOS in M1 transformants expressing the GHR-gp130 chimeric receptor. GH was expected to induce the homodimerization of the GHR-gp130 chimeric receptor because it has been well established that GH can induce the homodimerization of GHR through its two binding sites for the extracellular domain of GHR (Cunningham *et al.*, 1991). It is unlikely that the IL-6R α-chain is also stimulated by GH in M1 transformants expressing the GHR-gp130 chimeric receptor, because only the complex of IL-6R α-chain and IL-6, but not the IL-6R α-chain alone, can associate with gp130 through the extracellular domain of gp130 (Taga *et al.*, 1989). Therefore, our results suggest that homodimerization of gp130 without the IL-6R α-chain is enough

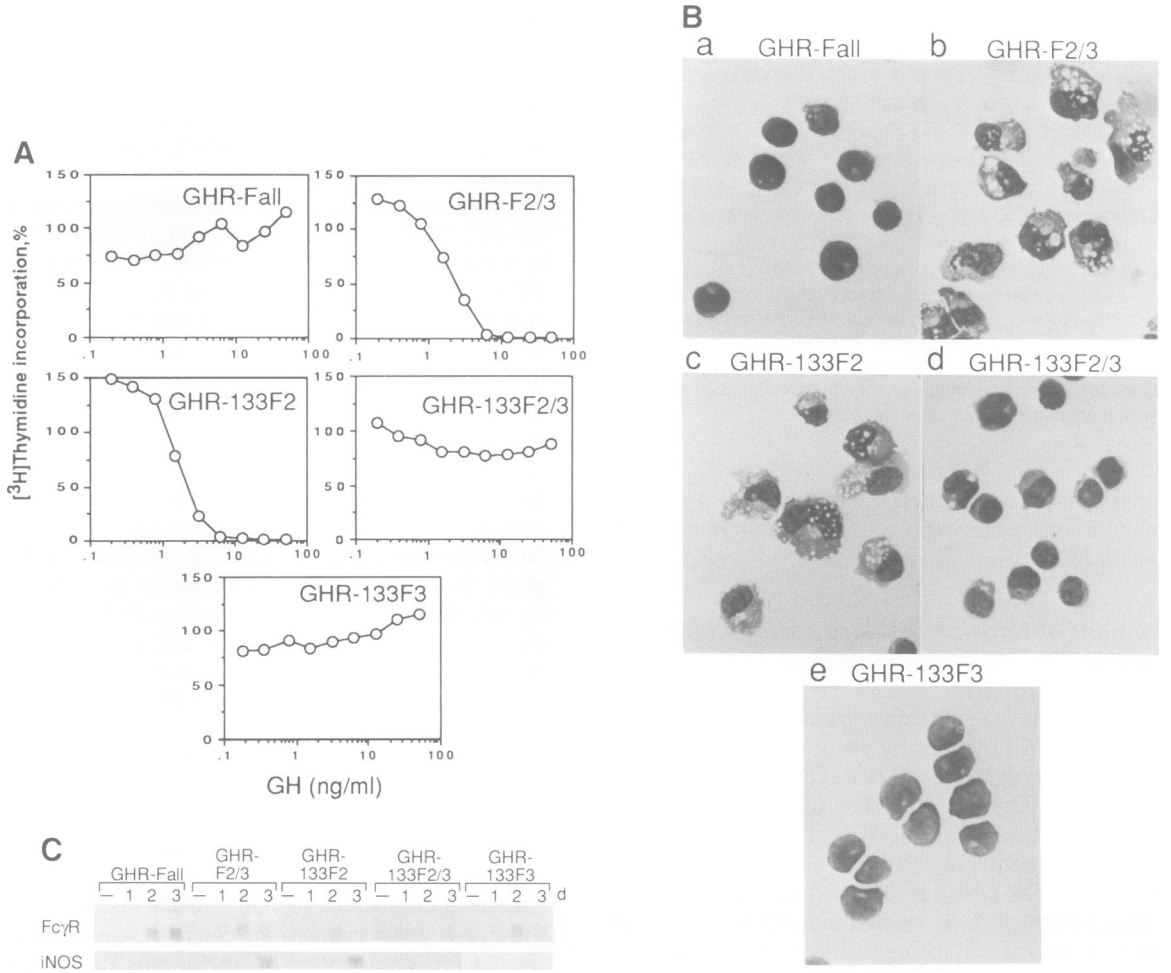

Fig. 6. Growth arrest and macrophage differentiation through the tyrosine-mutated chimeric receptors. (**A**) Growth arrest induced by GH through tyrosine-mutated receptors. Transformants that express tyrosine-mutated chimeric receptors (GHR-Fall, GHR-F2/3, GHR-133F2, GHR-133F2/3 and GHR-133F3; described in Figure 1) were cultured with various concentrations of GH for 4 days, then pulsed for 4 h with 0.5 μCi/well [^{3}H]thymidine. Incorporated [^{3}H]thymidine was determined and expressed as a ratio of incorporated radioactivity in stimulated versus unstimulated cells. (**B**) May–Grunwald–Giemsa staining of transformants (a, GHR-Fall; b, GHR-F2/3; c, GHR-133F2; d, GHR-133F2/3; e, GHR-133F3) stimulated with GH (50 ng/ml) for 4 days. (**C**) Expression patterns of FcγR and iNOS mRNA in transformants expressing the tyrosine-mutated receptors were analysed by Northern blotting as described in Figure 2C.

to generate the signals for growth arrest and macrophage differentiation.

Using transformants expressing mutant GHR-gp130 chimeric receptors with progressive C-terminal truncations and point mutations, we identified the first 133 amino acid residues of gp130 as the minimal region necessary for growth arrest and differentiation. While the membrane-proximal region of 61 amino acids has been shown to be sufficient for promoting transient DNA synthesis in the gp130-transfected pro-B cell line BAF-B03 (Murakami *et al.*, 1991), our present study showed that additional, membrane-distal regions are required, and that the region of amino acids 108–133 is critical for generating signals for both differentiation and growth arrest in M1 cells. This region contains box 3, which is conserved among gp130, LIFRβ and G-CSFR and has been shown to play a critical role in inducing acute-phase protein synthesis in hepatic cells (Ziegler *et al.*, 1993; Baumann *et al.*, 1994).

Our results showed that distinct regions of the cytoplasmic domain of gp130 are involved in generating different signals. Generation of different signals from distinct cytokine receptor regions has already been shown

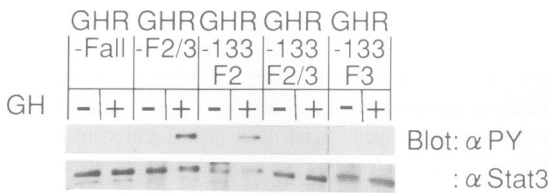

Fig. 7. Tyrosine phosphorylation of Stat3 through the tyrosine-mutated chimeric receptors. Transformants expressing tyrosine-mutated chimeric receptors were either stimulated (+) with GH (50 ng/ml) for 15 min or left unstimulated (–). Immunoprecipitated Stat3 was probed with anti-phosphotyrosine or anti-Stat3 antibodies, and detected by chemiluminescence.

in some cases. For instance, in G-CSFR, both the C-terminal region and the membrane-proximal region are required for the induction of the myeloperoxidase gene encoding a neutrophil differentiation-associated enzyme, although only the membrane-proximal region is necessary for generating growth signals (Fukunaga *et al.*, 1993). It has also been reported that different parts of receptors are required for generating multiple signals for DNA synthesis

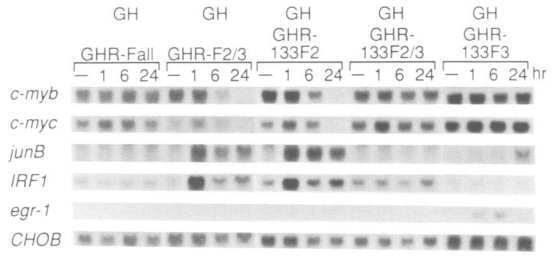

Fig. 8. Induction of the *junB*, *IRF1* and *egr-1* genes and repression of the c-*myb* and c-*myc* genes through the tyrosine-mutated receptors. Transformants expressing tyrosine-mutated chimeric receptors were stimulated with GH (50 ng/ml) for the number of hours indicated. Total RNA was extracted and the expression of c-*myb*, c-*myc*, *junB*, *IRF-1*, *egr-1* and *CHOB* was analysed by Northern blotting as described in Figure 5.

or cell growth, induction of a variety of genes, and prevention of apoptosis. These regional requirements have been observed for a variety of cytokine receptors, such as EpoR, IL-2Rβ, and the common β-chain for IL-3R, IL-5R and GM-CSFR (Sakamaki *et al.*, 1992; Sato *et al.*, 1993; Miura *et al.*, 1994; Kinoshita *et al.*, 1995; Miyazaki *et al.*, 1995). These observations, together with the results presented here, support the concept that cytokine receptors have the potential to generate distinct signals by activating different molecules capable of interacting with distinct regions of the cytokine receptor. This regional specificity may be the molecular basis behind the functional pleiotropy of cytokines.

One very important finding in our present study is that the activation of Stat3 is tightly correlated with the signals for growth arrest and macrophage differentiation. First, the minimal region of the cytoplasmic domain of gp130 required for these signals was also the minimal one for the activation of Stat3. The region of amino acids 108–133, which is critical for generating signals, contains a region called 'box 3', where the third tyrosine residue with the YXXQ motif is located at amino acid position 126. This motif is conserved among gp130, LIFRβ and G-CSFR and the tyrosine residue in the motif has previously been shown to play a role in activating Stat3. This activation occurs through the binding of the YXXQ motif to the SH2 domain of Stat3 (Stahl *et al.*, 1995). Secondly, the third tyrosine residue with the motif, but not the second one without the motif, was essential for the truncated GHR-gp130 chimeric receptor containing only the first 133 amino acid residues to generate the signals for growth arrest and differentiation. Finally, we showed that the other tyrosine residues, which reside in the C-terminal region downstream from amino acid 133, could substitute for the third tyrosine residue at amino acid position 126 in the context of the full-length chimeric receptor. Since all three tyrosine residues in the C-terminal distal region contain the YXXQ motif and since any of them is considered to be a docking site for Stat3 (Stahl *et al.*, 1995), all evidence supports the hypothesis that Stat3 plays an essential role in generating signals for growth arrest and macrophage differentiation, immediate early induction of *junB* and *IRF1*, and repression of c-*myb* and c-*myc* in M1 cells. However, the regulatory mechanisms for the expression of iNOS and FcγR do not appear to be simple. As described in the Results section, multiple signals may be required for the full expression of both of these genes.

The second tyrosine residue has been shown to be a docking site for a SH2-containing tyrosine phosphatase, PTP-1D (Stahl *et al.*, 1995). However, we have shown that this site is not required for generating the signals for growth arrest and differentiation. This site does appear to be required for induction of the *egr-1* gene and for delayed induction of the *junB* gene in M1 cells, since none of the M1 transformants expressing the chimeric receptors containing a mutation at the second tyrosine residue (GHR-133F2/3, GHR-133F2, GHR-F2/3 and GHR-F2) responded to GH by expressing *egr-1* mRNA. Furthermore, GH could induce the *junB* gene through GHR-133F3 at 24 h but not within 1 h after stimulation, whereas GH could not induce *junB* through GHR-133F2/3 at all. All results showed that the induction of the *egr-1* gene and the delayed induction of *junB* is regulated by the pathway that originates from the second tyrosine residue. The results suggest that *egr-1* may not be involved in IL-6-induced growth arrest and differentiation. This result appears to be inconsistent with the previous report by Nguyen *et al.* (1993) who showed an essential role for *egr-1* in macrophage differentiation in a particular M1 clone. Probably, as they also discussed, other gene product(s) may substitute for the role of *egr-1* in some M1 clones.

In this study, we could not separate the signals for growth arrest from those involved in differentiation. Differentiation-inducing signals usually accompany growth arrest, although growth arrest-inducing signals do not always induce cell differentiation: TGF-β, IFN-γ and LPS inhibit cell growth of M1 cells without inducing differentiation (Selvakumaran *et al.*, 1992, 1994). MyoD induces both myogenic differentiation and G_1 arrest, the latter by increasing the expression of cyclin-dependent kinase inhibitor p21Waf1/Cip1 (Halevy *et al.*, 1995; Parker *et al.*, 1995). Here, we observed the tight correlation among Stat3 activation, repression of c-*myc* and c-*myb*, growth arrest and differentiation. It has been suggested that repression of c-*myb* and c-*myc* is essential for the subsequent growth arrest and terminal differentiation, since deregulated expression of the c-*myb* or c-*myc* gene inhibits the IL-6-induced growth arrest and differentiation of M1 cells (Hoffman and Liebermann, 1991a; Selvakumaran *et al.*, 1992). Thus, it is possible to hypothesize that Stat3 induces differentiation by inducing a set of genes involved in macrophage differentiation and inhibits cell growth by inducing the expression of unknown genes, which both inhibit the expression of c-*myc* and c-*myb*, and interfere with the cell cycle progression. Our hypothesis that Stat3 plays a central role in cell differentiation and growth regulation is attractive, but still remains to be proved.

Materials and methods

Plasmid construction

pSP72-hgp130 was constructed by inserting the *Eco*RI–*Xba*I fragment of human gp130 (*Xba*I site was introduced downstream of the termination codon), which contains the transmembrane and cytoplasmic domain, into pSP72 (Promega). To construct C-terminal truncation mutants, fragments of gp130 were generated by the polymerase chain reaction (PCR) using pSP72-hgp130 as a template and subcloned into the *Eco*RI

and *Xba*I sites of pSP72. The fragment of the extracellular domain of rabbit GHR was also generated by PCR using the GHR cDNA (a gift from Dr W.I.Wood) as a template and subcloned into the *Xba*I and *Eco*RI site of Bluescript SK(+) (Strategene; pBS-GHRextra). The sequences of the primers are available on request. The fragments of GHR-gp130 chimeric receptors were constructed by inserting the *Eco*RI–*Hin*dIII fragments of gp130 or its truncation mutants from pSP72-hgp130 into the *Eco*RI, *Hin*dIII site of pBS-GHRextra. The expression vectors were obtained by subcloning of the *Xho*I fragments of GHR-gp130 chimeric receptors into pMSCVneoEB retroviral vector (a gift from Dr T.S.Hawley).

Cell and cell culture

The murine myeloid leukaemia cell line M1 cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% horse serum, 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere with 5% CO_2. The φ2 cells were grown in DMEM containing 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin.

Retroviral production and infection of the M1 cells

The pMSCVneoEB vectors carrying cDNAs encoding a variety of chimeric receptors were transfected into φ2 cells by the standard calcium phosphate method (Sambrook *et al.*, 1989). The transfectants were selected with 400 μg/ml G-418 (Gibco). Retroviral infection was performed by co-culturing M1 cells with the irradiated φ2 transfectants. A total of 2×10^5 M1 cells were placed on the adherent φ2 transfectants in a 60 mm tissue culture dish containing medium and incubated for 2 days. Infected cells were selected with 400 μg/ml G-418. G-418-resistant transformants were examined for GHR mRNA expression by Northern blot analysis and for the protein levels of GHR-gp130 chimera receptor by growth hormone binding assay.

Binding assay

Binding activity of the transformants for GH was examined by incubating 2×10^6 cells with 1 nM [^{125}I]GH at 4°C for 4 h in the presence or absence of a 500-fold excess of unlabelled GH in DMEM containing 20 mM HEPES, pH 7.3. The reaction mixture was placed on ice-cold FCS and the cell-bound GH fraction was separated from the unbound fraction by centrifugation at 10 000 *g* for 15 s. The radioactivity associated with the cell pellets was determined by a gamma-counter.

Thymidine incorporation

For thymidine incorporation, 5×10^3 cells/200 μl/well in 96-well microtitre plates were cultured in the presence of various concentrations of GH for 4 days and pulsed with 0.5 μCi/well of [^{3}H]thymidine for 4 h. Incorporated [^{3}H]thymidine was collected by an automated cell harvester and its radioactivity was determined by a liquid scintillation counter.

RNA extraction and Northern blot RNA analysis

Total cytoplasmic RNA was extracted by the NP-40 method (Sambrook *et al.*, 1989). In brief, 1×10^7 cells were washed twice with ice-cold Dulbecco's phosphate-buffered saline (DPBS) and lysed with 250 μl of lysis buffer (10 mM Tris–HCl, pH 8.6, 0.14 M NaCl, 1.5 mM $MgCl_2$, 0.5% NP-40). The cytoplasmic fraction was cleared by centrifugation at 10 000 *g* for 3 min and mixed with 250 μl of 2× Proteinase K buffer (0.2 M Tris–HCl, pH 7.5, 0.3 M NaCl, 25 mM EDTA, 2% SDS, 200 μg/ml Proteinase-K). The mixture was incubated at 37°C for 30 min and extracted twice with phenol–chloroform and once with chloroform; the RNA was then precipitated with ethanol.

Fifteen μg of total RNA was separated by electrophoresis in 1% agarose formaldehyde gels and transferred to Hybond N^+ (Amersham) nylon filters. Filters were hybridized in hybridization buffer (0.5 M $NaPO_4$, pH 7.0, 1 mM EDTA, 7% SDS, 1% BSA) at 65°C for 12–16 h and washed with 2× SSC, 0.1% SDS for 10 min at room temperature and with 0.1× SSC, 0.1% SDS at 65°C for 40 min and subjected to autoradiography. The probes used here were *junB* (2.1 kb, *Eco*RI fragment), c-*myc* (1.6 kb, the third exon, *Sac*I–*Hin*dIII fragment), c-*myb* (2.2 kb, *Eco*RI fragment), *IRF1* (2.0 kb, *Xba*I fragment, a gift from Dr T.Taniguchi), *egr*-1 (0.7 kb, *Eco*RI fragment), iNOS (0.8 kb, *Hin*dIII–*Eco*RI fragment, a gift from Dr J.Fujii), FcγRI (0.6 kb, nucleotides 601–1220 PCR fragment) and the B gene isolated from Chinese hamster ovary cells (CHOB, 0.6 kb, *Eco*RI–*Bam*HI fragment)

Immunoprecipitation and Western immunoblotting

Samples of 2×10^7 cells were harvested and washed twice with ice-cold DPBS containing 0.5 mM sodium vanadate and lysed in the lysis buffer (20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.1% NP-40, 0.8 mM sodium vanadate, 1 mM PMSF, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 5 μg/ml pepstatin). The lysate was cleared by centrifugation at 10 000 *g* for 15 min and then incubated with polyclonal anti-human Stat3 antibody recognizing a C-terminal region of Stat3 (H.Kojima *et al.*, unpublished results) for 2 h at 4°C. The immune complexes were collected by incubating with 10 μl of protein A–sepharose for 1 h. After washing the immune complexes with 1 ml of the lysis buffer five times, the complexes were eluted with 20 μl of Laemmli sample buffer, resolved in 7.5% SDS–PAGE, and transferred to Immobilon P (Millipore). The membrane was incubated with anti-phosphotyrosine antibody (4G10, MBI) or anti-Stat3 antibody for 1 h. After washing, the membrane was incubated with horseradish peroxidase-conjugated second-antibody for 1 h. The immune complex was detected by using an enhanced chemiluminescence system (ECL, Amersham).

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