Vol. 208, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 8, 1995 Pages 368-375

HIGH AFFINITY CHIMERIC HUMAN GRANULOCYTE-MACROPHAGE COLONY-STIMULATING FACTOR RECEPTOR CARRYING THE CYTOPLASMIC DOMAIN OF THE β SUBUNIT BUT NOT THE α SUBUNIT TRANSDUCES GROWTH PROMOTING SIGNALS IN Ba/F3 CELLS

Akihiko Muto, Sumiko Watanabe, Atsushi Miyajima‡, Takashi Yokota and Ken-ichi Arai§

Department of Molecular and Developmental Biology, the Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108, Japan

‡Department of Molecular Biology, DNAX Research Institute of Molecular and Cellular Biology, 901 California Avenue, Palo Alto, CA 94304

Received January 26, 1995

Granulocyte-macrophage colony-stimulating factor receptor (GMR) is composed of two distinct subunits α and β, and the cytoplasmic domains of both subunits are essential to transduce signals. We further analyzed the role of the cytoplasmic domain of each subunit by constructing chimeric subunits, designated α/β and β/α, by exchanging cytoplasmic domains of the α and β subunits of hGMR. Reconstituted high-affinity chimeric hGMRs, hGMR(α/β,β/α) and hGMR(α/β,β), as well as the wild type hGMR(α,β),transduced signals in Ba/F3 cells. These observations indicate that the original configuration between the extracellular and the cytoplasmic domains of the hGMR(α,β) subunits is not obligatory, and that hGMR(α/β,β) transduced signals through the cytoplasmic domain of the β subunit in an oligomeric form, without involvement of the cytoplasmic domain of the α subunit.

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a glycoprotein produced by a variety of cell types (1, 2). This cytokine stimulates proliferation, survival and differentiation of multi-lineage of hematopoietic cells. The human GM-CSF receptor (hGMR) consists of two distinct subunits designated α and β, and both subunits are the member of the cytokine receptor superfamily (3-6). The α subunit is specific to GMR and binds to GM-CSF with a low-affinity, whereas the β subunit is shared by the interleukin 3 receptor (IL-3R) and by the interleukin 5 receptor (IL-5R). The β subunit in itself shows no detectable affinity to any ligands but does contribute to form a high-affinity receptor with the α subunit (5, 7-9).

GM-CSF or IL-3 induces mRNAs of several immediate early genes (such as c-*fos*, c-*jun* and c-*myc*), tyrosine phosphorylation of cellular substrates and cell proliferation (4). We and others have shown that both the α and β subunits are essential and sufficient to reconstitute a high-affinity and functional hGMR in mouse cell lines (5, 10-13). Deletion analysis indicated that the cytoplasmic domains of both subunits are required to transduce hGM-CSF signals (14-

§To whom correspondence should be addressed. Fax: 81-3-5449-5424.

The abbreviations used are: GM-CSF, granulocyte-macrophage colony-stimulating factor; h, human; m, mouse; R, receptor; GMR, GM-CSF receptor; IL, interleukin; FCS, fetal calf serum; PAGE, polyacrylamide gel electrophoresis.

0006-291X/95 $5.00

16). However, a more specific role of the cytoplasmic domain of each subunit is not well known. Therefore, we examined roles of the cytoplasmic domains of the α and β subunits in signal transduction using two chimeric subunits of hGMR, *i.e.* α/β and β/α. We obtained evidence that hGMR(α/β,β) transduces signals through the oligomerized cytoplasmic domain of the β subunit, without the cytoplasmic domain of the α subunit.

Materials and Methods

Chemicals, genes and antibodies Recombinant hGM-CSF produced in *E. coli* was provided by the Schering-Plough Corporation. Mouse IL-3 (mIL-3) produced by silkworm (*Bombyx mori*) was purified as described previously (17). Anti phosphotyrosine mouse monoclonal antibody 4G10 was purchased from Upstate Biotechnology Inc. Mouse monoclonal antibody against hGMR α subunit, GMA-1, and rat monoclonal antibody against the human common β subunit, 5A5, were prepared by Dr. H. Kurata (Institute of Medical Science, The University of Tokyo) and by Dr. Y. Watanabe (DNAX Research Institute) (18), respectively. Mouse c-*fos* and rat c-*jun* cDNAs were kind gifts from Dr. T. Curran (Roche Institute of Molecular Biology). Mouse *c-myc* cDNA was provided by Dr. N. Arai (DNAX Research Institute). Human G3PDH cDNA was purchased from CLONTECH Laboratories, Inc.

Construction of chimeric receptors To construct the α/β and β/α chimeric hGMR subunits carrying the extracellular and transmembrane domains of the α and β subunits and the cytoplasmic domains of the β and α subunits, respectively, DNA fragments encoding the cytoplasmic domains of the α and the β subunits were replaced by the corresponding DNA fragments of the β and the α subunits. First, the α subunit cDNA was digested with *Eco*81I at amino acid position 350 followed by a fill-in reaction with the Klenow fragment, then the β subunit cDNA was digested with *Fsp*I at amino acid position 471. Both DNAs were then cleaved at the *Xba*I site in the vector (pME18S) sequence beside the 3' end of cDNA. Isolated blunt end-*Xba*I DNA fragments encoding the cytoplasmic domain of each receptor cDNA were exchanged and ligated to the vector containing the extracellular and transmembrane regions of the other subunit of the receptor. The resultant chimeric α/β product has one extra amino acid, serine, at the junction of the two polypeptide fragments derived from the α and β subunits. To establish stable transfectants, cDNAs were subcloned into pME18S containing a hygromycin or neomycin resistant gene.

Establishment of stable transfectants A mIL-3-dependent pro-B cell line, Ba/F3, was maintained in RPMI1640 medium containing 10% FCS, 50 U/ml penicillin, 50 μg/ml streptomycin and 0.25 ng/ml mIL-3. Ba/F3 cells were transfected by electroporation to establish stable transfectants, essentially as described elsewhere (11). Expression of the receptor(s) was confirmed by flow cytometry with FACScan (Becton Dickinson), using monoclonal antibodies directed against the α (GMA-1) and the β (5A5) subunits of hGMR.

Cell proliferation assay Harvested Ba/F3 cells were washed and resuspended in factor-free medium at 5 x 10^4 cells/ml, then 100 μl of aliquots of the cell suspension were seeded to a 96-well plate. After 24 h culture with various concentrations of mIL-3 or hGM-CSF, the MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay was performed as described by Mosmann (19).

Northern blot analysis The mRNAs of immediate early genes were analyzed by Northern blot analysis, as previously described (10). Briefly, cells were incubated in factor-free medium for 5 h prior for stimulation with mIL-3 or hGM-CSF. Total cellular RNA was extracted from the cells at indicated time points after stimulation by the acid guanidinium isothiocyanate-phenol-chloroform method (20). Each 10 μg of total RNA per lane was electrophoretically separated and transferred onto a nylon membrane. The RNA blot was hybridized with ^{32}P-labeled cDNA probes, exposed to an imaging plate and analyzed using a FUJIX Bio-imaging analyzer, model BAS-2000 (Fuji Film).

Analysis of tyrosine phosphorylated proteins After factor depletion for 5 h, the cells were resuspended at 4 x 10^6 cells/ml in the same medium followed by stimulation with or without either 1 ng/ml mIL-3 or 5 ng/ml hGM-CSF for 10 min at 37°C. The stimulated cells were rapidly precipitated by centrifugation and lysed in Laemmli's sample buffer (21) containing 1% 2-mercaptoethanol at 1 x 10^7 cells/ml. The proteins corresponding to 2 x 10^5 cells were subjected to SDS-PAGE and then Western blotting using an anti-phosphotyrosine antibody (4G10). The blots were developed with the ECL system (Amersham Japan Co. Ltd), according to the manufacturer's instructions.

Results

To examine the role of each cytoplasmic domain of hGMR α and β subunits, we generated the chimeric hGMR subunits α/β and β/α. The predicted structures of the chimeric receptors are schematically shown in Fig. 1. The chimeric subunit α/β consists of extracellular and transmembrane domains of the α subunit and the cytoplasmic domain of the β subunit. The chimeric subunit β/α consists of extracellular and transmembrane domains of the β subunit and the cytoplasmic domain of the α subunit. To examine the properties of signal transduction by chimeric hGMRs, we established Ba/F3 transfectants expressing the wild type and chimeric hGMR subunits, in various combinations. The expression of each subunit was confirmed by FACS analysis using antibodies against the extracellular domain of the hGMR α subunit (GMA-1) or the β subunit (5A5). The transfectant expressing the α/β subunit alone, designated Ba/F3-α/β, and transfectants expressing both subunits in various combinations, designated Ba/F3-α,β, Ba/F3-α/β,β/α, Ba/F3-α,β/α and Ba/F3-α/β,β, were used for subsequent studies. All of these transfectants, except for Ba/F3-α/β cells, expressed high- (Kd; 107-192 pM, 7000-11000 sites per cell) and low- (Kd; 6.3-10.8 nM, 91000-160000 sites per cell) affinity hGMR (data not shown). In contrast, Ba/F3-α/β cells exhibited only low-affinity hGMR (Kd; 8.2 nM, 306000 sites per cell, data not shown). These results are consistent with our previous observations that the extracellular domains of both the α and β subunits are sufficient to reconstitute high-affinity hGMR and that the cytoplasmic domains did not significantly affect the binding property of hGMR (14).

As we previously reported, hGM-CSF induced proliferation of Ba/F3 cells expressing the wild type hGMR, in a dose-dependent manner (11, 14). We asked whether the hGMRs containing chimeric subunits would transduce growth promoting signals in Ba/F3 cells (Fig. 2). Growth of Ba/F3 cells was measured using the MTT assay in the presence of various concentrations of hGM-CSF. As shown in Fig. 2A and 2B, Ba/F3-α/β,β/α cells proliferated in response to a low concentration (1 ng/ml) of hGM-CSF. Likewise, Ba/F3-α/β,β cells also proliferated with the property similar to that seen with Ba/F3-α,β cells expressing the wild type hGMR (Fig. 2D). These three transfectants continuously grew for more than a week in the

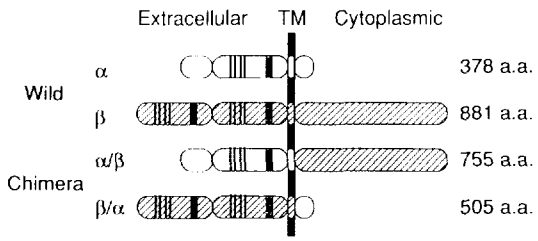

Fig. 1. Structure of wild and chimeric subunits of hGMRs.
The wild type α and β subunits and the chimeric subunits, α/β and β/α, are schematically represented. The extracellular, transmembrane (TM) and cytoplasmic domains are represented with ellipses as indicated at the top of the figure. The open and hatched ellipses represent domains derived from the α and β subunits, respectively. The vertical lines in the extracellular domain represent four conserved cysteine residues. The stippled boxes represent the WSXWS motif. The names and the predicted numbers of amino acids of each subunit are given on the left and right of the figure, respectively.

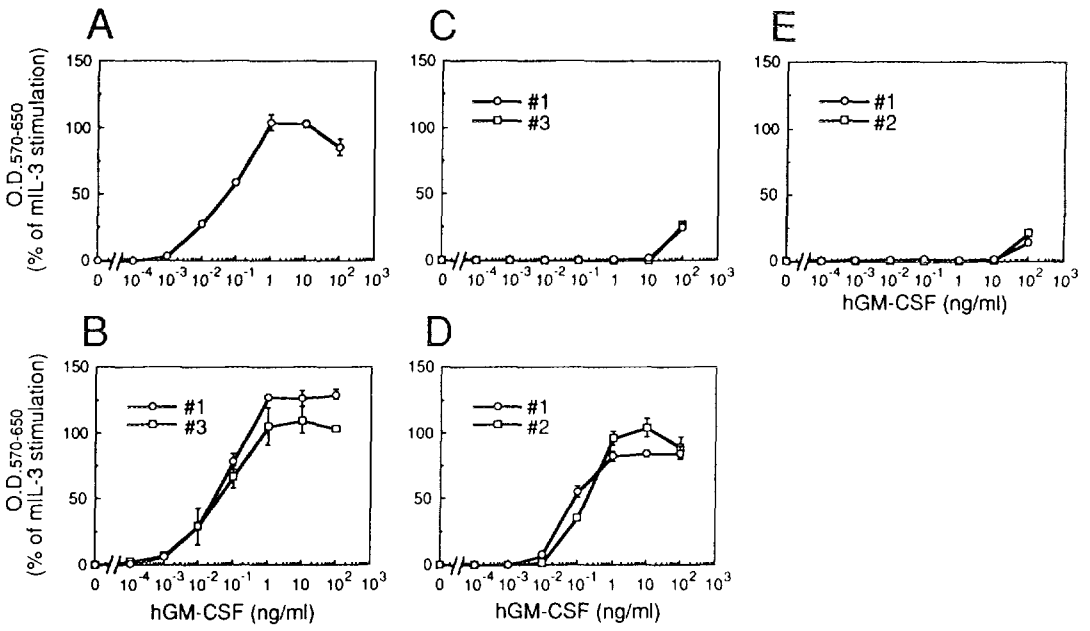

Fig. 2. Cell proliferation of Ba/F3 cells by hGM-CSF.
Proliferation of Ba/F3 transfectants was determined using the MTT assay following culture for 24 h in the presence of 0 to 100 ng/ml of hGM-CSF. As a control, the cells were cultured with 1 ng/ml mIL-3. Horizontal axis indicates the concentrations of hGM-CSF and vertical axis indicates the relative MTT reduction value normalized to the values for cells incubated with 1 ng/ml mIL-3. (A) Ba/F3-α,β, (B) Ba/F3-α/β,β/α, (C) Ba/F3-α,β/α, (D) Ba/F3-α/β,β and (E) Ba/F3-α/β. All values are the average of triplicated samples and standard deviations are shown as error bars. # indicates the clone number.

presence of a low concentration of hGM-CSF (data not shown). In contrast, Ba/F3-α,β/α and Ba/F3-α/β (Fig. 2C and 2E) cells proliferated only in the presence of a high concentration (100 ng/ml) of hGM-CSF.

Next, using Northern blot analysis, we examined whether or not hGMRs containing chimeric subunits could transduce signals to activate endogenous c-*fos*, c-*jun* and c-*myc* genes (Fig. 3). These genes were activated by hGM-CSF stimulation in Ba/F3 and NIH3T3 cells expressing the wild type hGMR (10, 22, 23). In all Ba/F3 transfectants expressing the high-affinity chimeric hGMR, with the exception of Ba/F3-α,β/α cells, hGM-CSF induced accumulation of c-*fos*, c-*jun* and c-*myc* mRNAs with kinetics similar to that of cells expressing the wild type hGMR. mIL-3 stimulation, used as a control, resulted in accumulation of these mRNAs in all the transfectants.

It has been well documented that hGM-CSF induces tyrosine phosphorylation of various intracellular proteins (14, 22, 24). Finally, we analyzed the pattern of protein tyrosine phosphorylation induced by hGM-CSF through the high-affinity chimeric hGMRs, by Western blotting, using the anti-phosphotyrosine antibody 4G10 (Fig. 4). In Ba/F3-α,β cells, hGM-CSF stimulated tyrosine phosphorylation of proteins (130, 95, 93, 76, 66, 55, 45 and 43 kDa) in a manner similar to that seen with mIL-3 (Fig. 4, lanes 2 and 3). These proteins were also phosphorylated in Ba/F3-α/β,β/α and Ba/F3-α/β,β cells in response to hGM-CSF, with a

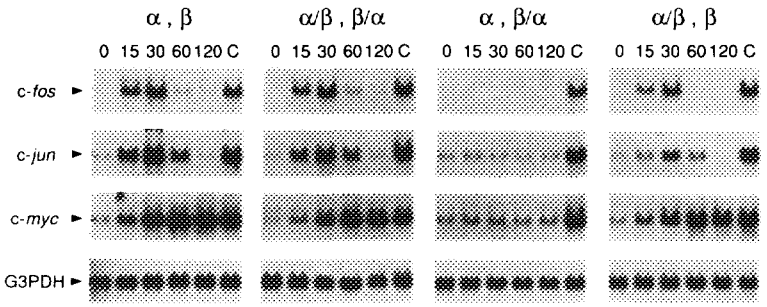

Fig. 3. Northern blot analysis of immediate early genes in Ba/F3 cells. Factor depleted Ba/F3 clones were stimulated with either 1 ng/ml mIL-3 or 5 ng/ml hGM-CSF. After incubation for 30 min with mIL-3 as a control (lane C) or for indicated times (min) with hGM-CSF (lanes 0 to 120), total RNA was extracted and 10 μg each was analyzed by Northern blots, as described in Materials and Methods. The names of transfectants and detected mRNAs are given at the top and left of the figure, respectively.

pattern indistinguishable from that for the Ba/F3-α,β cells (Fig. 4, lanes 5 and 9). In contrast to these transfectants, hGM-CSF failed to induce any detectable tyrosine phosphorylation in Ba/F3-α,β/α cells (Fig. 4, Lane 7).

Discussion

When we constructed chimeric α/β and β/α subunits of hGMR, we found that the high-affinity hGMR(α/β,β/α) composed of the chimeric α/β and β/α subunits was functional and transduced signals in Ba/F3 cells for activation of immediate early genes, tyrosine phosphorylation and cell proliferation. Thus, the original configuration between extracellular and cytoplasmic domains of the hGMR(α,β) subunits was not obligatory for reconstitution of an active hGMR. Based on this evidence, we did a series of experiments using the wild type (α and β) and chimeric (α/β and β/α) subunits of hGMR. Reconstitution studies indicated that the high-affinity hGMR(α/β,β) composed of the α/β and β subunits which carries no cytoplasmic portion of the α subunit was functional, whereas the hGMR(α,β/α) carrying only the cytoplasmic domain of the α subunit failed to form an active receptor. Thus, the cytoplasmic domain of the β subunit, in an oligomeric form, is sufficient to transduce growth promoting signals, and the cytoplasmic domain of the α subunit is dispensable to form a functional hGMR in the hGMR(α/β,β).

With regard to the manner in which hGM-CSF activates hGMR(α/β,β) without involving the cytoplasmic domain of the α subunit, at least two possibilities can be considered. One is that the cytoplasmic domain of the chimeric α/β subunit could substitute for the function of the cytoplasmic domain of the α subunit. The cytoplasmic domain of the α subunit has a proline-rich motif similar to box1 motif in the cytoplasmic domain of the β subunit, and both motifs were shown to be important for signal transduction (14, 25). In this context, the cytoplasmic domain of the α/β subunit in hGMR(α/β,β) may activate the β subunit by promoting a conformational change of the β subunit in a manner similar to the cytoplasmic domain of the α subunit in wild type hGMR. Alternatively, a molecule(s) associated with the cytoplasmic

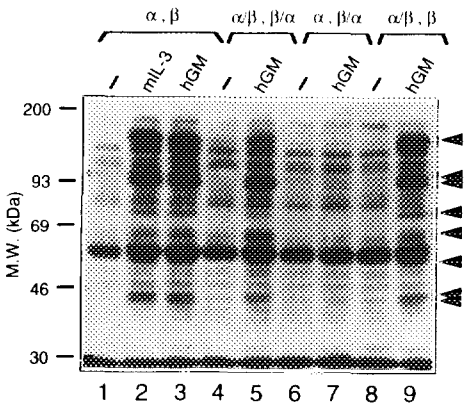

Fig. 4. Induction of tyrosine phosphorylation in response to hGM-CSF in Ba/F3 cells.

After factor depletion at the concentration of 4 x 10^6 cells/ml, the cultures were additionally incubated for 10 min without (lanes 1, 4, 6 and 8) or with 1 ng/ml mIL-3 (lane 2) or 5 ng/ml hGM-CSF (lanes 3, 5, 7 and 9). The cells were harvested and lysed in Laemmli's solution at 2 x 10^7 cells/ml. Ten μl of total cell extracts corresponding to 2 x 10^5 cells from Ba/F3-α,β (lanes 1 to 3), Ba/F3-α/β,β/α (lanes 4 and 5), Ba/F3-α,β/α (lanes 6 and 7) and Ba/F3-α/β,β (lanes 8 and 9) were separated by SDS-PAGE (8%) and tyrosine phosphorylated proteins were analyzed by Western blotting, using anti-phosphotyrosine antibody 4G10. The sizes of proteins are shown in kDa on the left of the figure. The positions of hGM-CSF induced tyrosine phosphorylated proteins are indicated by arrowheads on the right of the figure.

domain of the α subunit may be functionally mimicked by a molecule(s) associated with the cytoplasmic domain of the α/β subunit. The other possibility for activation of hGMR(α/β,β) is that the cytoplasmic domains of the chimeric α/β and wild type β subunits form a β-dimer-like structure, and the β-homodimer may be involved in signal transduction through a normal hGMR. This is not the case for hGM-CSF-induced dimerization of the β subunit, since we recently found that the β subunit formed a homodimer even in the absence of hGM-CSF but was activated by the ligand[1]. At present, we have no direct evidence to define which is a likely possibility.

As shown in Fig. 2, growth promoting signals were transduced in Ba/F3-α,β/α and Ba/F3-α/β cells, with a high dose (100 ng/ml) of hGM-CSF, findings similar to the case where Ba/F3 cells express the wild type α subunit alone (11). Ba/F3 cells express AIC2B, the mouse common β subunit, and this protein has been shown to form a functional but low-affinity hGMR with the human α subunit. In Ba/F3-α,β/α and Ba/F3-α/β cells, the wild type hGMR α subunit or the chimeric α/β subunit may have formed a functional hGMR with AIC2B. Consistent with this interpretation, in NIH3T3 cells which did not express AIC2B, the α/β subunit alone but not hGMR(α/β,β) failed to transduce growth signals, even in the presence of a high concentration of hGM-CSF (data not shown).

We showed here that growth promoting signals could be transduced through the cytoplasmic domain of the β subunit without involvement of the cytoplasmic domain of the α subunit in a

[1]A.Muto et al., manuscript in preparation.

chimeric hGMR system. However, this does not exclude the possibility that the cytoplasmic domain of the α subunit may also have alternative functions to transduce specific signals governing cellular responses other than proliferation. It is difficult to assess such functions because cell growth is also inhibited by truncation of the cytoplasmic domain of the α subunit. A comparison of signals transduced through hGMR(α,β) with those through hGMR(α/β,β) may shed more light on cellular events specifically induced by the cytoplasmic domain of the α subunit. Such signals, if any, may account for the specificity of GM-CSF, IL-3 and IL-5. The chimeric receptor system we have described is one method which will be useful to analyze functions of the cytoplasmic domain of the α subunit.

Acknowledgments

We thank T. Kinoshita, T. Kitamura, N. Sato, T. Itoh and H. Kurata for collaboration and helpful discussion and M. Ohara for comments on this manuscript. This work was supported in part by a grant-in-aid for scientific research on priority areas and cancer research from the Ministry of Education, Science and Culture of Japan. The DNAX Research Institute of Molecular and Cellular Biology is supported by the Schering-Plough Corporation.

References

1. Arai, K., Lee, F., Miyajima, A., Miyatake, S., Arai, N., and Yokota, T. (1990) Annu. Rev. Biochem. 59, 783-836.
2. Gasson, J.C. (1991) Blood 77, 1131-1145.
3. Miyajima, A., Kitamura, T., Harada, N., Yokota, T., and Arai, K. (1992) Annu. Rev. Immunol. 10, 295-331.
4. Miyajima, A., Mui, A.L.-F., Ogorochi, T., and Sakamaki, K. (1993) Blood 82, 1960-1974.
5. Hayashida, K., Kitamura, T., Gorman, D.M., Arai, K., Yokota, T., and Miyajima, A. (1990) Proc. Natl. Acad. Sci. USA 87, 9655-9659.
6. Bazan, J.F. (1990) Proc. Natl. Acad. Sci. USA. 87, 6934-6938.
7. Kitamura, T., Sato, N., Arai, K., and Miyajima, A. (1991) Cell 66, 1165-1174.
8. Tavernier, J., Devos, R., Cornelis, S., Tuypens, T., Van der Heyden, J., Fiers, W., and Plaetinck, G. (1991) Cell 66, 1175-1184.
9. Takaki, S., Murata, Y., Kitamura, T., Miyajima, A., Tominaga, A., and Takatsu, K. (1993) J. Exp. Med. 177, 1523-1529.
10. Watanabe, S., Mui, A.L.-F., Muto, A., Chen, J.X., Hayashida, K., Yokota, T., Miyajima, A., and Arai, K. (1993) Mol. Cell. Biol. 13, 1440-1448.
11. Kitamura, T., Hayashida, K., Sakamaki, K., Yokota, T., Arai, K., and Miyajima, A. (1991) Proc. Natl. Acad. Sci. U.S.A. 88, 5082-5086.
12. Sasaki, K., Chiba, S., Hanazono, Y., Mano, H., Yazaki, Y., and Hirai, H. (1993) J. Biol. Chem. 268, 13697-13702.
13. Eder, M., Griffin, J.D., and Ernst, T.J. (1993) EMBO J. 12, 1647-1656.
14. Sakamaki, K., Miyajima, I., Kitamura, T., and Miyajima, A. (1992) EMBO J. 11, 3541-3549.
15. Polotskaya, A., Zhao, Y., Lilly, M.L., and Kraft, A.S. (1993) Cell Growth & Differ. 4, 523-531.
16. Weiss, M., Yokoyama, C., Shikama, Y., Naugle, C., Druker, B., and Sieff, C. (1993) Blood 82, 3298-3306.
17. Miyajima, A., Schreurs, J., Otsu, K., Kondo, A., Arai, K., and Maeda, S. (1987) Gene 58, 273-281.
18. Watanabe, Y., Kitamura, T., Hayashida, K., and Miyajima, A. (1992) Blood 80, 2215-2220.
19. Mosmann, T. (1983) J. Immunol. Methods 65, 55-63.
20. Chomczynski, P., and Sacchi, N. (1987) Anal. Biochem. 162, 156-159.

21. Laemmli, U.K. (1970) Nature 227, 680-685.
22. Watanabe, S., Muto, A., Yokota, T., Miyajima, A., and Arai, K. (1993) Mol. Biol. Cell 4, 983-992.
23. Sato, N., Sakamaki, K., Terada, N., Arai, K., and Miyajima, A. (1993) EMBO J. 12, 4181-4189.
24. Kanakura, Y., Druker, B., Cannistra, S.A., Furukawa, Y., Torimoto, Y., and Griffin, J.D. (1990) Blood 76, 706-715.
25. Polotskaya, A., Zhao, Y., Lilly, M.B., and Kraft, A.S. (1994) J. Biol. Chem. 269, 14607-14613.