Biochemical and Biophysical Research Communications **277,** 195–199 (2000)
doi:10.1006/bbrc.2000.3662, available online at http://www.idealibrary.com on IDEAL®

Flt3 Ligand Induces Tyrosine Phosphorylation of Gab1 and Gab2 and Their Association with Shp-2, Grb2, and PI3 Kinase

Shuli Zhang and Hal E. Broxmeyer[1]

Department of Microbiology/Immunology, Department of Medicine, and Walther Oncology Center, Indiana University School of Medicine, Indianapolis, Indiana 46202; and Walther Cancer Institute, Indianapolis, Indiana 46206

Received September 7, 2000

The receptor tyrosine kinase Flt3 has been shown to play an important role in proliferation, differentiation, and survival of hematopoietic stem and progenitor cells. Although some postreceptor signaling events of Flt3 have been characterized, the involvement of Gab family proteins in Flt3 signaling is not known. In this study, we show that both Gab1 and Gab2 are rapidly tyrosine phosphorylated after Flt3 ligand stimulation of Flt3 ligand-responsive cells. They interact with tyrosine-phosphorylated Shp-2, p85, Grb2, and Shc. The results suggest that Gab proteins are engaged in Flt3 signaling to mediate downstream activation of Shp-2 and PI3 kinase pathways and possibly the Ras/Raf/MAPK pathway.

Key Words: **Flt3; Gab; tyrosine phosphorylation; signal transduction; hematopoietic cells.**

Flt3 ligand (FL) is a potent co-stimulatory cytokine that acts synergistically with a wide range of other colony stimulating factors and interleukins to stimulate proliferation and differentiation of hematopoietic stem and progenitor cells [1–4]. Its receptor Flt3 belongs to type III receptor tyrosine kinases that also include receptors for colony-stimulating factor 1, Steel factor, and platelet-derived growth factor [5]. Previous studies have shown that ligand binding of Flt3 results in phosphorylation of multiple cytoplasmic proteins such as Shc, Shp-2, Ship, Cbl and Cbl-b, and activation of several downstream signaling pathways including the Ras/Raf/MAPK and PI3 kinase cascades [6–12].

Gab1 and Gab2 (Grb2 associated binder 1 and 2) are newly identified scaffolding adapter proteins which share amino acid and structural homology with IRS-1 [13–15]. They have a PH domain and multiple potential binding sites for SH2 and SH3 domains. Recent studies have demonstrated that Gab1 and Gab2 are involved in the signaling of many cytokines, growth factors, and antigen receptors [15–17]. They are rapidly tyrosine phosphorylated upon stimulation and interact with several signaling molecules, such as Shp-2, Grb2, Shc, and PI3 kinase. Overexpression of Gab1 or Gab2 activates ERK kinase and enhances growth factor-mediated signaling events [15, 18, 19]. Mice lacking Gab1 die *in utero* and display developmental defects in the heart, placenta, and skin, which are similar to phenotypes observed in mice lacking signals of the hepatocyte growth factor, platelet-derived growth factor, and epidermal growth factor pathways [20].

For these reason, we evaluated the possibility that Gab1 and Gab2 might be involved in Flt3 signaling in three hematopoietic cell lines: Baf3/Flt3, THP-1, and RS4;11. FL induced rapid tyrosine phosphorylation of Gab1 and Gab2, and both Gab1 and Gab2 interacted with Shp-2, p85, Grb2, and Shc.

MATERIALS AND METHODS

Cytokines and antibodies. Recombinant human FL was generously provided by Immunex Corp. (Seattle, WA). Recombinant mouse IL-3 was purchased from R & D Systems (Minneapolis, MN). Rabbit polyclonal anti-Flt3, anti-Shp2, anti-Grb2, and anti-Shc antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit polyclonal anti-Gab1, anti-Gab2, anti-p85 antibody, and anti-phosphotyrosine mAb (4G10) were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY).

Cell lines and stimulation. The murine IL-3 dependent hematopoietic cell line Baf3/Flt3, a subline of Baf3 transduced with the human Flt3 receptor gene kindly provided by Immunex Corp., was cultured as described previously [10]. Human acute leukemia cell lines THP-1 and RS4;11 were obtained from ATCC and maintained in RPM I1640 medium supplemented with 10% fetal calf serum. Prior to FL stimulation, cells were washed with PBS and placed in serum-free RPMI1640 overnight. Cells were then washed once with

This work was supported by U.S. Public Health Service Grants R01 HL56416 and R01 DK53674 to H.E.B.

[1] To whom correspondence should be addressed at Walther Oncology Center, Indiana University School of Medicine, 1044 West Walnut Street, Indianapolis, IN 46202-5254. Fax: (317) 274-7592. E-mail: hbroxmey@iupui.edu.

0006-291X/00 $35.00

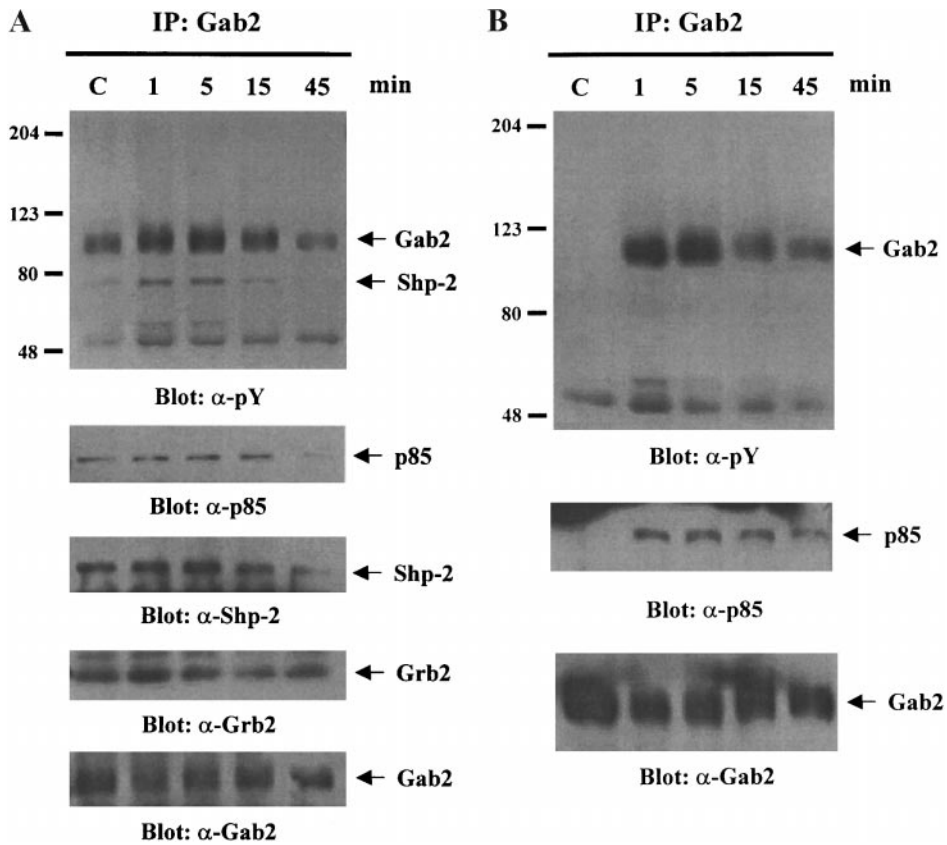

FIG. 1. FL induces tyrosine phosphorylation of Gab2 and its association with p85 and Shp-2 in both Baf3/Flt3 and THP-1 cells. Growth factor-starved Baf3/Flt3 (A) or serum-starved THP-1 (B) cells were stimulated with human FL (100 ng/ml) for various periods of time. Gab2 was immunoprecipitated from cell lysates and immunoblotted with anti-phosphotyrosine antibody (pY). The membranes were stripped and reblotted with the indicated antibodies. Results shown are representative of three experiments.

serum-free medium and stimulated with human FL (100 ng/ml) at 37°C for the indicated times.

Immunoprecipitation and immunoblotting. Cell extracts were prepared and subjected to immunoprecipitation and immunoblotting with the antibodies indicated as described previously [10]. Briefly, after stimulation at 37°C with human FL (100 ng/ml), cells were lysed in lysis buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 10% glycerol, 5 mM EGTA, 50 mM NaF, 1 mM Na_3VO_4, 1 μg/ml of aprotinin, leupepstin, and pepstatin, 100 μg/ml phenylmethylsulfonyl fluoride). For immunoprecipitations, antibodies were incubated with 0.5–1 mg of cell lysate for 1 h at 4°C. The immune complexes were recovered by incubation with protein A-agarose beads (Santa Cruz) for 1 h at 4°C. After washing three times in lysis buffer and once in PBS containing 1mM Na_3VO_4, the immune complexes were dissociated in SDS sample buffer and analyzed by SDS–PAGE and immunoblot. For immunoblotting, primary antibodies were detected with horseradish peroxidase-conjugated secondary antibodies and ECL (Amersham, Arlington, IL).

RESULTS AND DISCUSSION

FL induces transient tyrosine phosphorylation of Gab2 in both Baf3/Flt3 and THP-1 cells. To test whether Gab2 was involved in Flt3 signaling, we used Baf3/Flt3 cells transfected with full-length human Flt3 cDNA. This subline stably expresses human Flt3 receptor on the cell surface and proliferates in response to human FL [10]. THP-1 cells, which express endogenous human Flt3, were also used [9, 21]. Neither cell line expressed Gab1 as measured by immunoblotting. Gab2 was immunoprecipitated from cells treated with FL for various periods of time, separated by SDS–PAGE, transferred to the PVDF membrane and blotted with anti-phosphotyrosine antibody. As shown in Fig. 1, FL stimulation induced transient tyrosine phosphorylation of Gab2 in both cell lines, which peaked at about 5 min and then decreased. There was a basal level of phosphorylation of Gab2 in Baf3/Flt3 cells, and Gab2 constitutively associated with Shp-2, p85, and Grb2 (Fig. 1A). FL stimulation increased association of Gab2 with Shp-2, but not with p85. By 45 min, the phosphorylation of Gab2 returned to or was below the basal level, and there was little association of Gab2 with p85 and Shp-2, suggesting that phosphorylation of Gab2 was required for these interactions.

In THP-1 cells, in which there was no constitutive tyrosine phosphorylation, FL induced a similar pattern of Gab2 phosphorylation (Fig. 1B). In contrast to Baf3/Flt3 cells, FL did not induce tyrosine phosphorylation of Shp-2, and Shp-2 was not detected in Gab2 immu-

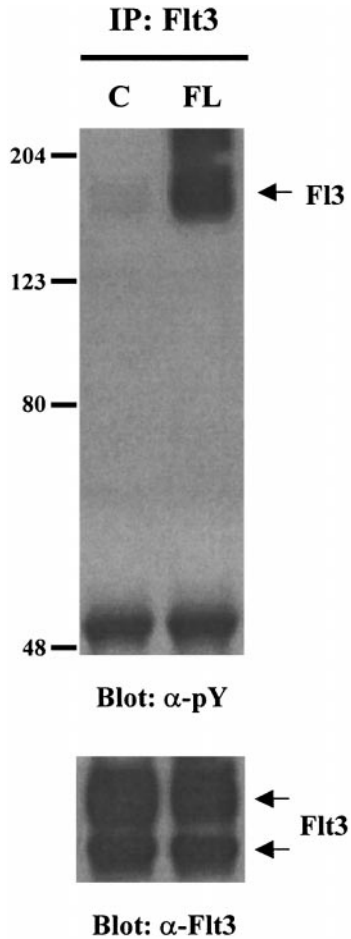

FIG. 2. FL induces tyrosine phosphorylation of Flt3 in RS4;11 cells. Serum-starved RS4;11 cells were stimulated with human FL (100 ng/ml) for 5 min. Flt3 was immunoprecipitated from cell lysates and immunoblotted with anti-phosphotyrosine antibody (pY). The membrane was stripped and reblotted with anti-Flt3 antibody. Results shown are representative of two experiments.

noprecipitates, suggesting that phosphorylation of Shp-2 is required for its association with Gab2. Also, Gab2 associated with p85 only after FL stimulation in THP-1 cells. No tyrosine phosphorylation of p85 was detected in Baf3/Flt3 or THP-1 cells after FL stimulation. Previously, we found that a p100 phosphorylated protein associated with both Shp-2 and p85 in these cells [9]. The results suggest that this p100 protein is Gab2. However, Flt3 was not detected in Gab2 immunoprecipitates, implying that Gab2 does not associate directly with Flt3.

FL induces transient tyrosine phosphorylation of both Gab1 and Gab2 in RS4;11 cells. To test whether Gab1 might also be involved in Flt3 signaling, RS4;11 cells, which express both Gab1 and Gab2 proteins, were used. This cell line was derived from the bone marrow of a patient with acute lymphoblastic leukemia [22]. RS4;11 cells express endogenous human Flt3 receptor as measured by both flow cytometry and immunoblotting (data not shown). FL stimulation induced autophosphorylation of Flt3 on tyrosine in this cell line (Fig. 2). As seen in Figs. 3 and 4, FL induced transient tyrosine phosphorylation of both Gab1 and Gab2, which peaked earlier than in Baf3/Flt3 and THP-1 cells. Both Gab1 and Gab2 associated with p85 and Shp-2, but only after stimulation with FL. Since Gab1 and Gab2 have been shown to be substrates for Shp-2 [15]. it is possible that Shp-2, once bound to Gab1 and Gab2, might dephosphorylate these two proteins, thus decreasing the interaction of Gab1 and Gab2 with either Shp-2 or p85. Both Gab1 and Gab2 constitutively associated with Grb2. Shc were also detected in Gab1 and Gab2 immunoprecipitates. Whether Shc binds directly to Gab1 and Gab2 or the binding is mediated through Grb2 is yet to be determined. As noted for

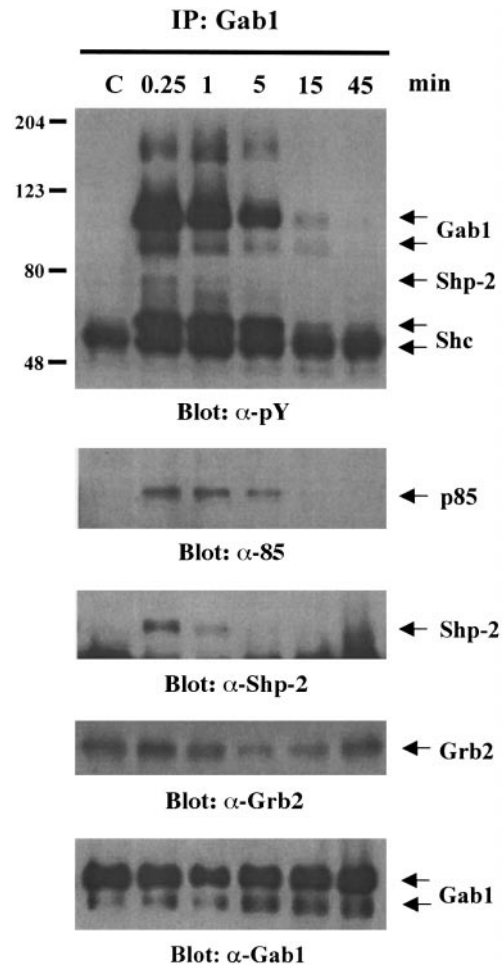

FIG. 3. FL induces tyrosine phosphorylation of Gab1 and its association with p85, Shp-2, and Grb2 in RS4;11 cells. Serum-starved RS4;11 cells were stimulated with human FL (100 ng/ml) for various periods of time. Gab1 was immunoprecipitated from cell lysates and immunoblotted with anti-phosphotyrosine antibody (pY). The membrane was stripped and reblotted with the indicated antibodies. Results shown are representative of three experiments.

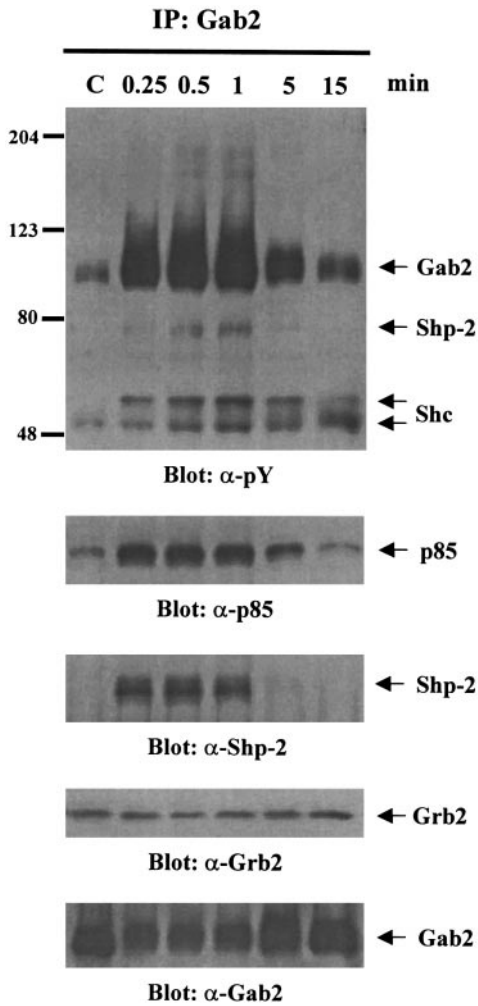

FIG. 4. FL induces tyrosine phosphorylation of Gab2 and its association with p85, Shp-2, and Grb2 in RS4;11 cells. Serum-starved RS4;11 cells were stimulated with human FL (100 ng/ml) for various periods of time. Gab2 was immunoprecipitated from cell lysates and immunoblotted with anti-phosphotyrosine antibody (pY). The membrane was stripped and reblotted with the indicated antibodies. Results shown are representative of three experiments.

Baf3/Flt3 and THP-1 cells, Flt3 was not detected in either Gab1 or Gab2 immunoprecipitates in RS4;11 cells, implying that Gab1 and Gab2 are not directly associated with Flt3.

Previous studies have implicated Gab1 and Gab2 in activation of the ERK pathway [15, 18–20, 23]. Overexpression of both Gab1 and Gab2 promoted mitogenic activation of ERK kinase activity. Since FL activates ERK [10], it is possible that Gab proteins may also link Flt3 to the ERK pathway. By interaction with p85, Gab1 and Gab2 may also modulate activation of PI3-kinase. Gab1 has been shown to mediate PI3-kinase activation and the promotion of cell survival by nerve growth factor [24]. Since p85 does not bind directly to human Flt3 receptor [9], it is possible that Gab1 or Gab2 may couple Flt3 to the PI3-kinase pathway. Thus Gab1 and Gab2 can act as scaffolding adapter proteins in Flt3 signaling and may participate in the activation of several downstream pathways. The exact roles of Gab1 and Gab2 in Flt3 signaling need to be determined.

REFERENCES

1. Lyman, S. D., James, L., Vanden Bos, T., de Vries, P., Brasel, K., Gliniak, B., Hollingsworth, L. T., Picha, K. S., McKenna, H. J., Splett, R. R., *et al.* (1993) Molecular cloning of a ligand for the flt3/flk-2 tyrosine kinase receptor: A proliferative factor for primitive hematopoietic cells. *Cell* **75,** 1157–1167.
2. Lyman, S. D., James, L., Johnson, L., Brasel, K., de Vries, P., Escobar, S. S., Downey, H., Splett, R. R., Beckmann, M. P., and McKenna, H. J. (1994) Cloning of the human homologue of the murine flt3 ligand: A growth factor for early hematopoietic progenitor cells. *Blood* **83,** 2795–2801.
3. Hannum, C., Culpepper, J., Campbell, D., McClanahan, T., Zurawski, S., Bazan, J. F., Kastelein, R., Hudak, S., Wagner, J., Mattson, J., *et al.* (1994) Ligand for FLT3/FLK2 receptor tyrosine kinase regulates growth of haematopoietic stem cells and is encoded by variant RNAs. *Nature* **368,** 643–648.
4. Broxmeyer, H. E., Lu, L., Cooper, S., Ruggieri, L., Li, Z. H., and Lyman, S. D. (1995) Flt3 ligand stimulates/costimulates the growth of myeloid stem/progenitor cells. *Exp. Hematol.* **23,** 1121–1129.
5. Rosnet, O., and Birnbaum, D. (1993) Hematopoietic receptors of class III receptor-type tyrosine kinases. *Crit. Rev. Oncog.* **4,** 595–613.
6. Dosil, M., Wang, S., and Lemischka, I. R. (1993) Mitogenic signalling and substrate specificity of the Flk2/Flt3 receptor tyrosine kinase in fibroblasts and interleukin 3-dependent hematopoietic cells. *Mol. Cell. Biol.* **13,** 6572–6585.
7. Rottapel, R., Turck, C. W., Casteran, N., Liu, X., Birnbaum, D., Pawson, T., and Dubreuil, P. (1994) Substrate specificities and identification of a putative binding site for PI3K in the carboxy tail of the murine Flt3 receptor tyrosine kinase. *Oncogene* **9,** 1755–1765.
8. Casteran, N., Rottapel, R., Beslu, N., Lecocq, E., Birnbaum, D., and Dubreuil, P. (1994) Analysis of the mitogenic pathway of the FLT3 receptor and characterization in its C terminal region of a specific binding site for the PI3′ kinase. *Cell. Mol. Biol.* **40,** 443–456.
9. Zhang, S., and Broxmeyer, H. E. (1999) p85 subunit of Pi3 kinase does not bind to human Flt3 receptor, but associates with Shp2, Ship, and a tyrosine-phosphorylated 100-kDa protein in Flt3 ligand-stimulated hematopoietic cells. *Biochem. Biophys. Res. Commun.* **254,** 440–445.
10. Zhang, S., Mantel, C., and Broxmeyer, H. E. (1999) Flt3 signaling involves tyrosyl-phosphorylation of SHP-2 and SHIP and their association with Grb2 and Shc in Baf3/Flt3 cells. *J. Leukocyte Biol.* **65,** 372–380.
11. Lavagna-Sevenier, C., Marchetto, S., Birnbaum, D., and Rosnet, O. (1998) The CBL-related protein CBLB participates in FLT3 and interleukin-7 receptor signal transduction in pro-B cells. *J. Biol. Chem.* **273,** 14962–14967.
12. Lavagna-Sevenier, C., Marchetto, S., Birnbaum, D., and Rosnet, O. (1998) FLT3 signaling in hematopoietic cells involves CBL, SHC and an unknown P115 as prominent tyrosine-phosphorylated substrates. *Leukemia* **12,** 301–310.
13. Holgado-Madruga, M., Emlet, D. R., Moscatello, D. K., Godwin, A. K., and Wong, A. J. (1996) A Grb2-associated docking protein in EGF- and insulin-receptor signalling. *Nature* **379,** 560–564.

14. Gu, H., Pratt, J. C., Burakoff, S. J., and Neel, B. G. (1998) Cloning of p97/Gab2, the major SHP2-binding protein in hematopoietic cells, reveals a novel pathway for cytokine-induced gene activation. *Mol. Cell* **2,** 729–740.
15. Nishida, K., Yoshida, Y., Itoh, M., Fukada, T., Ohtani, T., Shirogane, T., Atsumi, T., Takahashi-Tezuka, M., Ishihara, K., Hibi, M., and Hirano, T. (1999) Gab-family adapter proteins act downstream of cytokine and growth factor receptors and T- and B-cell antigen receptors. *Blood* **93,** 1809–1816.
16. Wickrema, A., Uddin, S., Sharma, A., Chen, F., Alsayed, Y., Ahmad, S., Sawyer, S. T., Krystal, G., Yi, T., Nishada, K., Hibi, M., Hirano, T., and Platanias, L. C. (1999) Engagement of Gab1 and Gab2 in erythropoietin signaling. *J. Biol. Chem*. **274,** 24469–24474.
17. Hibi, M., and Hirano, T. (2000) Gab-family adapter molecules in signal transduction of cytokine and growth factor receptors, and T and B cell antigen receptors. *Leuk. Lymphoma.* **37,** 299–307.
18. Takahashi-Tezuka, M., Yoshida, Y., Fukada, T., Ohtani, T., Yamanaka, Y., Nishida, K., Nakajima, K., Hibi, M., and Hirano, T. (1998) Gab1 acts as an adapter molecule linking the cytokine receptor gp130 to ERK mitogen-activated protein kinase. *Mol. Cell. Biol.* **18,** 4109–4117.
19. Zhao, C., Yu, D. H., Shen, R., and Feng, G. S. (1999) Gab2, a new pleckstrin homology domain-containing adapter protein, acts to uncouple signaling from ERK kinase to Elk-1. *J. Biol. Chem*. **274,** 19649–19654.
20. Itoh, M., Yoshida, Y., Nishida, K., Narimatsu, M., Hibi, M., Hirano, T. (2000) Role of Gab1 in heart, placenta, and skin development and growth factor- and cytokine-induced extracellular signal-regulated kinase mitogen-activated protein kinase activation. *Mol. Cell. Biol.* **20,** 3695–3704.
21. Dehmel, U., Zaborski, M., Meierhoff, G., Rosnet, O., Birnbaum, D., Ludwig, W. D., Quentmeier, H., and Drexler, H. G. (1996) Effects of FLT3 ligand on human leukemia cells. I. Proliferative response of myeloid leukemia cells. *Leukemia* **10,** 261–270.
22. Stong, R. C., Korsmeyer, S. J., Parkin, J. L., Arthur, D. C., and Kersey, J. H. (1985) Human acute leukemia cell line with the t(4;11) chromosomal rearrangement exhibits B lineage and monocytic characteristics. *Blood* **65,** 21–31.
23. Winnay, J. N., Bruning, J. C., Burks, D. J., and Kahn, C. R. (2000) Gab-1-mediated IGF-1 signaling in IRS-1-deficient 3T3 fibroblasts. *J. Biol. Chem*. **275,** 10545–10550.
24. Holgado-Madruga, M., Moscatello, D. K., Emlet, D. R., Dieterich, R., and Wong, A. J. (1997) Grb2-associated binder-1 mediates phosphatidylinositol 3-kinase activation and the promotion of cell survival by nerve growth factor. *Proc. Natl. Acad. Sci. USA* **94,** 12419–12424.