INTERLEUKIN-6 (IL-6), leukemia inhibitory factor (LIF), oncostatin M (OSM), IL-11, and ciliary neurotrophic factor (CNTF) exert pleiotropic functions on multiple cell types and constitute the IL-6 family of hematopoietic and neurotrophic cytokines. The receptors for the IL-6 family of cytokines share gp130 as a signal transducing subunit, explaining at least in part the molecular basis of the functional redundancy in the biologic activities exerted by these cytokines. Except for CNTF, all the IL-6 family of cytokines have been shown to play important roles in hematopoiesis and lymphopoiesis. The stimulation of the receptors by the IL-6 family of cytokines resulted in a rapid tyrosine phosphorylation of multiple molecules, including gp130 itself, but the cytoplasmic region of gp130 and other receptor subunits of the IL-6 family cytokines contain no intrinsic tyrosine kinase domain. The recent evidence showed that Janus kinase (Jak) family kinases constitutively associate with gp130 and are activated by the IL-6 family of cytokines, leading to the tyrosine-phosphorylation and activation of signal transducer and activator of transcription (STAT) family transcription factors. Furthermore, we showed that IL-6 induced the tyrosine-phosphorylation and activation of a STAT-associated novel tyrosine kinase, Sak (or p72Sak). However, the Jak-STAT signal pathway alone does not fully explain how the IL-6 family of cytokines exert their multiple biologic activities. Btk tyrosine kinase was identified as the responsible gene product of X-linked agammaglobulinemia (XLA) in human and X-linked immunodeficiency (XID) in mouse, showing that Btk is critically involved in B lymphopoiesis. Btk and Tec kinase together with Itk/Tsk and Dsrc28C constitute a novel subfamily of cytoplasmic tyrosine kinases whereas IL-3 and granulocyte colony-stimulating factor activated Tec but not Btk in a pro-B cell line. Furthermore, both Btk and Tec kinases were associated with gp130 without the ligand stimulation. Because Btk is a critical tyrosine kinase for B lymphopoiesis and Tec is considered to be involved in hematopoiesis, the results suggest the involvement of gp130-Btk-Tec signal pathway in early lymphohematopoiesis.
hours, then incubated for 10 minutes with IL-6 (200 ng/mL) and soluble IL-6 receptor α (IL-6Ra) (0.5 μg/mL). BAF-B03 and BAFhGCSFR cells were starved in serum-free medium for 1 to 2 hours, incubated with IL-3 (10% vol/vol WEHI-3B supernatants) and human G-CSF (100 ng/mL), respectively, for 10 minutes. The cells were then lysed in a lysis buffer (50 mmol/L Tris-HCl, pH 7.4, 0.15 mol/L NaCl, containing 1% NP-40, 1 mmol/L sodium orthovanadate, 1 mmol/L NaF, 1 mmol/L phenylmethylsulfonfluoride, and 10 μg/mL each of aprotinin, pepstatin, and leupeptin). In some experiments, cells were lysed under a mild detergent condition using a lysis buffer containing 0.1% NP-40. Proteins from the cell lysates were immunoprecipitated with respective antisera, resolved to 4% to 20% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to Immobilon (Millipore, Bedford, MA). The immunoblot filter was then immunoblotted with an antiphosphotyrosine antibody (4G10; UBI, New York, NY). The filter was then stripped and reprobed with respective antisera. Immunoreactive proteins were visualized by the enhanced chemiluminescence detection system (Amersham, Tokyo, Japan).

In-gel kinase assay. An in-gel kinase assay was performed as previously described. The immunoprecipitate with respective antisera from cytokine-stimulated cell lysates was resolved by 4% to 20% SDS-PAGE. After electrophoresis, the gel was washed twice with 50 mmol/L Tris-HCl, pH 7.4, 5 mmol/L 2-mercaptoethanol, incubated with 50 mmol/L Tris-HCl, pH 7.4, 5 mmol/L 2-mercaptoethanol, and 6 mol/L guanidine hydrochloride, and washed with 50 mmol/L Tris-HCl, pH 7.4, 5 mol/L 2-mercaptoethanol containing 0.04% Tween 40 (Nacalai Tesque, Kyoto, Japan). The gel was incubated in 20 mmol/L HEPES, pH 7.4, 20 mmol/L MgCl2, 20 mmol/L MnCl2, 2 mmol/L EDTA, and 5 μCi/mL γ-32P-adenosine triphosphate at 30°C for 30 minutes. Finally, the gel was washed several times with 5% trichloroacetic acid and 5% sodium pyrophosphate, followed by drying and autoradiography. Phosphorylated proteins were detected by Fuji BAS2000 Bio-image Analyzer (Fuji Film, Tokyo, Japan).

RESULTS

The stimulation of either IL-3R, gp130, or G-CSFR variably induced the tyrosine phosphorylation of several cytoplasmic signal transducing molecules. Because the bio- logic activities of IL-3, G-CSF, and the IL-6 family of cytokines are different, despite the fact that some are redundant, some signals generated through these cytokine receptors should be distinct. To identify a unique signal generated through gp130 molecule, we used an IL-3-dependent murine hematopoietic cell line, BAF-B03, and its stable transfectants, BAFm130 and BAFhGCSFR, which express murine gp130 and human G-CSFR, respectively. A murine hematopoietic cell line, BAF-B03, is a subclone of an IL-3-dependent cell line, BA/F3, which has properties of undifferentiated pro-B cells with their Ig genes in germline configuration. BAF-B03, BAFm130, and BAFhGCSFR proliferated equally well in response to IL-3, IL-6 plus soluble IL-6Ra, and G-CSF, respectively (data not shown). Figure 1 shows that a variety of proteins, such as Jak1, Jak2, 72-kD Sak, and Vav were variably tyrosine phosphorylated by different stimuli: IL-3 caused prominent tyrosine phosphorylation of Jak2 as previously reported, whereas the stimulation of either gp130 by IL-6 plus soluble IL-6Ra or G-CSFR induced the tyrosine phosphorylation of both Jak1 and Jak2. Tyrosine phosphorylation of Sak was induced by the stimulation of IL-3R, gp130, and G-CSFR as previously described. The stimulation of both IL-3R and G-CSFR induced the prominent tyrosine phosphorylation of Vav protein, which possibly functions as a signal transducer and a transcription factor. However, gp130-stimulation induced the minor tyrosine phosphorylation of Vav protein. We further examined whether the stimulation of these cytokine receptors induces the tyrosine phosphorylation of Btk and Tec tyrosine kinases. Figure 1 shows that the stimulation of IL-3R or gp130 induced the tyrosine phosphorylation of Tec tyrosine kinase. G-CSF induced the weak tyrosine phosphorylation of Tec tyrosine kinase. The tyrosine phosphorylation of Btk tyrosine kinase was only induced by the stimulation of gp130. Reprobing with antisera against respective proteins confirmed that the stimulation-induced changes were not caused by alterations of protein levels. The lower panels of Fig 1 also showed that the protein levels of each signal-transducing molecule were similar among BAF-B03, BAFm130, and BAFhGCSFR cells. The results showed that the stimulation of either IL-3R, gp130, or G-CSFR variably induced the tyrosine phosphorylation of several cytoplasmic signal-transducing molecules in a pro-B cell line, BAF-B03 and its stable transfectants, BAFm130 and BAFhGCSFR, suggesting that the combination of a variety of signal-transducing molecules that were variably activated may determine the specificity or the intensity of signals generated through various cytokine receptors. Among these signal-transducing molecules, Btk was tyrosine phosphorylated only by the stimulation of gp130, suggesting that in addition to the above-mentioned combination of a variety of signal-transducing molecules, Btk is involved in the generation of a unique signaling through gp130.

The stimulation of gp130 induced tyrosine phosphorylation of Jak1, Btk, and Tec tyrosine kinases in a time-dependent manner. Because both Btk and Tec kinases belong to the same subfamily of cytoplasmic tyrosine kinase and the defect of the former was shown to result in B-lymphocyte deficiency and the latter was suggested to be involved in hematopoiesis, we further examined the role of both Btk and Tec in gp130-signaling. At first, we investigated the time course of tyrosine phosphorylation of either gp130, Jak1, Btk, and Tec after gp130 stimulation in BAFm130 cells. As shown in Fig 2, tyrosine phosphorylation of both gp130 and Jak1 reached the maximum level at 5 to 15 minutes after gp130 stimulation and thereafter decreased. However, tyrosine phosphorylation of both Btk and Tec remained at the maximum level up to 60 minutes. Tyrosine phosphorylation of Btk and Tec seemed to be regulated differently from that of gp130 or Jak1. Figure 2, c and d shows that the 60-kD and 100-kD tyrosine phosphorylated proteins were coimmunoprecipitated with anti-Tec and anti-Btk, respectively after gp130 stimulation, although these 60-kD and 100-kD molecules showed no cross-reactivity with respective antiserum by Western blotting analysis. The molecular nature of these molecules was unknown.

The stimulation of gp130 enhanced in vitro kinase activity of Btk and Tec tyrosine kinases. We next examined whether gp130 stimulation actually activates the tyrosine kinase activity of both Btk and Tec kinases. The cell lysates of BAFm130 were immunoprecipitated with either anti-Btk or anti-Tec antiserum and kinase activity of the immunoprecipitate was examined by an in-gel kinase assay. Figure 3
Fig 1. Tyrosine phosphorylation of multiple signal transducing proteins by the stimulation of IL-3R, gp130, and G-CSFR. Cells of BAF-603.2, BAFml30, and BAF-GCSFR were stimulated with IL-3, IL-6 plus soluble IL-6Ra, and G-CSF, respectively, for 10 minutes, and the lysates of stimulated (+) or unstimulated (−) cells were immunoprecipitated with anti-Jak1 (a), anti-Jak2 (b), anti-STAT (c), anti-Vav (d), anti-Tec (e), and anti-Btk (f) and immunoblotted with antiphosphotyrosine antibody (anti-PY) (upper panel). The blot was stripped and reprobed with respective antisera as indicated (lower panel). Molecular weight markers on the left are in kilodaltons.

shows that the immunoprecipitate with each antiserum from the cells stimulated through gp130 contained higher in vitro kinase activity than that of unstimulated cells at the position corresponding to the molecular weight of Tec or Btk, showing that the stimulation of gp130 actually activated both Btk and Tec kinases.

Btk and Tec tyrosine kinases were constitutively associated with gp130. Because the results suggested that both Btk and Tec were directly activated by gp130 stimulation, we next examined whether Btk and Tec kinases are associated with gp130 as Jak kinase is. In the following experiments, we immunoprecipitated the cell lysates of BAFm130 under a mild detergent condition as described in Materials and Methods. Figure 4a shows that the immunoprecipitate with an antimmurine gp130 MoAb from either gp130-stimulated or unstimulated cells contained Tec and Btk, showing that both Tec and Btk were associated with gp130 independently of ligand stimulation. To further confirm this, we examined whether the immunoprecipitate with either anti-Tec or anti-Btk contains gp130. Figure 4b-1 and Fig 4c-1 show that under a mild detergent condition, in addition to Tec and Btk, several tyrosine-phosphorylated proteins (80 kD to 160 kD) were immunoprecipitated with either anti-Tec or anti-Btk antiserum after gp130 stimulation. Among these molecules, a 160-kD protein band contained gp130 (Fig 4b-2, c-2). gp130 was also detected in the immunoprecipitate with each antiserum from unstimulated cells. The results showed that both Tec and Btk are constitutively associated with gp130.

DISCUSSION

A variety of cytokines exert their functions by interacting with receptors that are members of the cytokine receptor superfamily. These receptors share extracellular motifs and possess restricted similarity in the cytoplasmic domains, lacking a catalytic kinase domain such as protein-tyrosine kinases. Therefore, they are designated as nontyrosine kinase receptor. Despite no intrinsic tyrosine kinase domains of cytokine receptors, a large number of cytokines induce the tyrosine phosphorylation of a variety of cellular proteins and, therefore, activation of tyrosine kinase is considered to be an initial step generating downstream signaling events. One of the central issues in cytokine receptor signaling has been to identify the kinases that couple ligand binding and...
Tyrosine phosphorylation. IL-2 receptor β chain was first shown to be associated with an src family tyrosine kinase, Lck. Furthermore, the members of Jak family of cytoplasmic protein tyrosine kinases have been shown to be associated with and activated by several cytokine receptors, including the receptors for erythropoietin, growth hormone, prolactin, G-CSF, IL-2, IL-3, granulocyte macrophage (GM)-CSF, and IL-6 family of cytokines. This provides an important mechanistic analogy between non-tyrosine kinase type cytokine receptors and tyrosine kinase type receptors, such as EGF and PDGF receptors. Jak kinase was shown to be essential to activate STAT family proteins. However, Jak-STAT signal transduction pathway alone can not fully explain the pleiotropy in biologic activities of cytokines and it is possible that several cytoplasmic tyrosine kinases are involved in cytokine-mediated signal transduction. In this report we demonstrate that in addition to Jak kinase, both Btk and Tec tyrosine kinases are activated by the stimulation of gp130 and constitutively associated with gp130.

The family of Btk-Tec-Itk/Tsk tyrosine kinases are abundantly expressed in hematopoietic lineage cells. Tec tyrosine kinase was recently shown to be involved in IL-3 signaling pathway. Here we showed that Tec was also involved in the gp130-mediated signaling pathway. Because the IL-6 family of cytokines are known to act on hematopoietic stem cells (IL-6, IL-11, and LIF were shown to work synergistically with IL-3, IL-4, stem cell factor), and GM-CSF to support the proliferation of multipotential hematopoietic progenitors, our current data may suggest that Tec is involved in the signaling of cytokines acting on stem cells. The genetic defect associated with human XLA and murine XID results in a failure of B-cell maturation and activation. Btk tyrosine kinase was identified as the responsible gene.

Fig 2. Time course of tyrosine phosphorylation of gp130, Jak1, Btk, and Tec in BAFm130 cells. The immunoprecipitate with either antimurine gp130 MoAb, HMp1 (a), anti-Jak1 (b), anti-Tec (c), or anti-Btk (d) antibody from BAFm130 cells (2 x 10⁶ cells) stimulated with IL-6 plus soluble IL-6Rα for the indicated times (min) was probed by antiphosphotyrosine antibody (anti-PY) (upper panel). The blot was stripped and reprobed with respective antibody as indicated (lower panel). Molecular weight markers on the left are in kilodaltons.

Fig 3. Activation of Tec and Btk tyrosine kinases by the stimulation of gp130. The immunoprecipitate with either anti-Tec (a) or anti-Btk (b) antiserum derived from BAFm130 cells (5 x 10⁶ cells) stimulated with IL-6 plus soluble IL-6Rα (+) or unstimulated cells (-) was subjected to an in-gel kinase assay as described in Materials and Methods. Molecular weight markers on the left are in kilodaltons.
and activation of Btk in mast cells. Furthermore, anti-Ig induces Btk activation in B cells. However, the molecular mechanism(s) of the involvement of Btk in B-lymphocyte development remains unknown. Both IL-6 and IL-11 together with stem cell factor were shown to be effective in the primary culture in the maintenance of B-lymphoid progenitors. Both IL-6 and IL-11 are involved in the differentiation of B lymphocyte into antibody-producing cells.

We showed that overexpression of IL-6 induces polyclonal plasmacytosis in IL-6 transgenic mice. All evidence showed that gp130-mediated signals contribute early B lymphopoiesis and terminal differentiation of B lymphocytes. Considering these facts and the critical role of Btk in B lymphopoiesis, Btk may play a role in the gp130-mediated signaling pathway regulating B-cell growth and differentiation, although the molecular mechanism(s) of Btk is still unknown.

We showed that gp130 used not only Jak but also Btk and Tec in the cells expressing these tyrosine kinases. Our present results suggest that nontyrosine kinase type cytokine receptors, such as gp130, can use a variety of cytoplasmic tyrosine kinases that potentially possess the binding ability to the cytoplasmic region of respective cytokine receptors. This model indicates that cytokine receptors can use a distinct cytoplasmic tyrosine kinase or different combination of tyrosine kinases in a variety of cells depending on the species of tyrosine kinases expressed in the respective target cells. This model may explain at least in part the mechanisms by which functional pleiotropy in cytokine activity is generated. Actually, Hck, an src subfamily of tyrosine kinase, is activated by gp130 stimulation and associated with gp130 in embryonic stem (ES) cells. The expression of Hck is apparently restricted to ES cells and hematopoietic cells, suggesting that ES and hematopoietic cells uniquely possess the gp130-Hck signaling pathway. It seems to be also true that gp130-Btk is restricted to B-lineage cells and gp130-Tec is more widely distributed among hematopoietic lineage and hepatic cells in contrast to the wide distribution of gp130-Jak signaling pathway.

Both Btk and Tec were constitutively associated with gp130, although it remains to be determined which region of gp130 binds with which region of Btk or Tec. Members of cytokine receptor family contains conserved box1 and box2 motifs within a membrane proximal cytoplasmic domain. This membrane-proximal region of cytokine receptors has been shown to be essential for the activation and association of Jak kinase by cytokine receptors including gp130. The Btk-Tec tyrosine kinase subfamily contains SH2 and SH3 domains and possesses a unique feature at their amino terminal region. This region is in distinct contrast to the src tyrosine kinase subfamily and designated as a pleckstrin homology (PH) domain. PH domain is present in a variety of signal transducing molecules including several serine-threonine kinases, guanosine-triphosphatases (GTPase), GTPase activating protein, and phospholipases. Although the function of PH domain is still unknown, this domain as well as the SH2 and SH3 domains may function in interaction with other signal transducing molecules. These features suggest that Btk and Tec interact with several other signaling molecules via these unique regions. Recent work for XLA and XID. The phenotypes associated with these immunodeficiencies indicate that Btk plays a critical role in B-cell development. Recent studies have also shown that FceRI cross-linking induced the tyrosine phosphorylation and activation of Btk in mast cells.

[Figure 4. Constitutive association of Btk and Tec kinases with gp130 in BAFl30 cells. The lysate of BAFl30 cells (1 x 10^6 cells) stimulated with IL-6 plus soluble IL-6Ra (+) or unstimulated (−) was obtained under a mild detergent condition as described in Materials and Methods. The lysate was immunoprecipitated with either antimurine gp130 MoAb, HMj31 (a), anti-Tec (b), or anti-Btk (c) and immunoblotted with either antiphosphotyrosine antibody (b-1, c-1), antimurine gp130 MoAb, RMj34 (a-1, b-2, c-2), anti-Tec (a-2, b-3), or anti-Btk (a-3, c-3). (●) indicates tyrosine-phosphorylated 160-kD, 140-kD, 100-kD, and 95-kD (b and c) and 80-kD (c) molecules. (●) indicates Tec (b) and Btk (c) molecules. Molecular weight markers on the left are in kilodaltons.]
showed that Btk interacts with SH3 domains of Fyn, Lyn, and Hck. However, mutations in either the PH domain, SH2 domain, or SH3 domain caused XLA, suggesting that multiple proteins associated with Btk are involved in Btk-mediated signaling pathways required for B-cell maturation and activation. From this point of view, any region of Btk could be the candidate for the binding site of gp130. Recently, Tec was actually shown to bind Lyn through its N-terminal unique domain directly. Furthermore, it is essential to determine the downstream effectors of Btk and Tec tyrosine kinases to elucidate the molecular mechanism of gp130-mediated signalings regulating hematopoiesis and lymphopoiesis. In this regard, the results presented in Fig 2, c and d and Fig 4, b and c may suggest the presence of several molecules associated with Btk or Tec in addition to gp130, although this possibility remains to be elucidated.

Taken together, the results reported in this article give us the insight of the molecular mechanism(s) of the functions of gp130 and both Btk and Tec kinases in lymphohematopoiesis.

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Association and activation of Btk and Tec tyrosine kinases by gp130, a signal transducer of the interleukin-6 family of cytokines

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