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IL-5 Receptor-mediated Tyrosine Phosphorylation of SH2/SH3-containing Proteins and Activation of Bruton's Tyrosine and Janus 2 Kinases

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Summary

Interleukin 5 (IL-5) induces proliferation and differentiation of B cells and eosinophils by interacting with its receptor (IL-5R) which consists of two distinct polypeptide chains, α and β (βc). Although both IL-5Rα and βc lack a kinase catalytic domain, IL-5 is capable of inducing tyrosine phosphorylation of cellular proteins. We investigated the role of IL-5Rα in tyrosine phosphorylation of molecules involved in IL-5 signal transduction, using an IL-5–dependent early B cell line, Y16 and transfectants expressing intact or mutant IL-5Rα together with intact βc. The results revealed that the transfectants expressing truncated IL-5Rα, which entirely lacks a cytoplasmic domain, together with βc, showed neither protein-tyrosine phosphorylation nor proliferation in response to IL-5. This confirms that IL-5Rα plays a critical role in protein-tyrosine phosphorylation which triggers cell growth. IL-5 stimulation results in rapid tyrosine phosphorylation of βc and proteins containing *Src* homology 2 (SH2) and/or SH3 domains such as phosphatidyl-inositol-3 kinase, Shc, Vav, and HS1, suggesting their involvement in IL-5–mediated signal transduction. IL-5 stimulation significantly enhanced activities of Janus 2 and B cell–specific Bruton's tyrosine kinases (JAK2 and Btk) and increased the tyrosine phosphorylation of JAK2 kinase. These results and recent data on signaling of growth factors taken together, multiple biochemical pathways driven by tyrosine kinases such as JAK2 and Btk are involved in IL-5 signal transduction.

Mouse IL-5 (mIL-5)[1] plays an important role in the growth and differentiation of B cells and eosinophils (1–3). IL-5, IL-3, and GM-CSF display a variety of overlapping actions in the production and activation of eosinophils (4). mIL-5 binds to a specific cell surface receptor (IL-5R) with both high (K_d, 10–150 pM) and low affinity (K_d, 2–10 nM). The receptor for IL-5 is composed of an α chain (IL-5Rα) of ~60 kD and a β chain (βc) of ~130 kD (5–7). The IL-5Rα alone binds IL-5 with low affinity. The βc does not bind IL-5 by itself, but does form a high affinity IL-5R in combination with IL-5Rα (6–10). The α chain for both mouse and human IL-5R is specific for IL-5 (6–11), whereas βc is common to receptors for IL-3 and GM-CSF, as well (9, 11–16). Both IL-5rα and βc are members of the cytokine receptor superfamily, containing the hallmark WSXWS consensus sequence and four conserved cysteine residues in the extracellular domain. The cytoplasmic domains share limited similarity with other cytokine receptors and lack detectable kinase catalytic domains. Using a series of βc mutants, two domains in the membrane-proximal portion of βc were found to be important for transducing human GM-CSF–mediated growth signals (17).

Growth factors trigger proliferation of cells when they bind to their specific receptors. Receptors bound to a ligand provide signals that are transmitted by means of biochemical reaction cascades (18). Phosphorylation of tyrosine residues on signal-transducing molecules is essential for activation of signaling pathways (18). Several tyrosine-phosphorylated effector

[1] *Abbreviations used in this paper:* βc, common β chain for IL-5R, IL-3R, GM-CSFR; BTK, Bruton's tyrosine kinase; GAP, Ras GTPase activating protein; GRF, guanine nucleotide releasing factor; JAK, Janus kinase; mIL-5, mouse IL-5; PI, phosphatidylinositol; PLC, phospholipase C; PTK, protein tyrosine kinase; PY, phosphotyrosine; SH, Src homology.

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molecules controlling growth have been identified on growth factor receptors carrying intrinsic protein tyrosine kinase (PTK) activity. These effector molecules are phospholipase C-γ (PLC-γ), phosphatidylinositol-3 (PI-3) kinase, and Ras-associated GTPase-activating protein (GAP) (18). They contain *Src*-homology 2 (SH2) and/or *Src*-homology 3 (SH3) domains, the former of which binds to phosphorylated tyrosine on protein (19), whereas the latter has been suggested to be responsible for the targeting of signaling molecules to specific subcellular locations (20).

The *shc* gene codes for three protein products of ~46, 52, and 66 kD (21) containing a single COOH-terminal SH2 domain. The tyrosine-phosphorylated Shc binds to Grb2 which activates Sos protein, a Ras nucleotide-exchange protein (22). Ras has been shown to work downstream of tyrosine kinases in the growth signaling pathway (23–25). $p95^{vav}$ (26) and $p75^{HS1}$ (27), which are expressed specifically in hematopoietic cells and have SH2 and/or SH3 domains, were recently demonstrated to be involved in B cell antigen receptor–mediated signaling (28, 29). Based on their unique sequences, these molecules were assumed to function as DNA binding proteins (26, 27).

Unlike several growth factor receptor families that possess intrinsic kinase domains, the receptors for cytokines have no cytoplasmic domain homology with any known enzymes involved in receptor-mediated signal transduction such as PTKs, protein serine/threonine kinases, or GAPs. However, tyrosine phosphorylation of cellular proteins has been observed in various cytokine/cytokine receptor systems (30) and is believed to be crucial in their signalling (31). A recently discovered family of nonreceptor tyrosine kinases, including Tyk2, Janus kinase 1 (JAK1), and JAK2, was suggested to be associated with cytokine receptors including GM-CSF and IL-3 (32, 33). We have shown that IL-5 induces distinct tyrosine phosphorylation of proteins migrating at ~130 to 140, 92, 53, 48, and 45 kD in the IL-5–dependent early B cell line, T88-M (34). Here we report that IL-5Rα is essential for tyrosine phosphorylation of cellular proteins and cell proliferation in response to IL-5. We demonstrate that IL-5 stimulation induces rapid tyrosine phosphorylation of βc, as well as proteins containing one or two SH2 and/or SH3 domains such as PI-3 kinase, Vav, Shc, and HS1. IL-5 also increases tyrosine phosphorylation of JAK2. Furthermore, IL-5 activates JAK2 and Bruton's tyrosine kinase (Btk) (35, 36).

Materials and Methods

Antibodies and Reagents. Hamster anti–mouse βc mAb (HB) was prepared according to previously described methods (37) and purified from supernatants with serum-free medium using a protein G–Sepharose 4B column (Pharmacia, Uppsala, Sweden). Mouse antiserum to human HS1 was raised using proteins with the full-length sequence of human HS1 expressed in *Escherichia coli*. Polyclonal anti-GAP, PLCγ-1, PI-3 kinase, and Shc antibodies were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-Vav antibody was obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Mouse anti–phosphotyrosine (PY) mAbs, 4G10 and PY20, were purchased from Upstate Biotechnology Inc. and ICN Biochemicals (Cleveland, OH), respectively. Rabbit anti-JAK1 and JAK2 sera were purchased from Upstate Biotechnology Inc. Rabbit anti-Btk serum was prepared by repeated injections of a peptide corresponding to its unique region, as described previously (35). Rabbit anti-Fyn antibody was generated against a synthetic peptide corresponding to amino acid residues 25-141 of the human Fyn protein sequence (38). Anti-Lyn mAb, Lyn-8, raised against a synthetic peptide corresponding to the NH_2-terminal–specific sequence (Arg-25 to Ala-119) of human Lyn protein (39) was kindly provided by Drs. Y. Yamanashi and T. Yamamoto (Institute of Medical Science, University of Tokyo, Tokyo, Japan). Anti-Fyn and anti-Lyn antibodies reacted with both human and mouse Fyn and Lyn, respectively (38, 39). mIL-5 was prepared and purified using anti-mIL-5 mAb coupled beads (5). Herbimycin A was purchased from Wako Pure Chemicals Co. Ltd. (Tokyo, Japan) and solubilized in DMSO. RPMI-1640, and Hepes buffers were obtained from Nissui Pharmaceutical Co. Ltd. (Tokyo, Japan).

Cell Lines and Cell Culture. A mIL-2–dependent cell line, CTLL-2, was maintained in RPMI-1640 medium supplemented with 10% FCS and 50 μM 2-ME and 5% conditioned medium from Con A–stimulated rat spleen cells. A mIL-3–dependent FDC-P1 cell line was maintained in RPMI-1640 medium supplemented with 5% FCS, 50 μM 2-ME, and 5 U/ml of mIL-3. mIL-5–dependent cell line, Y16 (6), was maintained in RPMI-1640 medium supplemented with 4% FCS and 50 μM 2-ME in the presence of mIL-5 (5 U/ml). Before being used for assay, cell lines were washed three times with HBSS and incubated for 8 h at 37°C in fresh medium without cytokines. The following transfectants were used in this study (6, 9, 16): FDC-5R, transfectants of IL-5Rα cDNA; FDC-5RαΔcyto, transfectants of the mutated α chain cDNA lacking the entire cytoplasmic domain of the α chain; CTLL-5Rα, CTLL-2 transfectants of IL-5Rα cDNA; and CTLL-5Rα/β, CTLL-2 transfectants of cDNAs for both IL-5Rα and βc.

Proliferation Assay. Y16 cells were harvested and washed with HBSS, then inoculated onto a 96-well microtiter plate at a concentration of 10^4/200 μl/well with RPMI-1640 containing 4% FCS at 37°C for 8 h and subsequently cultured with 2,000 U/ml mIL-5 at 37°C for 36 h (6). Herbimycin A (3 μg/ml) was dissolved in the culture medium containing DMSO and added at the beginning of the culture. As a control, DMSO-containing medium without herbimycin A was added. The cells were pulse labeled with [^{3}H]thymidine (0.2 μCi/well) during the last 12 h of the culture period and transferred onto fiberglass filters. [^{3}H]Thymidine incorporated into cells was measured with a liquid scintillation counter. The viability of Y16 cells after the herbimycin A treatment was determined by trypan blue dye exclusion test.

Preparation of Cell Lysates. Y16, FDC-5Rα, FDC-5RαΔcyto, CTLL-5Rα and CTLL-5Rα/β cells were deprived of cytokines for the 8 h of incubation before stimulation with the cytokine indicated. Subsequently, cells were cultured at 10^7 cells/ml with 2,000 U/ml IL-5, 1,500 U/ml IL-3, or 2,000 U/ml IL-2 for various periods of time at 37°C. They were then harvested by centrifugation and lysed in ice-cold lysis buffer (1–4 × 10^7 cells/ml) containing 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 2 mM EDTA, 100 U/ml aprotinin, 1 mM NaF, 1 mM PMSF, and 1 mM Na_3VO_4. Unsolubilized materials were removed by centrifugation for 15 min at 12,000 *g*. The cell lysates thus obtained were subjected to immunoprecipitation. Lysates prepared from 2 × 10^7 cells were mixed with an equal volume of 2X Laemmli sample buffer, and boiled for 5 min. Samples were electrophoresed on SDS-polyacrylamide gels (8%).

Immunoprecipitation. 1-ml quantities of the above cell lysates were precleared with protein G–Sepharose 4B and the resulting samples were incubated at 4°C for 60 min with 2–10 μg of the antibodies

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to be tested. Immune complexes were collected on protein G–Sepharose during a 60-min incubation at 4°C, washed three times with lysis buffer, and then two times with washing buffer containing 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 10% glycerol, and finally boiled for 5 min with 2X Laemmli's sample buffer.

Western Blotting. Samples were subjected to 8% SDS-PAGE and electrotransferred to a polyvinylidene difluoride (PVDF) filter (Millipore Corp., Bedford, MA) according to the manufacturer's instructions. Filters were blocked with buffer containing 20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.05% thymerosal, and 5% BSA (Fraction V; Sigma Chemical Co., St. Louis, MO). For Western blotting of PY-containing proteins, the filter was incubated with 1 μg/ml 4G10 or 2 μg/ml PY20 for 2 h, and washed four times with washing buffer containing 0.05% Tween 20. Phosphorylated proteins were then detected with enhanced chemiluminescence (ECL) assay kit (Amersham International, Amersham, Bucks, UK). In some of the experiments, ^{125}I-labeled PY20 (ICN Biochemicals) was used to detect tyrosine-phosphorylated proteins that were detected by autoradiography, as indicated.

In Vitro Kinase Assay. Cell lysates were mixed with anti-Lyn mAb, polyclonal anti-Btk, anti-Fyn, anti-JAK1 or anti-JAK2 antibodies and immune complexes were collected using protein-G–Sepharose and suspended in kinase buffer containing 40 mM Hepes, pH 7.4, 10 mM $MgCl_2$, 3 mM $MnCl_2$, 0.1% NP-40, and 30 μM Na_3VO_4. In some experiments, enolase (5 μg/each sample) denatured with acetic acid was added as an exogenous substrate. After addition of 4 μM ATP and 20 μCi γ[^{32}P] ATP (4,500 Ci/mM; ICN Biochemicals), the mixture was incubated for 5 min at 25°C. The samples were diluted twice with 2X Laemmli's sample buffer and boiled for 5 min. Eluates were subjected to 8% SDS-PAGE, the gels were treated with 1 M KOH for 2 h at 55°C, and phosphorylated proteins were visualized with a Bio-image analyzer, BAS2000 (Fuji Film Co. Ltd., Tokyo, Japan).

Results

Herbimycin A Inhibits the IL-5–induced Proliferation of an Early B Cell Line. Satoh et al. (40) and Sakamaki et al. (17) reported that herbimycin A, a specific inhibitor of tyrosine kinase, blocked IL-3 and GM-CSF–dependent cell proliferation and suggested the importance of tyrosine kinases in ligand-induced cell proliferation. To evaluate whether protein tyrosine phosphorylation is essential for IL-5–induced cell proliferation, the effects of herbimycin A on IL-5–induced tyrosine phosphorylation and cell proliferation were examined using an IL-5–dependent cell line Y16. Herbimycin A did not show a toxic effect sufficient to cause death in Y16 cells at concentrations <5 μg/ml under the conditions employed. As shown in Fig. 1, tyrosine phosphorylations of cellular proteins migrating at ~130, 90, and 60 kD were detected in IL-5–stimulated Y16 cells. The addition of herbimycin A, but not the DMSO used as a control, resulted in the inhibition of these tyrosine phosphorylations (Fig. 1 *A*) as well as the suppression of IL-5–dependent proliferation (Fig. 1 *B*). The inhibitory effects of herbimycin A were markedly reduced by the addition of 2-ME, an inhibitor of herbimycin A (data not shown). These results indicate that tyrosine phosphorylation of multiple intracellular substrates is a prerequisite for IL-5–dependent cell proliferation.

The Cytoplasmic Domain of the IL-5Rα is Involved in Generating a Signal for Tyrosine Phosphorylation. To envisage the roles of IL-5Rα *and* βc in IL-5–induced tyrosine phosphorylation, we reconstituted IL-5R in an IL-2–dependent T cell line, CTLL-2, by transfecting cDNAs for IL-5Rα either with or without βc. CTLL-5Rα/β transfectants, expressing both IL-5Rα and βc, proliferated in response to IL-5 as well as to IL-2 (16). IL-5 stimulation induced rapid protein tyrosine phosphorylation of subcellular proteins and these tyrosine phosphorylations had diminished slightly by 30 min (Fig. 2 *A*). IL-2 stimulation of the same transfectants induced a distinct protein tyrosine phosphorylation similar to that observed in response to IL-5, except that the tyrosine phosphorylation of the 130–140 kD protein was not observed. CTLL-5Rα transfectants expressing IL-5Rα alone showed no detectable levels of tyrosine phosphorylation in response to IL-5, although they had patterns of tyrosine phosphorylation in response to IL-2 similar to that observed in CTLL-5Rα/β transfectants (Fig. 2 *B*). These data indicate that βc, together with IL-5Rα, are required for induction of both protein tyrosine phosphorylation and cell proliferation.

We also investigated the role of IL-5Rα in IL-5–induced tyrosine phosphorylation. We stimulated FDC-P1 transfectants expressing either intact IL-5Rα (FDC-5Rα) or a mutant IL-5Rα (FDC-5RαΔcyto), in which the α chain has no cytoplasmic domain (6, 16), with IL-5 or IL-3. As described previously (16), both FDC-5Rα and FDC-5RαΔcyto expressed high affinity IL-5R, but FDC-5RαΔcyto showed no proliferative response to IL-5. As shown in Fig. 3 *A*, the rapid tyrosine phosphorylation of FDC-5Rα cellular proteins was observed in response to both IL-5 (Fig. 3 *A*) and IL-3 (Fig. 3 *B*). This tyrosine phosphorylation had diminished slightly by 30 min after IL-5 stimulation. In contrast, FDC-5RαΔcyto did not show a significant protein tyrosine phosphorylation in response to IL-5 (Fig. 3 *A*), while again

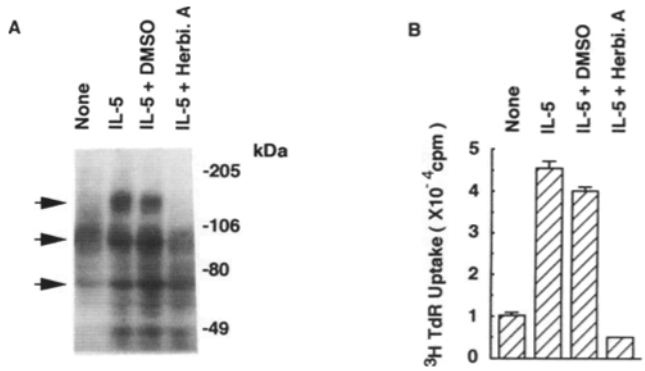

Figure 1. Inhibition of tyrosine phosphorylation and cell proliferation of Y16 cells by herbimycin A. (*A*) Y16 cells were incubated for 8 h in RPMI-1640 containing 4% FCS and 3 μg/ml herbimycin A, or DMSO as a control, in the absence of IL-5. Cells were then treated with 2,000 U/ml mIL-5 for 5 min at 37°C and lysed with 1% Triton X-100. Samples were separated by SDS-PAGE (8% polyacrylamide gel) and immunoblotted with ^{125}I-labeled PY20. The major tyrosine-phosphorylated proteins are indicated by arrows. The indicated marker proteins are given (*kDa*). (*B*) After being treated with herbimycin A or DMSO, cellular proliferation data were assessed in terms of [^{3}H]TdR incorporation into DNA after IL-5 stimulation.

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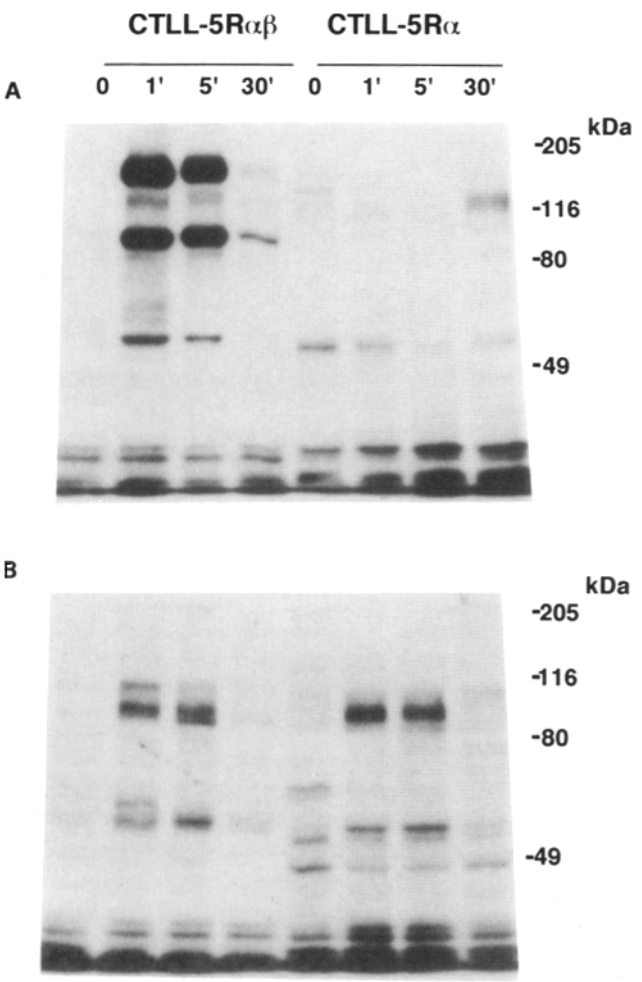

Figure 2. Protein tyrosine phosphorylation in IL-2– or IL-5–stimulated CTLL-2 transfectants. IL-2–dependent T cell line CTLL-2 transfectants (*CTLL-5Rα* and *CTLL-5Rαβ*) were treated with 2,000 U/ml mIL-5 (*A*) or 2,000 U/ml mIL-2 (*B*) for 0, 1, 5, or 30 min at 37°C. The cells were lysed with 1% Triton X-100 after stimulation. Cell lysates were separated by SDS-PAGE (8% polyacrylamide gel) and immunoblotted with the anti-PY mAb. The indicated marker proteins are given (*kDa*).

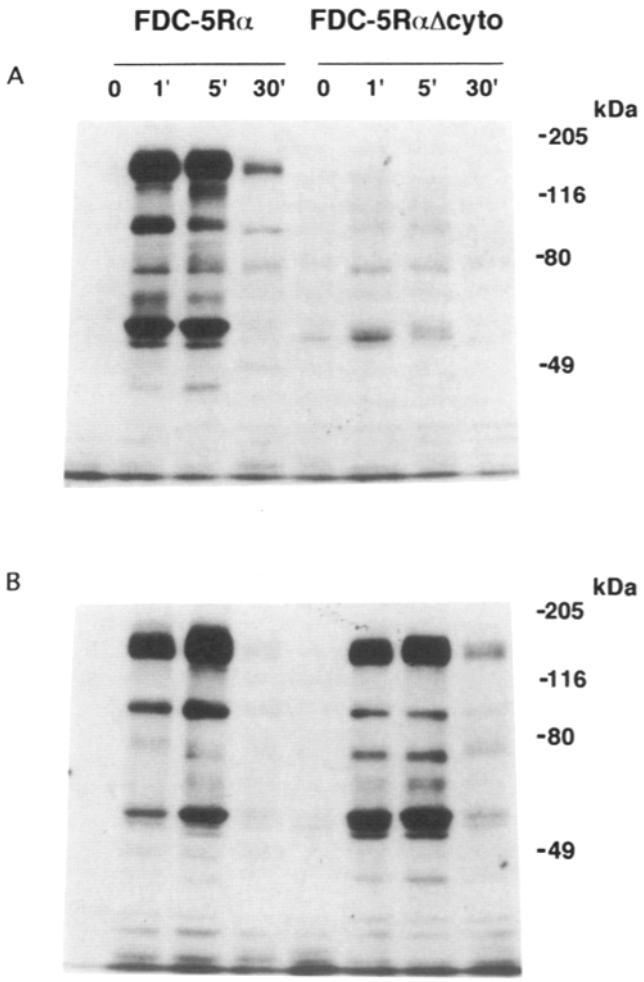

Figure 3. Protein tyrosine phosphorylation in IL-3– or IL-5–stimulated FDC-P1 transfectants. IL-3–dependent myeloid cell line, FDC-P1 transfectants (*FDC-5RαΔcyto* and *FDC-5Rα*) were treated with 2,000 U/ml mIL-5 (*A*) or 1,500 U/ml mIL-3 (*B*) for 0, 1, 5, or 30 min at 37°C. The cells were lysed with 1% Triton X-100 after the stimulation. Cell lysates were separated by SDS-PAGE (8% polyacrylamide gel) and immunoblotted with anti-PY mAb.

showing a pattern of tyrosine phosphorylation in response to IL-3 similar to that observed in FDC-5Rα (Fig. 3 *B*). These results indicate that the cytoplasmic region of IL-5Rα is essential, together with βc, for generating the signal for protein tyrosine phosphorylation in response to IL-5.

The Tyrosine-phosphorylated Protein Migrating at 130–140 kD is βc. One tyrosine-phosphorylated protein, initially migrating at ~130 kD, subsequently increased to 140 kD, over a period of 20 min, after stimulation of cells with IL-5 or IL-3 (Figs. 2 and 3, and Fig. 4 lanes *2* and *4*). As shown in our previous study (8), the molecular mass of βc is ~130 kD. Thus, the tyrosine-phosphorylated protein migrating at ~130 kD was assumed to be βc. The 130–140 kD protein phosphorylated in response to IL-5 was shown to be primarily βc by immunoprecipitation and absorption experiments using the anti-βc mAb, HB (37). When cell lysates of IL-5–stimulated Y16 cells were absorbed with the HB mAb, the tyrosine-phosphorylated band of 130–140 kD protein disappeared (Fig. 4, lane *3*), whereas most other tyrosine-phosphorylated bands remained. βc was then immunoprecipitated with HB and analyzed for tyrosine phosphorylation by SDS-PAGE followed by Western blotting using ^{125}I-labeled PY20. As shown in Fig. 4, βc was tryosine phosphorylated after IL-5 stimulation (lane *4*). We also observed that weakly tyrosine-phosphorylated proteins migrating at 40 and 100 kD coprecipitated with βc. These data clearly demonstrate that the markedly tyrosine-phosphorylated 130–140 kD protein is βc. The 40- and 100-kD proteins may be associated with βc.

PI-3 Kinase, Vav, Shc, and HS1 Protein are Tyrosine Phosphorylated upon IL-5 Stimulation. We then investigated IL-5–induced tyrosine phosphorylations of PLC-γ, GAP, PI-3 kinase, Shc, p95^{Vav} and p75^{HS1}, all of which contain SH2 and/or SH3 domains. Immunoprecipitation followed by immunoblot analysis of cell lysates of IL-5–stimulated Y16 cells,

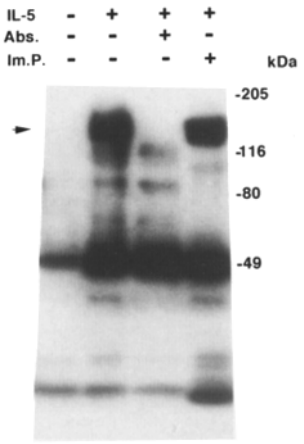

Figure 4. Immunoprecipitation of the 130-kD phosphoprotein with anti-IL-5Rβ chain mAb. Y16 cells were treated with 2,000 U/ml mIL-5 for 5 min at 37°C, and their cell lysates were preabsorbed (*Abs.*), immunoprecipitated (*Im.P.*) with HB mAb. Immunoprecipitates were separated by SDS-PAGE (8% polyacrylamide gel) and immunoblotted with ^{124}I-labeled PY20. The position to which the βc migrated is indicated by an arrow, and the indicated marker proteins are given (*kDa*).

using polyclonal or monoclonal antibodies specific for each of the above proteins, revealed that Y16 cells expressed all of the aforementioned molecules. The cell lysates of IL-5–stimulated Y16 cells immunoprecipitated with anti-PI-3 kinase antibody, followed by immunoblotting with anti-PY mAb, revealing that marked tyrosine phosphorylation of PI-3 kinase had occurred in response to IL-5 stimulation (Fig. 5). In contrast, no bands corresponding to tyrosine-phosphorylated PLC-γ or GAP were detected, under similar assay conditions, by immunoprecipitation with antibodies specific for each respective protein regardless of how long the cells were stimulated with IL-5 (1–30 min). No tyrosine-phosphorylated protein bands were observed even when the samples were immunoprecipitated with anti-PY mAbs (4G10 or PY20) and then immunoblotted with anti-PLC-γ or anti-GAP antibodies. It is intriguing that Vav, Shc, and HS1 proteins were tyrosine phosphorylated within 5 min of being stimulated with IL-5 (Fig. 6) but had disappeared by 30 min after IL-5 stimulation (data not shown). It was clear that the tyrosine-phosphorylated protein with an ~130-kD band was also coprecipitated with Shc by anti-Shc antibodies. No tyrosine phosphorylations of any of these SH2/SH3-containing proteins was observed in IL-5–stimulated FDC-5RαΔcyto (data not shown).

JAK2 is Tyrosine Phosphorylated and Activated by IL-5 Stimulation. The fact that tyrosine phosphorylation of cellular proteins including βc can be rapidly induced after IL-5 stimulation indicates the association of PTK with IL-5R along with IL-5 stimulation. However, we could not detect any tyrosine kinase activity in the immunoprecipitates using anti-βc mAb, HB, or anti-IL-5Rα mAb, H7 of the lysates from IL-5–stimulated Y16 cells. Recent studies have shown that the JAK family of cytoplasmic PTKs were associated with some of the cytokine receptors including IL-3R, and tyrosine phosphorylated just after the binding of ligand to the receptor. Tyrosine phosphorylation of a kinase is believed to be important in transmitting a signal to downstream of SH2-containing effector molecule(s). Thus, we examined whether the tyrosine phosphorylation of JAK1 and JAK2 kinases occurs upon IL-5 stimulation. Cell lysates of IL-5–stimulated Y16 cells were submitted to immunoprecipitation by antibodies specific for JAK1

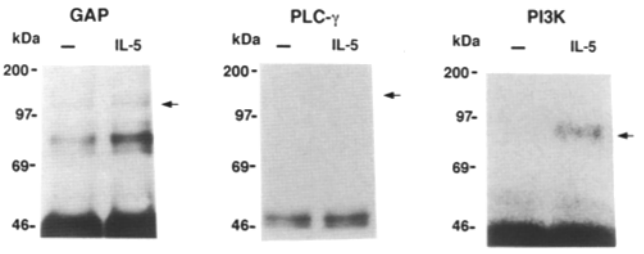

Figure 5. Detection of tyrosine-phosphorylated GAP, PLC-γ, and PI-3 kinase in IL-5–stimulated Y16 cells. Y16 cells were treated with 2,000 U/ml mIL-5 for 5 min at 37°C, lysed with 1% Triton X-100, and immunoprecipitated with anti-GAP, anti-PLC-γ, or anti-PI-3 kinase antibodies. Immunoprecipitates were subjected to SDS-PAGE analysis on 8–10% polyacrylamide gels, and immunoblotted with anti-PY mAb. The positions to which GAP, PLC-γ, and PI-3 kinase migrated are indicated by arrows, and marker proteins are given (*kDa*).

or JAK2 kinase and then precipitates were immunoblotted with anti-PY mAb. As shown in Fig. 7 *A*, marked tyrosine phosphorylation of JAK2 but not of JAK1 kinase, was observed upon IL-5 stimulation of Y16 cells. This phosphorylation was detected as early as 10 s after IL-5 stimulation (data not shown), suggesting that JAK2 kinase is located proximal to the activated IL-5R and function upstream of the IL-5 signaling. To assess the effects on kinase activities of JAK1 and JAK2, JAK1 or JAK2 was immunoprecipitated from the lysates of IL-5–stimulated Y16 cells and in vitro kinase assays were performed. No in vitro kinase activity was detected in immunoprecipitates with JAK1 antiserum from unstimulated or stimulated cells (Fig. 7 *B*). However, immunoprecipitates from IL-5–stimulated Y16 cells with JAK2 antiserum contained kinase activity and resulted in tyrosine phosphorylation of a protein of 130 kD that comigrated with JAK2. In contrast, the 130-kD band was not detected with unstimulated cells. This suggests that JAK2 is activated along with IL-5R ligation.

Btk Is Activated by IL-5 Stimulation. Btk was recently cloned as a B cell–specific cytosolic tyrosine kinase and identified

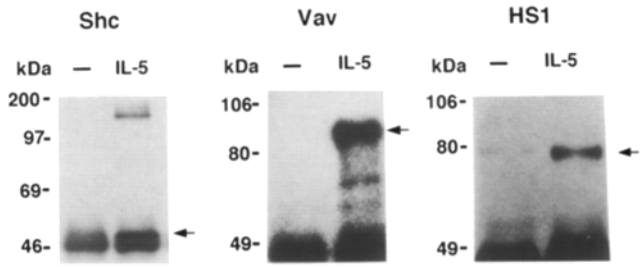

Figure 6. Detection of tyrosine-phosphorylated Sch, Vav, and HS1 in IL-5–stimulated Y16 cells. Y16 cells were stimulated with 2,000 U/ml mIL-5 for 5 min at 37°C, lysed with 1% Triton X-100, and immunoprecipitated with anti-Shc, anti-Vav, or anti-HS1 antibodies. Immunoprecipitates were analyzed by SDS-PAGE (8–10% polyacrylamide gel) and immunoblotted with anti-PY mAb for detection of phosphorylated Shc and HS1, or ^{125}I-labeled PY20, for detection of phosphorylated Vav. The positions to which Shc, Vav, and HS1 migrated are indicated by arrows, and marker proteins are given (*kDa*).

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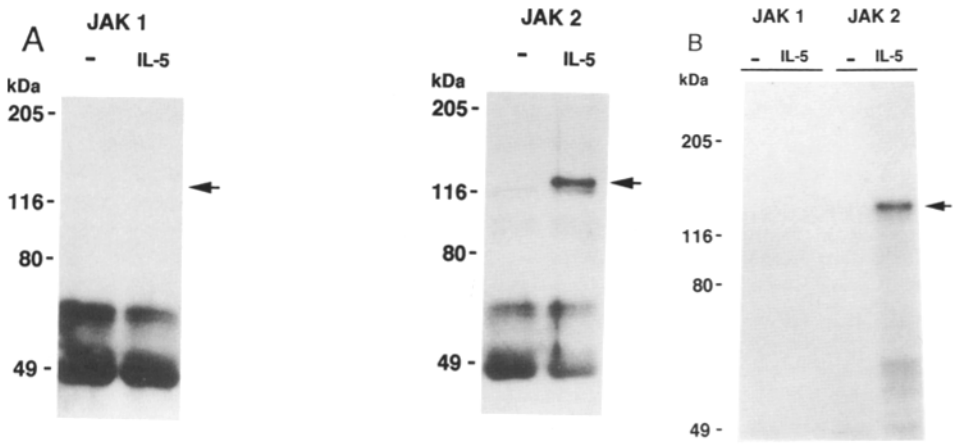

Figure 7. Tyrosine phosphorylation (*A*) and activation (*B*) or JAK2 tyrosine kinase upon IL-5 stimulation of Y16 cells. (*A*) Y16 cells were stimulated with 2,000 U/ml mIL-5 for 5 min at 37°C. The lysates were subjected to Western blotting using anti-PY mAb (4G10) and tyrosine-phosphorylated bands were detected by ECL assay. The positions to which JAK1 and JAK2 migrated are indicated by arrows and marker proteins are given (*kDa*). (*B*) Immunoprecipitates of (*A*) were subjected to in vitro kinase assay as described in Materials and Methods and separated by SDS-PAGE (8%). The gel was treated with 1 M KOH at 55°C for 2 h. Tyrosine-phosphorylated bands were detected by autoradiography. The position to which JAK2 migrated is indicated by arrows, and marker proteins are given (*kDa*).

as the molecule involved in X-linked human agammaglobulinemia (XLA) (35, 36). A single conserved residue within the NH_2-terminal unique region of Btk was shown to be mutated in XID mice (42, 43), in which B cells showed impaired responsiveness to IL-5 (44). Lyn and Fyn kinases were reported to be associated with B cell antigen receptors and to play roles in B cell development, and have also been suggested to be associated with the signaling cascade mediated by IL-3R (45) and GM-CSFR (34) in myeloid cell lines. Thus, we examined the effects of IL-5 on tyrosine phosphorylation of Btk, Lyn, and Fyn as described above. However, we could not detect any significant increase in phosphorylation of these kinases upon IL-5 stimulation (data not shown). Next, we investigated kinase activities for Btk, Lyn, and Fyn in IL-5–stimulated Y16 cells. Cell lysates were immunoprecipitated with anti-Btk, anti-Lyn, or anti-Fyn antibodies, and each of the immunoprecipitates was used for an in vitro kinase assay. We monitored kinase activities by autophosphorylation of each kinase and the phosphorylation of enolase, an exogenously added substrate. We confirmed that all kinase reactions

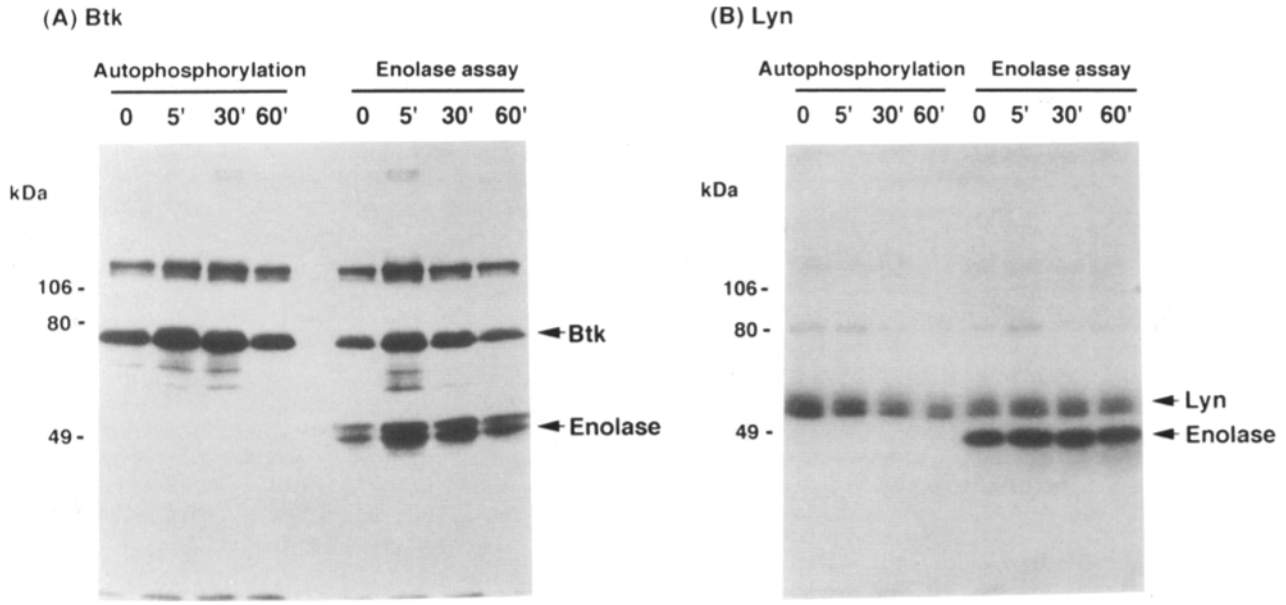

Figure 8. Time courses of Btk (*A*) and Lyn (*B*) kinase activities in IL-5–stimulated Y16 cells. Y16 cells were stimulated with 2,000 U/ml mIL-5 for 0, 5, 30, or 60 min. Cells were lysed with 1% Triton X-100 and immunoprecipitated with anti-Btk or anti-Lyn antibodies. Immune complexes were subjected to SDS-PAGE analysis on 8% polyacrylamide gels. Kinase assays were conducted as described in Materials and Methods. Kinase activity was detected by autoradiography. (*A*) Autophosphorylation and enolase phosphorylation resulting from anti-Btk immune complex kinase assay were revealed by autoradiography. The positions to which Btk and enolase migrated are indicated and marker proteins are given (*kDa*). (*B*) Autophosphorylation and enolase phosphorylation, resulting from the immune complex kinase assay of immunoprecipitates using anti-Lyn antibodies, were detected by autoradiography. The position of Lyn and enolase migrated are indicated and marker proteins are given (*kDa*).

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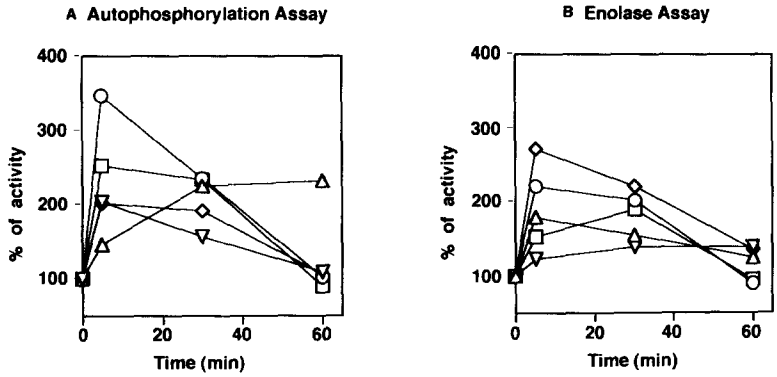

Figure 9. Time course of IL-5–induced increases in Btk kinase activity in Y16 cells. Y16 cells were treated with 2,000 U/ml mIL-5 for 0, 5, 30, or 60 min. The Btk (*A*) and enolase (*B*) bands seen in Fig. 8 (*A*) were excised from the gel and subjected to ^{32}P counting. Results from a series of five different experiments are shown.

proceeded linearly, for up to 20 min at 25°C under our assay conditions. As shown in Fig. 8 *A* and Fig. 9, *A* and *B*, significantly enhanced Btk kinase activity (two to three times higher than that of unstimulated controls) was detected in anti-Btk immunoprecipitates of Y16 cells stimulated with IL-5 for 5–20 min by both the autophosphorylation and the enolase assay. In contrast, no significant enhancement of either Lyn (Fig. 8 *B*) or Fyn (data not shown) kinase activity was detected under the conditions employed. No enhanced Btk activity was detected in IL-5–stimulated FDC-5R$\alpha\Delta$cyto (data not shown).

Discussion

We describe three major observations in this study: (*a*) The cytoplasmic domain of IL-5Rα is indispensable, together with βc, for IL-5–mediated signaling; (*b*) signal transducing molecules having SH2 and/or SH3 domains, such as PI-3 kinase, Shc, Vav, and HS1 proteins, are rapidly tyrosine phosphorylated in response to IL-5; and (*c*) the activity of JAK2 kinase and B cell–specific Btk is significantly enhanced after IL-5 stimulation. Tyrosine phosphorylation of JAK2 is rapidly induced upon stimulation with IL-5.

There is ample evidence that tyrosine phosphorylation of membrane receptors and cellular proteins is important for the regulation of receptor-mediated signal transduction. Tyrosine phosphorylation of cellular proteins have been reported in both IL-3- and GM-CSF–stimulation cells (34, 45–49) and patterns of protein tyrosine phosphorylation showed no apparent differences among these cytokines. As βc is shared among receptors for IL-3, IL-5, and GM-CSF (6–11) and is indispensable for their signal transduction pathways, IL-5 signal transduction is assumed to be similar to that of IL-3R and GM-CSFR systems. In fact, stimulation of IL-5–dependent T88-M cells with IL-5 induced tyrosine phosphorylation of multiple subcellular proteins similar to those induced by IL-3 (34). This was further confirmed in the present study using CTLL transfectants expressing recombinant IL-5Rα and βc (Fig. 2). The importance of tyrosine phosphorylation in IL-5 signal transduction was strengthened by experiments using herbimycin A. Treatment of IL-5–dependent Y16 cells with herbimycin A, a tyrosine kinase inhibitor, induced the inhibition of not only protein tyrosine phosphorylation, but also the cell growth induced by IL-5 (Fig. 1).

We reported that FDC-5R$\alpha\Delta$cyto, an IL-3–dependent FDC-P1 transfectant of a mutated IL-5Rα cDNA which lacks the entire cytoplasmic domain, shows no proliferative response to IL-5, although it can bind IL-5 with high affinity (16), indicating that the cytoplasmic domain of IL-5Rα is required for IL-5–induced cell proliferation. Furthermore, stimulation of FDC-5R$\alpha\Delta$cyto with IL-5 did not induce protein tyrosine phosphorylation (Fig. 3), although tyrosine phosphorylation did occur in response to IL-3 to an extent similar to that observed in its parental cell line, FDC-P1. These results indicate that IL-5Rα has two functions crucial for generating a growth signal: one is to bind IL-5 on the receptor and the other is to interact with βc to generate a signal for protein tyrosine phosphorylation. We cannot adequately explain the role of the cytoplasmic domain of IL-5Rα in IL-5–mediated signaling. Like receptors with tyrosine kinase domains in their cytoplasmic domains, binding of IL-5 to IL-5Rα might induce dimerization of βc, a signal transducer resulting in activation of PTKs. It is also possible that IL-5Rα, in combination with βc, might form the site for association with tyrosine kinase(s). We are currently approaching an understanding of these issues, based on the results of testing IL-5 responsiveness and tyrosine phosphorylation of transfectants expressing chimeric IL-5R containing the α chain in the extracellular and transmembrane domains and βc in the cytoplasmic domain together with intact βc.

The tyrosine phosphorylation of βc among cellular proteins was most abundant in IL-5–stimulated Y16 cells (Fig. 4). When we immunoprecipitated cell lysates with anti-βc mAb, two different phosphorylated proteins, of ~40 and 100 kD were also coprecipitated. These proteins may be associated with βc and may have been phosphorylated in response to IL-5 stimulation. Tyrosine phosphorylation of the βc of IL-3R or GM-CSFR has also been reported (47–49), although any molecules associated with βc were not identified. Sakamaki et al. (17), using a series of Baf transfectants of βc cDNA with deletions of various regions, described significant βc-mediated tyrosine phosphorylation of cellular proteins as apparently being distinct from proliferation. At this moment, we have no evidence for the dissociation of IL-5–induced protein tyrosine phosphorylation of βc and IL-5–induced cell proliferation.

Among signal-transducing molecules for cell growth, such as PLC-γ, GAP, and PI-3 kinase, which have SH2 domains

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for interacting with tyrosine kinase, we detected significant tyrosine phosphorylation of PI-3 kinase in response to IL-5 stimulation (Fig. 5). PI-3 kinase is a lipid kinase that phosphorylates the D3 position of phosphatidyl inositol, phosphatidyl inositol-4-phosphate and PI-4,5-P2 (50). PI-3 kinase consists of an 85-kD regulatory subunit and a 100-kD catalytic subunit. The p85 regulatory subunit harbors two tyrosine phosphate binding domains, SH2 domains, and the majority of the tyrosine residues in PI-3 kinase that serve as the substrates for various PTKs both in vivo and in vitro (51). Recent evidences indicate that PI-3 kinase is probably activated directly by the process of binding via its SH2 domains to specific tyrosine phosphate residues that are located in signaling complexes and are the products of receptor-stimulated PTKs. It was reported that IL-4 caused a strong association of PI-3 kinase with tyrosine-phosphorylated 170-kD protein whereas IL-3 induced an association of phosphorylated 97-kD protein with PI-3 kinase (52). Although it is unclear what role tyrosine phosphorylation of the p85 regulatory subunit plays in PI-3 kinase activation, the phosphorylation may indicate the interaction of PI-3 kinase with tyrosine-phosphorylated PTK through its SH2 domain. Yamanashi et al. (39) demonstrated that cross-linking of B cell antigen receptor induces tyrosine phosphorylation of the p85 regulatory subunit followed by activation of PI-3 kinase, which was associated with Lyn, a src-like PTK. It was recently shown that the universally expressed PKC-ζ is activated by the products of PI-3 kinase (53) and is critical for mitogenic signal transduction (54). The activation of PKC-ζ, mediated through phosphorylated PI-3 kinase after IL-5 stimulation, might be involved in the IL-5–mediated growth signal cascade and in the platelet-derived growth factor-mediated cascade as well (55).

It has been suggested that $P21^{ras}$ (Ras) functions as a signal-transducing molecule downstream from PTK(s) (22, 23). Ras has been reported to be activated after ligand stimulation in IL-3R-, GM-CSFR- and IL-5R–mediated signaling pathways (24, 25) and in antigen receptor–mediated signal transduction (56, 57). It was recently clarified (21) that the tyrosine-phosphorylated Shc protein functions as an adapter because of its association with Grb2, another adapter protein. Grb2 binds to both Shc and mSos through its SH2 and SH3 domains, respectively. mSos is a Ras nucleotide exchange protein and activates Ras (25). Satoh et al. (40) demonstrated that the increase in Ras-GTP, as well as cell proliferation induced by IL-3 and GM-CSF was diminished in cells treated with herbimycin A, suggesting the involvement of tyrosine kinase(s) in the Ras activation pathway mediated by these cytokines. We have shown that Shc protein ($p52^{Shc}$) is tyrosine phosphorylated in response to IL-5 stimulation (Fig. 6). In these experiments, we also detected a tyrosine-phosphorylated protein of ~130 kD which coimmunoprecipitated with Shc in the presence of anti-Shc antibodies. This protein might be βc, because its mol wt is similar to that of βc and we are currently testing this possibility. As tyrosine phosphorylation of Ras-GAP was not induced by IL-5 stimulation (Fig. 5); enhanced Ras activity may not be caused by tyrosine phosphorylation of GAP in conjunction with IL-5 stimulation. Therefore, activation of Ras in response to IL-5 stimulation is, at least in part, due to modulation of Shc by the tyrosine kinase(s) activated by IL-5 binding.

The $p95^{Vav}$(Vav), a proto-oncogene product specifically expressed in hematopoietic cells, contains a single SH2 and two SH3 domains as well as sequence motifs commonly found in transcription factors, such as the helix-loop-helix, leucine zipper, and zinc-finger motifs, and nuclear localization signals, in addition to being homologous with guanine nucleotide releasing factors (GRFs) (58). We observed that Vav was rapidly tyrosine phosphorylated upon stimulation with IL-5 (Fig. 6). Vav was also shown to be tyrosine phosphorylated upon cross-linkage of the antigen receptors on T and B cells (59). Recently, Gulbins et al. (58) demonstrated that both the tyrosine phosphorylation of Vav and the Vav-associated GRF activity for Ras were enhanced after TCR cross-linking. They proposed the concept that Vav is a tyrosine kinase–regulated GRF which is important in TCR-initiated signal transduction through activation of Ras. Therefore, the tyrosine phosphorylation of Vav observed in this study may be associated with the increase in the active form of Ras in IL-5–stimulated Y16 cells. It is also possible that, as expected from its structural homology, Vav plays other crucial roles as a transcriptional factor in the IL-5–induced gene expression associated with cell growth.

The HS1 gene is expressed specifically in cells of hematopoietic lineage (27). The NH_2-terminal moiety of HS1 protein has three copies of a 37-amino acid repeating motif, each of which contains a helix-turn-helix motif. The COOH-terminal portion has a potential α-helix containing an amphipathic region. In addition, an SH3 motif was identified near the COOH-terminus. HS1 is a major substrate of PTKs activated by binding of the B cell antigen receptors, mIgM and mIgD. As is evident from Fig. 6, the HS1 protein was also tyrosine phosphorylated upon stimulation with IL-5. Recently, an association of HS1 protein with Lyn tyrosine kinase was found in B cells activated by cross-linking of their mIgM with anti-IgM antibodies (29). Based on its structural homology, HS1 is assumed to function as a DNA binding protein. Our observations of the tyrosine phosphorylation of HS1 in response to IL-5 stimulation (Fig. 6) indicate that HS1 may transduce a signal generated by IL-5 ligation directly into the genetic loci involved in IL-5–induced cell growth. Recently, Larner et al. (60) demonstrated that treatment of human peripheral blood monocytes or basophils with IL-3, IL-5, IL-10, or GM-CSF activated the DNA binding proteins whose tyrosine residues were phosphorylated. They suggested that these cytokines, as well as IFN-α and IFN-γ, can modulate gene expression through activation of putative transcription factors by tyrosine phosphorylation. Both Vav and HS1 might be involved in the formation of such a DNA-binding complex.

As for tyrosine kinase(s) involved in IL-5R–mediated signaling, clarification of this process has been problematic. Torigoe et al. (45) have shown the activation of Lyn kinase to be associated with IL-3–, but not with IL-2–induced,

proliferation of myeloid-committed cells. Corey et al. (46) demonstrated that both Lyn and Yes kinases were activated and associated with PI-3 kinase in GM-CSF–stimulated, myeloid-derived cells. Hanazono et al. (49) reported that both GM-CSF and IL-3 can induce tyrosine phosphorylation and activation of c-*fps/fes* PTK in human erythroleukemic cell line. A new family of cytoplasmic PTKs, the JAKs (alternatively referred to as just another kinase family), consisting of JAK1, JAK2, and Tyk2, has been described and cloned (32, 61, 62). In addition to a kinase domain, these proteins are characterized by the presence of a second kinase-like domain and the absence of SH2, SH3, and membrane-spanning domains. The Tyk2 has been implicated in IFN-α receptor signal transduction (32). More recently, a variety of different cytokines including erythropoietin, growth hormone, IL-3, GM-CSF, G-CSF, and IFN-γ has been found to induce tyrosine phosphorylation and kinase activation of JAK2 (33, 63). In the current study, JAK2 was shown to be very rapidly (within 10 s) tyrosine phosphorylated and activated by IL-5 stimulation. This suggests that upon stimulation, JAK2 is located proximal to IL-5R and involved in an IL-5R–mediated signal transduction.

We demonstrated that Btk was activated by IL-5 stimulation of pre-B cell lines (Figs. 8 and 9), suggesting the involvement of Btk kinase in IL-5R–mediated signaling. We demonstrated that Btk was activated by IL-5 stimulation of pre-B cell lines (Figs. 8 and 9), suggesting the involvement of Btk kinase in IL-5R–mediated signaling. Recently, Kawakami et al. (64) showed that FcεRI cross-linking induces rapid activation and tyrosine phosphorylation of Btk in mouse bone marrow–derived mast cells. Although we could not detect significant enhancement of its tyrosine phosphorylation, these data indicate that Btk can be modulated by extracellular signals. The Btk protein, which belongs to a newly identified family of nonreceptor tyrosine kinases, may have the potential to interact in a novel way with protein(s) involved in an IL-5R–mediated signaling pathway. One amino acid substitution was reported in the NH_2-terminal domain unique to Btk in the XID mouse (42, 43), the B cells of which fail to respond to IL-5 (44), indicating the importance of the correct expression of this region of the protein in B cell responses to IL-5. Our findings may partly explain the B cell–specific defect in IL-5 responsiveness in XID mice. The discovery of cellular molecule(s) that can interact with Btk, through its NH_2-terminal unique region, should shed light on the complex signaling requirements of B cell differentiation in response to cytokines including IL-5.

In conclusion, we have demonstrated the data suggesting the involvement of multiple families of signal-transducing molecules such as Vav, Shc, HS1, and PI-3 kinase, and cytoplasmic PTKs such as JAK2 and Btk in IL-5R–mediated signaling pathways. We have failed to detect kinases associated with the IL-5R. However, published evidence suggests that the JAKs may well be associated, and our results also suggest the proximal location of JAK2 kinase to IL-5R. Successive immunoprecipitations using anti-Btk and anti-JAK2 kinase antibodies, reciprocally, followed by in vitro kinase assay, revealed no evidence for the association with each other (data not shown). It is still unclear at this moment whether the Btk kinase is a proximal mediator of IL-5 response or it could be downstream of IL-5R–mediated signaling pathway. Whereas our data provide important clues for clarifying the precise role of signaling molecules in the development, proliferation and differentiation of hematopoietic cells in response to IL-5, we need more experiments to clarify the roles of activation of Btk and JAK2 in IL-5 signaling. Furthermore, we should extend current studies to elucidate differences in IL-5R–mediated signal transduction cascades between cell proliferation and differentiation.

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