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A Critical Cytoplasmic Domain of the Interleukin-5 (IL-5) Receptor α Chain and Its Function in IL-5-Mediated Growth Signal Transduction

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Interleukin-5 (IL-5) regulates the production and function of B cells, eosinophils, and basophils. The IL-5 receptor (IL-5R) consists of two distinct membrane proteins, α and β. The α chain (IL-5Rα) is specific to IL-5. The β chain is the common β chain (βc) of receptors for IL-3 and granulocyte-macrophage colony-stimulating factor (GM-CSF). The cytoplasmic domains of both α and β chains are essential for signal transduction. In this study, we generated cDNAs of IL-5Rα having various mutations in their cytoplasmic domains and examined the function of these mutants by expressing them in IL-3-dependent FDC-P1 cells. The membrane-proximal proline-rich sequence of the cytoplasmic domain of IL-5Rα, which is conserved among the α chains of IL-5R, IL-3R, and GM-CSF receptor (GM-CSFR), was found to be essential for the IL-5-induced proliferative response, expression of nuclear proto-oncogenes such as c-*jun*, c-*fos*, and c-*myc*, and tyrosine phosphorylation of cellular proteins including JAK2 protein-tyrosine kinase. In addition, analysis using chimeric receptors which consist of the extracellular domain of IL-5Rα and the cytoplasmic domain of βc suggested that dimerization of the cytoplasmic domain of βc may be an important step in activating the IL-5R complex and transducing intracellular growth signals.

Interleukin-5 (IL-5) is a cytokine that regulates the production and function of B cells, eosinophils, and basophils (40). The IL-5 receptor (IL-5R) consists of two distinct membrane proteins, α and β. IL-5Rα alone specifically binds IL-5 but with low affinity (27, 39). While the β chain does not bind IL-5 by itself, it does form a high-affinity IL-5R in combination with IL-5Rα (6, 37, 38, 41). The β chain is the common β chain (βc) of receptors for IL-3 and granulocyte-macrophage colony-stimulating factor (GM-CSF) (23). βc forms high-affinity receptors for IL-3 and GM-CSF, the ligands of which bind specifically with IL-3Rα and the α chain of GM-CSF receptor (GM-CSFRα), respectively (7, 9, 10, 16, 29). βc is not only required for high-affinity binding but also essential for signal transduction. IL-5, IL-3, and GM-CSF have several overlapping functions, especially in eosinophils (23, 33, 40). The βc shared by the receptors of these three cytokines provides a molecular basis for the functional redundancy of these cytokines.

The extracellular domains of the α chains and βc are similar among members of the cytokine receptor superfamily. The α chains have a short cytoplasmic domain (~55 amino acid residues) with a short amino acid sequence which is conserved among these α chains (16, 39). In contrast, βc has a relatively large cytoplasmic domain (~440 amino acid residues). Although the cytoplasmic domains of the α chains and βc have no homology with signaling molecules such as kinases, phosphatases, nucleotide-binding proteins, and *src* homology domains, it has been well established that IL-5, IL-3, and GM-CSF induce rapid tyrosine phosphorylation of cellular proteins (13, 24, 28) as well as transcription of nuclear proto-oncogenes (3). Studies using a series of cytoplasmic deletion mutants of the human βc have revealed that there are at least two distinct regions in the cytoplasmic domain of βc required for growth signal transduction (31, 34). With a mutant IL-5Rα which lacks the entire cytoplasmic domain, it has been shown that the cytoplasmic region of IL-5Rα also has an important role in transmitting a growth signal through IL-5R (38). However, the critical cytoplasmic regions of IL-5Rα and their functions in signaling remain to be elucidated.

Recent studies have shown that some cytokine receptors form receptor dimers by means of ligand binding. One growth hormone molecule binds to two receptor molecules (4). The gp130 of IL-6R forms a homodimer, transducing the signal to the cell interior, upon binding IL-6 and p80 IL-6R (25). The dimerization of the receptor molecule, which has a large cytoplasmic domain, may be a common mechanism for activation of and signal transduction through cytokine receptors. Issues remaining to be resolved include how many receptor subunits the high-affinity IL-5R is composed of and how the IL-5R complex activates the signal transduction machinery in the cell interior.

In this study, we identified the critical cytoplasmic region of IL-5Rα and characterized its role in growth signaling. Our results demonstrate that the membrane-proximal proline-rich region is critical for induction of c-*fos*, c-*jun*, and c-*myc* and tyrosine phosphorylation of cellular proteins and JAK2 kinase. In addition, using a chimeric receptor molecule of IL-5Rα with βc, we analyze the activation mechanism of the IL-5R complex.

MATERIALS AND METHODS

Reagents. Murine IL-5 (mIL-5) was prepared and purified with anti-mIL-5 monoclonal antibody (MAb)-coupled beads as described previously (22). Rat MAb against mIL-5Rα (H7) (12, 44) was purified with protein G-coupled Sepharose (Pharmacia). A MAb against phosphotyrosine (4G10) and antisera

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against JAK2 were obtained from Upstate Biotechnology Inc. (Lake Placid, N.Y.).

Primers used in PCR. The primers used in constructing the mutant mIL-5Rαs are as follows. The 5R-S primer (CCTTTC CAGATCATTGC) is a forward primer corresponding to the sequence of mIL-5Rα cDNA. The following class A primers carry antisense sequences of the cytoplasmic portion of the mIL-5Rα, portions of which have deletion or substitution mutations: DC1-A, GTTACTCTTTGGGGCCCTGGTCCAT AAATGACACAC; DC2-A, TGGGGCCGGAACCGGTG GAAA; DC3-A, AAATCCAACCTCTTCAATTTTGGTTTC ATTCGAAGG; DC4-A, GACTTCAAATCCAACCTCTTC; AAV-A, AACCGCTGCAAACAACCT; PAVA-A, TGGGG CCGCAACCGCTGGAAACAACCT; and APVA-A, TGGG GCCGCAACCGGTGCAAACAACCT. The following class B primers carry sequences complementary to class A primers, portions of which have deletion or substitution mutations: DC1-S, GCCCCAAAGAGTAACATCAAA; DC2-S, CCGGT TCCGGCCCCACTCCCTGTGGTTACTGAATAT; DC3-S, GAAGAGGTTGGATTTGAAGTC; DC4-S, GTTGGATTT GAAGTCTGATGGCATTTTGCCATTCTG; AAVP-S, TGT TTGCAGCGGTTCCGGCCCC; AAVA-S, TGTTTGCAGC GGTTGCGGCCCC; and VA-S, CGGTTGCGGCCCCAAA GAGT. The 5RUT-A primer (AAGCGGCCGCCTGGCTCT GAGTG) is a reverse primer which has an antisense sequence of mIL-5Rα cDNA and a *Not*I recognition site.

The primers used in constructing the chimeric receptors between mIL-5Rα and murine βc(AIC2B) [mβc(AIC2B)] are as follows. The 5R-S primer is a forward primer. The following class A′ primers carry antisense sequences of the mIL-5Rα: 5REC-A, CCACATAAATAGGTTGACTCC; and 5RTM-A, ACTCTGCAGATGAGTGAGAA. The following class B′ primers carry hybrid sequences consisting of sequences complementary to class A′ primers and sense strand sequences of mβc: 5REC/2B-S, AACCTATTTATGTGGGAACTGACTG GGTGATGCCC; and 5RTM/2B-S, ACTCATCTGCAGAGT GTGTGTCTCTGTATACAGG. The 2BIC-A (GAGAGGTG ACACGTTGTTGT) primer is a reverse primer which has an antisense sequence of mβc cDNA.

Construction of expression plasmids. The cDNA fragments of mIL-5Rα (39) were inserted downstream from the SRα promoter in the expression vector pME18 (kindly provided by A. Miyajima, DNAX Research Institute), and a hygromycin-resistant selection marker was added to the vector. The resulting plasmid was designated pME18hyg-m5R.

The plasmids which expressed the deletion mutations of mIL-5Rα were constructed by recombinant PCR (11) with the following primer combinations: DC1, DC1-A, and DC1-S; DC2, DC2-A, and DC2-S; DC3, DC3-A, and DC3-S; DC4, DC4-A, and DC4-S; AAvp, AAV-A, and AAVP-S; AAvA, AAV-A, and AAVA-S; pAvA, PAVA-A, and VA-S; and ApvA, APVA-A, and VA-S. In the first PCR, one part of the mIL-5Rα cDNA in pME18hyg-m5R was amplified by using 5R-S as the forward primer and a class A primer as the reverse primer. Another part of the mIL-5Rα was amplified by using a class B primer as the forward primer and 5RUT-A as the reverse primer. PCR was carried out in 20 mM Tris-HCl (pH 8.3)–50 mM KCl–3 mM $MgCl_2$–0.1 mg of gelatin per ml–50 μM each deoxynucleoside triphosphate–1 μM primers–1 ng of template DNA–2.5 U of *Taq* polymerase (GIBCO BRL) in a total volume of 50 μl. The PCR conditions were 1 min at 93°C, 1 min at 56°C, and 1 min 72°C for 20 cycles. Products from the first PCR were isolated by agarose gel electrophoresis and mixed 1:1. Then the secondary PCR proceeded as described above, with 5R-S and 5RUT-A primers. The product was digested with restriction enzymes and then ligated with DNA fragments of the appropriate 5′ and 3′ portions of mIL-5Rα. The plasmids which expressed the chimeric receptors consisting of IL-5Rα and βc were also constructed by recombinant PCR using the following primer combinations: ααβ, 5RTM-A, and 5RTM/2B-S; and αββ, 5REC-A, and 5REC/2B-S. In the first PCR, one part of the mIL-5Rα cDNA was amplified with 5R-S as the forward primer and a class A′ primer as the reverse primer. Part of mβc was amplified with a class B′ primer as the forward primer and 2BIC-A as the reverse primer. The secondary PCR proceeded with 5R-S and 2BIC-A primers. The product was digested with restriction enzymes and then ligated with DNA fragments of the appropriate 5′ portion of mIL-5Rα and 3′ portion of mβc. All of the structures of the resultant mutated or hybrid cDNAs were determined by DNA sequencing.

Cells and transfection. A mouse IL-3-dependent cell line, FDC-P1, was maintained in RPMI 1640 medium supplemented with 5% fetal calf serum, 50 μM 2-mercaptoethanol, and 5 U of mIL-3 per ml. FDC-P1 cells were transfected with expression plasmids as described previously (38). Transfectants were cultured for selection in a medium containing 500 μg of hygromycin (Wako Pure Chemical Industries, Osaka, Japan) per ml. The expression of the cDNA products was examined by flow cytometric analysis using MAbs against mIL-5Rα with a FACScan instrument (Becton-Dickinson).

IL-5 binding assay and chemical cross-linking. Binding of mIL-5 to transfected cells was achieved with ^{125}I-labeled mIL-5 as described previously (22). Binding data were analyzed by Scatchard analysis with the EBDA and LIGAND computer programs (Elsevier-BIOSOFT, Cambridge, United Kingdom). Cross-linking experiments were performed by a method described previously (22). In brief, 5×10^6 cells were incubated with ^{125}I-labeled mIL-5, collected, and resuspended in 500 μl of Hanks balanced salt solution (HBSS) containing 1 mM disuccinimidyl tartarate (Pierce Chemical Co.). After incubation at 4°C for 30 min, cells were solubilized with lysis buffer (phosphate-buffered saline containing 1% Triton X-100 and protease inhibitors). After the insoluble fraction was removed by centrifugation, cell lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (7.5% polyacrylamide) under reducing conditions. The gel was fixed and dried and then analyzed with a Fujix BAS2000 Bioimaging Analyzer (Fuji Photo Film, Tokyo, Japan).

Proliferation assay. For the proliferation assay, transfectants were harvested and washed with HBSS and then inoculated into 96-well microtiter plates at a concentration of 10^4/0.2 ml per well with various concentrations of mIL-5. The cells were pulse-labeled with [^{3}H]thymidine (0.2 μCi per well) during the last 6 h of a 48-h culture period, and incorporated [^{3}H]thymidine was measured with a MATRIX 96 Direct Beta Counter (Hewlett-Packard).

Northern (RNA) blots. Transfectants were washed three times with HBSS and cultured at a density of 6×10^6/6 ml per dish in RPMI 1640 medium containing 5% fetal calf serum for 12 h. After starvation, cells were stimulated with 2 nM mIL-5 for the periods indicated below. Cells were collected, and total RNA was prepared by the acid guanidine isothiocyanate-phenol-chloroform method (2). A 10-μg sample of total RNA was denatured and separated through a 1% agarose gel containing 2.2 M formaldehyde. The fractionated RNA was transferred to nylon membranes (GeneScreen; NEN) and hybridized with ^{32}P-labeled DNA fragments. As probes, 2.1-kb *Eco*RI-*Not*I fragments from murine c-*fos* cDNA, 1.8-kb *Eco*RI fragments from rat c-*jun* cDNA, and 1.5-kb *Cla*I-*Eco*RI fragments from murine c-*myc* cDNA were used.

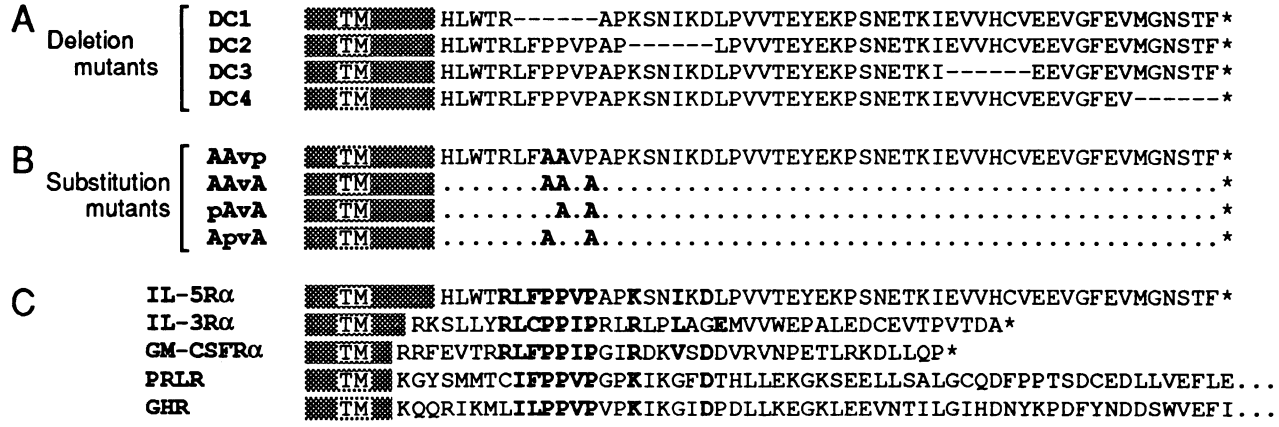

FIG. 1. Cytoplasmic domains of the mutant mIL-5Rα used in this study. All mutants of IL-5Rα have the wild-type extracellular domain. (A) Deletion mutants. Deleted amino acid residues (dashes) are indicated. (B) Substitution mutants. Amino acid residues replaced with alanine (boldface) and the amino acid residues that are the same as those of the wild-type mIL-5Rα (dots) are shown. (C) Comparison of the amino acid sequences of the cytoplasmic domains of murine IL-5Rα, IL-3Rα, GM-CSFRα, prolactin receptor (PRLR), and growth hormone receptor (GHR). Conserved and related amino acid residues (boldface) and the transmembrane portion (TM) are indicated.

Western blots (immunoblots) and immunoprecipitation. Cells were washed three times in HBSS and cultured in RPMI 1640 medium containing 5% fetal calf serum for 8 to 12 h. The cells were then collected and stimulated with 2 nM mIL-5. Finally, cells were collected and lysed on ice in lysis buffer (1% Triton X-100, 10% glycerol, 150 mM NaCl, 20 mM Tris [pH 7.4], 5 mM EDTA, 10 mM NaF, 1 mM sodium orthovanadate, 100 U of aprotinin per ml, 10 mM iodoacetoamide, 25 μg of *p*-nitrophenyl *p'*-guanidinobenzoate [pNpGB] per ml). After centrifugation at 15,000 × *g* for 20 min, clear cell lysates were obtained and subjected to SDS–8% PAGE. For JAK2 immunoprecipitation, cell lysates were preincubated with rabbit serum and protein G-Sepharose for 1 h at 4°C and further incubated with antiserum against JAK2 for 2 h at 4°C. The immune complex was then precipitated with 20 μl of protein G-Sepharose, washed three times with lysis buffer, and subjected to SDS–7.5% PAGE. The proteins were separated and transferred to an Immobilon-P membrane (Millipore). After being blocked with Tris-buffered saline (TBS) containing 5% bovine serum albumin (Fraction V; Sigma), the membrane was incubated with the appropriate primary antibody and washed in TBS containing 0.1% Tween 20 (TBS-T). After incubation with goat anti-mouse or anti-rabbit second antibodies coupled with horseradish peroxidase, the membrane was washed four times with TBS-T and subjected to an enhanced chemiluminescence detection system (Amersham).

RESULTS

Construction and expression of deletion mutants of IL-5Rα. A murine IL-3-dependent cell line, FDC-P1, intrinsically expresses βc. FDC-P1 transfectants (FDC-5R) expressing the wild-type IL-5Rα become responsive to IL-5 (37, 39). The FDC-P1 transfectants expressing the mutant IL-5Rα, which lacks the entire cytoplasmic domain, reconstitute high-affinity IL-5R with βc. The transfectants do not, however, show a proliferative response to IL-5 (38), indicating that the cytoplasmic region of IL-5Rα is indispensable for IL-5-mediated growth signal transduction. To identify the regions of the cytoplasmic domain of IL-5Rα which are critical for signaling, we created four different mutant cDNAs of IL-5Rα, each of which had a deletion of 6 amino acid residues in its cytoplasmic domain (Fig. 1A). DC1 had a deletion in the region rich in proline residues, which is highly conserved among the α chains of IL-5R, IL-3R, and GM-CSFR, prolactin receptor, and growth hormone receptor (Fig. 1C). DC2 had a deletion in the region adjacent to the proline-rich region. DC3 and DC4 had deletions in the carboxy-terminal region that is unique to IL-5Rα. These mutants were expressed in FDC-P1. The level of expression of the mutant IL-5Rα in each mutant was evaluated by staining with anti-IL-5Rα MAb (12, 44) followed by flow cytometric analysis with a FACScan. Regardless of the cytoplasmic deletions, all four different transfectants expressed similar levels of IL-5Rα on their cell surfaces. Each transfectant was then examined for IL-5 binding, and the results are shown in Table 1. All four transfectants expressed high-affinity IL-5 binding sites with K_d values which were essentially equivalent to those of transfectants expressing the wild-type IL-5Rα. These results suggest that mutations induced in the cytoplasmic domain of IL-5Rα do not affect the interactions of IL-5Rα with IL-5 or βc, thereby facilitating reconstitution of the high-affinity IL-5R. We also confirmed by cross-linking experiments that the mutated IL-5Rα proteins were processed properly and were the correct size and that they formed the

TABLE 1. Binding profiles of mIL-5R on FDC-P1 transfectants[a]

Transfectant	High-affinity receptor		Low-affinity receptor	
	K_d (pM)	No. of binding sites/cell	K_d (nM)	No. of binding sites/cell
Wild type	28–47	2,000–4,000	6.1–9.5	45,000–92,000
DC1	53–110	3,000–7,000	7.3–12.0	31,000–100,000
DC2	30–58	3,000–8,000	6.5–8.2	63,000–120,000
DC3	25–63	2,000–5,000	4.6–8.9	37,000–81,000
DC4[b]	27–57	800–4,000	5.9–9.2	41,000–100,000
AAvp	36–55	3,000–5,000	4.2–7.2	36,000–65,000
AAvA	32–61	2,000–5,000	4.7–7.7	42,000–94,000
pAvA	46–76	2,000–6,000	3.9–6.3	54,000–87,000
ApvA	24–58	2,000–4,000	3.4–7.4	33,000–61,000
ααβ[c]	92–130	600–1,000	ND[d]	
αββ[c]	88–140	800–2,000	ND	

[a] Two or three independent transfectants were analyzed for each mutant.
[b] Clone 2 exhibited only high-affinity receptor with 800 binding sites.
[c] Transfectants expressing ααβ or αββ exhibit only high-affinity receptor.
[d] ND, not detected.

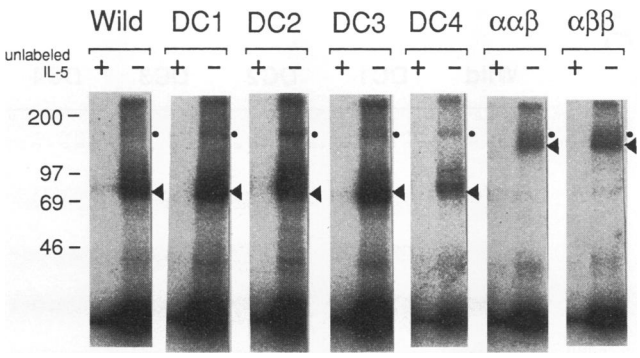

FIG. 2. Chemical cross-linking of ^{125}I-labeled mIL-5 to FDC-P1 transfectants. Cells were incubated with 2 nM ^{125}I-labeled mIL-5 at 4°C for 2 h in the presence (+) or absence (−) of a large molar excess of unlabeled mIL-5 and were cross-linked with disuccinimidyl tartarate. The cell lysates were subjected to SDS-PAGE under reducing conditions and were analyzed with a Fujix BAS2000 Bioimaging Analyzer. Complexes of IL-5 and the wild-type, mutant, or chimeric IL-5Rα proteins (arrowheads) and complexes of IL-5 and βc (dots) are indicated. Molecular mass markers (in kilodaltons) are shown on the left.

high-affinity receptor with βc which is intrinsically expressed in FDC-P1 cells (Fig. 2).

Cytoplasmic regions rich in proline residues of IL-5Rα are indispensable for signaling. Next we examined IL-5-induced proliferation of FDC-P1 transfectants expressing deletion mutants of IL-5Rα. As shown in Fig. 3, DC3 and DC4 transfectants proliferated well upon stimulation with IL-5. Dose-response curves of these transfectants with IL-5 were comparable to those of transfectants expressing the wild-type IL-5Rα. Moreover, they proliferated continuously in the presence of 10 pM IL-5. In contrast, DC1 transfectants did not respond to IL-5. DC2 transfectants proliferated only with high concentrations of IL-5 in the culture. These results indicate that the region rich in proline residues in the cytoplasmic domain of IL-5Rα is essential for IL-5-mediated growth signal transduction. It is also clear that the proximal region downstream from the proline-rich area is important, though not essential.

The proline-rich regions are essential for proto-oncogene expression and protein-tyrosine phosphorylation. After ligand binding to cytokine receptors, several biochemical events are

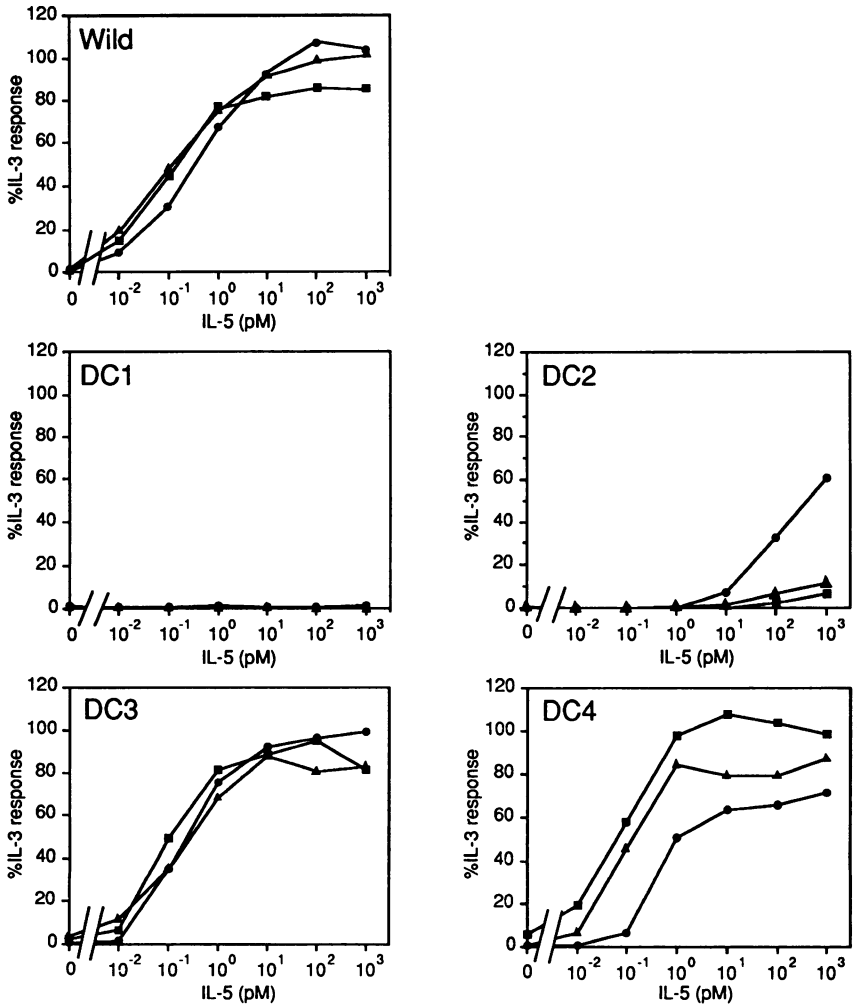

FIG. 3. Proliferation of FDC-P1 transfectants expressing the deletion mutants of IL-5Rα. Transfectants were incubated for 48 h in the presence of various concentrations of IL-5, and incorporated [^{3}H]thymidine was measured. Results are expressed as percentages of [^{3}H]thymidine incorporation in the same cells incubated with 100 U of IL-3 per ml. Three independent clones (▲, ●, and ■) were examined for each mutant IL-5Rα transfectant.

induced in the cell interior (15). It is well established that induction of protein-tyrosine phosphorylation and nuclear proto-oncogene expression is critical for cytokine signal transduction. We examined the induction of c-*fos*, c-*jun*, and c-*myc* transcripts in response to IL-5 by Northern blot analysis. In the wild-type IL-5Rα transfectants, expression of c-*fos*, c-*jun*, and c-*myc* proto-oncogene mRNAs was rapidly induced upon stimulation with IL-5 (Fig. 4A). Increased levels of expression of these proto-oncogenes were also observed in DC3 and DC4 transfectants and were comparable to those observed in transfectants of the wild-type IL-5Rα. In contrast, the proto-oncogene expressions described above were not induced in DC1 transfectants in response to stimulation with IL-5. In DC2 transfectants, induction of these proto-oncogenes was initially weak but became significant with prolonged membrane exposure (data not shown). We observed that the induction of c-*fos*, c-*jun*, and c-*myc* mRNAs upon stimulation with IL-3 and the expression levels of these proto-oncogenes were nearly the same in all four deletion mutant transfectants (data not shown).

Next, we analyzed protein-tyrosine phosphorylation in the transfectants. In the wild-type IL-5Rα transfectants, several cellular proteins (130 to 140, 120, 95, 70, 55, 50, and 42 kDa) were phosphorylated within 10 min of stimulation with IL-5 (Fig. 4B). Their phosphorylation patterns were essentially the same as those induced by IL-3, except for the tyrosine phosphorylation of proteins with molecular masses of 130 to 140 kDa; the molecular mass of the corresponding phosphorylated protein was slightly lower (120 to 130 kDa) in the case of IL-3 stimulation. In DC3 and DC4 transfectants, similar sets of cellular proteins were phosphorylated in response to IL-5 stimulation. In contrast, no phosphorylated cellular proteins were detected in DC1 and DC2 transfectants following IL-5 stimulation (Fig. 4B), while IL-3 stimulation induced comparable levels of tyrosine phosphorylation of cellular proteins in all four deletion mutant transfectants (data not shown).

Recently, it was reported that IL-3 stimulation results in rapid and specific tyrosine phosphorylation of JAK2 protein-tyrosine kinase in IL-3-dependent hematopoietic cells (35). We therefore investigated tyrosine phosphorylation of JAK2 in mutant IL-5Rα transfectants. As shown in Fig. 4C, JAK2 kinase was tyrosine phosphorylated upon stimulation of the wild-type IL-5Rα transfectants with both IL-5 and IL-3. In DC3 and DC4 transfectants, IL-5 stimulation resulted in marked tyrosine phosphorylation of JAK2. In DC1 and DC2 transfectants, however, tyrosine phosphorylation of JAK2 was hardly detectable following IL-5 stimulation. These results clearly demonstrate that the cytoplasmic region rich in proline residues plays a critical role in IL-5-mediated signal transduction, represented by the induction of nuclear proto-oncogene expression and tyrosine phosphorylation of cellular proteins, including JAK2 protein-tyrosine kinase. The adjacent region downstream from the proline-rich residues is also necessary for effective transduction of IL-5-mediated signals. In DC2 transfectants, an IL-5-induced proliferative response and induction of proto-oncogene expression, though at quite low levels, were observed. IL-5 stimulation may also induce tyrosine phosphorylation of cellular proteins which are not detectable with the immunoblot assay used in the present study.

Identification of critical amino acid residues in the cytoplasmic region rich in proline residues by substitution analysis. To identify the critical amino acid residues in the cytoplasmic region of IL-5Rα, we generated several substitution mutants of IL-5Rα. First, we made IL-5Rα mutants with a single amino acid substitution, alanine replacement of Arg-349, Phe-351, Pro-353, Pro-355, Lys-358, or Asp-363. These mutants were expressed in FDC-P1 and tested for IL-5-induced proliferation. All transfectants showed proliferative responses comparable to those of wild-type IL-5Rα transfectants (data not shown). Next, we generated mutants in which alanine was

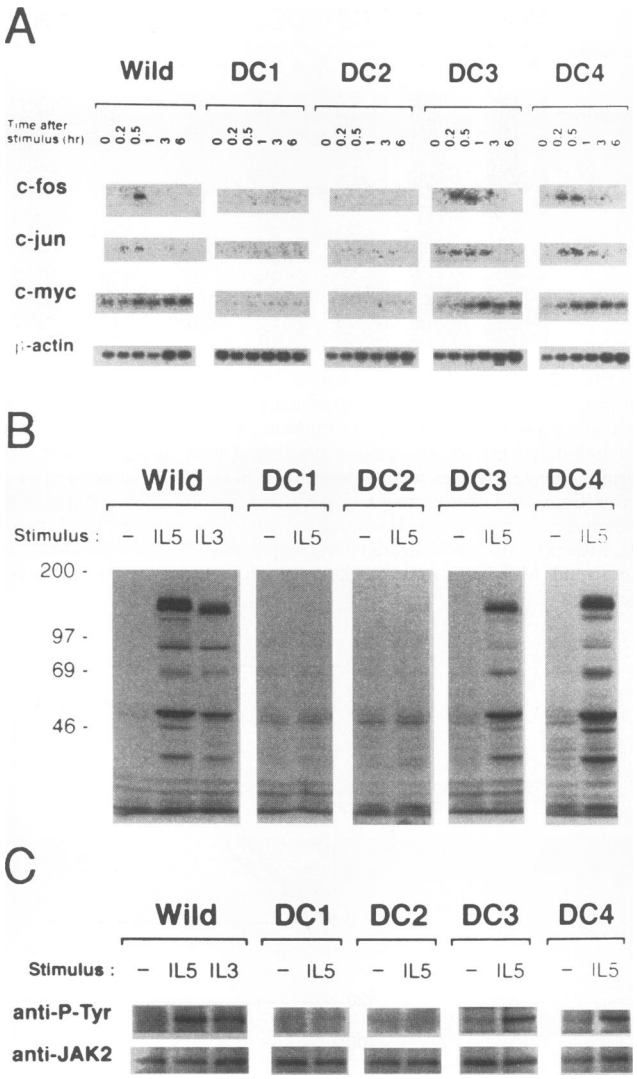

FIG. 4. IL-5-induced cellular responses in FDC-P1 transfectants expressing the IL-5Rα deletion mutants. (A) Induction of early-response proto-oncogenes. Cytokine-starved cells were stimulated with 2 nM IL-5 for the indicated times. Total RNA (10 μg) was separated through a 1% agarose gel and transferred onto nylon membranes. The membranes were hybridized with radiolabeled DNA probes, washed, and analyzed with a BAS2000 Bioimaging Analyzer. (B) Tyrosine phosphorylation of cellular proteins. Cytokine-depleted cells were either unstimulated (−) or stimulated with 2 nM IL-5 for 5 min. Transfectants expressing the wild-type IL-5Rα were also stimulated with 2,000 U of IL-3 per ml. Cell lysates (equivalent to 10^6 cells per lane) were separated by SDS–8% PAGE and immunoblotted with the monoclonal anti-phosphotyrosine antibody 4G10. Molecular mass markers (in kilodaltons) are shown on the left. (C) Tyrosine phosphorylation of JAK2 protein tyrosine kinase. Cell lysates (equivalent to 2×10^7) prepared as described above were incubated with antiserum against JAK2, and the immune complexes were precipitated. Protein samples were subjected to SDS–7.5% PAGE and transferred to polyvinylidene difluoride membranes. The membranes were immunoblotted with 4G10 (anti-P-Tyr) and with antiserum against JAK2 (anti-JAK2).

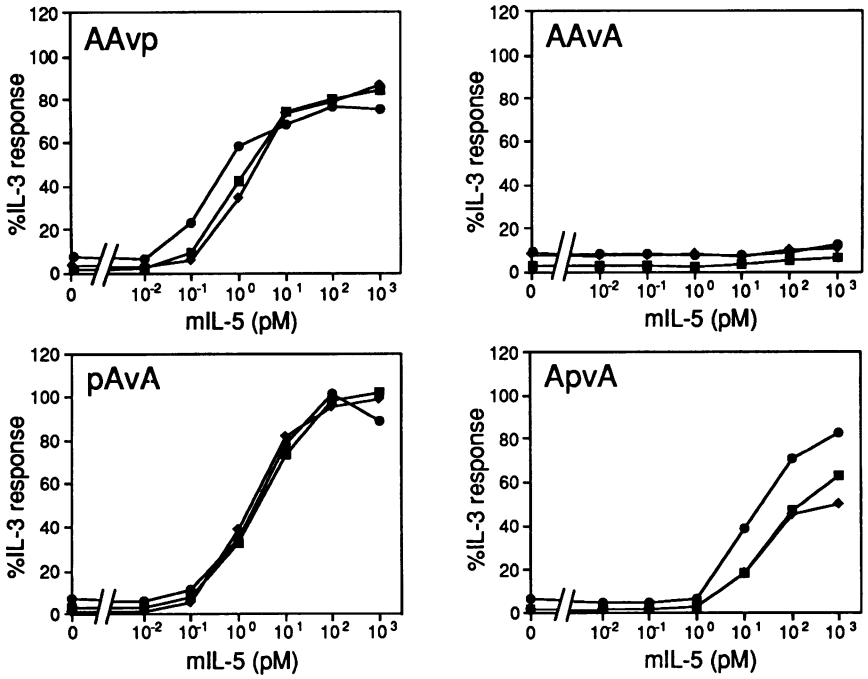

FIG. 5. Growth response of FDC-P1 transfectants expressing the substitution mutants of IL-5Rα. Cells were incubated for 48 h in the presence of various concentrations of IL-5, and incorporated [^{3}H]thymidine was measured. Results are expressed as percentages of [^{3}H]thymidine incorporation in the same cells incubated with 100 U of IL-3 per ml. Three independent clones (▲, ●, and ■) were examined for each mutant IL-5Rα transfectant.

substituted for two or three proline residues (Fig. 1B). Transfectants of these IL-5Rα mutants expressed high-affinity IL-5 binding sites and K_d values nearly the same as those of transfectants expressing the wild-type IL-5R (Table 1). As shown in Fig. 5, substitution of Pro-352 and Pro-353 for Ala (AAvp mutant) or Pro-353 and Pro-355 for Ala (pAvA mutant) resulted in transfectants showing a proliferative response to IL-5. Substitution of Pro-352 and Pro-355 for Ala (ApvA mutant) led to a slightly impaired proliferative response to IL-5. Substitution of all of three proline residues for Ala (AAvA) resulted in complete loss of IL-5-induced proliferation. These results indicate that the existence in IL-5Rα of one of these three proline residues conserved among IL-3Rα, GM-CSFRα, prolactin, and growth hormone receptors is adequate for IL-5 signal transduction. In addition, Pro-352 and Pro-355, in the proline-rich region, appear to have more critical functions in the cytoplasmic domain of IL-5Rα than does Pro-353.

Signal transduction mediated by chimeric molecules of IL-5Rα with βc. In addition to growth factor receptors having intrinsic tyrosine kinase activity, some cytokine receptors, such as growth hormone receptor, gp130 of IL-6R, and G-CSFR, are activated by forming receptor dimers (4, 14, 25). As described above, all functions of the cytoplasmic domain of IL-5Rα in signal transduction depend on the short proline-rich region. It is therefore reasonable to speculate that IL-5Rα may only promote dimerization of βc in IL-5-mediated growth signal transduction and that dimerization of βc is mediated through cytoplasmic proline-rich regions of IL-5Rα. To evaluate this hypothesis, we generated chimeric receptor molecules consisting of the extracellular domain of IL-5Rα with the cytoplasmic domain of βc. As the transmembrane portion may contribute to growth signal transduction, two chimeric receptors with transmembrane portions from IL-5Rα (ααβ mutant) or βc (αββ mutant) were generated (Fig. 6A).

FDC-P1 transfectants of these two chimeric receptors exhibited high-affinity IL-5 binding sites, and their K_d values were slightly lower than those of high-affinity IL-5 binding sites on transfectants expressing the wild-type IL-5R (Table 1). The expression levels of the chimeric molecules, evaluated by fluorescence-activated cell sorter (FACS) analysis with an anti-mIL-5Rα MAb, were considerably lower than those of the wild-type receptor. No low-affinity IL-5 binding sites were detected on the transfectants, because of the low levels of expression of the chimeric receptors. Cross-linking experiments showed that the chimeric molecules interacted with endogeneously expressed βc and formed a high-affinity receptor (Fig. 2). In these transfectants, it is considered that IL-5 binding induces heterodimerization (heteromultimerization) of extracellular domains of IL-5Rα and βc, resulting in homodimerization of the cytoplasmic domain of βc. These transfectants were capable of proliferating in response to IL-5 (Fig. 6B). Expression of c-*fos*, c-*jun*, and c-*myc* transcripts was also induced by IL-5 stimulation (Fig. 7A), although the level of expression of c-*fos* mRNA was lower than those of c-*jun* and c-*myc*. In addition, a set of cellular proteins was tyrosine phosphorylated upon stimulation with IL-5 (Fig. 7B). These intracellular changes, induced by IL-5 stimulation, appeared to be weak in the chimeric receptor transfectants compared with those in the wild-type IL-5Rα transfectant. The patterns of proto-oncogene and tyrosine phosphorylation were, however, similar to these observed in transfectants of the wild-type IL-5Rα. The IL-5-dependent growth signals, including induction of proto-oncogene expression and tyrosine phosphorylation of cellular proteins, appeared to be transduced by dimerization (multimerization) of the cytoplasmic domain of βc without that of IL-5Rα. We also stably expressed the αββ mutant with or without βc in an IL-2-dependent T-lymphocyte line, CTLL-2. Only CTLL-2 transfectants that express both αββ and βc showed IL-5-dependent growth like FDC-P1

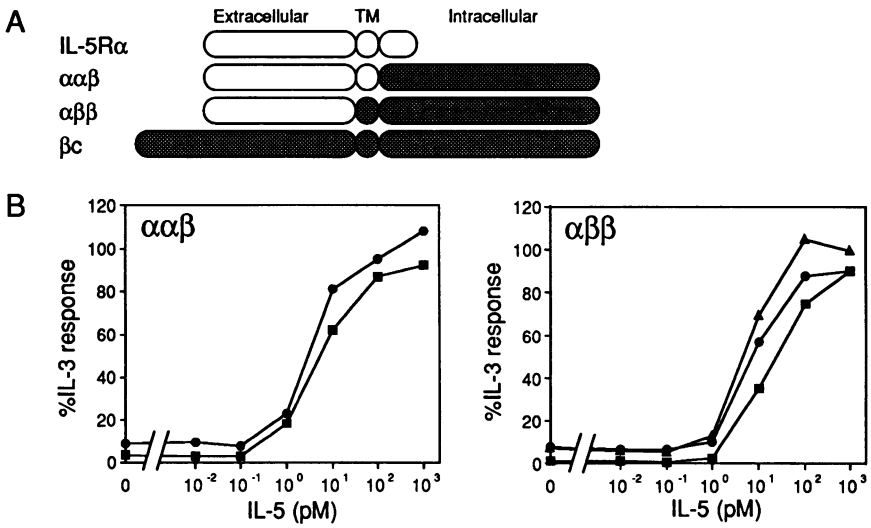

FIG. 6. (A) Schematic representation of the chimeric receptors consisting of IL-5Rα (open boxes) with βc (shaded boxes). (B) Proliferation of FDC-P1 transfectants expressing the chimeric receptors. Transfectants were incubated for 48 h in the presence of various concentrations of IL-5, and incorporated [^{3}H]thymidine was measured. Results are expressed as percentages of [^{3}H]thymidine incorporation in the same cells incubated with 100 U of IL-3 per ml. Two and three independent clones were examined for the ααβ and αββ transfectants, respectively.

transfectants (data not shown). This indicates that αββ alone is not functional and that the receptor complexes with αββ and βc are functional in CTLL-2 cells as well as in FDC-P1 cells.

DISCUSSION

By using a mutant IL-5Rα lacking the entire cytoplasmic domain, we have demonstrated the importance of the cytoplasmic domain of IL-5Rα in the transduction of an IL-5-dependent growth signal (38). In this study, we identified two distinct regions of the IL-5Rα cytoplasmic domain required for IL-5-mediated signal transduction, a membrane-proximal, proline-rich region and an adjacent region downstream of the proline-rich region (Fig. 3 and 4). These two regions are well conserved among IL-5Rα, IL-3Rα, and GM-CSFRα and prolactin and growth hormone receptors. It is speculated that these regions play a critical role in transducing common growth signals through these cytokine receptors, such as induction of nuclear proto-oncogene expression and tyrosine phosphorylation of cellular proteins, including JAK2 protein kinase. The proline-rich region of IL-5Rα contains a Pro-X-Pro sequence that exists in the membrane-proximal box 1 region of other members of the cytokine receptor family (26). βc also contains a box 1 region. A truncated mutant of βc that lacks the box 1 region does not transduce GM-CSF-induced growth signals in BaF3 cells (31), suggesting that the box 1 region of βc is also important in IL-5-mediated signal transduction. In the case of the IL-6R gp130 subunit, mutation of two proline residues in the Pro-X-Pro sequence results in complete loss of receptor activity (26). In contrast, on the basis of our analysis of substitution mutations of IL-5Rα, neither of the proline residues of the Pro-X-Pro sequence is required for growth signal transduction. However, one of the proline residues in the well-conserved cytoplasmic region common to IL-5R, IL-3R, and GM-CSFR (from Leu-350 to Pro-355 in mIL-5Rα) does appear to be required for interaction with βc for growth signal transduction. This is demonstrated by the pAvA mutation, which does not greatly affect receptor-mediated growth signal transduction (Fig. 5). These results suggest that the proline-rich sequences of α chains of IL-5R, IL-3R, and GM-CSFR may function differently in growth signal transduction from the proline-rich box 1 regions of βc and other members of the cytokine receptor family.

The interaction between proline-rich sequences of various proteins and the SH3 domains of adapter proteins has attracted considerable attention as a possible mechanism for signal transduction through growth factor receptors (8, 30, 45). In addition to the Pro-X-Pro motif, the critical proline-rich sequence of IL-5Rα also contains a Pro-X-X-Pro motif that is conserved among various SH3-binding proteins (45). We speculate, however, that the proline-rich sequence of IL-5Rα is not likely to interact with the SH3 domains of cellular proteins, or that the interaction of the proline-rich sequence of IL-5Rα and SH3 domains is not critical for transduction of IL-5-mediated signals, for the following two reasons. First, the proline-rich sequence of IL-5Rα lacks the additional proline residue usually found one or two residues amino terminal of the Pro-X-X-Pro motif (45). Second, substitution of either of the proline residues of the Pro-X-X-Pro motif with alanine (AAvp and pAvA mutants), which is expected to greatly reduce the affinity of binding to a target SH3 domain (30, 45), did not impair IL-5-mediated signal transduction. Instead, the proline residues in the critical region of IL-5Rα may contribute to the flexibility of its cytoplasmic domain, which is important for signal transduction, or may be important for the interaction with certain intracellular signaling protein(s) which do not have an SH3 domain, such as members of the JAK tyrosine kinase family. However, we must also consider the possibility that Pro-357 in IL-5Rα, which is not conserved in IL-3Rα and GM-CSFRα, might have affected our results. Further studies will be required to explore the functional differences of the proline-rich residues among IL-5Rα, IL-3Rα, and GM-CSFRα.

Recent studies by many investigators have revealed the essential role of the JAK family of protein-tyrosine kinases in signal transduction through cytokine receptors (1, 18, 35, 36, 43). JAK kinases are associated with the box 1 regions of the members of the cytokine receptor family. It has been reported that the JAK family kinases induce tyrosine phosphorylation and translocation of ~91-kDa cellular transcription factors, as

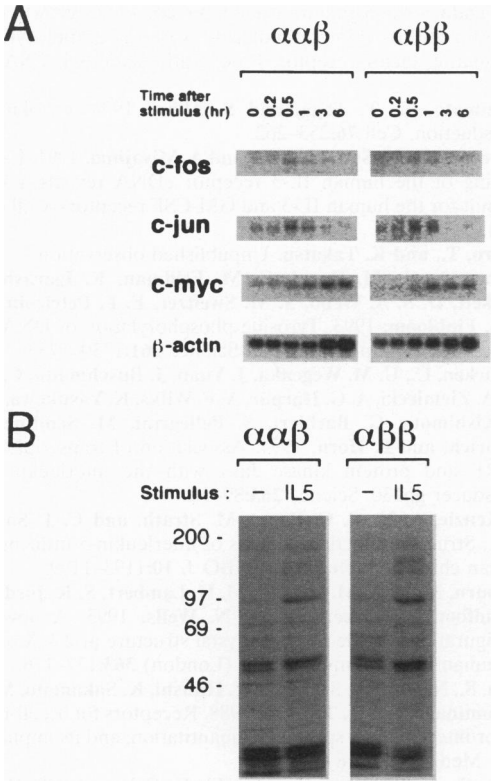

FIG. 7. IL-5-induced cellular responses in FDC-P1 transfectants expressing the chimeric receptor of IL-5Rα with βc. (A) Induction of early-response nuclear oncogenes. Cytokine-starved cells were stimulated with 2 nM IL-5 for the indicated times. Total RNA (10 μg) was separated through a 1% agarose gel and transferred onto nylon membranes. The membranes were hybridized with radiolabeled DNA probes, washed, and analyzed with a BAS2000 Analyzer. (B) Tyrosine phosphorylation of cellular proteins. Cytokine-depleted cells were either unstimulated (−) or stimulated with 2 nM IL-5 for 5 min. Cell lysates (equivalent to 10^6 cells per lane) were separated by SDS–8% PAGE and immunoblotted with the monoclonal anti-phosphotyrosine antibody 4G10. Molecular mass markers (in kilodaltons) are shown on the left.

well as their translocation into the nucleus, upon stimulation with interferons or IL-6 (18, 36, 42). In this study, we demonstrated that JAK2 kinase was rapidly phosphorylated on tyrosine residues upon IL-5 stimulation in FDC-P1 transfectants expressing IL-5Rα. Tyrosine phosphorylation of the JAK2 kinase was also observed in an IL-5-dependent early B-cell line, Y16 (16a). In addition, treatment of human basophils with IL-5 induces tyrosine phosphorylation and activation of DNA-binding proteins that recognize the gamma interferon response region (17). Therefore, it is likely that the JAK2 tyrosine kinase may play an important role in the activation of transcription factors involved in IL-5-mediated signal transduction. As shown in Fig. 4, the IL-5 signal transduction pathway via JAK2 kinase also appears to require the cytoplasmic proline-rich sequence of IL-5Rα.

Results obtained with chimeric receptors of IL-5Rα with βc indicated that dimerization of the cytoplasmic domain of βc leads to the ability to transduce growth signals in the absence of the cytoplasmic domain of IL-5Rα. The dimerization of βc in signal transduction has been examined by using chimeric receptors consisting of the extracellular domain of erythropoietin R (Epo-R) and the cytoplasmic domain of β_{IL-3}, which is highly homologous to βc. Zon et al. reported that the Epo-R/β_{IL-3} chimeric receptor conferred Epo-dependent growth in both BaF3 and CTLL-2 cells (46). In contrast, Sakamaki et al. reported that this chimeric receptor is nonfunctional in CTLL-2 cells and suggested that additional components may be required for growth signal transduction (32). Our results are consistent with those of the first report. It is possible that a conformational change involving the extracellular domains of IL-5Rα and βc may be important for the function of the βc cytoplasmic domain or that our CTLL-2 cells have some signal transduction component which is missing in the CTLL-2 cells used by Sakamaki et al.

Taken together with the observation that the cytoplasmic proline-rich region of IL-5Rα is essential, two models for the mechanism of activation of the IL-5R complex can be postulated. In one model, IL-5 induces multimerization of IL-5R subunits and dimerization of the cytoplasmic domain of βc. The proline-rich regions of the cytoplasmic domain of IL-5Rα interact directly or indirectly with βc, and this interaction is essential for either dimerization or conformational change of the cytoplasmic domain of βc. This dimerization of βc brings the two JAK2 kinases, which are associated with the box 1 region of βc, in sufficiently close proximity to cross-phosphorylate and result in activation of the JAK2 kinases. In the second model, the proline-rich region of IL-5Rα interacts with an intracellular protein kinase, such as JAK2 or other members of the JAK family. The IL-5Rα-associated kinase and the βc-associated JAK2 kinase cross-phosphorylate and activate each other. The function of the cytoplasmic region of IL-5Rα can be substituted by βc and the βc-associated JAK2 kinase in the chimeric receptor transfectants. At present, we have no definite evidence supporting either of these models. In our previous studies using chemical cross-linking of radiolabeled IL-5 to IL-5-responsive cells, we detected only two complexes, consisting of IL-5 and IL-5Rα or of IL-5 and βc (21, 22, 37, 38). Though it has been demonstrated that one IL-5 molecule binds to a single soluble IL-5Rα molecule in the absence of βc (5), it is still unclear how many receptor subunits form the high-affinity IL-5R complex. Mutation analysis of IL-5 revealed that the amino-terminal and carboxy-terminal portions of IL-5 bind to βc and IL-5Rα, respectively (19). As the structure of IL-5 consists of an antiparallel disulfide-linked dimer, the IL-5 homodimer thus contains two binding sites for IL-5Rα and two binding sites for βc (20). In contrast, a GM-CSF monomer is able to bind to its receptor and transduce signals. There is probably a common binding stoichiometry among IL-5, IL-3, and GM-CSF and the respective receptors. We need to clarify the molecular structures of IL-5 and the IL-5R complex in order to understand the mechanisms of activation and signal transduction through IL-5R.

Deletions in the carboxy-terminal region of the cytoplasmic domain of IL-5Rα (DC3 or DC4) did not affect its ability to transduce an IL-5-mediated growth signal. As the carboxy-terminal regions of the α chains of IL-5R, IL-3R, and GM-CSFR do not share significant homology and since they represent the only unique part of the cytoplasmic domains of these three cytokine receptor complexes, it is possible that these regions may contribute to the transduction of ligand-specific signals. In this study, we investigated the role of the critical cytoplasmic region of IL-5Rα in the growth signal transduction cascade. IL-5 did not induce significant morphological changes or expression of cell surface molecules on FDC-P1 cells transfected with the wild-type IL-5Rα (38a). As IL-5 induces not only proliferation but also differentiation of B cells and eosinophils, we are interested in clarifying the signal transduction pathways of mutant IL-5Rα, especially DC3,

DC4, and chimeric receptors, in the differentiation of B cells. We are currently investigating B-cell lines capable of differentiating into immunoglobulin M-producing cells in response to IL-5. These studies are designed to determine whether an IL-5-specific signal transduction pathway exists and, if so, which portions of the cytoplasmic domain of IL-5Rα contribute to the IL-5-specific signaling pathway.

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