Critical Cytoplasmic Domains of Human Interleukin-9 Receptor α Chain in Interleukin-9-mediated Cell Proliferation and Signal Transduction*

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Interleukin-9 receptor (IL-9R) complex consists of ligand-specific α chain and IL-2R γ chain. In this study, two regions in the cytoplasmic domain of human IL-9Rα were found to be important for IL-9-mediated cell growth. A membrane-proximal region that contains the BOX1 consensus sequence is required for IL-9-induced cell proliferation and tyrosine phosphorylation of Janus kinase (JAK). Deletion of this region or internal deletion of the BOX1 motif abrogated IL-9-induced cell proliferation and signal transduction. However, substitution of the Pro-X-Pro in the BOX1 motif with Ala-X-Ala failed to abolish IL-9-induced cell proliferation but decreased IL-9-mediated tyrosine phosphorylation of JAK kinases, insulin receptor substrate-2, and signal transducer and activator of transcription 3 (STAT3) and expression of c-myc and junB. Another important region is downstream of the BOX1 motif and contains a STAT3 binding motif YLPQ. Deletion of this region significantly impaired IL-9-induced cell growth, activation of JAK kinases, insulin receptor substrate-2, and STAT3 and expression of early response genes. A point mutation changing YLPQ into YLPA greatly reduced IL-9-induced activation of STAT3 and expression of c-myc but did not affect cell proliferation. These results suggest that cooperation or cross-talk of signaling molecules associated with different domains of IL-9Rα other than STAT3 is essential for IL-9-mediated cell growth.

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†The abbreviations used are: IL, interleukin; R, receptor; h, human; m, murine; JAK, Janus kinase; STAT, signal transducer and activator of transcription; IRS-2, insulin receptor substrate-2; SIE, c-sis-inducible element; APRF, acute-phase response factor; CMV, cytomegalovirus; PVDF, polyvinylidene difluoride.

The involvement of IL-9 in lymphomagenesis has been suggested by in vivo studies (8) in which a higher susceptibility to T cell lymphoma was observed in transgenic mice expressing IL-9 constitutively and by in vitro experiments (9) in which IL-9 has been shown to protect mouse lymphoma cells from dexamethasone-induced apoptosis. The functions of IL-9 are mediated by the IL-9 receptor (IL-9R), which consists of a ligand-specific α chain and IL-2 receptor (IL-2R) β chain. IL-2R γ chain, normally referred to as the common γ chain (γc, chain), is shared by receptors for IL-2, IL-4, IL-7, IL-9, and IL-15 (10–13). Some of the signaling pathways elicited by these cytokines are quite similar, which probably explains in part the functional redundancy of these cytokines.

IL-9R α chain (IL-9Rα) is IL-9-specific and is responsible for IL-9 binding. The cDNAs encoding mouse and human IL-9Rα have been cloned (14–15). IL-9Rα belongs to the hematopoietic receptor superfamily and has no intrinsic tyrosine kinase motif in its cytoplasmic region. Several homologous sequences, such as the BOX1 consensus sequence and a serine-rich region, which were demonstrated to be important for IL-2Rβ function (16), are also present in the cytoplasmic region of human IL-9Rα (hIL-9Rα). Recently, it has been shown that several biochemical events including tyrosine phosphorylation of JAK kinases, activation of signal transducers and activators of transcription (STAT) proteins, and expression of nuclear proto-oncogenes are involved in IL-9-mediated signal transduction (17–19). In the present study, a series of deletions and point mutations were made in the cytoplasmic region of hIL-9Rα. These mutants were transfected into a mouse IL-9-dependent T cell line, TS1, to investigate the functions of different domains of hIL-9Rα in IL-9-mediated cell proliferation and activation of JAK/STAT and primary response genes.

MATERIALS AND METHODS

Plasmid Construction—The full-length cDNA for hIL-9Rα was provided by Dr. Ming-Shi Chang at Amgen (Thousand Oaks, CA). The hIL-9Rα cDNA fragments containing different C-terminal truncations of the cytoplasmic region were generated by polymerase chain reaction. The full-length and C-terminal-truncated cDNAs for hIL-9Rα were subcloned into blunt-ended XhoI site in pReCMV vector (Invitrogen). Site-directed mutations in the BOX1 and STAT3 binding motifs (BOXm and STAT3 m) within the cytoplasmic domain of hIL-9Rα were generated using the Altered Sites II in vitro mutagenesis systems (Promega). Internal deletion of the BOX1 motif (between amino acids 298 and 315) in hIL-9Rα (BOXd) was performed using the polymerase chain reaction method as described previously (20). To construct pFlagIL-9R plasmids, an NdeI-XhoI restricted fragment from pFlag-CMV-1 expression vector (Eastman Kodak Co.) that contained part of the CMV promoter and the coding sequence for preprotrypsin leader peptide and flag epitope tag was subcloned into various pReCMV-IL-9 constructs to replace NdeI-XhoI-restricted DNA fragments. The resulting plasmids were designated as pFlagIL-9R W, pFlagIL-9R D1, pFlagIL-9R D2, pFlagIL-9R D3, pFlagIL-9R D4, pFlagIL-9R BOXm, pFlagIL-9R STAT3 m, and...
pFlagIL-9R BOXd (see Fig. 1). All plasmid constructs were confirmed by DNA sequencing.

Cell Cultures and Transfection—Murine T cell clone, TS1, was maintained in Cibick’s medium (Irvine Scientific) supplemented with 10% fetal calf serum and 1 mg/ml mIL-9 (R & D Systems). For stable expression of hIL-9R, TS1 cells in exponential growth phase were suspended at 2 × 10^7 cells/ml in culture medium. 0.3 ml of these cells was transferred into a 0.4-cm cuvette with 30 μl of pFlagIL-9R DNA. Following a single 250-V/960-microfarad pulse with Bio-Rad Gene Pulser, each sample was cultured in 5 ml of medium with mIL-9 (1 ng/ml) at 37 °C. Stable transfectants were isolated by selection in 1 mg/ml G418 for 3 weeks. Cells expressing high levels of FlagIL-9R proteins were further enriched by Fluorescence-activated Cell Sorter using an anti-Flag M2 monoclonal antibody (Kodak) and goat anti-mouse IgG1-fluorescein isothiocyanate (Southern Biotechnology Associates, Inc.).

Proliferation Assay—Transfectants expressing different forms of hIL-9Rα were washed three times, seeded in triplicates at 5 × 10^4 cells/200 μl of culture medium/well either without growth factors or supplemented with various concentrations of hIL-9. After incubation at 37 °C for 3 days, 1 μCi of [3H]thymidine (Amersham Life Science, Inc., specific activity 5 Ci/mmol) was added per well and further incubated for 4 h at 37 °C before harvest. The incorporated [3H]thymidine was determined using a liquid scintillation counter (Beckman Instruments, LS 6000IC).

Western Blots and Immunoprecipitation—Cells were starved and stimulated with hIL-9α as described previously (18). Briefly, about 2 × 10^5 cells were starved for 8 h at 37 °C in the absence of hIL-9 and serum, then cells were collected and stimulated without or with hIL-9α (30 ng/ml) for 5 min. Cells were then lysed in 0.5 ml of 1% Nonidet P-40 lysis buffer containing different proteinase inhibitors (21). After centrifugation, clear cell lysates were obtained and incubated with anti-JAK1, anti-JAK3, anti-IRS-2, or anti-STAT3 antibody (Upstate Biotechnology, Inc.) overnight at 4 °C. Then protein A-agarose was added, and the mixture was rotated for 1 h. The immunoprecipitates were washed three times with lysis buffer and separated by 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes and immunoblotted sequentially with anti-phosphotyrosine (Upstate), anti-JAK1, anti-JAK3, anti-IRS-2, and anti-STAT3 antibodies and finally detected with the ECL techniques according to manufacturer instructions. For detection of FlagIL-9R expression, total cell lysates were prepared from 2 × 10^5 cells and immunoprecipitated with 2 μg of anti-Flag M2 antibody. The immunoprecipitates were separated by 10% SDS-polyacrylamide gel electrophoresis, transferred to PVDF membranes, and immunoblotted with anti-hIL-9R antibody (R & D Systems).

Northern Blot Analysis—Northern blot analysis was performed as described previously (22). For detection of primary response gene expression, transfectants expressing different mutant forms of hIL-9Rα were starved and stimulated with hIL-9α (30 ng/ml) for 1 h. Total RNAs were isolated from cells using a guanidinium isothiocyanate method (23). 5 μg of total RNA was used for Northern blot and hybridized with 32P-labeled c-myc, junB, e-jun, and c-fos cDNAs. The mouse glyceraldehyde-3-phosphate dehydrogenase probe was hybridized to the same filters to ensure equal loading in each lane. The expression of different pFlagIL-9R constructs in TS1 transfectants was determined using the hIL-9α cDNA as the probe.

Gel Shift and Gel Supershift Assays—Nuclear extracts were prepared from unstimulated and stimulated TS1 cells as described previously (24). A 32P-labeled oligonucleotide corresponding to a high affinity dyed symmetrical acute-phase response factor (APRF) binding site (5'-CTTCCCCGGAATTC-3') was used as the probe (25). For the gel shift assay, 10 μg of nuclear proteins were incubated with 2 μg of poly(dI-dC) before the addition of 2 × 10^5 cpm of probe. After incubation at room temperature for 30 min, the reactions were analyzed with a 5% native polyacrylamide gel. For the gel supershift assay, nuclear extracts were preincubated with 2 μg of anti-Stat3 antibody or the same amount of preimmune IgG at 4 °C for 2 h and then incubated with the probe for 30 min at room temperature. In oligonucleotide competition experiments, a 100-fold molar excess of various competitors were preincubated with nuclear extracts for 10 min before the binding reactions. The sequences of c-sis-inducible element (SIE), AP-1, and Oct-1 competitors are: SIE, 5'-GTCGACATTCTGGAAATCTT-3'; AP-1, 5'-TGTGAGTCGAGGCCGAA-3' (Promega); Oct-1, 5'-TCTGGAATGCA-3' (Santa Cruz Biotechnology).

RESULTS

Expression of Mutant hIL-9Rα Proteins—To identify structural elements critical for IL-9-mediated cell proliferation and signal transduction in the cytoplasmic region of hIL-9Rα, a series of mutants with deletions or point mutations in hIL-9Rα were constructed and stably transfected into TS1 cells. TS1 is a mouse IL-9-dependent T cell line that does not respond to hIL-9 stimulation. It has been shown previously that transfection of hIL-9Rα cDNA into TS1 cells conferred responsiveness of these cells to hIL-9 (14). A schematic diagram of mutant receptor constructs used in the present study is shown in Fig. 1. To determine the expression of different pFlagIL-9R constructs in transfected TS1 cells, Northern blot and immunoprecipitation analysis were performed to detect hIL-9R mRNAs and proteins in different transfectants. As shown in Fig. 2, A and B, the parental TS1 cells did not express hIL-9Rα transcripts or proteins and cells transfected with different pFlagIL-9R constructs expressed various sizes of hIL-9Rα mRNAs and proteins. It was noticed that two different sizes of hIL-9Rα proteins were expressed in each transfectant, which may represent different glycosylated forms of hIL-9Rα. Cell surface expression of FlagIL-9Rα proteins was further confirmed by flow cytometric analysis. As shown in Fig. 2C, cells transfected with pRc/CMV vector alone (negative control) showed a similar fluorescence intensity to untransfected cells, whereas
transfectants expressing various pFlagIL-9R α cDNAs had a higher relative fluorescence intensity than control cells. The percentage of positive cells was very similar among different transfectants.

Two Regions in the Cytoplasmic Domain of IL-9R α Play Important Roles in IL-9-induced Cell Growth—To understand the role of different regions of the cytoplasmic domain in hIL-9R α for IL-9-mediated cell growth, TS1 cells transfected with different hIL-9R α cDNAs were tested for their proliferative response to various concentrations of hIL-9. As shown in Fig. 3, cells transfected with vector alone did not grow in the presence of hIL-9; in fact, >95% of cells were dead before the addition of [³H]thymidine. TS1 cells transfected with wild-type hIL-9R α (pFlagIL-9R W) showed a strong response to hIL-9 induction,
with cell proliferation detected even at a very low concentration of hIL-9. This suggests that the FlagIL-9R fusion protein functioned like the natural form of hIL-9Rα in response to hIL-9 stimulation in TS1 cells. Transfectants harboring pFlagIL-9Rα D1 and pFlagIL-9Rα D2 (D1 and D2) showed a proliferative response comparable to wild-type transfectants, suggesting that distal C terminus and the serine-rich region of hIL-9Rα are dispensable for IL-9-induced cell proliferation. This is in part different from IL-2Rβ1 in which the serine-rich region has been shown to be critical for IL-2-induced cell growth (27). Two regions in the cytoplasmic domain of hIL-9Rα were implicated to be critical for IL-9-mediated cell proliferation in the present study. A membrane-proximal domain between amino acids 295 and 338 including a BOX1 consensus sequence, was shown to be essential for IL-9-induced cell proliferation. The proliferative response to IL-9 was abrogated when this region was further truncated (D4) or if the BOX1 motif in this region was deleted (BOXd), indicating that the BOX1 consensus sequence in hIL-9Rα is required for IL-9-mediated cell growth. However, unlike other receptor systems, mutation of the Pro-X-Pro motif in BOX1 consensus sequence of hIL-9Rα (BOXm) did not abolish but slightly reduced IL-9-mediated cell growth. The second important region is located downstream of the BOX1 consensus sequence between amino acids 338 and 422. This region contains a STAT3 binding site and was found to play a crucial role in promoting IL-9-induced cell proliferation. When this region was truncated (D3), cells only proliferated at a higher concentration of hIL-9 (1 ng/ml). Since STAT3 has been shown to be the only STAT protein activated by IL-9 in TS1 cells (18), we also mutated STAT3 binding site within the hIL-9Rα to evaluate the role of STAT3 in IL-9-mediated cell growth. Interestingly, point mutation at the STAT3 binding site from YMPQ to YLPA (STAT3 m transfectants) did not significantly affect IL-9-induced cell proliferation, although a similar point mutation (from YMPQ to YMPA) has been shown to abolish tyrosine phosphorylation of STAT3 induced by gp130 family of cytokines (28). We also noticed that wild type, D1, D2, and STAT3 m transfectants had a higher incidence of becoming factor-independent compared with D3, D4, BOXm, and BOXd transfectants, implicating that overexpression of specific domains of IL-9Rα may result in transformation of TS1 cells.

Deletion of Two Regions in the Cytoplasmic Domain of hIL-9Rα Abrogates Tyrosine Phosphorylation of JAK Kinases, IRS-2, and STAT3 Induced by hIL-9—Accumulated evidence has revealed that molecular cascades involved in cytokine-mediated cell growth are associated with protein tyrosine phosphorylation. JAK1, JAK3, IRS-2, and STAT3 have been demonstrated to be activated by IL-9 stimulation through tyrosine phosphorylation in our previous studies (17, 18). To examine the role of hIL-9Rα in IL-9-induced growth-promoting signals, the status of hIL-9-induced tyrosine phosphorylation of JAK kinases, IRS-2, or STAT3 in TS1 cells expressing wild-type and mutant hIL-9Rαs were tested. As illustrated in Fig. 4, tyrosine phosphorylation of JAK1, JAK3, IRS-2, and STAT3 was comparable in wild-type hIL-9Rα transfectants (W) and in parental TS1 cells stimulated with hIL-9 and mIL-9, respectively, indicating that transfected hIL-9Rα transduces similar signals as its murine counterpart following hIL-9 stimulation. Deletion of the C terminus and the serine-rich region of hIL-9Rα (D1 and D2) failed to change hIL-9-induced tyrosine phosphorylation of JAK kinases, IRS-2, and STAT3. The BOX1 consensus sequence in the membrane-proximal region was shown to be absolutely required for IL-9-induced signaling, since truncation (D4) or internal deletion (BOXd) of this region completely abolished IL-9-induced tyrosine phosphorylation of JAK kinases, IRS-2, and STAT3. Furthermore, point mutation of Pro-X-Pro motif into Ala-X-Ala (BOXm) within the BOX1 consensus sequence also decreased IL-9-induced tyrosine phosphorylation of JAK kinases, IRS-2, and STAT3. It was also found that tyrosine phosphorylation of JAK kinases and IRS-2 was greatly decreased when the BOX1 downstream region of hIL-9Rα was truncated (D3). This observation is consistent with a recent report, in which the BOX1 downstream region of hIL-9Rα was found to be absolutely required for IL-9-induced JAK1 activation in BW 5147 cells (29). This region was also found to be indispensable for activation of STAT3, since truncation of this region eliminated IL-9-induced tyrosine phosphorylation of STAT3 (D3). Point mutation of the STAT3 binding motif from YLPQ to YLPA in this region (Stat3 m) greatly decreased IL-9-induced tyrosine phosphorylation of STAT3, suggesting activation of STAT3 is most likely through this YLPQ motif.

To further confirm the status of STAT3 activation by hIL-9 in different hIL-9Rα transfectants, gel shift and gel supershift assays were performed to detect specific DNA-protein complexes following hIL-9 stimulation. As indicated in Fig. 5A, the nuclear extracts from wild-type transfectants stimulated by hIL-9 specifically bind to the APRF sequence that has a high affinity binding site for STAT3 proteins (25). This binding activity was induced within 1 min after hIL-9 stimulation and persisted for at least 15 min. The competition experiments (Fig. 5B) showed that the APRF binding activity induced by IL-9 could be completely blocked with 100-fold excess of cold APRF or SIE competitor but not with 100-fold excess of AP-1 or Oct-1 oligonucleotide. It was further shown by the gel supershift assay (Fig. 5B) that most of the IL-9-induced APRF-protein complexes can be specifically supershifted by anti-STAT3 antibody, indicating that the major proteins in IL-9-induced APRF-protein complexes are STAT3 or STAT3-related molecules. IL-9-induced APRF binding activities were also examined in different hIL-9Rα transfectants (Fig. 5C); it was found that the induction of APRF binding activities correlated with STAT3 tyrosine phosphorylation in these transfectants following IL-9 stimulation.
**FIG. 4.** Tyrosine phosphorylation of JAK1, JAK3, IRS-2, and STAT3 in TS1 transfectants expressing hIL-9Rα. Cells were starved in serum-free medium for 8 h. 2 × 10^6 cells were not stimulated or stimulated with hIL-9 (30 ng/ml) for 5 min and immunoprecipitated with 1.5 μg of anti-JAK1, JAK3, or STAT3 or 4 μg of anti-IRS-2 antibody at 4 °C overnight. The immunoprecipitates were separated by 7.5% SDS-polyacrylamide gel electrophoresis and transferred to PVDF membranes. The membranes were immunoblotted with anti-phosphotyrosine (P-Tyr), anti-JAK1, anti-JAK3, anti-IRS-2, and anti-STAT3.

**DISCUSSION**

In the present study, we have demonstrated the importance of the cytoplasmic domain of hIL-9Rα in IL-9-mediated cell proliferation and signal transduction. We identified two regions, a membrane-proximal region containing BOX1 consensus sequence and the adjacent downstream region containing a STAT3 binding site, as required for IL-9-mediated signal transduction. In our study, the membrane-proximal region including 47 amino acids of the cytoplasmic domain of hIL-9Rα (D3) was shown to support IL-9-induced cell proliferation and tyrosine phosphorylation of JAK kinases even though the response of this mutant to IL-9 was greatly reduced compared with wild type hIL-9Rα. This observation is different from a recent report by Demoulin et al. (29) in which a mouse IL-3-dependent cell line, Ba/F3, transfected with truncated hIL-9Rα plasmid encoding 115 amino acids of the cytoplasmic region containing the BOX1 motif no longer responds to hIL-9. The difference between the two studies could be due to the difference in the cell types used in each of these experiments. The function of the membrane-proximal region in hIL-9Rα is most likely to be mediated by the BOX1 motif, which is also found in the membrane-proximal region of many cytokine receptors and has been demonstrated to be important for JAK1/JAK2 activation and cell growth in several cytokine receptor systems (30–34). In our study, truncation or internal deletion of the BOX1 motif in the membrane-proximal region of hIL-9Rα totally abolished hIL-9-induced cell proliferation and tyrosine phosphorylation of JAK1 and JAK3, suggesting the BOX1 consensus sequence in hIL-9Rα plays a preeminent role in IL-9-induced activation of JAK kinases and cell growth. Since mutation of the conserved Pro-X-Pro motif in the BOX1 consensus sequence inactivated the receptor and JAK1 activation in certain receptor systems (30, 35, 36), we also introduced point mutations (Pro→Ala-X-Ala) in the BOX1 consensus sequence of hIL-9Rα (BOXm). To our surprise, these mutations did not totally abolish IL-9-mediated cell growth, but decreased tyrosine phosphorylation of JAK1 and JAK3. Although activation of JAK3 has been demonstrated to be mainly associated with IL-2R γ chain (37, 38), tyrosine phosphorylation of JAK3 was shown to be affected by mutations in the membrane-proximal and the BOX1 downstream regions of hIL-9Rα in our study. Recently, a membrane-proximal region of human IL-2Rβ was shown to be critical for JAK3 activation (35), raising the possibility that JAK3 may bind to receptor subunits other than IL-2R γ chain for its activation. In this study, we also investigated tyrosine phosphorylation of IRS-2 by IL-9 in various transfectants. It appears that tyrosine phosphorylation of IRS-2 is correlated with tyrosine phosphorylation of JAK kinases, which is consistent with our earlier observation that JAK kinases are responsible for IL-9-induced tyrosine phosphorylation of IRS proteins (18). In agreement with the study by Demoulin et al. (29), our data also indicated that the BOX1 downstream region in hIL-9Rα plays an important role in IL-9-mediated cell growth. Deletion of this region not only greatly reduced hIL-9-stimulated cell proliferation and tyrosine phosphorylation of JAK kinases and IRS-2 but also abrogated tyrosine phosphorylation of STAT3. A
were performed with nuclear extracts from wild-type hIL-9R analyzed by the gel shift assay.

the indicated period of time, and the APRF DNA binding activity was prepared from wild-type hIL-9R transfectants treated with hIL-9 for 5 min in the presence of 100-fold excess of unlabeled competitors or 2 μg of pre-immune IgG or anti-STAT3 antibody. Comp., competitor. C, gel shift assays were performed with nuclear extracts from TS1 cells stimulated by mIL-9 for 5 min (as a positive control) or from different hIL-9Rα transfectants stimulated by hIL-9 for 5 min.

STAT3 binding site (YLPQ) was found within this region. YXXQ has been shown to be important for tyrosine phosphorylation of STAT3 in gp130 family receptors (28), and the important role of the tyrosine residue within YLPQ motif in hIL-9Rα has recently been reported (29). Since the tyrosine residue within this motif was shown to play a key role in IL-9-mediated cell growth and activation of STAT1α, STAT3, and STAT5, STAT factors were suggested to be involved in IL-9-mediated cell proliferation. However, in our study, a point mutation in this STAT3 binding site from YLPQ to YLPA did not significantly affect IL-9-induced cell proliferation but greatly reduced tyrosine phosphorylation and activation of STAT3, suggesting that STAT3 may not be the major signaling molecule in IL-9-induced cell growth in TS1 cells. In the gp130 system, a recent report suggested that STAT3 may be involved in cell differentiation and growth arrest mediated by IL-6 (39).

Finally, we also investigated primary response gene expression in hIL-9R-transfected cells. The importance of primary response genes as potential critical targets for cytokine-induced proliferation has been extensively studied (40–42). Although our previous study has shown that IL-9 could induce activation of junB and c-myc in a human growth factor-dependent cell line (19), the correlation between the activation of these two genes and IL-9-mediated cell proliferation has never been demonstrated. Our present study indicated that activation of c-myc upon stimulation with IL-9 depends on the presence of both the membrane-proximal and the BOX1 downstream regions in the cytoplasmic domain of hIL-9Rα, suggesting that IL-9-induced expression of c-myc may require JAK kinases and signals associated with the BOX1 downstream region. This is different from IL-2Rβ, in which the serine-rich region is important for the activation of c-myc (27). In conjunction with the functional study, c-myc is not likely to play an important role in IL-9-mediated cell proliferation in TS1 cells, since the induction of c-myc by IL-9 was greatly impaired in STAT3m and BOXm transfectants that can proliferate in response to hIL-9. Interestingly, tyrosine phosphorylation and activation of STAT3 by IL-9 were also decreased in both STAT3m and BOXm transfectants, suggesting that STAT3 may be involved in IL-9-mediated activation of c-myc. hIL-9-induced junB activation appears to be attributed to both regions of hIL-9Rα and correlates with IL-9-induced cell proliferation. Although STAT3 was recently shown to be involved in the transcriptional regulation of the junB promoter through an IL-6 response element (43, 44), the induction of junB by IL-9 was not significantly decreased in STAT3m transfectants, implicating that additional transcription factors may be induced by IL-9 in the activation of junB.

In conclusion, two important regions in the cytoplasmic domain of hIL-9Rα have been demonstrated to play important roles in IL-9-induced cell growth and signal transduction. A membrane-proximal region between amino acids 338 and 338 containing the BOX1 motif is absolutely essential for IL-9-induced cell proliferation and tyrosine phosphorylation of JAK kinases and a BOX1 downstream sequence between amino acids 338 and 422 including a YLPQ motif is critical for IL-9-induced cell growth, activation of STAT3, and up-regulation of c-myc and junB gene expression. Since both of these two regions are important for optimal cellular proliferation induced by IL-9, we suggest that the reconstitution of a fully functional
hIL-9R requires the cooperation or cross-talk between different signaling transducers associated with hIL-9R cytoplasmic domain, even though activation of distinctive signaling molecules may be mediated by individual cytoplasmic regions of hIL-9R.

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REFERENCES
1. Renault, J. C., Houssiau, F., Louahed, J., Vink, A., Van Snick, J., and Uyttenhove, C. (1993) Adv. Immunol. 54, 79–97
2. Yang, Y.-C., Ricciardi, S., Ciarettta, A., Calvetti, J., Kelleher, K., and Clark, S. C. (1989) Blood 74, 1880–1884
3. Uyttenhove, C., Simpson, K., and Van Snick, J. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 6943–6948
4. Dugas, B., Renault, J. C., Pene, J., Bonneley, J. Y., Peti-France, C., Braguet, P., Bousquet, J., Van Snick, J., and Mencia-Huerta, J. M. (1993) Eur. J. Immunol. 23, 1687–1692
5. Hultner, L., Moeller, J., Schmitt, E., Jager, G., Reishbach, G., Ring, I. L., and Broxmeyer, H. E. (1990) Blood 75, 746–749
6. Eeklund, K. K., Ghildyal, N., Austen, F. K., and Stevens, R. L. (1993) J. Immunol. 151, 4266–4273
7. Williams, D. E., Anderson, D., Cosman, D., Boswell, H. S., Cooper, S., Grabstein, K. H., and Bronxwexler, H. E. (1990) Blood 79, 906–911
8. Renault, J. C., van der Lugt, N., Vink, A., van Roon, M., Godfraind, C., Warnier, G., Merz, H., Peller, A., Berns, A., and Van Snick, J. (1994) Oncogene 9, 1237–1332
9. Renault, J. C., Vink, A., Louahed, J., and Van Snick, J. (1995) Blood 85, 1303–1305
10. Takeshita, T., Asoo, H., Ohitani, K., Ishii, N., Kumaki, S., Tanaka, N., Munatata, H., Nakamura, M., and Sugamura, K. (1992) Science 257, 379–382
11. Sugamachi, M., Nakamura, Y., Russell, S. M., Ziegler, S. F., Tsang, M., Cao, X., Giri, J. G., Andieh, M., Eisenman, J., Shanebeck, K., Grabstein, K., Kumaki, S., Namen, A., Park, L. S., Cosman, D., and Anderson, D. (1994) EMBO J. 13, 2822–2830
12. Kimura, Y., Takeshita, T., Kondo, M., Ishii, N., Nakamura, M., Van Snick, J., and Sugamura, K. (1995) Int. Immunol. 7, 115–120
13. Renault, J. C., Druez, C., Kermouni, A., Houssiau, F., Uyttenhove, C., Van Roost, E. N., and Van Snick, J. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 5690–5694
14. Chang, M.-S., Engel, G., Benedict, C., Basu, R., and McNinch, J. (1994) Blood 83, 3195–3205
15. Minami, Y., Kono, T., Miyazaki, T., and Taniguchi, T. (1993) Annu. Rev. Immunol. 11, 245–267
16. Yin, T., Yang, L., and Yang, Y.-C. (1995) Blood 85, 3101–3106
17. Yin, T., Keller, S. R., Quelle, F. W., Witthuhn, B. A., Tsang, M. L., Lienhard, G. E., Ihle, J. N., and Yang, Y.-C. (1995) J. Biol. Chem. 270, 20497–20502
18. Kang, L. Y., and Yang, Y.-C. (1995) J. Cell. Physiol. 163, 623–630
19. Vallette, F., Mege, E., Reiss, A., and Adesnik, M. (1989) Nucleic Acids Res. 17, 723–733
20. Yin, T., Yasukawa, K., Taga, T., Kishimoto, T., and Yang Y.-C. (1994) Exp. Hematol. 22, 467–472
21. Yang, L., and Yang, Y.-C. (1995) Blood 86, 2526–2533
22. Komarowsky, P., and Sack, N. (1987) Anal. Biochem. 162, 156–159
23. Dignam, J. D., Lebovitz, R. M., and Roeder, R. G. (1983) Nucleic Acid Res. 11, 1475–1489
24. Akira, S., Nishio, Y., Inoue, M., Wang, X-J., Wei, S., Matsusaka, T., Yoshida, K., Sudo, T., Naruto, M., and Kishimoto, T. (1994) Cell 77, 63–71
25. Herrea, R. E., Shaw, P. E., and Nordheim, A. (1989) Nature 340, 68–70
26. Hatakeyama, M., Mori, H., Dei, T., and Taniguchi, T. (1989) Cell 59, 837–845
27. Stahl, N., Fagguggella, T., Boulton, T., Zions, Z., Darnell, J., and Yancopoulos, G. (1995) Science 267, 1349–1353
28. Demoulin, J.-B., Uyttenhove C., Van Roon, E., and Donckers, D., Van Snick, J., and Renauld, J. C. (1996) Mol. Cell. Biol. 16, 4710–4716
29. Morukami, M., Narazaki, M., Hibi, M., Yawata, H., Yasukawa, H., Hamaguchi, M., Taga, T., and Kishimoto, T. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 11349–11353
30. Narazaki, M., Witthuhn, B. A., Yoshida, K., Silvennoinen, O., Yasukawa, K., Ihle, J. N., Kishimoto, T., and Taga, T. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 2285–2289
31. Miura, O., Cleveland, J. L., and Ihle, J. N. (1993) Mol. Cell. Biol. 13, 1768–1775
32. Dallavina, L., Howard, O. M., Rui, H., Kirken, R. A., and Farrar, W. L. (1994) J. Biol. Chem. 269, 18267–18270
33. Ihle, J. N. (1995) Adv. Immunol. 6, 1–35
34. Kirken, K. A., Rui, H., Malabarba, M. G., Howard, O. M., Kawamura, M., O'Shea, J. J., and Farrar, W. L. (1995) Cytooki 7, 689–700
35. Picchi, M., Asao, H., Tanaka, N., Oka, K., Takeshita, T., Nakamura, M., Van Snick, J., and Sugamura, K. (1996) Eur. J. Immunol. 26, 1322–1327
36. Russell, S. M., Johnstone, J. A., Sugami, M., Kawamura, M., Baas, C. M., Friedmann, M., Berg, M., McVicar, D. W., Witthuhn, B. A., Silvennoinen, O., Golden, A. S., Schmalstieg, F. G., Ihle, J. N., O'Shea, J. J., and Leonard, W. J. (1994) Science 266, 1042–1045
37. Miyazaki, T., Tawahara, A., Fujii, H., Nakagawa, Y., Minami, Y., Liu, Z. J., Oishi, I., Silvennoinen, O., Witthuhn, B. A., Ihle, J. N., and Taniguchi, T. (1994) Science 266, 1045–1047
38. Yamanaka, Y., Nakajima, K., Fukuda, T., Hibi, M., and Hirano, T. (1996) EMBO J. 15, 1557–1565
39. Horie, M., and Bronxwexler, H. E. (1993) J. Biol. Chem. 268, 968–973
40. Hunter, T. (1991) Cell 64, 249–260
41. Lui, J., Clegg, C. H., and Shoayb, M. (1992) Cell Growth Differ. 3, 307–313
42. Kojima, H., Nakajima, K., and Hirano, T. (1996) Oncogene 12, 547–554
43. Coffer, P., Lutticken, C., van Puijenbroek, A., Klop-de Jonge, M., Harn, P., and Krujiver, W. (1995) Oncogene 10, 985–994