Vol. 190, No. 3, 1993 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 15, 1993 Pages 992-1000

THE CHIMERIC RECEPTOR BETWEEN INTERLEUKIN-2 RECEPTOR β CHAIN AND INTERLEUKIN-4 RECEPTOR TRANSDUCES INTERLEUKIN-2 SIGNAL

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Received December 9, 1992

SUMMARY: Transfectants expressing the chimeric receptor which has human interleukin-4 receptor (hIL-4R) in the extracellular domain and human interleukin-2 receptor β chain (hIL-2Rβ) in the cytoplasmic domain showed continuous growth in response to human interleukin-4 (hIL-4), whereas transfectants expressing hIL-4R responded only transiently to hIL-4. The pattern of tyrosine phosphorylation induced by hIL-4 in transfectants expressing the chimeric receptor was very similar to that in transfectants expressing hIL-2Rβ, but different from that in transfectants expressing hIL-4R, indicating that the chimeric receptor transduces the interleukin-2 like growth signal by hIL-4.

Interleukin-2 (IL-2) plays a crucial role in the clonal expansion of T cells and also participates in growth and differentiation in other cell lineages. These biological activities are mediated by the binding of IL-2 to specific, high affinity IL-2 receptor (IL-2R) complex on the cell surface (1-3). The high affinity ($Kd=10^{-11}$ M) IL-2R complex is composed of at least two distinct polypeptides, the IL-2R α chain (IL-2Rα) and the IL-2R β chain (IL-2Rβ), each of which is capable of binding IL-2 with low affinity ($Kd=10^{-8}$ M) and with intermediate affinity ($Kd=10^{-9}$ M), respectively. It has been demonstrated that the coexpression of IL-2Rα and IL-2Rβ on the surface of IL-2R negative

Abbreviations:
IL, interleukin; IL-2Rβ, interleukin-2 receptor β chain; IL-4R, interleukin-4 receptor; MAP kinase, mitogen-activated protein kinase; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide-tetrazolium.

0006-291X/93 $4.00

lymphoid cells confers high affinity ($Kd=10^{-11}$ M) binding on IL-2 (4). IL-2Rα has only 13 amino acid residues in the cytoplasmic domain which are not essential for IL-2 signal transduction (5-7), whereas IL-2Rβ, which has 288 amino acid residues in the cytoplasmic domain, has been demonstrated to transduce the IL-2 signal in stably transfected IL-3 dependent mouse pro-B cell line, Ba/F3 cells, and to have the critical region in the cytoplasmic domain for IL-2 signal transduction (8). It has also been demonstrated that IL-2Rβ transduces IL-2 signal in the absence of IL-2Rα (9). However, IL-2Rβ belongs to the cytokine receptor family and does not have any catalytic motifs in the cytoplasmic domain such as protein tyrosine kinase which are thought to be important for signal transduction (4). These findings suggest that other component(s) with important roles in IL-2 signal transduction may associate with IL-2Rβ via the cytoplasmic domain. However, we can not exclude the possibility that the other component(s) may associate via the extracellular domain. In order to address this issue, we have constructed the chimeric receptor between human IL-2Rβ (hIL-2Rβ) and human interleukin-4 receptor (hIL-4R), and analyzed the quality of the signal transduced by hIL-2Rβ, hIL-4R and the chimeric receptor.

Materials and Methods

Construction of the chimeric receptor and transfection. To construct the chimeric receptor, a unique EcoRV site was generated at the cytoplasmic domain of hIL-2Rβ cDNA and a unique SspI site was generated at the cytoplasmic domain of hIL-4R cDNA by the site-directed mutagenesis. For hIL-2Rβ cDNA, EcoRI-XbaI digested hIL-2Rβ cDNA was inserted into the Bluescript vector. A single-stranded template was isolated and annealed with mutagenic oligonucleotide, and second-strand DNA synthesis was performed as previously described (10). For the hIL-4R cDNA, the EcoRI-XbaI digested hIL-4R cDNA was inserted into the pSELECT vector (Promega, in vitro mutagenesis kit), and the preparation of a single-stranded template, annealing of mutagenic oligonucleotide and second-strand synthesis were carried out according to the manufacturer's manual. The following oligonucleotides were synthesized on an Applied Biosynthesis 380A DNA synthesizer:
EcoRV: 5'-GTTCCTGCAGTTGATATCGATCAGCAA-3'
SspI: 5'-AGCATCACCAATATTAAGAAAGA-3', and used to construct mutant cDNA. The mutant hIL-2Rβ cDNA excised from Bluescript by EcoRI and HindIII was filled in at both ends and ligated into the mammalian expression vector pME18S (K. Maruyama and A.

Miyajima, unpublished), a derivative vector of pCEV4 (11), which was cleaved by XhoI and filled in at both ends. The EcoRI-NotI fragment from pME18ShIL-2Rβ was isolated and inserted into a *neo*-resistant gene carrying vector, pME18S*neo*, which was cleaved with EcoRI and NotI. The chimeric receptor, which has hIL-4R at the extracellular domain and hIL-2Rβ at the cytoplasmic domain, was constructed by isolating the EcoRI-SspI fragment from mutated pSELECThIL-4R and inserting it into the the EcoRI-EcoRV cleaved pME18S*neo* hIL-2Rβ cDNA. The mutant cDNAs were sequenced by the dideoxy sequencing method to confirm the introduced mutation.

Transfection.
Plasmid DNAs were transfected into IL-3 dependent mouse pro-B cell line Ba/F3 cells by the electroporation method, as described proviously (12). Briefly, ten million cells growing exponentially were transfected with 100 μg of cDNA linearized with KpnI. Electroporation was carried out at 960 μF and 400 V using a Gene Pulser (Bio-Rad). Transfectants were inoculated into 96 well plates to obtain clones by limiting dilution, and selected in 1.5 mg/ml G418.

Cell culture and the proliferation assay.
Transfectants were maintained in RPMI 1640 medium supplemented with 10% FCS, recombinant mouse IL-3 derived from silkworm (13), 100 u/ml penicillin, 100 μg/ml streptomycin, and 0.4 mg/ml G418. For the short-term proliferation assay, 1 x 10^4 cells were cultured in 96 well plates with either recombinant human IL-2 (hIL-2) (Genzyme, Boston, MA) or recombinant human IL-4 (hIL-4) (provided by Dr. Paul Trotta, Schering-Plough Corp. Bloomfield, NJ), and after a 2 day culture, proliferation rates were measured with the MTT method (14). For the long-term proliferation assay, 5 x 10^4 cells were cultured in 24 well plates with factors, and cell numbers were counted at indicated times.

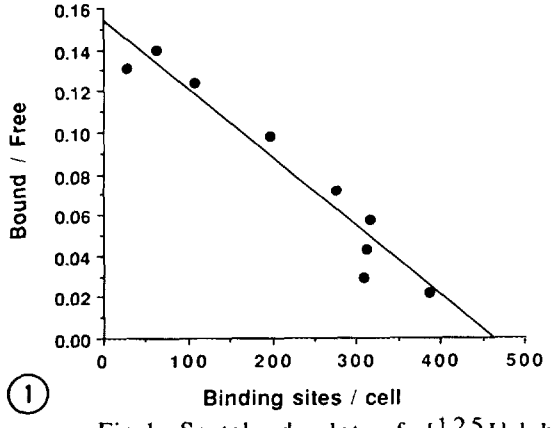
Binding Assays and cross-linking of [^{125}I]-IL-4.
Radiolabeling of recombinant hIL-4, binding assays, and chemical cross-linking were carried out as previously described (15).

Western blotting for tyrosine phosphorylated protein.
Cells (1 x 10^6 cells/ml) were starved in RPMI 1640 containing 0.075% BSA for 6 hr at 37 °C. After starvation, cells were harvested, resuspended in RPMI 1640 containing 0.075% BSA and 50 μM sodium orthovanadate at 4 x 10^6 cells/ml, and further incubated for 30 min at 37 °C. Cells were then stimulated with factors for the indicated time at 37 °C. After stimulation, cells were harvested and lysed on ice for 30 min with 25 μl of 1% Triton X-100 containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 50 mM sodium fluoride, 30 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 0.1% aprotinin and 2 mM PMSF. Cell lysates were then centrifuged and clear

supernatants equivalent to 4 x 10^6 cells were subjected to SDS-PAGE. Proteins were transferred onto nitrocellulose filters (Hybond-ECL, Amersham) and probed with polyclonal rabbit anti-phosphotyrosine antibody (Zymed, CA), and developed using the ECL system (Amersham).

Results and Discussion

Expression on transfectants of the chimeric receptor between hIL-4R and hIL-2Rβ, designated as hIL-42R was examined by ligand binding analysis using [^{125}I]-labeled hIL-4. As shown in Fig. 1, Schatchard analysis revealed that one transfectant expressed approximately 460 high affinity binding sites for hIL-4 with a dissociation constant of 25.2 pM which was consistent with the dissociation constant previously reported (16). Two other transfectants showed approximately 360 binding sites with a



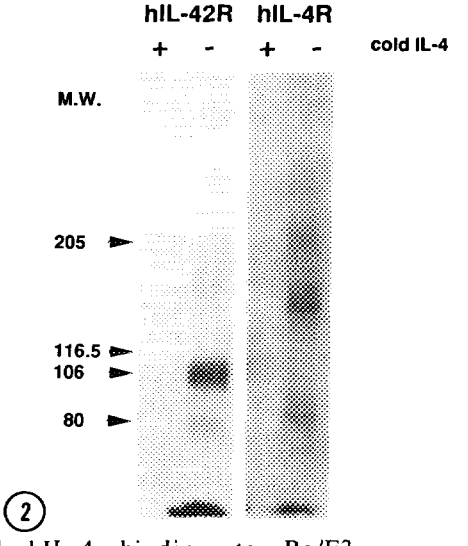


Fig.1. Scatchard plot of [^{125}I]-labeled hIL-4 binding to Ba/F3 transfectant expressing chimeric receptors. Cells were incubated with various concentrations of [^{125}I]-hIL-4 for 3 hr at 4 °C. Bound and free ligand was separated by centrifugation through oil gradient. Nonspecific binding was measured by adding a 150-fold molar excess of nonradiolabeled hIL-4.

Fig. 2. Chemical crosslinking of [^{125}I]-labeled hIL-4 to hIL-42R and hIL-4R. Ba/F3 transfectants expressing hIL-42R or hIL-4R were incubated with 150 pM of [^{125}I]-hIL-4 for 2 hr at 4 °C in the presence or absence of cold hIL-4. Cells were then washed and a crosslinker (BS^3, Pierce) was added. Cell lysates were subjected to SDS-PAGE and autoradiography. The mobilities of size markers are indicated.

dissociation constant of 78 pM and 290 binding sites with a dissociation constant of 48 pM, respectively. The chemical crosslinking experiments demonstrated that the transfectant which expressed hIL-42R expressed the 90 kDa IL-4 binding protein as predicted (Fig. 2), since the molecular weight of the extracellular domain of hIL-4R was calculated approximately as 55 kDa with glycosylation, and the molecular weight of the cytoplasmic domain of hIL-2Rβ was calculated as 35 kDa. These results indicated that the cytoplasmic domain of hIL-2Rβ does not affect the affinity of the extracellular domain of hIL-4R, and the extracellular domain of hIL-4R is sufficient to determine the ligand specificity of hIL-4R.

The proliferative responses of several transfectants expressing hIL-42R were examined by the MTT assay. As shown in Fig. 3. A, all transfectants responded to hIL-4, indicating that hIL-42R is able to transduce growth signal. In order to analyze the difference of growth signal transduced by each receptor, each transfectant was examined in the long-term assay since transfectants expressing hIL-2Rβ or hIL-4R also respond to hIL-2 or hIL-4, respectively, in the MTT assay (data not shown). Both transfectants expressing hIL-2Rβ and hIL-42R grew continuously during the culture period. However,

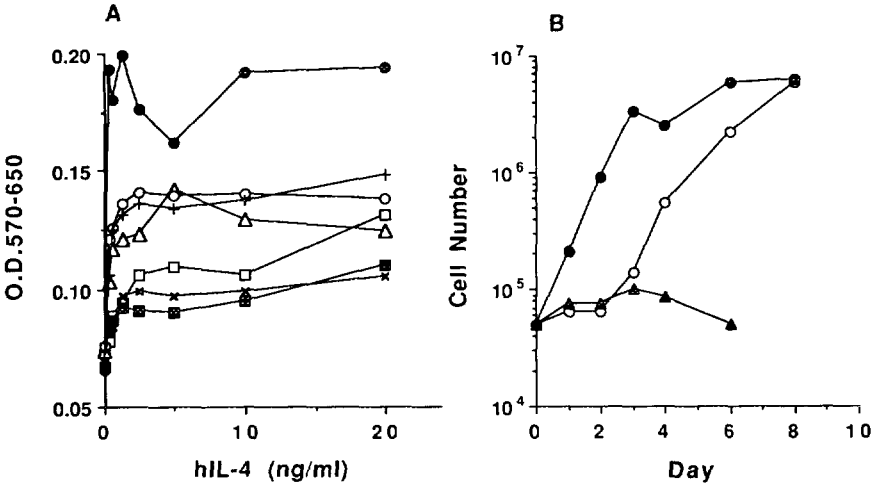


Fig. 3. Proliferative responses of Ba/F3 transfectants to lymphokines. (A) Seven Ba/F3 transfectants expressing hIL-42R were incubated with various concentrations of hIL-4. Proliferative responses of these cells were assayed 2 days after stimulation by MTT. (B) Ba/F3 transfectants expressing hIL-2Rβ (●) were incubated with 10 ng/ml hIL-2. Ba/F3 transfectants expressing hIL-42R (○) and hIL-4 (▲) were incubated with 10 ng/ml human IL-4. Cell numbers were counted at indicated days.

transfectant expressing hIL-4R did not grow continuously (Fig. 3. B). Transfectant expressing hIL-42R showed a lag phase, whereas transfectant expressing hIL-2Rβ grew without a lag phase. This result may suggest that hIL-2Rβ associates with other molecule(s) through the extracellular domain, which may not be essential for growth signal transduction but necessary for maximum response to hIL-2. Nevertheless, this result indicated that hIL-42R transduced a signal similar to hIL-2Rβ.

To further characterize the signal transduced by each receptor, the patterns of tyrosine phosphorylated proteins were analyzed in each transfectant, since it has been shown that IL-2 and IL-4 induce tyrosine phosphorylation of a distinct set of proteins (3, 17, 18). As shown in Fig. 4, hIL-2 induced tyrosine phosphorylation of five proteins (pp160, pp110, pp90, pp70 and pp44) in Ba/F3 cells expressing hIL-2Rβ. We also observed a weak protein tyrosine phosphorylation in pp54 which migrated very close to a dense band around the molecular weight of 53 kd. This pattern of protein tyrosine phosphorylation is very similar to that previously reported (19). In contrast, transfectant expressing hIL-4R showed weak tyrosine phosphorylation of three proteins (pp145, pp98 and pp87) in response to hIL-4. In transfectant expressing hIL-42R, hIL-4 induced the same protein tyrosine phosphorylations as that in transfectants expressing hIL-2Rβ except pp160 (Fig. 4). This result suggested that the pp160 may be a specific tyrosine phosphorylated molecule by the IL-2R system which may require the extracellular domain of IL-2Rβ. Since hIL-4 induced a growth profile in transfectant expressing hIL-42R similar to the one induced by hIL-2 in transfectant expressing hIL-2Rβ without tyrosine phosphorylation in pp160, pp160 does not seem to be essential for growth signal transduction of IL-2. Alternatively, pp160 may be required for maximum growth signal transduction, as discussed above. Collectively, these results indicate that signal transduction induced by hIL-42R is the same as that induced by hIL-2Rβ, suggesting that the cytoplasmic domain of hIL-2Rβ is enough to determine the specificity of transduced signal. In the present study, we cannot exclude the possible involvement of the γ chain of human IL-2R (IL-2Rγ), which has been cloned recently (20). IL-2Rγ also belongs to the cytokine receptor family; it does not have the catalytic domain, but has a partial src-homology 2 (SH2) domain in the cytoplasmic domain, suggesting that some tyrosine phosphorylated proteins may associate with IL-2Rγ through the SH2 domain. However, if IL-2Rγ is involved in growth signal transduction in this system, either IL-2Rγ

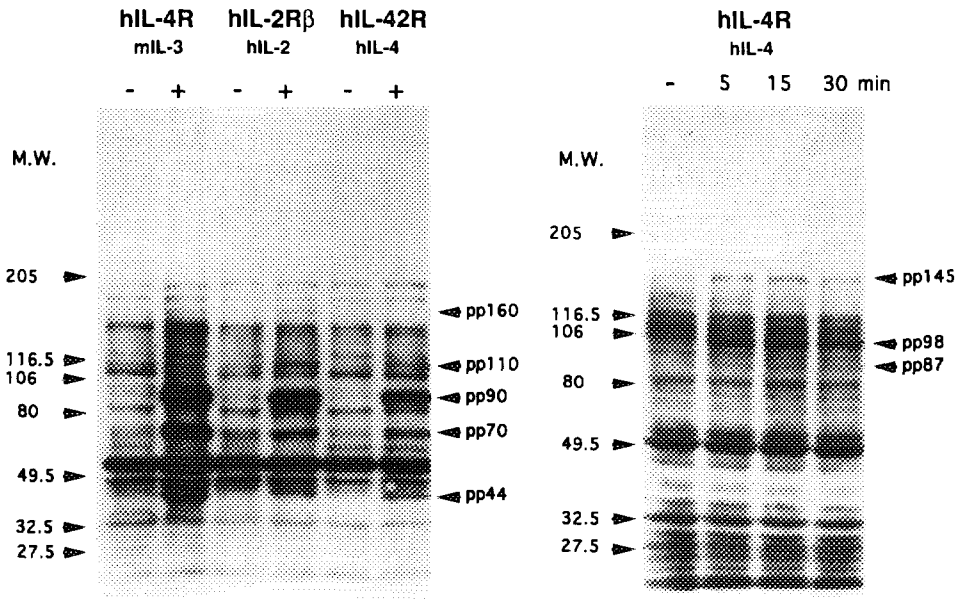


Fig. 4. Protein tyrosine phosphorylation in Ba/F3 transfectants induced by lymphokines. Ba/F3 transfectants expressing hIL-4R, hIL-2Rβ or hIL-42R were stimulated with 10 ng/ml mouse IL-3, hIL-2 or hIL-4, respectively, for 10 min at 37 °C (left panel). Ba/F3 transfectants expressing hIL-4R were stimulated with 10 ng/ml hIL-4 for the indicated time at 37 °C. Cell lysates were subjected to western blotting, and tyrosine phosphorylated proteins were detected as described in Materials and Methods. The mobilities of size markers and tyrosine phosphorylated proteins are indicated.

may associate with IL-2Rβ in the cytoplasmic portion, or may not be necessary for the growth signal transduction of IL-2.

Interestingly, when Ba/F3 cells were stimulated with mouse IL-3, Western blotting for protein tyrosine phosphorylation showed five common proteins, pp160, pp90, pp70, pp54 and pp44, which were observed upon hIL-2 stimulation in transfectant expressing hIL-2Rβ, although the intensity of signals induced by IL-3 was stronger than that induced by IL-2. These observations are consistent with the report that IL-3 and IL-2 may partly share a common signal pathway (21). Indeed, it has been recently reported that src-family tyrosine kinase, lyn, is activated with either IL-2 (22) or IL-3 (23). IL-2 and IL-3 have also been shown to induce rapid phosphorylation and activation of Raf-1 kinase (24-26). The tyrosine phosphorylated protein of 70 kDa is likely to be Raf-1 kinase. It has been demonstrated that IL-3 phosphorylates mitogen-activated protein kinase (MAP kinase) (27, 28). The tyrosine phosphorylated

protein of 44 kDa is likely to be MAP kinase. Although there is no direct evidence that IL-2 phosphorylates MAP kinase, our results suggest that IL-2 may induce tyrosine phosphorylation of MAP kinase. Our present study also indicates that IL-4-induced tyrosine kinase may be different from tyrosine kinases activated by IL-2 or IL-3. Further investigations will be required to identify any tyrosine kinase involved in signal transduction of IL-4.

Acknowledgments

We thank Debra Robison for synthesizing oligonucleotides. We also thank Dr. Dovie Wylie for commenting on earlier drafts of the manuscript, and Drs. Maureen Howard and Jacques Chiller for critical review of the manuscript and encouragement. The DNAX Research Institute of Molecular and Cellular Biology is supported by Schering-Plough Corporation.

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