Molecular cloning of a novel type 1 cytokine receptor similar to the common gamma chain

Keishi Fujio, Tetsuya Nosaka, Tetsuo Kojima, Toshiyuki Kawashima, Takashi Yahata, Neal G. Copeland, Debra J. Gilbert, Nancy A. Jenkins, Kazuhiko Yamamoto, Takashi Nishimura, and Toshio Kitamura

In a complementary DNA (cDNA) screening of murine Th2-skewed lymphocytes with our recently developed signal sequence trap method termed SST-REX, a novel type 1 cytokine receptor, Delta1 (δ1), was identified. Although δ1 is ubiquitously expressed in multiple tissues, the expression level is higher in Th2-skewed lymphocytes than in Th1-skewed ones. The δ1 cDNA encodes a 359-amino acid type 1 membrane protein. The extracellular domain of 206 amino acids showed 24% identity with the murine common γ receptor that is shared among the receptors for interleukin(IL)-2, IL-4, IL-7, IL-9, and IL-15. The membrane-proximal region of δ1 includes a box1 motif, which is important for association with Janus kinases (JAKs), and showed a significant homology with that of the mouse erythropoietin receptor (EPOR). A box2 motif was also found in close proximity to the box1 region. Dimersization of the cytoplasmic region of δ1 alone did not transduce proliferative signals in IL-3-dependent cell lines. However, the membrane-proximal region of δ1 could substitute for that of human EPOR in transmitting proliferative signals and activating JAK2. These results suggest that δ1 is a subunit of cytokine receptor that may be involved in multiple receptor systems and play a regulatory role in the immune system and hematopoiesis. (Blood. 2000; 95:2204-2211)

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Materials and methods

Reagents

Recombinant murine IL-3 (mIL-3) was produced in silkworm. Recombinant human thrombopoietin (hTPO) was obtained from R & D Systems (Minneapolis, MN). Antibodies directed against TYK2, Janus kinase (JAK)1, and JAK2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies raised against JAK3 and the 4G10 antibody, raised against phosphotyrosine, were purchased from UBI (Lake Placid, NY). The anti-FLAG M2 antibody was purchased from Eastman Chemical Company (Kingsport, TN).

Cell lines and cell culture

Ba/F3,24 FDC-P1,25 LG, LGM,26 and 32D27 cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 2 mM/L glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, and 1 ng/mL mIL-3. CTLL-2 cells28 were maintained in RPMI 1640 supplemented with 10% FCS, 2 mM/L glutamine, 100 µg/mL 2-mercaptoethanol, 100 U/mL penicillin, 100 µg/mL streptomycin, and 20 ng/mL mIL-2. NIH3T3 fibroblast cells were cultured in DMEM containing 10% FCS (DMEM/10%FCS) and guanine phosphoribosyl transferase selection reagent (Speciality Media, Lavallette, NJ). The cells were transfected to DMEM/10%FCS without guanine phosphoribosyl transferase selection reagents 2 days before transfection.

Construction of the cDNA library for SST-REX

Induction of antigen-specific activation of Th1/Th2 cells was performed using DO11.10 transgenic mice as described,30 and poly(A)+ RNA samples were prepared from resting or activated Th1 and Th2 cells using FastTrack2.0 Kit (Invitrogen Carlsbad, CA). Complementary DNA (cDNA) was synthesized from poly(A)+ RNA of resting Th1 and Th2 cells with random hexamers using SuperScript Choice System (Gibco-BRL, Rockville, MD) according to the manufacturer’s instructions. The synthesized cDNA was size-separated through SizeSep 400 Spun Column (Pharmacia, Uppsala, Sweden) and inserted into BstXI sites of pMX-SST using BstXI adaptors (Invitrogen). The ligated DNA was electroporated into DH10B cells (Electromax, Gibco-BRL) using an E-coli Pulser (Bio-Rad, Hercules, CA) at 1.8 kV. Plasmid DNA was prepared using JETstar (Genomed, Research Triangle Park, NC) from 200-ml Escherichia coli cultures. Infection of Ba/F3 cells with high-titer retroviruses derived from the library for SST and, also, isolation of cDNA fragments by PCR from factor-independent clones were performed as described.20

Construction of a cDNA library from resting Th2 cells

Complementary DNA was synthesized from poly(A)+ RNA from resting Th2 cells with oligo-dT primers, using SuperScript Choice System, as described above, and inserted into BstXI sites of pME18S,31 using BstXI adaptors. The ligated DNA was electroporated, and plasmid DNA was prepared as described above.

Cloning of δ1 cDNAs

A biotinylated probe of 407 base pairs (bp) was made by PCR from the δ1 cDNA with oligoprimers 5'GGTGTTGCTGACATGCATGCATG3' and 5' TCAGCAGGGTCTCCTCAGTCTT3' using 50-µM Biotin-21-dUTP (Clontech Laboratories, Palo Alto, CA), 200-µM dATP, 200-µM dGTP, 200-µM dCTP, and 10-µM dTTP. An oligo-dT–primed cDNA library of resting Th2 cells was screened using RecA protein coated with biotinylated linear DNA probes as described.32-34

Northern blot analysis

A Mouse Multiple Tissue Northern Blot (Clontech) was probed with the 407-bp δ1-specific PCR fragments generated with the same set of primers as described above. The blots were hybridized overnight in 50% formamide, 5 x SSC, 1% sodium dodecyl sulfate (SDS), 6 x Denhardt’s reagent, and 100 µg/mL of salmon sperm DNA at 42°C with a 32P-labeled randomly primed probe and washed at 55°C in 0.1 x SSC and 0.1% SDS, followed by exposure to radiographic film. Poly(A)+ RNA samples (3 µg) prepared from various mouse cell lines using FastTrack2.0 Kit were separated by electrophoresis through a 1% agarose formaldehyde gel and transferred to Hybond-N+ (Amersham, Arlington Heights, IL) nylon filter. The Northern blot was hybridized and washed as described above.

Construction and expression of chimeric receptors consisting of the human receptor for EPO or TPO and δ1

For expression of the wild type hEPOR (hEPO receptor), a full-length hEPOR35 was cloned into the EcoRI and NotI sites of the pmx-puro retrovirus vector.36 For construction of chimeric receptors hEPOR-δ1R and hMPL-δ1R, an EcoRI site was inserted between the extracellular and transmembrane domains of each cDNA. Then, the cytoplasmic domain of δ1 cDNA generated by PCR with high-fidelity DNA polymerase Pyrobest (Takara Otsu, Shiga, Japan) was cloned into pmx-puro at 5’-EcoRI and 3’-NotI sites, and the EcoRI fragment containing the extracellular domain of hEPOR or hMPL was cloned into an EcoRI site. The primers used to create EcoRI and NotI sites in the δ1 cDNA were 5’-AAAGATATCCCGCCCTCCTGCCTGGG-3’ and 5’-GGTAGGGAA TTCCGGAA TTTCCTCGA-3’. The primers used to create EcoRI sites in hEPOR cDNA were 5’-AAAGAATTCCGGGCTGTTATCATGGAC-3’ and 5’-AAAGAATTCG GGGTCAGTGCTGCTAGG-3’. The primers used to create EcoRI sites in hMPL cDNA were 5’-GGTGGAGGATCCCGAATTCCTCGA GTGGG-3’ and 5’-AAAGAATTCGAGAGTCGTGGCTGGTGGG-3’. To construct a chimeric receptor EDER, a 57-amic acid stretch covering the transmembrane domain and the box1 region of hEPOR was replaced with the corresponding region of δ1; first, an HpaI site was inserted between the extracellular and transmembrane domains, and an XhoI site was inserted downstream of the conserved tryptophan residue in the cytoplasmic domain of hEPOR. Second, the box1 region of δ1 cDNA was cloned into the EcoRI and NotI sites of the pmx-puro vector. Then, the cytoplasmic region of hEPOR was cloned into the XhoI and NotI sites, and the extracellular domain of hEPOR was cloned into the EcoRI and HpaI sites. The primers used to create 5’-EcoRI and HpaI sites and 3’-XhoI and NotI sites at the end of the δ1 cDNA spanning the transmembrane and the membrane-proximal region were 5’-AAAAAAATCTGTTACCCGGCCCTGGG-3’ and 5’-AAAGCGGCCGCCTCGAAGTCGTGGG-3’. The primers used to create 5’-XhoI and 3’-NotI sites in the cytoplasmic region of hEPOR were 5’-AAATCCTGAGCTTACCCAGAAATGGGTC-3’ and 5’-AAAGCGGCCGCCTCGAAGTCGTGGG-3’. The primers used to create 5’-EcoRI and 3’-HpaI sites in the extracellular region of hEPOR were 5’-AAAGAATTCG GGGGTCGTATCAGAGGCCTGCTGCTAGG-3’ and 5’-AAAGTTAAGGGGGCTCAGTGGCTGCTCAGG-3’. All constructs were confirmed by restriction enzyme analyses and sequencing.

The resulting expression plasmids were introduced into FDC-P1 via retrovirus infection as described.35 Infected cell lines were selected in the presence of 1 µg/mL of puromycin (Sigma, St. Louis, MO) to obtain a stable transfectant.

Construction and expression of a tagged molecule of δ1

To construct the tagged δ1, δ1-FLAG was designed by fusing the FLAG-sequence (DYKDDDK) to the C-terminus of δ1. A PCR fragment of the δ1-FLAG was cloned into pmx-puro retrovirus vector at 5’-EcoRI and 3’-NotI sites. The primers used to add the FLAG sequence to the C-terminus of δ1 was 5’-GGAGAATTCGAGAGTCGTGGCTGGTGGG-3’ and 5’-AAAGGCGGCCGTACACTGTTGCTCAGGTCTGGTGTAAATCC- AGGGTCATGAGGCCCTGCTG-3’. Immunoprecipitation and Western blotting

Cells were lysed in the lysis buffer (50 mM Tris-HCl, pH 7.5; 150 mM NaCl, 1% Triton X-100; 1 mM EDTA; 0.2 mM Na2VO4; and 2 mM PMSF). For surface labeling of the cells, NHS-LC-Biotin (Pierce, Rockford, IL)
was added prior to cell lysis. Lysates were cleared of debris by centrifugation at 12,000 g for 40 minutes, and the supernatants were incubated with antibodies at 4°C for 2 hours. Immune complexes were precipitated with protein A-Sepharose (Pharmacia), washed with the lysis buffer, and proteins were eluted with the sample buffer (62.5 mM Tris-HCl, pH 6.8; 2% SDS; 10% glycerol; 5% 2-mercaptoethanol; and 0.02% bromophenol blue) for SDS-PAGE (SDS-polyacrylamide gel electrophoresis). The eluted proteins were electrophoresed on an 8%–to-16% gradient gel (GradiGene, North Ryde, Australia). Membranes were probed with antibodies or streptavidin-horseradish peroxidase (Vector Laboratories, Burlingame, CA) and visualized using the enhanced chemiluminescence detection system (Amersham) as described by the manufacturer.

Chromosomal localization

Interspecific backcross progenies were generated by mating (C57BL/6J × Mus spretus) F1 females and C57BL/6J males. A total of 205 N2 mice were used to map the 61 locus. DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, Southern blot transfer, and hybridization were performed essentially as described. All blots were prepared with Hybrid-N+ nylon membrane (Amersham). The probe, a 407-bp fragment of mouse cDNA, was labeled with [α-32P]dCTP using a random primed labeling kit (Stratagene, La Jolla, CA); washing was done to a final stringency of 1.0 × standard saline citrate (SSC), 0.1% SDS, 65°C. C57BL/6J and M. spretus DNAs were digested with several enzymes and analyzed by Southern blot hybridization for informative restriction fragment length polymorphisms (RFLPs) using a mouse cDNA probe. An Scal fragment of 8.7 kilobases (kb) or 11.0 kb was detected in C57BL/6J DNA or M. spretus DNA, respectively. The presence or absence of 11.0-kb Scal M. spretus–specific fragment was followed in backcross mice.

A description of the probes and RFLPs for the loci linked to δ1, including Fig5, Dmp1, Gfi1, and Crybb2 has been reported elsewhere. Recombination distances were calculated using Map Manager, version 2.6.5. Gene order was determined by minimizing the number of recombination events required to explain the allele distribution patterns.

Results

Isolation and sequencing of the δ1 cDNA clone encoding a novel cytokine receptor

In the screening using the SST-REX system, 20 Ba/F3 clones, which are transduced with cDNAs containing signal sequences, showed factor-independent growth through surface expression of a constitutively active MPL. As a fusion protein. Among cDNA clones that we obtained from either a Th1 or a Th2 cell library using SST-REX, 1 of the clones, F215, showed a significant homology with the murine common receptor (γc) at the amino acid level. A full-length cDNA was obtained from an oligo-dT–primed cDNA library derived from resting Th2 cells using the RecA screening system and was confirmed to contain a sequence that was identical to F215 (Figure 1A).

This cDNA contains an open reading frame of 1077 nucleotides, which encoded a putative protein of 359 amino acids (Figure 1B). The predicted amino acid sequence included a potential hydrophobic signal peptide, followed by a classical cytokine receptor module containing 2 of the 4 conserved cysteine residues, fibronectin type III–like domains, a WS boxlike sequence PSEWT, a module containing 2 of the 4 conserved cysteine residues, fibronec-tin type III–like domains, and a 106–amino acid cytoplasic domain. Examination of the cytoplasmic domain of δ1 identified a membrane-proximal box1 motif (Apolar-X-X-A1phatic-Pro-X-Pro) (residues 262-269), which is known to be important for association with JAKs.

δ1 showed a moderate homology at the extracellular domain with the mouse γc (24% identical, 36% similar; Figure 2A). The membrane-proximal region of δ1, including the box1 region, showed a significant homology with those of the mouse EPOR and other receptor subunits, including the common β chain (β2) (Figure 2B), and harbored the conserved tryptophan W287, which localized downstream of the box1 and is required for JAK2 association. The 12–amino acid sequence from A311 to L322 had a considerable similarity with the box2 region, which is also important for signal transduction in many cytokine receptors (Figure 2B).

A truncated form of δ1 was also cloned from an oligo-dT–primed Th2 cDNA library (Figure 1). The short form lacked membrane-proximal and transmembrane domains, and potentially encoded a soluble form of δ1.
Expression of $d_1$ was examined by Northern blots for several mouse cell lines and Th1- and Th2-skewed cells (Figure 3A). A single transcript of 1.4 kb was identified in all myeloid cell lines examined, CTLL2, and resting and activated murine Th1 and Th2 cells but not in 2 fibroblast cell lines, including NIH3T3 cells. Th2-skewed cells showed a much higher expression than Th1-skewed cells. Slight up-regulation of the expression by T-cell activation was observed in both T-cell cultures.

In Northern blot analysis of multiple tissues, a single transcript of 1.4 kb was identified in heart, brain, spleen, lung, kidney, and testis but not in skeletal muscle (Figure 3B).

**Functional analysis of $d_1$**

First, we overexpressed $d_1$ in Ba/F3 cells that proliferate or survive in response to IL-3 or IL-4, respectively. However, the overexpression of $d_1$ did not enhance Ba/F3 response to these cytokines. Moreover, these cytokines did not induce tyrosine phosphorylation of $d_1$-FLAG in Ba/F3 cells overexpressing $d_1$, suggesting that $d_1$ was not involved in IL-3 and IL-4 signaling (data not shown). We next examined the function of the cytoplasmic domain of $d_1$; chimeric receptors between $d_1$ and hEPOR or hMPL were generated to characterize the effect of homodimerization of the cytoplasmic domain of $d_1$. EDER chimera was made by substitution of the membrane-proximal region of hEPOR with that of the $d_1$ (Figure 4A) to evaluate function of the membrane-proximal region of $d_1$, including box1, as described in “Materials and methods.” With regard to mitogenic properties, FDC-P1 cells expressing the EDER proliferated efficiently in response to hEPO (Figure 4B), while FDC-P1 cells expressing a chimeric receptor hEPO-$d_1$R or hMPL-$d_1$R did not transmit proliferative signals in response to EPO or TPO, respectively.

**Phosphorylation of Janus kinases by the EDE chimeric receptor**

The mitogenic activity of the EDER suggested that JAK was activated by hEPO-induced dimerization. Indeed, Western blot analysis detected hEPO-dependent JAK2 phosphorylation in cells expressing the EDER (Figure 5). JAK1, JAK3, and TYK2 were not phosphorylated in response to hEPO stimulation in the cells expressing the EDER. None of the 4 JAKs were phosphorylated in response to EPO or TPO stimulation in cells expressing a chimeric receptor hEPO-$d_1$R or hMPL-$d_1$R, respectively (data not shown).

**Expression of $d_1$ protein**

A retrovirus construct, pMX-puro $d_1$-FLAG, was designed to express a tagged molecule of $d_1$ and was introduced into Ba/F3 cells via retrovirus infection. The cell lysate was immunoprecipitated by the anti-FLAG antibody (Figure 6). The expressed $d_1$ protein had a molecular mass of 50 kd, which is larger than the...
calculated molecular mass of 37 791 d. The difference is probably due to attachment of sugar moieties to some of the 2 predicted potential N-linked glycosylation sites found in the extracellular domain of δ1.

Chromosomal mapping of the δ1 gene

The mouse chromosomal location of δ1 was determined by interspecific backcross analysis using progeny derived from matings of (C57BL/6J x M. spretus) F1 x C57BL/6J mice. This interspecific backcross mapping panel has been typed for more than 2900 loci that are well distributed among all the autosomes as well as the X chromosome. The 11.0-kb Sca1 M. spretus RFLP

Figure 4. Structure of chimeric receptors and factor responsiveness of FDC-P1 transfectants. (A) Shown schematically are the wild type δ1 and EPOR together with the chimeras hEPO-δ1R, hEDER, and hMPL-δ1R. Fused domains are designated by restriction sites generated within cognate cDNAs. (B) Time course of growth of the FDC-P1 cells retrovirally transduced with various receptor constructs. Parental FDC-P1 and FDC-P1 cells expressing hEPOR, hEPOR-δ1R, EDER, and hMPL-δ1R were cultivated in the presence of 1 ng/mL of mIL-3, 2 U/mL of hEPO, 2 U/mL of hEPO, 2 U/mL of hEPO, and 100 ng/mL of hTPO, respectively, and the cell number was counted at the indicated times.

Figure 5. Activation of JAK2 kinase through the ectopically expressed chimeric EDE receptor in FDC-P1 cells. The ability of the chimeric receptor EDER to mediate hEPO-dependent activation of JAK-kinases was assayed by immunoprecipitation with antibody to JAK2 and Western blotting with antibody to phosphotyrosine (monoclonal antibody 4G10). Reprobing was carried out using the anti-JAK2 antibody after stripping. Similar analyses were also performed using anti-JAK1, anti-JAK3, and anti-TYK2 antibodies.
Discussion

We isolated and characterized a novel member of the type 1 cytokine receptor family, δ1, from a cDNA library of the murine Th2-skewed cells using SST-REX; δ1 has a WS box-like sequence, PSEWT, and the box1 and box2 motifs in its membrane-proximal region. 44

When compared to other members of the type 1 cytokine receptor family, δ1 showed the closest similarity to the murine γc in the extracellular domain, with a 24% identity at the amino acid level. Its overall structure is also similar to that of γc, not only in total amino acid length (δ1 359 vs γc 369) but also in length of the extracellular domain (δ1 206 vs γc 232) and the cytoplasmic domain (δ1 106 vs γc 86). Moreover, the expression of δ1 is ubiquitous in hematopoietic cells, as is the case for γc. Although the WS box of δ1 is diversified from the typical sequence of WSXWS, the presence of box1 and box2 domains and the similarity to the γc indicated that δ1 constitutes a member of the type 1 cytokine receptor family. Because a membrane-proximal 50-amino acid segment of the cytoplasmic domain includes both box1 and box2 regions that are essential for supporting signaling and cell proliferation,45-49 it is likely that δ1 is involved in signal transduction.

We were not able to obtain a human homologue of δ1 in cross-hybridization screening using the mouse δ1 as a probe under low stringent conditions; nor did we find any human ESTs homologous to δ1 (unpublished results), suggesting that the human counterpart of δ1 has a low homology with that of the mouse or that there is not a corresponding gene in the human. The central region of mouse chromosome 5, where we identified δ1, shares regions of homology with human chromosomes 4q, 1p, and 22q (summarized in Figure 7), suggesting that the human homologue of δ1, if any, will map to 1 of these chromosomal regions.

In the functional analysis of δ1, hEPOR-δ1R or hMPL-δ1R, the 2 chimeric receptors in which the extracellular domain of δ1 was replaced by the human EPO or TPO receptors, did not transduce proliferative signals in response to hEPO or hTPO, respectively. These results suggested that homodimerization of δ1 could not transduce proliferating signals, while other cytokine receptors, including glycoprotein130, βc, and IL-2Rβ, containing both the box1 and box2 regions were able to transduce proliferating signals upon homodimerization.50-52 On the other hand, the EDER chimera, in which the box1 region of the human EPOR was replaced with that of δ1, induced phosphorylation of JAK2 and cell proliferation in response to human EPO, indicating that the membrane-proximal region of δ1 can substitute for that of the EPOR. It is known that EPO stimulation induces tyrosine phosphorylation of JAK2c53 and that the box1 motif of EPOR is required for association with JAK2.46 In addition, the membrane-proximal region of the EPOR specifies JAK2 activation within a heterologous IL-2 receptor complex.54 The present data indicate that the box1 motif of δ1 is capable of activating JAK2 in heterologous receptor complexes. We speculate that δ1 may be a shared receptor subunit like γc and is involved in signal transduction through JAK2 activation. Whether δ1 has its own ligand remains to be determined.

Although the expression of δ1 is ubiquitous in the immune and the hematopoietic cells, it is clearly up-regulated in Th2-skewed cells, when compared to Th1-skewed cells, suggesting that up-regulation of δ1 expression has something to do with differentiation into Th2 cells. In addition, activation of these cells by antibody

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![Figure 7](image-url)
stimulation significantly enhanced the expression of δ both in Th1- and Th2-skewed cells, implicating δ₁ in the regulation of the immune system. These data lead us to examine whether δ₁ did not alter cellular response to IL-4, and tyrosine phosphorylation of δ₁-FLAG was not induced in response to IL-4 (data not shown). These results suggested that δ₁ was not involved in the IL-4 receptor system, but potential involvement of δ₁ in IL-4 signaling cannot be ruled out at present.

Thus, it is suggested that a novel cytokine receptor δ₁, which is similar to γc in its structure and ubiquitously expressed, is a receptor subunit that plays some roles in signal transduction of the immune system and hematopoiesis.

Acknowledgments

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References


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Following publication of the article “A primitive hematopoietic cell is the target for the leukemic transformation in human Philadelphia-positive acute lymphoblastic leukemia” in the February 1, 2000, issue of Blood, by Cobaleda, Gutiérrez-Cianca, Pérez-Losada, Flores, García-Sanz, González, and Sánchez-García (Blood. 2000;95:1007-1013), large portions of the text were found to be plagiarized from an article previously published by Bonnet and Dick, “Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell” (Nature Medicine. 1997;3:730-737). The essential research and the findings of the article by Cobaleda et al are original and worthy of publication; however, the improper use of text from the article in Nature Medicine is a direct violation of copyright law and betrays the assumed professional conduct of all authors submitting manuscripts to Blood.
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