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Molecular Cloning and Characterization of CRLM-2, a Novel Type I Cytokine Receptor Preferentially Expressed in Hematopoietic Cells[1]

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A murine expressed sequence tag (EST) showing homology with erythropoietin receptor (EPOR) was identified in the EST database. Cloning of the full-length cDNA revealed a 359 amino acid novel type I cytokine receptor, designated cytokine receptor like molecule-2 (CRLM-2). While CRLM-2 lacks typical WS*X*WS motif, it has a significant homology with EPOR, IL-2 receptor β and γ, and IL-9 receptor α. The murine *CRLM-2* gene is composed of 8 exons, and an alternative mRNA splicing generates a variant transcript encoding a soluble CRLM-2. CRLM-2 is preferentially expressed in hematopoietic cells, particularly in hematopoietic progenitors and myeloid cells. Furthermore, CRLM-2 is constitutively associated with JAK2, a well-known tyrosine kinase that transmits signals from cytokine receptors. These data strongly suggest that CRLM-2 is a novel cytokine receptor involved in the regulation of hematopoietic system. © 2000 Academic Press

Homeostasis of hematopoietic system is maintained by continuous proliferation and differentiation of progenitor cells generated from the pluripotent hematopoietic stem cells. These progenitors undergo rapid expansion and maturation under the influence of various cytokines (1, 2). Cytokines play vital roles in immune and hematopoietic systems by way of signaling via cytokine receptors (3, 4). Cytokine receptors compose a growing superfamily that is divided into four subfamilies based on characteristic structure in their extracellular domains (4, 5). Among those, type I cytokine receptors mediate the signals of many cytokines including those that regulate proliferation and differentiation of hematopoietic cells. Members of this family share several structural features such as codistributed cysteine residues, WS*X*WS motif, and fibronectin type III-like domains in the extracellular domain. In the cytoplasmic domain, they have membrane-proximal Box 1 motif (Apolar-X-X-X-Aliphatic-Pro-X-Pro) responsible for binding and activation of JAK family members (4–6). Most of the class I cytokine receptors form heterodimers or higher-order oligomers made up of 2 or more different subunits (e.g., IL-2 receptor and IL-6 receptor), while receptors for erythropoietin (EPO) and growth hormone are homodimers of a single subunit (4, 5).

In a search of ESTs that show homology with known type I cytokine receptors, we identified a new cytokine receptor-like molecule, CRLM-2. Characterization of full-length cDNA and genomic structure of the *CRLM-2* gene revealed a novel type I cytokine receptor preferentially expressed in hematopoietic cells. Our data provide a new regulatory signaling pathway for hematopoiesis as well as for immune functions.

MATERIALS AND METHODS

Cloning of CRLM-2 cDNA and genomic DNA. Bidirectional RACE was performed using a total mouse embryo cDNA library (Clontech, Palo Alto, CA) using primers specific for EST AA018020 and phage vector. Full-length cDNA was amplified using new primers directed to the 5′ and 3′ ends of RACE products. The genomic DNA fragment was amplified by PCR from the embryonic stem cell (TT2) genomic DNA using specific primers (nucleotides 1 to 19; 5′-GGC GAC ATG GCA TGG GCA C-3′ and nucleotides 1166 to 1186; 5′-GCC TGT CTG TAT GCA AAT GTG-3′). The exon–intron bound-

The nucleotide sequence of mouse CRLM-2 has been deposited in the GenBank database under the Accession No. AB039945.

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aries were determined by sequencing and sizes of introns were assessed by PCR.

Northern blotting and RT-PCR. Northern blot analysis was performed as previously described (7). Total RNA was isolated by guanidium isothiocyanate extraction followed by CsCl gradient purification. RNA samples (15 μg/lane) were resolved by agarose formaldehyde gel electrophoresis and transferred to Biotrans nylon membranes (ICN, Costa Mesa, CA). [α-^{32}P]dCTP-labeled full-length CRLM-2 cDNA was used as a probe. RT-PCR was performed as previously described using purified hematopoietic cells by cell sorting (8–10). The cDNAs representing about 2500 cells were amplified for 40 cycles (30 s at 94°C, 30 s at 60°C, and 30 s at 72°C) using CRLM-2-specific primers (nucleotides 440 to 472; 5′-TGG ACT ACG AGG TGC AGC ACC-3′ and nucleotides 851 to 870; 5′-GGA ACT TCC AGG CCT GGA TT-3′). PCR products were gel electrophoresed and visualized by ethidium bromide staining.

Stable transfectants and proliferation assay. The full-length and soluble CRLM-2 cDNAs were tagged on the C-terminus with a FLAG and 6× His, respectively. The leader sequence of the mouse EPOR was replaced by that of preprotrypsin followed by a FLAG epitope. Chimeric receptor genes were constructed by replacing the transmembrane and cytoplasmic domains of EPOR with those of CRLM-2, mouse βc (AIC2A), or mouse IL-3Rα (11). All constructs were subcloned into an expression vector pCAGGS and introduced into a mouse IL-3-dependent cell line, Ba/F3 (12) by electroporation. Cells were stained with anti-FLAG antibody (M2) (Sigma, St. Louis, MO), and clone-sorted by FACS Vantage (Becton–Dickinson, San Jose, CA). Cell proliferation was assayed based on the rates of stimulated reduction of the tetrazolium compound WST-1 to formazan (Takara, Ohtsu, Japan). Stable Ba/F3 lines, deprived of IL-3 for 12 h, were plated onto 96-well plates at 1×10^4 cells/well. Cells were incubated in the presence of human EPO or mouse IL-3 (50 ng/ml) for 60 h, then further incubated for 4 h in the presence of WST-1. WST-1 reduction was assessed by absorbance at wavelength 450 nm using a multiwell-ELISA reader.

Cell surface biotinylation. 293T cells transiently transfected with FLAG-CRLM-2 cDNA were biotinylated on the cell surface using ECL protein biotinylation system (Amersham, Buckinghamshire, UK). Briefly, transfected cells in PBS were labeled with biotinylation reagent on ice for 30 min. The reaction was stopped by removing the biotin solution, washing two times with PBS and incubating 10 min with Tris-buffered saline (20 mM Tris–HCl, pH 7.6, 150 mM NaCl). Cells were solubilized with lysis buffer and protein in cell lysates was immunoprecipitated with anti-FLAG antibody, resolved by SDS–PAGE and immunoblotted with streptavidin-HRP. Proteins were visualized by the ECL system.

Immunoprecipitation and Western blotting. Lysate from 3×10^7 Ba/F3 cells stably transfected with CRLM-2 was immunoprecipitated with anti-FLAG antibody (M2). Immune complexes were collected on protein G–Sepharose, resolved by SDS–PAGE, then transferred to a PVDF membrane (Millipore, Bedford, MA). The membrane was probed with anti-JAK2 polyclonal antibody (UBI, Lake Placid, NY). Proteins were visualized by the ECL system (Amersham).

RESULTS AND DISCUSSION

cDNA Cloning of CRLM-2

In a search of ESTs that show homology with known type I cytokine receptors, we identified several ESTs encoding cytokine receptor-like molecules (CRLM) in the database EST. Among them, EST AA018020 was identified by using EPOR as a bait of homology search. Further database search identified 13 additional ESTs that represent the same gene as EST AA018020. The composite of these ESTs revealed to encode a novel type I cytokine receptor. We designated this receptor CRLM-2, and isolated a full-length cDNA from total mouse embryo cDNA library. The CRLM-2 cDNA encodes 359 amino acid type I transmembrane protein with signal peptide and single transmembrane domain (Fig. 1A). The primary structure of CRLM-2 contains several motifs characteristic of type I cytokine receptors, including conserved cysteine residues and membrane-proximal Box 1 motif, which is necessary for binding and activation of JAKs (4–6, 13). However, typical WS*X*WS and Box 2 motifs are missing. On the C-terminal tail, there is one tyrosine residue that is potentially important for the binding and activation of STATs (13). Alignment of deduced amino acid sequence with other members of type I cytokine receptors showed that CRLM-2 is most homologous to IL-2Rβ, IL-2Rγ, EPOR, and IL-9Rα (Fig. 1B). CRLM-2 was expressed as a 50 kDa protein on the cell surface (Fig. 3, lane 2), which is larger than the predicted molecular mass of 35 kDa probably due to the posttranslational modification by N-glycosylation.

Genomic Structure and Generation of Soluble CRLM-2 by Alternative Splicing

In the process of cDNA cloning, we identified a variant transcript encoding soluble CRLM-2. To understand the mechanism that generates variant transcripts, we isolated 4.5 kb DNA fragment containing the *CRLM-2* gene. The overall structure of the mouse *CRLM-2* gene was determined by sequencing all exon–intron boundaries and the entire coding region. Comparison of the CRLM-2 genomic sequence with that of the cDNA revealed that the gene is composed of 8 exons (Figs. 2A and 2B). All the sequences of exon–intron junctions exhibit consensus sequences of eukaryotic splice junctions (14) (Fig. 2B). The first exon encodes 5′-untranslated region, signal peptide, and N-terminal amino acids. The next four exons (exons 2 to 5) encode the extracellular region. Exons 2 and 3 contain cysteine residues conserved among cytokine receptor superfamily. The transmembrane domain is encoded by exon 6. Exon 7 encodes cytoplasmic domain including "BOX 1" domain that is necessary for signal transduction. Exon 8 contains C-terminal amino acids and 3′-untranslated region, including a polyadenylation signal. The gene structure of CRLM-2 shows a high similarity in genomic structure to the previously reported class I cytokine receptor genes, particularly to IL-2Rγ (15) and EPOR (16). The characteristic motifs of cytokine receptor family are similarly organized on the genome, including conserved cysteines and Box 1, indicating the co-evolution of the *CRLM-2* gene with other cytokine receptor genes (6). The variant transcript revealed to arise from alternative mRNA splicing of exon 6 encoding the transmembrane domain,

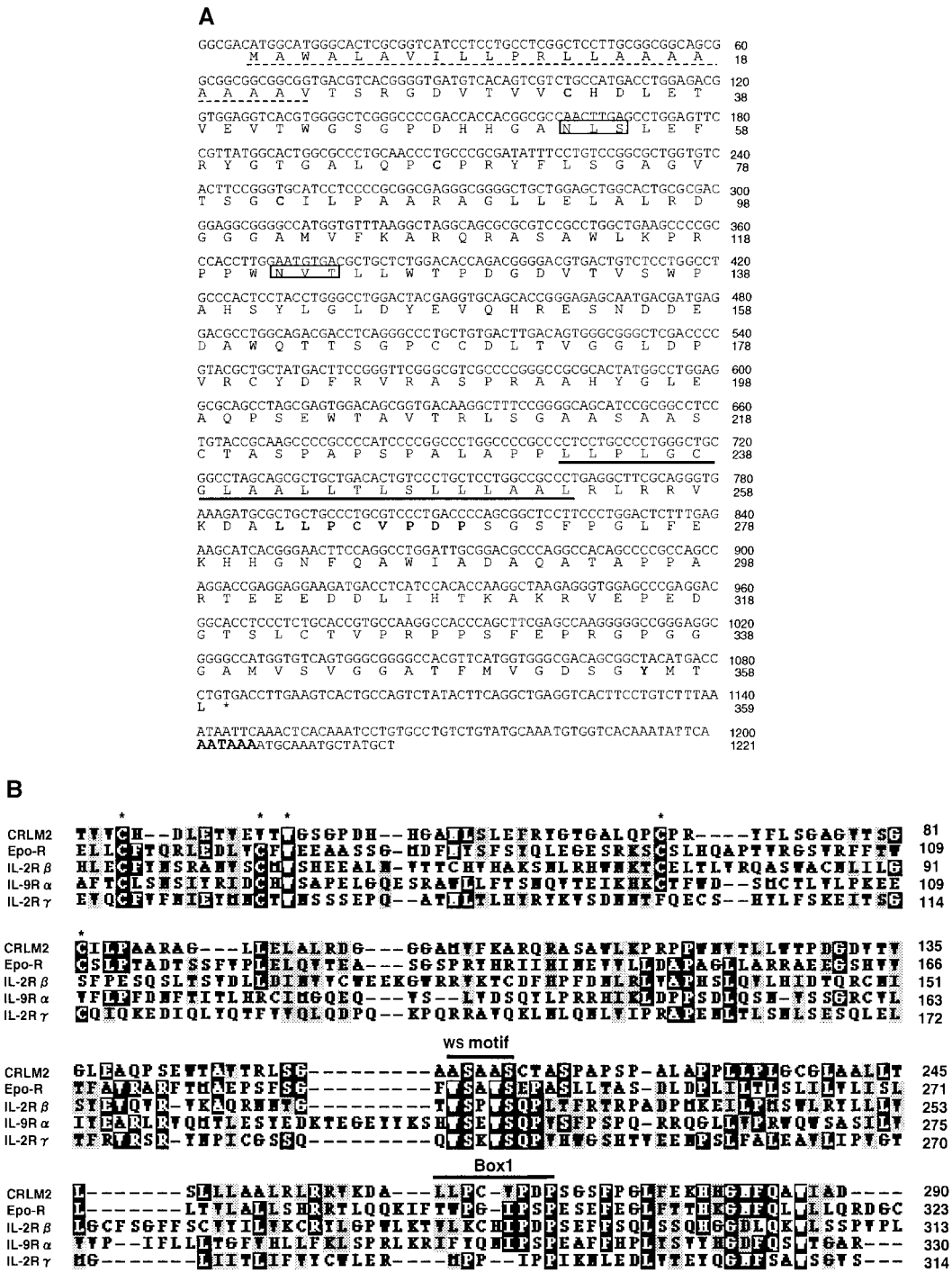

FIG. 1. (A) The nucleotide and deduced amino acid sequences of mouse CRLM-2. The predicted signal sequence and the transmembrane domain are indicated by a dotted line and a thick line, respectively. Potential N-glycosylation sites (N-X-S/T) are boxed. Cysteine residues and Box 1 motif conserved among type I cytokine receptors, a tyrosine residue in the cytoplasmic domain, and a polyadenylation signal are displayed in bold letters. (B) Alignment of the deduced amino acid sequences of CRLM-2 with those of known type I cytokine receptors. Only the parts that show highest homology are shown. Gaps were introduced to maximize the homology. Conserved amino acids are boxed. Conserved cysteine residues are marked with asterisks. The WS*X*WS and Box 1 motifs are indicated by overhead lines.

which results in the frame shift and early termination of translation (Fig. 2C). Soluble CRLM-2 protein was readily detected in the culture media from the transfectants as a 35 kDa protein (Fig. 3, lane 3).

Expression of CRLM-2

Northern blot analysis showed that 1.4 kb CRLM-2 mRNA is preferentially expressed in lymphohemato-

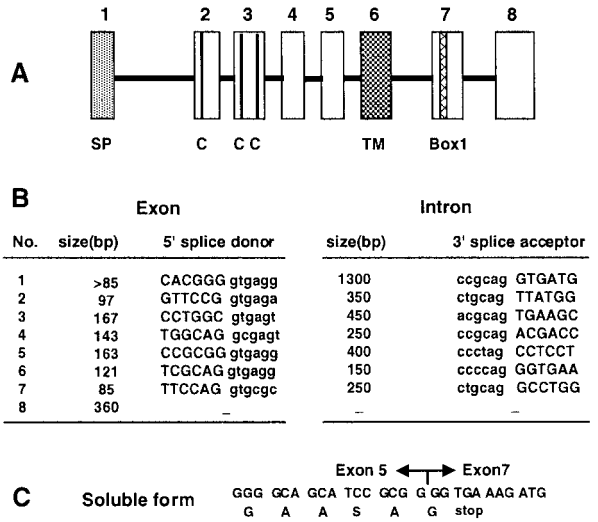

FIG. 2. Genomic structure of the mouse *CRLM-2* gene. (A) Schematic representation of exon/intron structure. Signal peptide (SP), conserved cysteine residues (C), transmembrane domain (TM), and Box 1 are depicted. (B) Exon–intron boundaries of the mouse *CRLM-2* gene. (C) The nucleotide and deduced amino acid sequences of soluble CRLM-2. The boundary of exons 5 and 7 is presented.

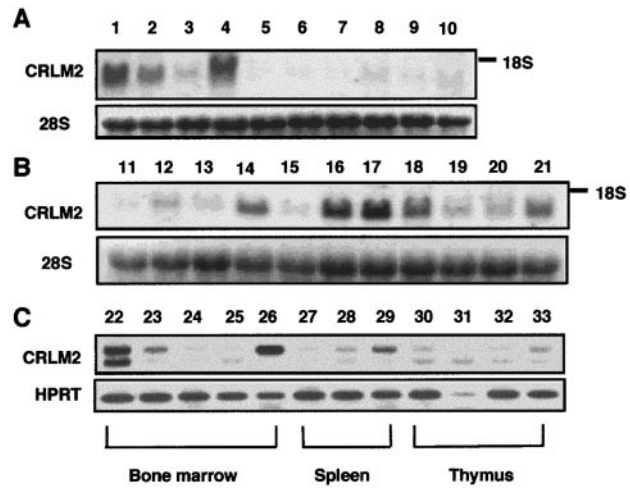

FIG. 4. Expression of CRLM-2 mRNA. (A, B) Northern blot analysis of mRNA of CRLM-2 in mouse tissues (A) and mouse hematopoietic cell lines (B). Ethidium bromide-stained 28S rRNA is shown to demonstrate equivalent RNA loading. (C)RT-PCR analysis of CRLM-2 expression in mouse lymphohematopoietic cells. HPRT (hypoxanthine guanine phosphoribosyl transferase) was used as a reference gene for semiquantitative analysis. Tissues and cells studied are bone marrow (lane 1), spleen (lane 2), thymus (lane 3), testis (lane 4), muscle (lane 5), kidney (lane 6), liver (lane 7), lung (lane 8), heart (lane 9), brain (lane 10), BW5147 (lane 11), EL4 (lane 12), Ba/F3 (lane 13), EML (lane 14), MEL (lane 15), 32D (lane 16), 32D stimulated with G-CSF for 1 day (lane 17), NFS60 (lane 18), M1 (lane 19), WEHI3 (lane 20), RAW274.6 (lane 21), c-Kit$^+$Sca-1$^+$Lin$^-$ progenitors (lane 22), Lineage marker-negative cells (lane 23), TER119$^+$ erythroblasts (lane 24), B220$^+$ B cells (lane 25), Gr-1$^+$ granulocytes (lane 26), CD3$^+$ T cells (lane 27), B220$^+$ B cells (lane 28), NK1.1$^+$ NK cells (lane 29), CD4$^+$CD8$^-$ T cells (lane 30), CD4$^-$CD8$^+$ T cells (lane 31), CD4$^+$CD8$^+$ T cells (lane 32), and CD4$^-$CD8$^-$ T cells (lane 33).

poietic tissues, particularly in the bone marrow and spleen (Fig. 4A). Other than lymphohematopoietic tissues, CRLM-2 mRNA is abundantly expressed in the testis. Among hematopoietic cell lines, CRLM-2 is highly expressed in a progenitor cell line, EML (17) and myeloid cell lines (32D, NFS60, and RAW274.6). Expression of CRLM-2 was upregulated during the granulocytic differentiation of 32D cells induced by G-CSF (18) (Fig. 4B, lanes 16 and 17). RT-PCR analysis of primary hematopoietic cells again showed its predominant expression in progenitor cells (c-Kit$^+$Sca-1$^+$Lin$^-$) (10) and granulocytic cells (Gr-1$^+$) (Fig. 4C). In addition, CRLM-2 was expressed moderately in NK cells and at a very low level in all other cell fractions analyzed. The predominant expression of CRLM-2 in progenitors and myeloid cells suggests its important role in these cells. Among known cytokines, G-CSF and GM-CSF specifically act on both progenitors and myeloid cells (3). Even though the number of granulocytes significantly decrease in the G-CSF deficient mice, the myeloid cells still normally differentiate in the mice (19). In the case of GM-CSF, no defect in myeloid cell development has been identified in gene deficient mice (20). These findings are suggestive of compensatory signaling pathways for myeloid development, and CRLM-2 might be one of the candidate pathways for it.

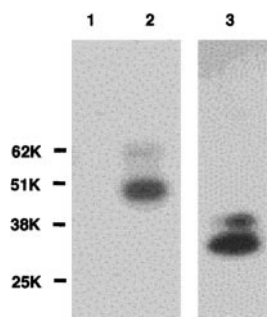

FIG. 3. Detection of CRLM-2 protein. 293T cells transiently transfected with CRLM-2-FLAG cDNA were biotinylated on the cell surface. CRLM-2 was immunoprecipitated with anti-FLAG antibody, resolved by SDS–PAGE and immunoblotted with streptavidin-HRP. (lane 1, mock; lane 2, CRLM-2-FLAG). 293T cells were transiently transfected with soluble CRLM-2 cDNA. The culture supernatant was resolved by SDS–PAGE and immunoblotted with anti-histidine antibody (lane 3).

mRNA for soluble CRLM-2 was readily detected by RT-PCR in primary hematopoietic cells except for Gr-1-positive granulocytes (Fig. 4C). Production of a significant amount of soluble CRLM-2 may indicate its important role in the regulation of CRLM-2-mediated signaling. Among many soluble cytokine receptors, biological meanings of soluble IL-6Rα and IL-13Rα2 have been well characterized (21). Soluble IL-6Rα positively acts for the IL-6 signal transduction by recruiting IL-6 to gp130, the signal transducer of IL-6 (22). On the other hand, soluble IL-13Rα2 neutralizes IL-13 by

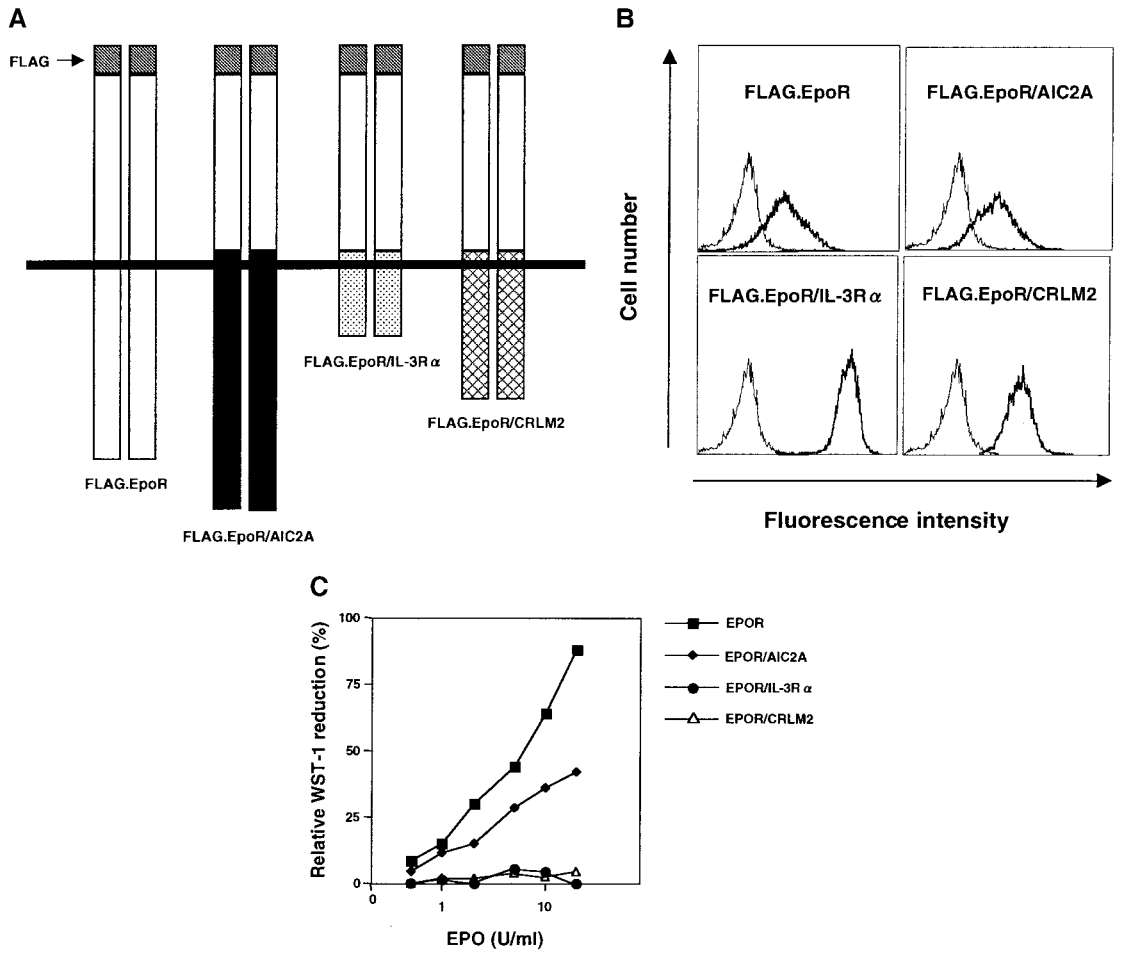

FIG. 5. Mitogenic activity of CRLM-2 cytoplasmic domain in Ba/F3 cells. (A) Schematic representation of wild-type and chimeric cytokine receptors. (B) Flow cytometric analysis of receptor expression in stable transfectants. Ba/F3 cells were transfected with wild-type or chimeric cytokine cDNAs. The cells were stained with biotinylated monoclonal anti-FLAG antibody, followed by phycoerythrin-conjugated streptavidin (thick lines). As a control, the staining profiles of parental cells are also shown (thin line). (C) Evaluation of mitogenic activity of CRLM-2. Ba/F3 cells transfected with the full-length EPOR or chimeric receptors were washed free of IL-3 and incubated with indicated concentrations of EPO. Mitogenesis was assayed based on stimulated rates of WST-1 reduction. Data are plotted as the percentages of maximal response of cells cultured with IL-3.

trapping IL-13 away from its cell surface receptor (23). Although further characterization is required for soluble CRLM-2, the fact that Gr-1-positive granulocytes, which express the highest level of CRLM-2 mRNA, do not express soluble form suggests its antagonistic function against its putative ligand.

Characterization of CRLM-2 as a Signal Transducer

Among several receptor subunits that compose type I cytokine receptors, receptors for EPO, G-CSF and thrombopoietin, and β subunits including βc and gp130 can transmit mitogenic signals by homodimerization, whereas most α subunits such as IL-3Rα cannot (11, 22, 24). To test whether CRLM-2 is a signal transducer or not, we constructed chimeric receptors in which the transmembrane and cytoplasmic domains of EPOR were replaced with those of CRLM-2 (Fig. 5A). As shown in Fig. 5B, Ba/F3 cells stably transfected with EPOR/IL-3Rα or EPOR/CRLM-2 chimeras expressed higher levels of receptors than those with full-length EPOR or EPOR/AIC2A. In contrast with full-length EPOR and EPOR/AIC2A chimera, however, EPOR/IL-3Rα and EPOR/CRLM-2 chimeras did not stimulate proliferation of Ba/F3 cells in response to EPO (Fig. 5C). Autodimerization of CD8/CRLM-2 chimera by virtue of the CD8α extracellular domain, which forms disulfide-linked dimer, did not stimulate proliferation, either (data not shown). As predicted from its Box1 motif (25), however, CRLM-2 was constitutively associated with JAK2 (Fig. 6). JAK2 is a major Janus kinase functional in lymphohematopoietic system (26) and is known to constitutively associate with conventional α subunits, such as IL-5Rα (27). These data suggest that CRLM-2 has functions similar to those of

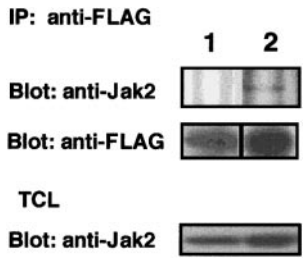

FIG. 6. Physical interaction of CRLM-2 with JAK2. FLAG-tagged TMTSP, a transmembrane protein with no homology to cytokine receptors (negative control), and CRLM-2 were expressed in Ba/F3 cells and immunoprecipitated with anti-FLAG antibody. Immunoprecipitates and total cell lysates (TCL) were resolved by SDS–PAGE and immunoblotted with anti-JAK2 or anti-FLAG antibody. Lane 1, BaF3/TMTSP; lane 2, BaF3/CRLM2.

conventional α subunits. Thus, upon stimulation by its putative ligand, CRLM-2 is likely to form receptor complex with its partner, and transmit signals other than mitosis through JAK family kinases on its Box 1 motif.

CONCLUDING REMARKS

Homeostasis of hematopoietic systems are precisely regulated by orchestration of multiple signaling pathways. Our data suggest that CRLM-2, a novel type I cytokine receptor, is a subunit of a receptor complex for as yet unknown cytokine. Identification of its ligand and characterization of CRLM-2-mediated signals should help our understanding of the complex regulatory network of hematopoietic system. During the preparation of this manuscript, Fujio *et al.* has reported the cloning of the same murine cytokine receptor as CRLM-2, designated Delta 1, from mouse Th2 lymphocytes (28). Although detailed expression of Delta 1 in hematopoietic cells has not been determined, Delta 1 is reportedly expressed in Th2 lymphocytes at a high level. Taken together with our findings, CRLM-2 could be a signal transducer that mediates important signals for both hematopoietic and immune regulation.

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