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Cytokine-Like Factor-1, a Novel Soluble Protein, Shares Homology with Members of the Cytokine Type I Receptor Family

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In this report we describe the identification, cloning, and expression pattern of human cytokine-like factor-1 (hCLF-1) and the identification and cloning of its murine homologue. Human CLF-1 and murine CLF-1 shared 96% amino acid identity and significant homology with many cytokine type I receptors. CLF-1 is a secreted protein, suggesting that it is either a soluble subunit within a cytokine receptor complex, like the soluble form of the IL-6R α-chain, or a subunit of a multimeric cytokine, e.g., IL-12 p40. The highest levels of hCLF-1 mRNA were observed in lymph node, spleen, thymus, appendix, placenta, stomach, bone marrow, and fetal lung, with constitutive expression of CLF-1 mRNA detected in a human kidney fibroblastic cell line. In fibroblast primary cell cultures, CLF-1 mRNA was up-regulated by TNF-α, IL-6, and IFN-γ. Western blot analysis of recombinant forms of hCLF-1 showed that the protein has the tendency to form covalently linked dimer and tetramers. These results suggest that CLF-1 is a novel soluble cytokine receptor subunit or part of a novel cytokine complex, possibly playing a regulatory role in the immune system and during fetal development. The Journal of Immunology, 1998, 161: 1371–1379.

Cytokines and growth hormones are secreted molecules controlling important cell functions, such as proliferation, differentiation, and survival (1–3). They exert their effects via specific receptors located on the target cell surface. These receptors are grouped into families according to both structural and amino acid sequence similarities (4–6). The cytokine receptor superfamily comprises the receptors for many families, including IFN, TNF, and hematopoietic growth factors. The cytokine type I receptors make up the largest subclass of this superfamily and are characterized by the presence of a conserved extracellular region of approximately 200 amino acids containing two fibronectin type III modules (7). This region, known as the cytokine receptor-like domain, has been shown to play an essential role in receptor function in terms of ligand binding (8) and receptor complex formation (9). It is characterized by four conserved cysteine residues in the first module and a W-S-x-W-S motif in the second (5).

The receptors for the IL-6-type cytokines (10), including IL-6, ciliary neurotrophic factor (CNTF), IL-11, leukemia inhibitory factor (LIF), oncostatin M, and cardiotoxin-1 are typical of the cytokine type I receptor family, forming multicomponent complexes comprising ligand binding and shared signaling subunits (11). Pleiotropy and redundancy within this family can be accounted for by the fact that the receptors for many of these cytokines use the signaling subunits gp130 (12) or the LIF receptor (13), both with long intracytoplasmic tails and involved in the induction of the JAK-STAT signaling pathway (14, 15). Receptor specificity is provided by the ligand binding subunit. In the case of the IL-6, IL-11, and CNTF receptors (IL-6R, IL-11R, and CNTFR), a soluble form of this subunit is capable of rendering cells sensitive to the appropriate ligand provided the correct signaling subunit (gp130 or LIF receptor) is present on the cell surface (16–19). In the case of the receptors for LIF and cardiotoxin-1, LIF receptor serves as both a ligand binding and a signaling subunit (13, 20, 21), as is the case for gp130 within the oncostatin M receptor complex (22, 23).

Another interesting configuration is that demonstrated by the organization of IL-12 and its receptor. IL-12 has been shown to be composed of two covalently linked polypeptide chains known as p35 and p40. Certain regions of p35 share homology with IL-6 and granulocyte CSF, while p40 shares homology with the extracellular region of IL-6R and CNTFR (24). p40 also displays the hallmark of the cytokine type I receptor family, containing the four conserved cysteine residues and a WSEWAS sequence, resembling the W-S-x-W-S motif (5). This has led to the suggestion that IL-12 may have originated from a cytokine/soluble cytokine receptor complex, a suggestion supported by the homology between the IL-12R and gp130 (25).

5 Abbreviations used in this paper: CNTF, ciliary neurotrophic factor; LIF, leukemia inhibitory factor; CNTFR, ciliary neurotrophic factor receptor; CLF-1, cytokine-like factor-1; EST, expressed sequence tag; hCLF-1, human cytokine-like factor-1; mCLF-1, murine cytokine-like factor-1; hIL-13Rα1, human IL-13 receptor α1-chain.
In this report, bioinformatics was used to identify human and murine homologues of a novel cytokine-like factor, CLF-1, which was subsequently cloned and analyzed. Human and murine CLF-1 share 96% amino acid identity, suggesting an important function for the protein. CLF-1 shares significant homology with many members of the cytokine type I receptor family. Analysis of CLF-1 mRNA distribution and recombinant forms of the CLF-1 protein provide the initial characterization for this novel member of the cytokine type I receptor family.

Materials and Methods

Cloning of human and mouse cDNA for CLF-1

The amino acid sequence N K L C F D D N K L W S D W S E A Q S I G K E Q N from the murine IL-13r (26) was used to search the GenBank database with expressed sequence tags (ESTs) using TBLASTN to identify cDNAs encoding receptors with related sequence. BLASTN searches of the GenBank database with the identified EST allowed the identification of ESTs with overlapping sequence homology.

The mouse cDNA clone 4790243 was purchased from Research Genetics (Birmingham, AL). 5'-RACE on poly(A)+ RNA extracted from mouse lung allowed the cloning of 310 bp of the murine cDNA upstream of the 5' end of the cDNA clone using the Marathon cDNA amplification kit (Clontech, Palo Alto, CA) following the manufacturer's guidelines. Primers used in the PCR amplification (along with the adapter protein-1 primer provided) were 5'-GGTAGATCCCTCGAGTTAGTCTG-3' for 5' PstI and 5'-GGTAGATCCCTCGAGTTAGTCTG-3' for 3' PstI. The amplified products were cloned into the vector pCR II (Invitrogen, Leek, The Netherlands), and colonies were screened by hybridization with the oligonucleotide probe 5'-AAGGATCTACAGGGCGACCCGGGCGCGTGATGGTTT-3'.

A portion of the hCLF-1 cDNA was amplified by PCR using cDNA derived from human lung poly(A)+ RNA with the primers 5'-ACCCGCCAGGGGCCCTTACTGG-3' and 5'-TTGAGGAGTAGTTGGTGTG-3'. The amplified product was cloned into the vector pCR II and subsequently used as a 32P-labeled probe to screen a human placental cDNA library in g10. The largest cDNA identified (1740 bp) was recloned in pBluescript II SK+ (Stratagene, La Jolla, CA).

DNA and protein sequence analysis

Sequences obtained from cDNA clones as well as all relevant ESTs were imported into and analyzed by the sequence analysis software Sequencher (GeneCodes, Ann Arbor, MI). The signalP server (http://www.cbs.dtu.dk/ signalp/cbsignlap.html) was used to identify the predicted cleavage site of the signal peptide for CLF-1. DNA and amino acid sequence alignments as well as prediction of hydrophobic regions were analyzed with the Wisconsin package version 8.1 (Genetics Computer Group, Madison, WI).

Source of cells and culture conditions

The cell lines HEK-293 and CHO were obtained from the American Type Culture Collection (Manassas, VA). Palmar fibromatosis lesion and mammary gland fibroblasts were gifts from Prof. G. Gabbiani (University Medical Center, Geneva, Switzerland). Tonsillar fibroblasts were derived from whole tonsils obtained from surgery following a previously described protocol (27). All the above cell types were maintained in DMEM and Ham's F-12 nutrient solution supplemented with 10% FCS. Fibroblastic cells and cell lines were stimulated with the appropriate cytokines at a final concentration of 10 ng/ml/cytokine.

Detection of the CLF-1 gene by Southern blot analysis

Genomic DNA was isolated from cells or tissues with the DNAzol reagent (Life Technologies, Grand Island, NY) following the manufacturer's guidelines. The source of the genomic DNA was as follows: HEK-293 human fibroblastic cells, African green monkey COS-7 cells (American Type Culture Collection), C57BL/6 mouse tails, rat liver, rabbit liver, cow liver, and chicken embryo liver. Aliquots of genomic DNA (5 μg) were digested with either SacI or BamHI and subjected to Southern blot analysis using a 32P-labeled 1.2-kb fragment of hCLF-1 cDNA as a probe. Hybridization was performed in 5× SSC, 5× Denhardt's solution, and 5% SDS at 55°C. Filters were washed with 0.1x SSC and 1% SDS at 55°C and exposed at 70°C.

Detection of hCLF-1 mRNA by Northern blot analysis

The same 32P-labeled 310-bp hCLF-1 cDNA fragment that was used to screen the human placental cDNA library (see above) was used to identify hCLF-1 mRNA transcrits in the Human, Human II, Human Immune System, Human Endocrine, and Human Fetal Multiple Tissue Northern Blots (Clontech). Hybridization was performed in ExpressHyb solution (Clontech). Hybridization and washing were performed according to the manufacturer's guidelines.

Detection of hCLF-1 mRNA by RT-PCR

Total RNA was purified from the appropriate source using TRIzol reagent solution (Life Technologies) following the manufacturer's guidelines. Five micrograms of total RNA was reverse transcribed using the first strand synthesis kit (Pharmacia LKB Biotechnology, Uppsala, Sweden) following the manufacturer's guidelines, and 1/10th of the cDNA was amplified by 32 cycles of PCR using the primers 5'-GGATGAGAGGCCCACACAGC-3' and 5'-AGGGCCAGATGAATCAGTGC-3' for hCLF-1 cDNA and 5'-GGCGAGCACGCCCAGAGCAAG-3' and 5'-CGATTTCGCCCTGGGCGTG-3' for β-actin cDNA. PCR products were analyzed by agarose gel electrophoresis.

Production of recombinant soluble hCLF-1

To generate recombinant soluble protein, hCLF-1 cDNA in pBluescript II SK- was digested with EcoRI and Stul and cloned into pFASIL-1 (Life Technologies) digested with the same enzymes. The oligonucleotides 5'-GGCCTGATCCCTTAGCCGGAGGCCCACACAGC-3' and 5'-CCATGATCCCTTAGCCGGAGGCCCACACAGC-3' were annealed and cloned into pFASIL-1 containing the hCLF-1 cDNA digested with MluI and Stul. The annealed oligonucleotides encoded six histidines and the CLEPHTACD tag, an epitope recognized by mAb 179 (Affymax, Palo Alto, CA), at the 3' end of the hCLF-1 cDNA.

Recombinant baculovirus was generated using the BAC-TO-BAC kit (Life Technologies) and was used to infect Sf9 cells (American Type Culture Collection) expanded in SF900II medium (Life Technologies). Recombinant protein was purified from the Sf9 culture medium using a column packed with 50 ml of Ni-NTA (Qiagen, Basel, Switzerland) following the manufacturer's guidelines. The recovered fraction contained approximately 30% monomeric hCLF-1 as determined by SDS-PAGE under reducing conditions.

Expression of cell membrane-bound hCLF-1 in Sf9 cells

To generate membrane-bound hCLF-1, pBluescript II SK- containing hCLF-1 cDNA was digested with SacI. The human IL-13Rα1 (hIL-13rα1) (28) transmembrane and cytoplasmic domains were amplified by PCR from pBluescript II SK- containing the hIL-13rα1 cDNA using the oligonucleotides 5'-TCCCCCGGTGATACATACCATGATTTCTGTT-3' and 5'-TCCATCCACTAGGAGGTCGACTTT-3'. The PCR product was digested with SacI and ligated into the SacI-digested pBluescript II SK- containing hCLF-1 cDNA. DNA encoding the polyoma virus middle T Ag epitope (ATATCTG) was inserted at the 5' end of the hCLF-1 cDNA by digesting the plasmid with Xhol and BamHI and cloning into these restriction enzyme sites the two annealed oligonucleotides (5'-TGGTCTCGAGGCACGGAGGCCAGGAGGAGGAC-3' and 5'-GATCTCGAGGACTGAGAGGAGGAGGAGGAC-3'). The cDNA encoding the tagged fusion protein was excised from pBluescript II SK- by digestion with SpeI (whose site was subsequently blunt ended using the Klenow fragment of DNA polymerase I) and EcoRI, and ligated into pFASIL-1 containing the mellitin signal peptide digested with Bsp120I (whose site was subsequently blunt ended using the Klenow fragment of DNA polymerase I) and EcoRI.

Recombinant baculovirus was generated using the BAC-TO-BAC kit and used to infect Sf9 cells. Expression of the fusion protein on the surface of the Sf9 cell line was confirmed at 24 and 48 h by flow cytometry using a mAb recognizing the tee tag. Briefly, cells were washed with FACS buffer (1% BSA and 0.01% sodium azide in PBS) and successively incubated for 30 min with the anti-EE mAb at 10 μg/ml in FACS buffer and FITC-labeled sheep anti-mouse F(ab')2 fragments (Silenius Laboratories, Hawthorn, Australia) diluted 1/100 in FACS buffer. Fluorescence was measured using a FACScan (Becton Dickinson, Erembodegen, Belgium).

Expression of cell membrane-bound hCLF-1 in CHO cells

To generate membrane-bound hCLF-1, pBluescript II SK- containing the hCLF-1/hIL-13rα1 cDNA without the EE tag was digested with SacI and EcoRI and inserted into the expression vector pCDNA3 (Invitrogen) at the same restriction enzyme sites. To insert the native signal peptide from hCLF-1 at the 5' end of the fusion construct, pBluescript II SK- containing the full-length hCLF-1 was digested with KpnI and BamHI and the excised fragment (encoding the
signal peptide) was inserted into pcDNA3 containing the hCLF-1/hIL-13Rα1 cDNA digested with the same enzymes. The expression vector was linearized and introduced into CHO cells by electroporation using previously described conditions (28).

Transfected cells were selected using 500 μg/ml geneticin (Life Technologies). Control cells were transfected with pcDNA3 containing the full-length hIL-13Rα1 cDNA. Expression of hCLF-1 on the surface of the CHO cells was detected by flow cytometry with a mAb recognizing hCLF-1 (see below) using the conditions described above. Positive cells were selected and sorted by flow cytometry and replaced in culture. After three rounds of sorting, a population of CHO cells expressing high levels of membrane-bound hCLF-1 was obtained.

**Generation of mouse anti-hCLF-1 mAbs**

A BALB/c mouse was immunized on days 0, 7, and 28 s.c. in the limbs and behind the neck with 100 μg of purified recombinant hCLF-1 in carbonate buffer, pH 9.6. Plates were washed, blocked with 1% BSA in PBS and incubated for 2 h with 200 μl of hybridoma supernatant, and washed again. Bound Ab was revealed with horseradish peroxidase-coupled goat anti-mouse IgG (Southern Biotechnology Associates, Birmingham, AL). Specificity was checked using an ELISA with recombinant soluble hIL-13Rα1 that had the same recognition tags as recombinant hCLF-1 at 1 μg/ml. Specific positive supernatants were further screened by flow cytometry on Sf9 cells either mock infected or infected with baculovirus harvest medium, and 7 to 10 days after fusion, the supernatants were harvested for screening.

**Screening hybridoma supernatants for anti-hCLF mAbs**

Ninety-six-well plates (Falcon 3912, Becton Dickinson Labware Europe, Meylan, France) were coated at 4°C with 1 μg recombinant hCLF-1 in carbonate buffer, pH 9.6. Plates were washed, blocked with 1% BSA in PBS, incubated for 2 h with 200 μl of hybridoma supernatant, and washed again. Bound Ab was revealed with horseradish peroxidase-coupled goat anti-mouse IgG (Southern Biotechnology Associates, Birmingham, AL). Specificity was checked using an ELISA with recombinant soluble hIL-13Rα1 that had the same recognition tags as recombinant hCLF-1 at 1 μg/ml. Specific positive supernatants were further screened by flow cytometry on Sf9 cells either mock infected or infected with baculovirus encoding the membrane-bound hCLF-1/hIL-13Rα1 fusion protein or on transfected CHO cells expressing hIL-13Rα1 or the hCLF-1/hIL-13Rα1 fusion protein.

**Purification of anti-hCLF-1 mAbs**

Abs were purified by chromatography on protein A-Sepharose Fast Flow in PBS and eluted in 0.1 M citrate, pH 4.5. Eluates were then subjected to gel filtration on Superdex-200 (Pharmacia) equilibrated in PBS.

**Detection of hCLF-1 by Western blot analysis**

The supernatants or cell lysates containing the recombinant protein were diluted 1/1 with Tris-glycine-SDS sample buffer (Novex, San Diego, CA) and heated to 95°C for 5 min. To reduce proteins, samples to a final concentration of 375 mM. The proteins were resolved on an 8% polyacrylamide gel (Novex) and electrotransferred onto a nitrocellulose membrane (32). The membrane was blocked in PBS containing 5% dried milk and 0.15% Tween-20 for 1 h at room temperature and incubated for 1 h at room temperature with PBS containing 2.5% dried milk and 5 μg/ml of the appropriate mAb. Bound mAb was detected with horseradish peroxidase-labeled sheep anti-mouse Ab and ECL (Amersham Life Sciences, Aylesbury, U.K.) following the manufacturer’s guidelines.

**Results**

**Identification and characterization of a new CLF**

The GenBank database with ESTs was searched using TBLASTN with a 20-amino acid sequence surrounding the W-S-x-S motif of murine IL-13 (26). ESTs showing significant homology were then translated, and the open reading frames were used to search the Swissprot database using BLASTP for homologous proteins. The amino acid sequence from the murine EST W66776 shared a high level of homology with the prolactin receptor (33) and the receptors for the IL-6-type cytokines (10). Using the sequence of W66776 to search the GenBank database allowed the identification of overlapping homologous sequences (of both murine and human origin), which, in turn, were run against the GenBank database to identify more overlapping sequences (Table I). This allowed the assembly of overlapping sequences encoding the human and mouse cDNAs. The cDNA clone 479043, which gave rise to the mouse EST EST found further 5’ in the sequence assembly was obtained from the IMAGE consortium. After sequencing, it was found to contain an insert of 1 kb, including a 3’ poly(A) tail. The rapid amplification of 5’ cDNA ends on murine lung cDNA allowed the cloning of a further 308 bp upstream.

To clone the human cDNA encoding CLF-1, a 310-bp PCR product was amplified from human lung cDNA using primers designed from the human ESTs. The PCR product was, in turn, used as a probe to screen a human placental cDNA library, resulting in the isolation of a full-length clone of 1740 bp, which included a 3’ poly(A) tail. The human cDNA encoded a precursor protein of 422 amino acids with a putative signal peptide of 37 amino acids.

**Table I. ESTs corresponding to human and mouse CLF-1 in GenBank**

<table>
<thead>
<tr>
<th>Receptor</th>
<th>EST Accession Number</th>
<th>Source of Origin</th>
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</tr>
<tr>
<td></td>
<td>W17583</td>
<td>Embryo</td>
</tr>
<tr>
<td></td>
<td>W66776</td>
<td>Embryo</td>
</tr>
<tr>
<td>Human</td>
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<td>Pregnant uterus</td>
</tr>
<tr>
<td></td>
<td>AA043001</td>
<td>Pregnant uterus</td>
</tr>
<tr>
<td></td>
<td>AA121532</td>
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<td></td>
<td>AA377893</td>
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<td></td>
<td>AA406406</td>
<td>Melanocyte/fetal heart/uterus</td>
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<td></td>
<td>H14009</td>
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</tr>
<tr>
<td></td>
<td>W46604</td>
<td>Fibroblast</td>
</tr>
</tbody>
</table>

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The murine cDNA encoded a protein of 383 amino acids. The cDNA was incomplete at the 5’ end as the first amino acid of the translated sequence aligned to amino acid 39 of the human sequence, and no starting methionine or putative signal peptide could be identified. Both mouse and human CLF-1 contained 11 cysteine residues and 6 potential N-linked glycosylation sites. Sequence analysis of the human and murine cDNAs showed 85% nucleic acid identity and 96% amino acid identity (Fig. 1A). Human and murine CLF-1 showed close homology to the prolactin receptor and receptors of the IL-6-type cytokines (10) (Table II). The N-terminal region of both sequences appeared to represent an Ig-like domain, most closely resembling the C2-set sequence (34). This domain was followed by two fibronectin type III modules of approximately 100 amino acid residues each. Alignment of the human and mouse amino acid sequences to members of the IL-6-type cytokine receptor family showed regions of conserved homology within these two functionally important modules known as the cytokine receptor-like domain, and both sequences contained the highly conserved four cysteine residues and the W-S-x-W-S motif characteristic of this domain (5) (Fig. 1B). We were unable to identify any transmembrane domains within the amino acid sequence of the mature proteins or hydrophobic region at the C-terminus of the sequence, characteristic of glycosyl phosphatidylinositol-anchored proteins such as the CNTFR α-chain (CNTFRα).
FIGURE 1. A. Alignment of the human and mouse CLF-1 amino acid sequences. Residues sharing identity between the two proteins are boxed in black. Unknown residues at the N-terminus of mCLF-1 due to the incomplete cloning of the 5' end of the cDNA are indicated by a dash. The four conserved cysteines and the W-S-x-W-S motif are indicated above the alignment. B. Alignment of the Ig-like and cytokine receptor-like domains of human and mouse CLF-1 with related cytokine type I receptors. Regions of amino acid identity are boxed in black. The four conserved cysteines and the W-S-x-W-S motif are indicated above the alignment. hgp130, human gp130.
This suggested that the cloned human and mouse cDNAs encode soluble proteins.

Characterization of the hCLF-1 gene

Using the hCLF-1 cDNA sequence to search the GenBank database we identified the hCLF-1 gene sequence, under the accession number AC003112. The gene was encoded by nine exons contained within a 14-kb region of the chromosome 19-specific cosmid R30292 mapping to 19p12 (confirming the location predicted by the EST with accession no. H14009). The erythropoietin receptor (36), sharing homology with CLF-1, and the soluble protein EBI3 (37) are the only known members of the cytokine type I receptor family shown to be localized on this arm of chromosome 19. The intron/exon organization of the gene for hCLF-1 is in agreement with the predicted structure of the domains within the hCLF-1 protein, following the rule proposed for the cytokine receptor-like domain (Fig. 2) (38). The first exon encodes the signal peptide, exon 2 encodes the Ig-like domain, exons 3 and 4 encode the N-terminal cytokine receptor-like domain, and exons 5 and 6 encode the C-terminal cytokine receptor-like domain. Exons 7 and 8 showed no homology with any other family members, while exon 9 had the stop codon close to its 5’ end and the polyadenylation signal close to its 3’ end. We were unable to identify anything resembling an exon encoding a putative transmembrane domain within the 17.4 kb of DNA sequence contained within the contig downstream of the exon containing the W-S-x-W-S motif (exon 6).

Conservation of the CLF-1 gene

The very high amino acid identity between the human and mouse protein sequences for CLF-1 suggested that the gene had been highly conserved during recent evolution. As the cDNAs encoding human and mouse CLF-1 shared 85% homology, we examined the conservation of the gene in a number of animal species by Southern blot analysis of genomic DNA using an hCLF-1 cDNA probe (Fig. 3). As expected, human and monkey genomic DNA gave the strongest signals, but signals could be detected in all six mammalian species studied, with faint bands also seen with chicken genomic DNA, indicating that the gene is highly conserved within these species.

Tissue and cellular distribution of hCLF-1 mRNA

hCLF-1 mRNA expression was studied in human tissues by Northern blot analysis. The mRNA transcript migrated as a 1.7-kb species, a size close to that predicted from the clone obtained by library screening. Expression of the 1.7-kb transcript could be detected in several tissues (Fig. 4). The strongest expression of the hCLF-1 mRNA was detected in the spleen, thymus, lymph node, appendix, bone marrow, stomach, placenta, heart, thyroid, and ovary. Interestingly, a strong level of expression was also detected in fetal lung. We studied the level of mRNA expression by Northern blot analysis in several cell lines, including the fibroblastic cell line HEK 293, the monocyte cell line THP-1, JY lymphoblastoid cells, RPMI 8226 myeloma cells, the mast cell line HMC-1, HBE-140 bronchial epithelial cells, and HUVEC. A detectable level of expression could be seen in JY cells, HMC-1 cells, and PMA-stimulated THP-1 cells. The strongest expression, however, was detected in HEK-293 cells (data not shown).

Table II. Homology between CLF-1 and IL-6-type cytokine receptors

<table>
<thead>
<tr>
<th>Cloned Receptor</th>
<th>Alignment with</th>
<th>Identical Residues</th>
<th>Similar Residues</th>
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<tr>
<td>Human hGP130</td>
<td>87/306</td>
<td>120/306</td>
<td></td>
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<tr>
<td>hCNTFR</td>
<td>79/306</td>
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<td>hGCSFR</td>
<td>85/306</td>
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<tr>
<td>Mouse mGP130</td>
<td>89/304</td>
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<tr>
<td>mGCSFR</td>
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<tr>
<td>mIL-6Rα</td>
<td>71/304</td>
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*Homology was ascertained using Bestfit in the Wisconsin package, version 8.1.
The expression of hCLF-1 mRNA in HEK 293 cells and fibroblast primary cell cultures was studied by RT-PCR following stimulation with proinflammatory cytokines. Whereas no effect was seen on the constitutive mRNA level found in HEK 293 cells (Fig. 5A), an up-regulation of hCLF-1 mRNA was observed in all three fibroblast primary cell cultures under stimulation with IFN-γ, TNF-α up-regulated hCLF-1 mRNA in palmar fibromatosis lesion and mammary gland fibroblasts, while IL-6 up-regulated hCLF-1 mRNA in palmar fibromatosis lesion and tonsillar fibroblasts. Interestingly, IL-1β had a down-regulatory effect on hCLF-1 mRNA production in two of the three fibroblast cultures (Fig. 5, B–D).

**Generation of mAbs recognizing hCLF-1**

A BALB/c mouse was immunized with recombinant soluble hCLF-1 generated with the baculovirus expression system. Supernatants from the resulting hybridomas were tested by ELISA using the same recombinant protein. An ELISA using a recombinant soluble form of hIL-13Rα1 with the same recognition tags as the recombinant hCLF-1 was performed on the positive hybridomas to eliminate Abs raised against the tags or conserved cytokine receptor epitopes. Specific positive mAbs were then tested by flow cytometry using Sf9 cells infected with baculovirus encoding the hCLF-1/hIL-13Rα1 membrane-bound fusion protein or CHO cells stably transfected with an expression vector encoding the same fusion construct. A strong signal was seen with one hybridoma, 92A10, with no signal detected on mock-transfected Sf9 cells or hIL-13Rα1-transfected CHO cells (Fig. 6). The mAb expressed by this hybridoma was determined to be an IgG2a.

**Characterization of recombinant forms of hCLF-1**

The recombinant forms of hCLF-1 expressed in Sf9 and CHO cells were examined by Western blot analysis under nonreducing and reducing conditions using either the appropriate anti-tag mAb or the anti-hCLF-1 mAb. The mAb 179 revealed that in the supernatant of Sf9 cells infected with baculovirus encoding recombinant soluble hCLF-1, the protein formed covalently linked tetramers. This was demonstrated by the presence of a high molecular mass band (~200 kDa) detected under nonreducing conditions. Under reducing conditions, only the band corresponding to the monomeric form of the protein was detected (~48–49 kDa; Fig. 7A). A similar pattern was detected using the anti-EE mAb on the cell lysate of baculovirus-infected Sf9 cells expressing the hCLF-1/hIL-13Rα1 fusion protein. Western blotting revealed a predominant band corresponding to covalently linked homodimeric protein (~120 kDa) when performed under nonreducing conditions. A larger band with a molecular mass of >200kDa was also detected, probably corresponding to covalently linked tetrameric protein. Under reducing conditions, only the band corresponding to monomeric protein could be detected (~48–49 kDa; Fig. 7A). A similar pattern was detected using the anti-EE mAb on the cell lysate of baculovirus-infected Sf9 cells expressing the hCLF-1/hIL-13Rα1 fusion protein. Western blotting revealed a predominant band corresponding to covalently linked homodimeric protein (~120 kDa) when performed under nonreducing conditions. A larger band with a molecular mass of >200kDa was also detected, probably corresponding to covalently linked tetrameric protein.

In contrast to the di- and tetrameric forms of hCLF-1, the monomeric form of the protein was not recognized by the anti-hCLF-1 mAb by Western blotting (Fig. 7, A–C).
Discussion

In this report we describe the identification and cloning of the human and mouse homologues of a novel gene, CLF-1. The human cDNA encodes a 422-amino acid precursor protein with a 37-amino acid putative signal peptide. Based on their deduced amino acid sequences, human and murine CLF-1 would appear to constitute new members of the cytokine type I receptor family. The expression pattern of hCLF-1 suggests a role for the protein in the immune system and in fetal development.

Human and murine CLF-1 were seen to share 96% amino acid identity (Fig. 1A) and also show highest homology with the prolactin receptor (33) and gp130 (12). It is interesting to note that amino acid identity between human and mouse gp130 is 77%, and that between the human and mouse prolactin receptors is 69%. Southern blot analysis, using hCLF-1 cDNA as a probe, indicated that the high conservation of the CLF-1 gene sequence between human and mouse can be extended to monkey, rat, rabbit, cow, and chicken (Fig. 3). The hybridization and washing conditions were relatively stringent, thus reducing the risk of nonspecific signals. This extremely high level of conservation during recent evolution suggests a functionally important role for CLF-1.

Information concerning the homology between the N-terminal region of human and mouse CLF-1 and the extracellular region of members of the cytokine type I receptor family is provided in Figure 1B and Table II. The four conserved cysteines and the W-S-x-W-S motif are shown above the alignment in Figure 1B. These sequence motifs characterize members of the type I cytokine receptor family (5) and are located within two fibronectin type III modules (7) that have been shown to play an important role in receptor function (8, 9). This homology suggests that CLF-1 constitutes a new member of the cytokine type I receptor family. The CLF-1 gene structure is also typical of those found within this family of receptors (Fig. 2) (38).

Although CLF-1 appears to exist uniquely as a soluble protein, there are numerous examples of soluble forms of receptor subunits in the cytokine type I receptor family. These soluble subunits can exhibit either antagonistic effects in terms of ligand signaling, such as those shown by soluble gp130 (39, 40) and the soluble IL-5Rα chain (IL-5Rα) (41), or agonistic effects, such as those shown by the IL-6Rα chain (IL-6Rα) (16, 17), the IL-11Rα chain (IL-11Rα) (18), and CNTFRα (19). Receptor chains within the cytokine type I receptor family generally function as receptor signaling or ligand binding subunits (11). If we are to assume that CLF-1 exists solely in soluble form and is indeed a soluble receptor chain, it is likely that it serves as a ligand binding subunit. Further analysis must be...
undertaken to determine whether any of the known ligands is able to bind CLF-1.

Human CLF-1 mRNA is predominantly expressed in adult spleen, thymus, lymph node, appendix, bone marrow, stomach, placenta, heart, thyroid, and ovary (Fig. 4). We can also predict that the transcript is well expressed in pregnant female uterus when looking at the source of origin of hCLF-1 ESTs (Table I). Expression of hCLF-1 mRNA was also studied by Northern blot analysis in a number of different primary and transformed cells. Strongest expression was observed in the fibroblastic cell line HEK 293 with detectable levels in the B cell line JY, the immature mast cell line HMC-1, and the monocyte cell line THP-1 following stimulation (data not shown). The expression seen in HEK 293 cells prompted us to study the modulation of hCLF-1 mRNA levels in HEK 293 cells and fibroblast primary cell cultures in response to the proinflammatory cytokines IL-1β, TNF-α, IL-6, and IFN-γ. Although these cytokines had no real effect on the constitutive level of hCLF-1 mRNA expression in HEK 293 cells, a significant up-regulation of mRNA was observed in the primary fibroblast cultures in response to IFN-γ with an up-regulation in two of the three cultures in response to TNF-α and IL-6, as detected by RT-PCR (Fig. 5, A–D). The tissue distribution of hCLF-1 mRNA and its up-regulation by proinflammatory cytokines in fibroblast cultures suggest that hCLF-1 may be involved in regulation of the immune system during an inflammatory response.

A strong expression of hCLF-1 mRNA was also observed in fetal lung (Fig. 4). In addition, mCLF-1 mRNA transcripts were detected by Northern blot analysis in total mouse embryos on days 11, 15, and 17 days postcoitum, with no expression seen on day 9 postcoitum (data not shown). Furthermore, many ESTs encoding human and mouse CLF-1 were derived from fetal tissues (Table I). This points to an additional role for CLF-1 in mediating regulatory signals during fetal development.

Western blot analysis of the recombinant forms of hCLF-1 baculovirus-infected Sf9 cell culture supernatants, membrane-bound hCLF-1 fusion protein from baculovirus-infected Sf9 cell lysates and membrane-bound hCLF-1 fusion protein from stably transfected CHO cell lysates under nonreducing and reducing conditions, provided two significant observations (Fig. 7, A–C). Firstly, the recombinant protein had a tendency to exist in covalently linked homomeric forms when comparing the difference in molecular mass under nonreducing or reducing conditions. Secondly, it appeared that the anti-hCLF-1 mAb recognized the nonreduced form of the protein, while reduced (i.e., monomeric) forms of the protein were poorly recognized. These findings suggested that the mAb bound to a structural epitope, formed when two or more hCLF-1 chains are covalently linked together, as opposed to a sequence epitope on the protein. Furthermore, HEK 293 cells (which constitutively expressed hCLF-1 mRNA) were used in an attempt to detect hCLF-1 protein in its native form using the mAb raised against the recombinant protein. No evidence was obtained of hCLF-1 being expressed on the cell surface by flow cytometry or in the cell supernatant by Western blot or immunoprecipitation, suggesting one of three possibilities. Firstly, a tight translational control could result in a level of protein production below the threshold of detection of the Ab. Secondly, hCLF-1 could be secreted from the cell in monomeric form, rendering itself undetectable to the mAb. Thirdly, hCLF-1 could form a covalently linked heterodimeric structure with a different subunit, again rendering itself invisible to the mAb.

The cytokine IL-12 is composed of two disulfide-linked A and B glycosylated polypeptides of approximately 40 kDa (p40) and 35 kDa (p35). Certain regions of the p35 amino acid sequence show homology to IL-6 and granulocyte CSF, suggesting that p35 is derived from a cytokine-like molecule. The p40 amino acid sequence, however, shows homology with the extracellular domain of members of the cytokine type I receptor family, most notably with IL-6Rα and CNTFRα. Interestingly, recombinant p40 can form a covalently linked homodimer, a form that in the mouse acts as a physiologic antagonist of IL-12, probably competing for IL-12 binding to its receptor (24, 42, 43).

From these observations, it is tempting to compare CLF-1 with IL-12 p40. Firstly, both proteins share homology with the extracellular regions of IL-6Rα and CNTFRα. Secondly, when recombinant protein is expressed in cells transfected with the appropriate cDNA, both CLF-1 and IL-12 p40 can form covalently linked homodimers. If, indeed, as with the IL-12 p40 subunit, CLF-1 exists in its native functional form as a covalently linked heterodimer, this could well account for our failure to detect the protein using the anti-hCLF-1 mAb in cells expressing hCLF-1 mRNA as discussed earlier.

The generation of Abs recognizing the native form of CLF-1 could allow the purification and thus structural characterization of the native protein, leading to the identification of covalently linked heterologous subunits. The identification of a cell membrane-bound structure for CLF-1 is ongoing and will also further the characterization of this novel protein.

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