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Four New Members Expand the Interleukin-1 Superfamily*

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We report here the cloning and characterization of four new members of the interleukin-1 (IL-1) family (FIL1δ, FIL1ϵ, FIL1ζ, and FIL1η, with FIL1 standing for "Family of IL-1"). The novel genes demonstrate significant sequence similarity to IL-1α, IL-1β, IL-1ra, and IL-18, and in addition maintain a conserved exon-intron arrangement that is shared with the previously known members of the family. Protein structure modeling also suggests that the *FIL1* genes are related to IL-1β and IL-1ra. The novel genes form a cluster with the IL-1s on the long arm of human chromosome 2.

The cytokine interleukin-1 (IL-1)[1] elicits a wide array of biological activities that initiate and promote the host response to injury or infection, including fever, sleep, loss of appetite, acute phase protein synthesis, chemokine production, adhesion molecule up-regulation, vasodilatation, the pro-coagulant state, increased hematopoiesis, and production and release of matrix metalloproteinases and growth factors (1). It does so by activating a set of transcription factors that includes NFκB and AP-1, which in turn promote production of effectors of the inflammatory response, such as the inducible forms of cycloxygenase and nitric oxide synthase (2, 3). Interleukin 1 activity actually resides in each of two molecules, IL-1α and IL-1β, which act by binding to a common receptor composed of a ligand binding chain, the type I IL-1 receptor, and a required signaling component, the IL-1R accessory protein (AcP) (4–7). A third member of the family, the IL-1 receptor antagonist (IL-1ra), also binds to the type I IL-1 receptor but fails to bring about the subsequent interaction with AcP, thus not only not signaling itself but also, by blocking the receptor, preventing the action of the agonist IL-1s (8, 9). Additional regulation is provided by the type II, or decoy, IL-1 receptor, which binds and sequesters the agonist IL-1s (especially IL-1β) without inducing any signaling response of its own (10–13). The two agonist IL-1s (IL-1α and IL-1β) are synthesized as larger precursors which undergo proteolytic removal of their pro-domains to generate the mature cytokines (14). At least for IL-1β, this processing is coupled to secretion (15, 16). IL-1ra, in contrast, contains a signal peptide and is secreted by the more traditional route through the endoplasmic reticulum (9).

Recently, another cytokine, interleukin 18 (17, 18) was recognized to be related to the interleukin-1 family based on the similarity of its amino acid sequence and predicted tertiary structure (19). IL-18 induces the production of γ-interferon from T cells, especially in combination with IL-12, and stimulates the killing activity of cytotoxic T lymphocytes and NK cells by up-regulating Fas ligand (20). Like the agonist IL-1s, IL-18 contains a prodomain that is removed by the same protease, caspase-1, that processes IL-1β (21, 22). Consistent with its being related to the IL-1s, IL-18 binds a receptor which is homologous to the IL-1 receptor. The ligand-binding chain IL-1Rrp1 (or IL-18Rα) (23, 24) was cloned initially on the basis of its homology to the IL-1R (25). The signaling subunit (IL-18Rβ) was originally named AcPL (AcP-like) for its similarity to the IL-1R signaling subunit (26). The IL-18 receptor subunits are encoded in the same gene cluster on chromosome 2 as are the type I and II IL-1 receptors (25–27).

We have searched for novel members of the IL-1 family. We report here the sequences and some of the characteristics of four genes that appear to have descended from the same common ancestor as did IL-1α, IL-1β, IL-1ra, and IL-18. We propose that these novel molecules be designated FIL1δ, -ϵ, -ζ, and -η, with FIL1 being an acronym for Family of IL-1.

EXPERIMENTAL PROCEDURES

Cloning of Novel Human IL-1 Family Members

The following details supplement the general descriptions given under "Results" for the cloning of the individual IL-1 family members.

FIL1δ—A 469-base pair single-stranded ^{32}P-labeled PCR product spanning the entire mouse FIL1δ coding region (found in GenBank™ W08205) was used to probe a human placenta cDNA library (in λUniZAP XR; Stratagene number 937225) (hybridization in 40% formamide at 42 °C; wash in 0.3 M NaCl at 55 °C). Several clones were isolated, all of which appeared to lack the full open reading frame by comparison with mouse FIL1δ. Vector-anchored PCR on DNA from the same library was used to isolate the remaining coding sequence.

FIL1ϵ—A human genomic library (Stratagene catalog number 946205; in λFixII) was screened using a ^{32}P-labeled single-strand DNA probe corresponding to the entire IL-1-like coding sequence present in GenBank™ EST AA030324 (hybridization in 45% formamide at 42 °C; wash in 0.3 M NaCl at 63 °C). The insert from one positive plaque was mapped to locate the hybridizing region, sequencing of which then revealed the 3′-most exon of the human *FIL1ϵ* gene. 5.3 kilobases of human genomic DNA to the 5′ side of this exon was isolated using the CLONTECH Human GenomeWalker kit (catalog number K1803-1). Sequencing of this DNA allowed identification of the remaining coding exons. The structure of the gene was confirmed by isolation of a PCR product in which the predicted exons were indeed spliced, using as template first-strand cDNA from the cell lines HL60 and THP1, and from human thymic tissue. Interestingly, while the original genomic DNA sequence coded for glutamine at amino acid 12, cDNA clones from all three sources contain arginine at amino acid 12.

FIL1ζ—The FIL1ζ open reading frame was identified in a cDNA library made from the pancreatic tumor cell line HPT-4.

FIL1η—A human genomic DNA sequenced to identify the *FIL1η* 3′ exon was obtained using the CLONTECH Human GenomeWalker kit (catalog number K1803-1).

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The nucleotide sequences reported in this paper for FIL1δ, FIL1ϵ, FIL1ζ, and FIL1η have been deposited in GenBank™ with accession numbers AF201830, AF201831, AF201832, and AF201833.

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[1] The abbreviations used are: IL, interleukin; AcP, accessory protein; IL-1ra, interleukin 1 receptor antagonist; PCR, polymerase chain reaction; PDF, probability density function; LPS, lipopolysaccharide.

TABLE I

PCR primer sequences used for analyzing expression of novel IL-1 family member mRNA

On some occasions, a second PCR reaction using nested primers was performed for FIL1δ and FIL1ε. Cycle numbers and annealing temperatures are also given.

Molecule	Primary or nested	Sense/anti	Sequence	No. cycles	Anneal °C
FIL1δ	Primary	Sense	GGGAGTCTACACCCTGTGGAGCTCAA	30	58
FIL1δ	Primary	Antisense	CTGCTGGAAGTAGAAGTCTGTGATGG		
FIL1δ	Nested	Sense	GGAGCTCAAGATGGTCCTGAGTGGGGCGCT	30	58
FIL1δ	Nested	Antisense	GCATTCCAGCCACCATTCTCGGGAAGCT		
FIL1ε	Primary	Sense	GACACACCTCAGCAGGGGAGCATTCAGG	40	60
FIL1ε	Primary	Antisense	AACAGCATAGTTAACCCAAAGTCAGTAG		
FIL1ζ	Primary	Sense	TGAGATCCTATGTCAGGCTGTGATAGG	40	60
FIL1ζ	Primary	Antisense	TGCTATGAGATTCCCAGAGTCCAGGACC		
FIL1η	Primary	Sense	ACATCATGAACCCACAACGGGAGGCAGCAC	35	60
FIL1η	Primary	Antisense	CTCTATCCTGGAACCAGCCACCCACAGC		
FIL1η	Nested	Sense	CCAAATCCTATGCTATTCGTGATTCTCGAC	35	58
FIL1η	Nested	Antisense	GGATTTATTCCACAGAATCTAAGTAGAAG		

Structure Modeling

Template sequences for structure modeling were extracted from the Protein Data Bank (28). A sequence alignment for the superfamily was generated based on that proposed by Bazan *et al.* (19) for the IL-1s and IL-18, which appeared valid by examination of both cysteine and real *versus* predicted sheet alignments. Preliminary analysis using the program Gene Fold (29) demonstrated that the experimentally determined structures for IL-1β (Protein Data Bank designations 1hib, 2i1b, 1iob, 1itb, and 21bi) and IL-1ra (Protein Data Bank designations 1ilr, 1ira, and i1rp) were valid templates for the new IL-1 family members FIL1δ, FIL1ε, and FIL1ζ. Although the sequence identity of the new IL-1-like cytokines is greater to IL-1ra, Gene Fold showed a stronger match to the structure of IL-1β. In addition, the various IL-1β structures appear better aligned structurally (as seen by superposition) than do the IL-1ra structures, so both templates were used for modeling. Modeler (30) was used to generate a family of 20 structures for each query sequence. All structures showed a well defined core β-trefoil, with higher variability in the outer loops; the per molecule probability density function (PDF) used by modeler varied from 1194 to 1984. The structure with the lowest overall PDF violation was visualized using a variable width ribbon based on the per residue PDF violation and showed that the highest violation was in the region of highest structural difference between IL-1ra and IL-1β. At this point the cysteine positions for the three models were revisited to ensure that no disulfide links were missed. A representative structure for each model was chosen by first ordering the models within a family by total PDF violation. After discarding structures with obvious problems, such as knots, the remaining members were then superimposed onto their mean. The structure with the lowest all atom root mean square deviation from the mean was chosen as the representative structure. Finally, the models were analyzed using Procheck (31) and it was shown that the structures are valid at 2.0-Å resolution with no major structural problems. The structure models for FIL1δ, FIL1ε, and FIL1ζ can be examined by contacting the authors.

Intron/Exon Mapping

Intron placement was determined by direct cloning or amplification of genomic DNA from the various novel IL-1 loci. Primers were designed so that the PCR products overlapped one another to ensure that small introns were not overlooked. The sequence of the PCR products from genomic DNA was compared with the cDNA sequence in order to determine the exon/intron junctions.

Chromosome Mapping

Chromosome mapping was performed using the Genebridge 4 radiation hybrid panel (32) (catalog number RH02.05 from Research Genetics) which consists of 93 human/hamster hybrid cell lines. Genomic DNA from the cell lines was amplified using PCR primers specific for the human version of each novel IL-1 family gene. Products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining. Each hybrid was scored in the following manner: 0 was assigned if there was clearly no amplification; 1 was assigned where there clearly was amplification; a score of 2 was assigned where the data was ambiguous. Scores were then submitted to the Whitehead Institute/MIT for chromosomal assignment and placement relative to known framework markers on the radiation hybrid map. Scores for each gene are as follows: *FIL1δ*, 1000000100000000100012010010012-0000110110000100000000010100001000112000000010001000010001-0100; *FIL1ε*, 1010001000010001000110112100110000111010010-0000100010101002101111000000100001000110001010 0; *FIL1ζ*, 1010-0001000010001000120122100100000110110100100000100010101001-1021110000001000010001120010100; *FIL1η*, 1220002000100000100-0000100100100012000110020100000000010100000100022000000010-0001000110001010 0. Scores used for concomitant map placement of IL-1α, IL-1β, and IL-1ra were obtained from the NCBI (*IL-1α*, X02851; *IL-1β*, D20737 and AA150507; *IL-1ra*, H50548, R49297, and T72887).

Expression Analysis

First-strand cDNAs present in CLONTECH (Palo Alto, CA) Human Multiple Tissue cDNA Panels I (catalog number K1420-1) and II (catalog number K1421-1) and the Human Immune Panel (catalog number K1426-1) were screened by PCR amplification using primers given in Table I. The primers were designed to span introns so that products arising from genomic DNA and cDNA could be distinguished. In some cases, nested primers were used in a second PCR reaction. The presence of an amplification product for each gene/tissue combination was determined by analysis on agarose gels stained with ethidium bromide.

Alternatively, individual cell types from human peripheral blood were isolated from multiple donors, and stimulations were performed as described (33, 34, 41). In brief, NK cells were incubated with IL-12 (R & D Biosystems; 1 ng/ml) for either 2 or 4 h. T cells were unstimulated or stimulated with anti-CD3 (OKT-3 antibody, immobilized on plastic at 5 μg/ml) or with the combination of anti-CD3 and anti-CD28 (the anti-CD28 antibody was CK248, used in soluble form as a 1:500 dilution of ascites fluid), for 4 h. Monocytes were unstimulated, or stimulated with LPS (Sigma, 1 μg/ml) for 2 or 3 h. B cells were unstimulated, or stimulated with a combination of 005% SAC + 500 ng/ml CD40L trimer (Immunex) + 5 ng/ml IL-4 (Immunex) for 3.5 or 4 h. Dendritic cells were stimulated with LPS as for monocytes for 2 or 4 h. After isolation of RNA and synthesis of first-strand cDNA, PCR amplifications and gel analysis were performed as described above.

Expression of Novel IL-1 Family Proteins for Receptor Binding

Novel IL-1 family members, as well as control IL-1β and IL-18 molecules, were generated by transfection of expression vector constructs into COS cells using DEAE-dextran (35). Expression vectors used were pDC409 (36) for FIL1ε and FIL1ζ, or pDC412, a close relative, for FIL1δ and FIL1η. The unmodified open reading frames were used for FIL1δ, ε, and η. For FIL1ζ, the sequence beginning with Lys^{27} (KNLN. . . . .) was fused downstream of the human immunoglobulin κ light chain signal peptide. IL-1β, with an ATG codon added to the N terminus of the mature form (beginning with Ala^{117}), was expressed in pDC409. Human IL-18 was expressed as the mature form fused to the IL-7 signal peptide in the expression vector pDC206 (37). For radiolabeling, 48 h after transfection cells were starved of cysteine and methionine for 60 min, then labeled with 70 μCi/ml of a [^{35}S]cysteine/methionine mixture (Amersham; >1000 Ci/mmol) for 4–6 h. It is perhaps of interest that FIL1δ, FIL1ε, and FIL1η appear to be secreted from the COS cells despite the absence of either signal peptide or prodomain. C-terminal FLAG-tagged FIL1δ and -ε were partially purified from the conditioned medium using the tags, and their N termini sequenced. The N-terminal amino acid of the secreted FIL1ε was methionine 1; it had been modified by N-terminal acetylation. The N-terminal amino acid of the secreted FIL1δ is valine 2. Thus, there does not appear to have been

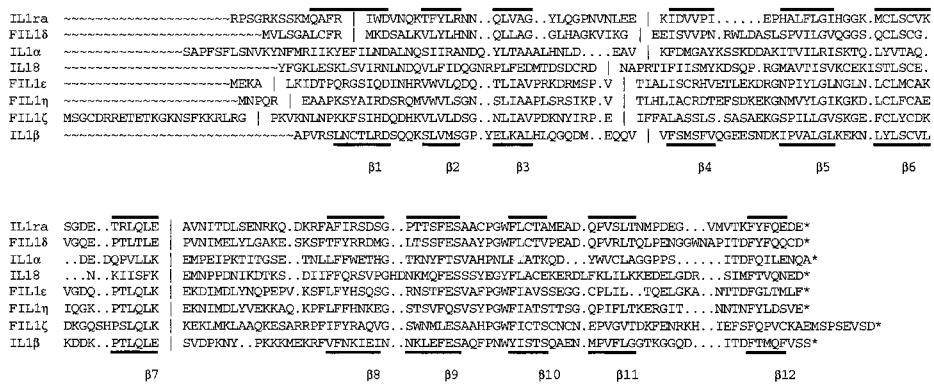

FIG. 1. **Alignment of amino acid sequences for human members of the IL-1 superfamily.** The full-length predicted translation products are shown for FIL1δ, -ε, -ζ, and -η, whereas the mature peptides, without prodomains or signal peptide, are given for IL-1α, IL-1β, IL-1ra, and IL-18. The alignment is based on that presented by Bazan *et al.* (19) with slight modifications. *Bars above* and *below* the sequence indicate regions of experimentally determined (IL-1β and IL-1ra) or proposed β-strand. The *vertical lines* within each sequence indicate intron positions. Some of these are taken from GenBank™ (*IL-1α*, accession number X03833; *IL-1β*, accesssion number X04500; *IL-1ra*, accession number X64532; *IL-18*, accession number E17138). The rest were determined for this paper. The sequences of FIL1δ, FIL1ε, FIL1ζ, and FIL1η have been deposited in GenBank™ (accession numbers AF201830, AF201831, AF201832, and AF201833).

cleavage of an unrecognized signal peptide or prodomain in either molecule. There are a number of proteins which, when transfected into COS cells, do not later appear in the medium, so this is not a general phenomenon attributable to leaky cells. However, the intracellular version of IL-1ra (icIL-1ra, a kind gift of William Arend, University of Colorado) also appears in the medium following transfection of COS cells. The significance of these findings is currently unknown.

Receptor Binding Assays

The novel IL-1 family members, present as ^{35}S-labeled proteins in conditioned medium from transfected COS cells, were tested for binding to Fc fusion proteins of the IL-1 receptor superfamily members (see Footnote 2 for general methods)[2] IL-1R type I, IL-1R AcP, IL-1Rrp1, IL-1Rrp2, IL-1R AcPL, and T1/ST2 as follows: 0.5–1.0 ml of conditioned medium was pre-cleared for 2 h at 4 °C with 50 μl of protein G-Sepharose (Amersham Pharmacia Biotech; 50% solution in phosphate-buffered saline) containing 1% Triton X-100, 0.02% NaN_3, and protease inhibitors (Roche Molecular Biochemicals catalog number 1 836 145). After a brief spin (3 min, 1000 rpm), the supernatant was transferred to a fresh tube and incubated overnight at 4 °C with 1 μg of receptor/Fc fusion protein plus another 50 μl of protein G-Sepharose. The mixture was centrifuged briefly, and the supernatant mixed with 0.5 ml of phosphate-buffered saline containing 5% glucose and protease inhibitors and spun again. The pellet was washed four times with 1 ml of a solution containing 0.4 M NaCl, 0.05% SDS, 1% Nonidet P-40, and protease inhibitors, and resuspended in 25 μl of 2 × reducing sample buffer (Zaxis, catalog number 220-2110106). Samples were run on 4–20% Tris glycine gels (Novex) and autoradiographed.

RESULTS

The four previously known members of the IL-1 family (IL-1α, IL-1β, IL-1ra, and IL-18), while possessing a low overall fractional amino acid identity, share certain common amino acid sequence motifs, the most obvious of which can be summarized as F(X_{10-12})F*X*S(AVS)*XX*(PE)*XX*(FY)(LI)(CAS)(TC) where *X* is any amino acid, and parentheses indicate that one of the included amino acids is present at that position. There are similarities in intron placement within the family as well. Relying on the sequence similarity, we searched public EST data bases and found sequences corresponding to three novel IL-1 family members, described below as FIL1δ, FIL1ε, and FIL1ζ. A fourth novel family member, described below as FIL1η, was originally revealed in a published patent application. Examination of the sequence (called IL-1δ by the inventors) in the patent application suggested that it was derived from an aberrantly spliced mRNA. We searched for and found an alternative form of mRNA that contains the conserved family sequence motif in the extreme 3′ exon. A brief description of the cloning and characteristics of each of the family members is given below. The sequences, and a comparison with the previously known IL-1 superfamily members, are given in Fig. 1.

FIL1δ

A search of GenBank™ revealed a murine EST, accession number W08205, that resembled the known IL-1s but was not identical to any. The IMAGE clone corresponding to the EST was sequenced and found to contain the entire open reading frame of an IL-1-like molecule. Unlike the known family members, this novel polypeptide (called FIL1δ) appeared to contain neither a signal peptide nor a prodomain at the N terminus. A human FIL1δ cDNA was then isolated from a human placenta cDNA library, using mouse FIL1δ as a probe. The human sequence predicted an open reading frame similar to that of mouse FIL1δ. Multiple FIL1δ cDNA clones from both species were subsequently isolated, and all had the same predicted open reading frame, with no evidence for isoforms containing either signal peptide or prodomain. Interestingly, among the cDNA clones from both species were found several different 5′-untranslated region sequences (data not shown). These different 5′ sequences appear to derive from separate exons, in that they can be found (separately) in genomic sequence upstream of the *FIL1δ* coding region, and have potential splice donor sites at their 3′ ends. Presumably the *FIL1δ* gene is transcribed from at least two promoters.

FIL1ε

A later search of GenBank™ revealed a murine EST, accession number AA030324, that resembled a second novel IL-1 family member. Sequencing of the IMAGE clone corresponding to the EST showed an open reading frame that appeared to encode the C-terminal portion of an IL-1 molecule, but which could not be extended in the 5′ direction. The mouse sequence was used to probe a human genomic library, and a positive clone was identified and the insert sequenced. The sequence revealed a 212-base pair region with homology to the 3′-most exon of mouse *FIL1ε*. There was a potential splice acceptor site at the 5′ end of this region, and a stop codon at the 3′ end of the 70-amino acid open reading frame. More human genomic DNA to the 5′ side of this open reading frame was then isolated and sequenced, revealing three additional putative exons with sequence similarity to the mouse EST and to other IL-1 family members. On the assumption that the four putative exons were

[2] Born, T. L., Morrison, L. A., Esteban, D. J., VandenBos, T., Thebeau, L. G., Chen, N., Spriggs, M. K., Sims, J. E., and Buller, M. L. (2000) *J. Immunol.*, in press.

spliced to form a single coding region, PCR primers were designed and used successfully to amplify the predicted product from several different human RNA sources. As for FIL1δ, the predicted FIL1ϵ reading frame contains neither a signal peptide nor a prodomain.

FIL1ζ

A third EST, accession number AI014548, was found in GenBank™ that appeared to encode an IL-1-like molecule. However, further sequencing revealed that the corresponding (human) IMAGE clone contained a stop codon upstream of the open reading frame but no initiating methionine. Screening of two other cDNA libraries resulted in isolation of a second, distinct aberrant clone, as well as a clone that contained an open reading frame that did begin with a methionine and that extended for 192 amino acids. This last clone was named FIL1ζ. Sequence comparison with other family members suggests that FIL1ζ has a prodomain of some 15–30 amino acids.

Analysis of genomic DNA demonstrated that an intron lies between the nucleotides encoding the 23rd and 24th amino acids of the 192 amino acid open reading frame form (see Figs. 1 and 4). The stop codon-containing sequences found in the aberrant cDNA clones lie within this intron, and appear to be incorporated into mRNA by cryptic splicing events. Since we had found three different cDNA isoforms for FIL1ζ, only one of which appeared to contain a functional open reading frame, it was important to determine the relative levels of the different transcripts. This was done by designing PCR primers that would amplify and distinguish the three isoforms, and using them to examine expression in a panel of first-strand cDNAs. The (presumably functional) FIL1ζ transcript was found in lymph node, thymus, bone marrow stroma, lung, testis, and placenta (Table II). We could not detect the form of mRNA represented by the EST in any tissue, whereas that represented by the other form of "aberrant" mRNA was present in bone marrow stroma (from which we had originally isolated it), lung, and placenta but not in the other tissues (not shown). The mRNA encoding that form appeared to be much less abundant than the functional FIL1ζ mRNA.

FIL1η

A cDNA clone containing part of the FIL1η sequence was originally identified in an osteoclastoma library (38). The DNA sequence presented in this document appeared to encode the N-terminal half of an IL-1 like molecule, which then diverged in the C-terminal portion. Since the C-terminal regions of the different IL-1 family members contain the greatest sequence conservation, including the motif described above, and since the point of divergence lay exactly at the position of a conserved intron in the IL-1 family (see below), we searched for an alternative transcript that might encode a more typical member of the family.

PCR with first strand cDNA templates from various tissue sources, using primers lying entirely within the 5′-half of the osteoclastoma coding sequence (38), gave products from tonsil, bone marrow, heart, placenta, lung, testis, and colon. Only very faint bands were obtained, and only in tonsil and testis, when a 5′ primer from the 5′-half and a 3′ primer from the 3′-half of the osteoclastoma coding sequence were used, consistent with our interpretation. Human genomic DNA containing the 5′-half of the osteoclastoma sequence and extending further downstream was then isolated and sequenced. A putative exon was found 823 base pairs downstream of the point of divergence of the osteoclastoma sequence from other family members; this putative exon contained the expected sequence motifs for the C-terminal portion of an IL-1 family member, as well as a potential splice acceptor site at its 5′ end. PCR primers designed to amplify a hypothetical cDNA formed by splicing of the 5′ portion of the osteoclastoma sequence with this 3′ exon did indeed give a product from human tonsil first strand cDNA, which when sequenced contained the predicted 157-amino acid open reading frame. The open reading frame, and the gene encoding it, were named FIL1η. Like FIL1δ and FIL1ϵ, FIL1η does not contain an apparent signal peptide or prodomain.

TABLE II

Expression data for novel IL-1 family members

The table summarizes all available expression data on the novel IL-1 family members. "–" indicates that the mRNA was looked for but not found; a blank space indicates that the analysis was not done for that particular gene/RNA combination. Positive results were derived as follows: "a", by PCR analysis from a panel of first strand cDNAs (Clontech); "b", by cDNA library screening; "c", by the existence of an EST; "d", by PCR analysis of an individual RNA. "e" indicates that expression of the gene was increased by LPS. In the source column for tissues, "pool" was a mixture of fetal lung, testis, and B cell. In the source column for human cell lines, MoT and HUT102 are T-cell lines; Raji is a B cell line; THP-1 and U937 are macrophage lines; IMTLH is an unpublished bone marrow stromal cell line, derived at Immunex; HL60 is an early hematopoietic precursor line; HPT-4 is a pancreatic tumor line; T84 is a colon tumor line. For FIL1δ in lung, (a) indicates that the PCR product lacks exon 2. The PCR products for FIL1δ, FIL1ϵ, and FIL1η were especially strong from tonsil RNA, while that for FIL1ζ was strong in placenta and testis.

Source	FIL1δ	FIL1ϵ	FIL1ζ	FIL1η
Human tissue				
Spleen	–	a	–	–
Lymph node	a	a	a	–
Thymus	a	a	a	–
Tonsil	a	a	–	a
Bone marrow	–	b	a,b	a
Fetal liver	–	–	–	–
Leukocyte	–	a	–	–
Heart	–	–	–	a
Brain	a	–		–
Placenta	a,b	–	a	a
Lung	(a)	–	a	a
Liver	–	–	–	–
Skeleton muscle	a	–	–	–
Kidney	–	–	–	–
Pancreas	–	–	–	–
Prostate	a	–	–	–
Testis	a	–	a	a
Ovary	–	–	–	–
Small intestine	–	–	–	–
Colon	–	–	–	a
Fetal brain		b		
NK cells	b			
Parathyroid tumor	c			
Colon tumor			c	
Pool			c	
Human cell lines				
Mo-T		b		
HUT-102		b		
Raji		b		
THP-1		d	d	
U937			d,e	
IMTLH		d	d	
HL60		d	d	
HPT-4		b	b	
T84		b		
Mouse tissue				
Spleen	d,e	d,e		
Kidney	b			
Placenta/yolk sac	d	c		
Embryo	c			
Stomach	c	c		
Tongue	c	c		
Skin	c			
Mouse cell lines				
Macrophage (RAW)	d,e	b,d,e		

TABLE III
Sequence identity

The numbers represent percent sequence identity between the indicated IL-1 superfamily members, as determined by using the program "gap" (Wisconsin Package Version 10.0, Genetics Computer Group (GCG)). For IL-1α, IL-1β, IL-18, and IL-1ra, the mature peptide (lacking signal sequence or prodomain) was used for the comparison. For FIL1ζ, the mature form was assumed to start with Lys27, based on primary sequence alignment and analysis of predicted eight-dimensional structure, and this sequence was used.

	IL-1α	IL-1β	IL-1ra	IL-18	FIL1δ	FIL1ϵ	FIL1ζ	FIL1η
IL-1α	…	24	20	21	20	23	21	26
IL-1β		…	31	17	32	27	24	32
IL-1ra			…	22	50	30	29	30
IL-18				…	27	20	21	21
FIL1δ					…	31	35	37
FIL1ϵ							36	46
FIL1ζ						…	…	44
FIL1η								…

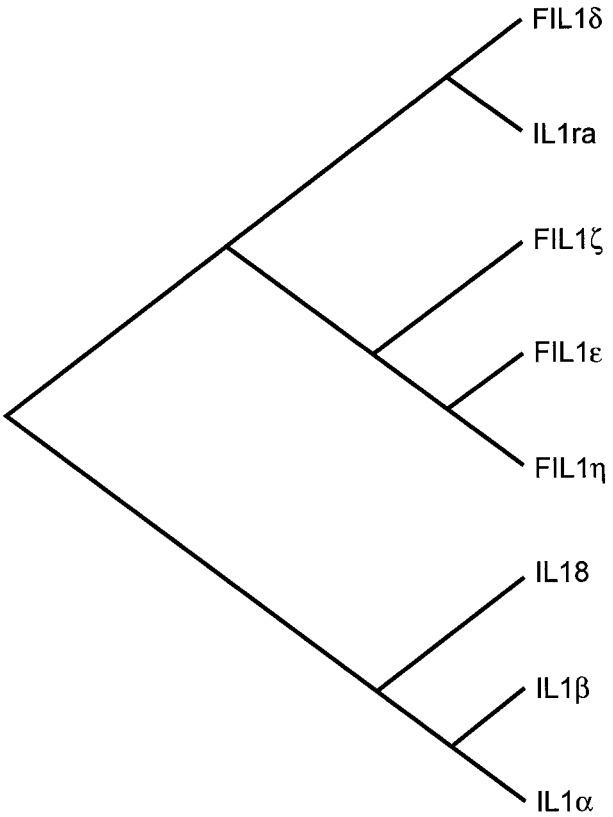

FIG. 2. **Dendrogram illustrating the relationship between members of the IL-1 superfamily.** The dendrogram was generated by the program Treeview (40) using the amino acid sequence alignment produced by the program Pileup (Wisconsin Package Version 10.0, Genetics Computer Group (GCG), Madison, WI).

A Gene Family

Sequence Comparison—The novel members of the IL-1 family are approximately as similar to one another in sequence as they are to the classical IL-1s; this level of identity is in turn similar to that shown by the classical IL-1s among themselves. Typically any given pair of family members shows 20–35% sequence identity (Table III). Those that stand out as being more similar to one another than average are FIL1δ/IL-1ra, FIL1ϵ/FIL1η, and FIL1ζ/FIL1η. These relationships can also be seen in the dendrogram presented in Fig. 2, in which it appears that IL-1α, IL-1β, and IL-18 form one sequence subfamily; FIL1ϵ, FIL1ζ, and FIL1η form a second subfamily, and IL-1ra and FIL1δ form a third. The novel members can easily be placed onto the structure-based sequence alignment presented by Bazan *et al.* (19) (Fig. 1).

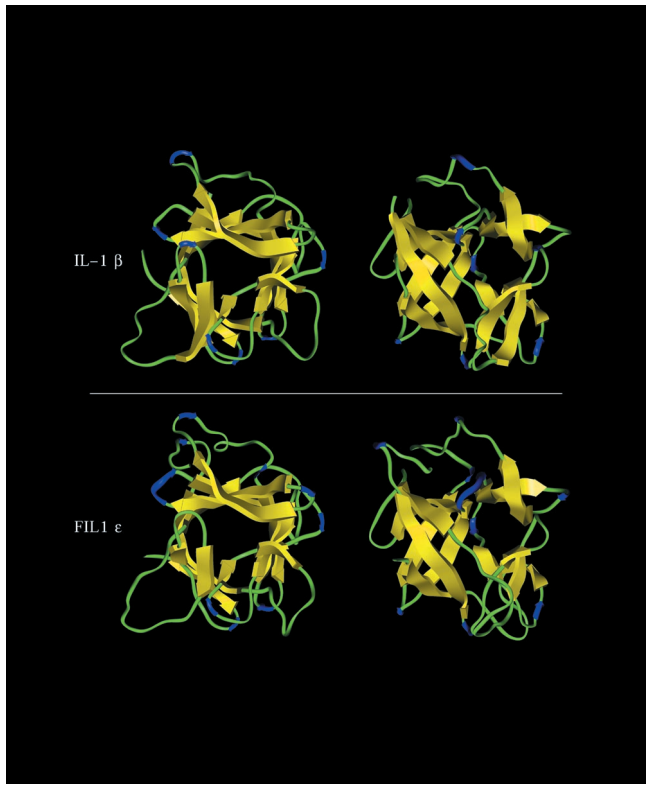

FIG. 3. **Structure models.** Structural models of IL-1β and FIL1ϵ were generated as described under "Experimental Procedures." The figure shows views looking down the opening of the barrel in the β-trefoil structures, as well as views of the models rotated by 90° along the *x* axis. *Yellow*, β-strand; *green*, coil; *blue*, β-turn.

Three-dimensional Protein Structure Prediction—The structures of FIL1δ, FIL1ϵ, and FIL1ζ have been modeled using as templates the experimentally determined structures of IL-1β and IL-1ra. The novel IL-1 superfamily member amino acid sequences could with minimal energy violations be folded into structures which superimpose well onto the IL-1β and IL-1ra crystal structures. In particular, the core 12-stranded, β-trefoil structure appears well conserved (see Fig. 3 for FIL1ϵ). The major points of difference between the FIL1δ, FIL1ϵ, and FIL1ζ models, and between them and the experimental structures, lie in the loops connecting the β strands, where IL-1β and IL-1ra also differ most from each other.

Genomic Structure—The known genes of the IL-1 family display a conserved pattern of intron placement and intron/exon junctions, clearly indicating their derivation from a common ancestor. The novel IL-1 family members presented here demonstrate the same pattern. The most C-terminal intron in the coding region always falls between codons, and lies immediately after the predicted β-strand 7. By analogy to the structure of the *IL-1α* and *IL-1β* genes, we have called this intron 5, even though the rest of the family has only three introns within the coding sequence (except *IL-18*, which has four). Intron 4 (the intron N-terminal to intron 5) falls between the first and second nucleotides of the codon, and lies just N-terminal to β-strand 4. Intron 3 is more variable in placement. In *IL-1α*, *IL-1β*, *IL-18*, and probably in *FIL1ζ*, it lies within the prodomain, not far upstream of the site of processing. In the other family members, it appears to lie not far downstream of the initiating methionine. It is also more variable in placement within the codon, falling after either the first (*IL-1α*, *IL-1β*, *IL-18*, *FIL1ϵ*, *FIL1ζ*, *FIL1η*) or second (*IL-1ra, FIL1δ*) nucleo-

tide of the codon.

Chromosomal Location—The novel IL-1 family members have been mapped using the radiation hybrid method. They cluster on human chromosome 2q, between the framework markers D2S121 and D2S110 (not shown). The *IL-1α/IL-1β/IL-1ra* cluster lies within this same interval. At the level of resolution seen with the Genebridge 4 panel, the novel and classical IL-1 genes appear to be interspersed.

Expression Pattern

We have analyzed the expression pattern of the novel IL-1 family members in several ways. Using a panel of first strand cDNAs derived from various tissues as templates for PCR, we find that the novel family members are all expressed in lymphoid organs, although the detailed pattern differs somewhat from cytokine to cytokine (Table II). RNA for each is also present in a small number of non-lymphoid tissues. Table II also summarizes expression data obtained from cDNA library screening, from searching EST data bases, and from PCR analysis of individual RNA samples. No easy generalization about expression patterns, either for the individual cytokines or for the family, is obvious.

Hematopoietic Subsets—We wanted to look specifically at expression of each of the novel IL-1 family members in individual cell types from peripheral blood. Cells were prepared from human blood and cultured for a short time in various conditions. RNA was made and analyzed by RT-PCR for the presence of FIL1δ, -ϵ, -ζ, and -η (Table IV). All family members were expressed in activated monocytes and B cells. In most cases, there was some expression present in these cells even without stimulation. FIL1δ was also expressed in activated dendritic cells. The only one of the family members expressed by T cells was FIL1ϵ.

Receptor Binding—We asked whether any of the novel IL-1 family members could bind to the known members of the IL-1 receptor family. Conditioned medium from COS cells transfected with each of the different novel ligands and labeled with [^{35}S]Cys/Met, as well as from COS cells transfected with IL-18 as a positive control, were incubated with Fc fusions of the characterized IL-1R family members (IL-1R type I, IL-1R AcP, IL-1Rrp1, IL-1Rrp2, AcPL, and T1/ST2), followed by precipitation of the Fc proteins using protein G. The precipitates were electrophoresed on SDS gels, and autoradiographed to see whether any of the ligands were able to bind to any of the receptors. While IL-18 was seen consistently to bind to IL-1Rrp1, no other complexes were observable using this technique (data not shown).

TABLE IV

Expression of novel family members in hematopoietic cell subsets

Expression of IL-1 family members was determined by PCR analysis of RNA isolated from subsets of peripheral blood mononuclear cells, obtained as described under "Experimental Procedures."

Cell subset	FIL1δ	FIL1ϵ	FIL1ζ	FIL1η
NK cell + IL-12	ND[a]	ND	+	ND
T cell	−	+	−	−
Monocyte	++	+	ND	+
Monocyte + LPS	++	++	++	++
B cell	++	+	−	+
B cell stimulated	++	+	+	+
Dendritic cell + LPS	++	−	ND	ND

[a] ND, not done.

DISCUSSION

We describe here the discovery of novel genes that double the size of the IL-1 superfamily. Assessment of the *FIL1δ*, *FIL1ϵ*, *FIL1ζ*, and *FIL1η* genes as paralogs of *IL-1α*, *IL-1β*, *IL-1ra*, and *IL-18* is based on several factors. First, sequence alignment (Fig. 1) reveals certain conserved amino acid motifs. Not only is there easily recognizable conservation of primary structure, but the amino acid sequences readily allow modeling into a predicted three-dimensional structure that is conserved with the known IL-1s (Fig. 3). In addition, intron placement is highly conserved in these new genes and is similar to that found in the "traditional" IL-1s as well as *IL-18* (Figs. 1, 4). This provides an independent measure of evolution from a common ancestor. Finally, consistent with evolution by gene duplication, the new IL-1 superfamily members are all clustered in the same region of human chromosome 2q that contains *IL-1α*, *IL-1β*, and *IL-1ra*. *IL-18* is the only superfamily member that does not map to this location.

The novel IL-1 family members are expressed in a variety of hematopoietic and non-hematopoietic cell types. It is not easy to formulate generalizations about expression patterns, except to say that FIL1ϵ appears to be the only one of these putative cytokines routinely expressed in T cells, and (not unexpectedly) all of the family members are expressed in activated monocytes and B cells. From the infrequency of ESTs corresponding to these genes in GenBank™, as well as the number of PCR cycles required to detect an amplification product in positive RNA sources, it would appear that they are all expressed at relatively low abundance. Nevertheless, FIL1δ, FIL1ϵ, and

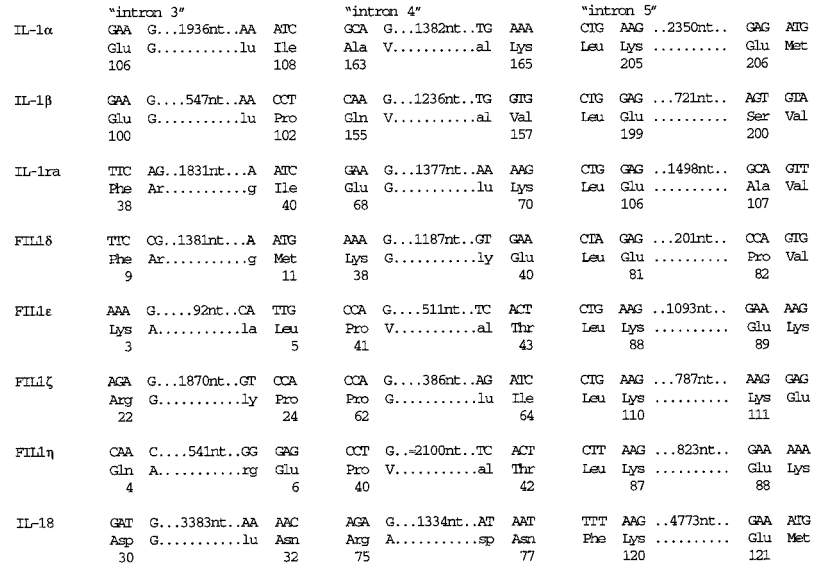

FIG. 4. **Intron positions.** Intron positions were taken from GenBank™ (*IL-1α*, accession number X03833; *IL-1β*, accession number X04500; *IL-1ra*, accession number X64532; *IL-18*, accession number E17138) or were determined for this paper by sequencing of genomic DNA (either directly cloned, or PCR amplified) and comparison to the cDNA sequences. The "intron 3," "intron 4," "intron 5" designations are by analogy with *IL-1α* and *IL-1β*, and do not imply that there are five introns in each of the other genes. Intron sizes, where known, are indicated. Amino acid numbers are given *below* the lines, and refer to the primary translation product.

FIL1ζ can be regulated by LPS (and most likely other agents) in monocytes and spleen cells, and FIL1δ appears to be transcribed from at least two different promoters, indicating that regulation of expression in this family is active.

It might be expected that the new IL-1 superfamily members would bind to members of the IL-1 receptor superfamily. However, we have been unable to demonstrate this in co-precipitation assays using Fc fusions of the known receptors and orphan receptor homologs. It could be that there are as yet undiscovered members of the IL-1R superfamily. Alternatively, unlike the case with the IL-1s and IL-18, high affinity binding detectable by co-precipitation may require two receptor subunits to be present. Finally, of course, it is possible that these cytokines bind to a different type of receptor. IL-18, for example, was recently shown to be capable of binding with high affinity to a soluble protein that has little similarity to other IL-1R family members (39).

The biological activity of the novel IL-1 family members remains to be characterized. IL-1α, IL-1β, and IL-18 are all capable of activating gene expression programs that enhance immune responses and promote inflammation. It is obvious to speculate that FIL1δ, FIL1ϵ, FIL1ζ, and FIL1η might have similar actions. On the other hand, IL-1ra acts to block the actions of the agonist IL-1s, and it is possible that one or more of the novel family members might similarly play an antagonist role. In this context, however, it should be noted that none of them binds to either the type I IL-1R or to IL-1Rrp1, and therefore they presumably do not regulate signaling by either IL-1 or IL-18. It is also possible that the resemblance of these molecules to IL-1 says nothing at all about their receptor binding or the type of biological responses they might invoke. These issues will be clarified by further investigation.

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