Identification and Initial Characterization of Four Novel Members of the Interleukin-1 Family

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The interleukin (IL)-1, fibroblast growth factors (FGFs), and their homologues are secreted factors that share a common β-barrel structure and act on target cells by binding to cell surface receptors with immunoglobulin-like folds in their extracellular domain. While numerous members of the FGF family have been discovered, the IL-1 family has remained small and outnumbered by IL-1 receptor homologues. From expressed sequence tag data base searches, we have now identified four additional IL-1 homologues, IL-1H1, IL-1H2, IL-1H3, and IL-1H4. Like most other IL-1/FGFs, these proteins do not contain a hydrophobic leader sequence. IL-1H4 has a prepeptide sequence, while IL-1H1, IL-1H2, and IL-1H3 encode only the mature protein. Circular dichroism spectra and thermal stability analysis suggest that IL-1H1 folds similarly to IL-1α. The novel homologues are not widely expressed in mammals. IL-1H1 is constitu­tively expressed only in placenta and the squamous epithelium of the esophagus. However, IL-1H1 could be induced in vitro in keratinocytes by interferon-γ and tumor necrosis factor-α and in vivo via a contact hypersensitivity reaction or herpes simplex virus infection. This suggests that IL-1H1 may be involved in pathogenesis of immune mediated disease processes. The addition of four novel IL-1 homologues suggests that the IL-1 family is significantly larger than previously thought.

The interleukin (IL)-1/fibroblast growth factor (FGF) family is a growing family of proteins that share a common β-barrel structure consisting of 12 β-strands (1–4). There are currently 19 members of the FGF family (FGF1–19) (5–7) and four members of the IL-1 family (IL-1α, IL-1β, IL-1ra, and IL-18) (8, 9). With a few exceptions, most IL-1/FGFs are synthesized as intracellular proteins without a characteristic hydrophobic leader peptide but nevertheless are released from cells and act on target cells by binding and signaling through cell surface receptors. In some cases, such as IL-1α and IL-18, proteolytic cleavage of a precursor form of the protein is required for elaboration of the active form of the ligand (10–13). The IL-1 and FGF receptors also share structural homology in their extracellular ligand binding domain, since they both consist of multiple immunoglobulin-like repeats (3, 4, 14), and this appears to lead to a similar mode of ligand recognition (15).

While the overall folds of the ligands and receptor extracellular domains have been maintained evolutionarily, the family has evolved into two distinct arms, the IL-1s and the FGFs, which show no significant sequence homology and have distinct biological properties. The limited sequence homology is also seen between IL-1 family members and results from the small number of internal residues required to maintain the β-barrel structure and the extensive use of backbone elements in receptor recognition (3, 4). The distinction in biological activities reflects differences in the intracellular regions of their cognate receptors. The FGF receptors encode a tyrosine kinase within the intracellular domain that is stimulated upon ligand binding and initiates a signaling cascade that results in cell proliferation. This contributes to their role in stimulating tissue growth during development and repair. In contrast, the IL-1 receptors do not encode any enzymatic activity in their intracellular domains, but upon ligand binding, they recruit two serine/threonine protein kinases, the interleukin-1 receptor-associated kinases, through motifs shared with the highly evolutionarily conserved toll receptor family (16). These stimulate intracellular signaling pathways, leading to the activation of NF-κB and AP-1 transcription factors (17, 18) that in turn induce genes involved in the initiation of immune and inflammatory responses. Thus, IL-1α and β stimulate the production of chemokines, cytokines, and adhesion molecules that serve to recruit leukocytes to sites of infection or injury and initiate an inflammatory response. IL-18 stimulates the production of TH1 helper T cells resulting in a cell-mediated immune response that can lead to cancer immunity and delayed type hypersensitivity responses (19).

IL-1α activate their target receptors by bringing together two different receptor subunits. IL-1α and IL-1β bind with subnanomolar affinity to the cell surface type I IL-1R and recruit IL-1R accessory protein in order to stimulate target cells (17). Similarly, IL-18 binds to the IL-18 receptor (IL1rrp) with 20 nM affinity and recruits the IL-18R accessory protein (20, 21). The activity of both IL-1 and IL-18 is modulated by the soluble or...
membrane-bound decoy receptors, IL-1R type II and IL-18BP, respectively (22, 23). IL-1 activity is also moderated by the presence of a natural receptor antagonist, IL-1ra, which binds to the type I IL-1R but does not recruit IL-1RAcP. However, there are some IL-1R-like receptors that have yet to be paired with ligands, such as ST2/T1 and IL-1rrp2 (24–27). This suggests that there may be additional members of the IL-1 family. In this paper, we describe the discovery of four novel members of the IL-1 family and the initial biological and biochemical characterization of IL-1H1.

MATERIALS AND METHODS

Cells, Coll Culture, and Reagents—Human keratinocytes were obtained from Clonetics (Walkersville, MD) and cultured in growth medium supplied by the vendor. A431 cells were obtained from ATCC (Manassas, VA) and grown in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum. IL-1β and TNFα were produced at SmithKline Beecham, and INFγ was purchased from R&D Systems (Minneapolis, MN).

Identification and Cloning of Novel IL-1 Homologues—Potential full-length cDNAs corresponding to two new members of the IL-1 family (IL-1H1 and IL-1H4) were initially identified through a search of an

![Sequence alignment and schematic representation of various members of the IL-1 family. A, mature regions of various members of the IL-1 family were first aligned using the Clustal algorithm of the MEGALIGN program of Lasergene software (DNASTAR Inc., Madison, WI). The alignment was then subsequently refined manually. Residues matching the majority are boxed and shaded. The arrow bars below the alignment indicate the predicted β-strands for IL-1α and IL-1β that appear to be conserved among other members. B, various members of the IL-1 family are represented schematically to show the precursor, mature region, and cleavage site (arrow) of each protein. In the case of IL-1ra, the differential splicing using alternate exons is also shown. Note that IL-1H4 encodes a putative cleavage site similar to IL-1α, IL-1β, and IL-18. The experimentally determined (arrow) and putative cleavage site (arrow and question mark) and flanking sequence for IL-1β, IL-1H4, and IL-18 are also shown.](http://www.jbc.org/)
assembled version of the public and commercial EST data bases, respectively, using BLAST or FASTA and known members of the IL-1 family. Two additional members (IL-1H2 and IL-1H4) were subsequently found by searching with complete sequences of IL-1H1 and IL-1H3, respectively. The cDNAs corresponding to these ESTs were obtained from Human Genome Sciences (Gaithersburg, MD) (IL-1H1 and IL-1H2) or the IMAGE consortium (mouse IL-1H3: accession no. W08205, IMAGE clone no. 323723; IL-1H4: accession nos. AI014548 and AI343258, IMAGE clone no. 1628761). Complete sequencing of these cDNAs showed that they contained an entire coding region for each homologue. A portion of the sequence of IL-1H1 matched with human STS CHLC.GAAT11C03.F3330 clone GAAT11C03 (accession no. G942011), which has been mapped to chromosome 2q approximately 142 centimorgans from the end of the chromosome. A partial, aberrantly spliced mouse homologue of IL-1H1 (accession no. AA030324) was identified in an assembled mouse EST data base in a search with human IL-1H1. A correctly spliced mouse IL-1H1 cDNA homologue was obtained via reverse transcriptase-polymerase chain reaction from RNA isolated from TNFα/INF-γ-stimulated murine keratinocyte PAM 212 cells.

**Expression and Purification**—The full-length cDNA encoding IL-1H1 was subcloned into pET16B (Novagen Inc., Madison, WI) vector at the NdeI site. To ensure an authentic N terminus following factor Xa cleavage, removal of the NdeI site was performed using the Quick-Change System (Stratagene, La Jolla, CA) according to the manufacturer’s recommendations. Human IL-1 H1 was then expressed as an N terminus His6-factor Xa-tagged protein in *Escherichia coli* BL21(DE3) with 1 mM isopropyl-1-thio-β-galactopyranoside at 25 °C for 16 h according to the vendor’s recommendations. *E. coli* cells expressing human and murine IL-1H1 were suspended at a 1:10 ratio in 50 mM Tris-HCl, pH 8, 300 mM NaCl, 1 mM phenylmethylsulfonyl fluoride. The cells were lysed and purified using Nε-νitrilotriacetic acid resin according to the manufacturer’s instructions (Qiagen, Inc., Valencia, CA). The His tag was cleaved off by incubating IL-1H1 overnight at room temperature with factor Xa at a factor Xa/IL-1H1 ratio of 1:100. The mixture of cleaved and uncleaved proteins was passed over a Superdex 75 sizing column in phosphate-buffered saline (PBS) for final purification and to remove any endotoxin. Purified proteins were used to immunize rabbits to generate polyclonal antisera according to standard protocols.

**Circular Dichroism**—Circular dichroism data were measured on a Jasco J-710 CD Spectropolarimeter at 0.3 mg/ml cytokine in a 0.1-cm path length water-jacketed cuvette at 22 °C in 10 mM sodium phosphate, 150 mM NaCl, 0.3 mM EDTA, pH 7.0. Wavelength scans were taken at 0.5 nm/min, and eight spectra were averaged in each case. Concentrations of cytokines were determined by absorbance at 280 nm using extinction coefficients calculated from sequence (28). Thermal stability curves were measured by monitoring at a single wavelength while raising scanning temperature at about 1 degree/min.

**Biophysical Characterization of IL-1H1**—Far-UV circular dichroism spectra of IL-1ra (dashed), IL-1β (dotted), and IL-1H1 (solid) shown as mean residue ellipticity versus wavelength. The shapes and amplitudes of the IL-1ra and IL-1β spectra agreed well with previous reports (35, 36). B, thermal unfolding stability of IL-1ra (open symbols) and IL-1H1 (closed symbols) shown as the fraction of unfolded cytokine versus temperature. Unfolding was monitored by ellipticity at 215 nm. Half-unfolded Tm values determined from the data in the figure are 59 and 61 °C for IL-1ra and IL-1H1, respectively.

**In Vivo Models**—BALB/c mice (*n* = 6) were treated with oxazolone as described previously (30). Briefly, mice were sensitized on day 0 by a single application of 10 μl of a 1.6% solution of oxazolone (Sigma) in ethanol on the left ear. To characterize the early response to oxazolone, the animals were challenged on day 7 with 10 μl of 0.8% solution of oxazolone on the left ear. Ear thickness was measured with a dial micrometer (L. S. Starrett Co., Athol, MA), and tissue samples were collected at various time points after challenge over a 24-h period. In

### Table I

#### Percentage of amino acid similarity between various IL-1 family members

| Cytokine | IL-1ra | IL-1β | IL-1H1 | IL-1H2 | IL-1H3 | IL-1H4 | IL-18 |
|----------|--------|-------|--------|--------|--------|--------|-------|
| IL-1ra   | 100    | 28.4  | 29.1   | 25.0   | 22.8   | 24.5   | 15.7  |
| IL-1β    | 100    | 35.7  | 27.0   | 34.0   | 37.8   | 18.8   | 28.6  |
| IL-1H1   | 100    | 31.1  | 29.1   | 54.1   | 24.5   | 25.0   |       |
| IL-1H2   | 100    | 32.1  | 30.6   | 30.6   | 18.9   |       |       |
| IL-1H3   | 100    | 29.1  | 17.9   | 19.9   |       |       |       |
| IL-1H4   | 100    | 26.0  | 30.6   | 12.2   |       |       |       |
| IL-18    | 100    |       |       |       |       |       |       |

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**Fig. 2.** Biophysical characterization of IL-1H1. A, far-UV circular dichroism spectra of IL-1ra (dashed), IL-1β (dotted), and IL-1H1 (solid) shown as mean residue ellipticity versus wavelength. The shapes and amplitudes of the IL-1ra and IL-1β spectra agreed well with previous reports (35, 36). B, thermal unfolding stability of IL-1ra (open symbols) and IL-1H1 (closed symbols) shown as the fraction of unfolded cytokine versus temperature. Unfolding was monitored by ellipticity at 215 nm. Half-unfolded Tm values determined from the data in the figure are 59 and 61 °C for IL-1ra and IL-1H1, respectively.
Results and Discussion

Identification and Characterization of Novel IL-1 Homologues—A search of public and private EST data bases for sequence homologues of the IL-1 family revealed four new members, which we have designated IL-1H1, IL-1H2, IL-1H3, and IL-1H4. Complete predicted sequences of the four proteins and their alignment with known IL-1 family members is shown in Fig. 1A. The percentage amino acid similarity of the IL-1Hs to known members of the family including IL-18 varies between 12 and 54%, which can be compared with the 28–36% similarity seen among IL-1α, IL-1β, and IL-1ra, all of which bind to the same receptor (Table I). Murine paralogues of the known IL-1s are typically >60% identical, whereas murine IL-1H1 is 53.2% identical and 63% similar to human IL-1H1. The closest homologues of murine IL-1H3 are murine IL-1ra (59.1% similarity, 52.5% identity) and human IL-1ra (54.1% similarity, 47.9% identity). Based on homology, IL-1H3 is a relatively recent duplication of IL-1ra and is not a paralogue of IL-1H2 or IL-1H4. Indeed, the likely human orthologue of IL-1H3 (IL-1HY1) was recently published while this manuscript was in preparation (34). Human IL-1HY1 and murine IL-1H3 are ~90% identical and differ in only few amino acids in the central and C-terminal regions (34). IL-1H4 seems to be the most distantly related protein to known IL-1s.

IL-1H1 was mapped to chromosome 2q12–21, which is close to the region where IL-1α, IL-1β, and IL-1ra have been localized, further supporting an evolutionary relationship within the IL-1 family. Interestingly, human IL-1H3 (IL-1HY1) was also mapped to chromosome 2q14 in close proximity to IL-1ra (34). The chromosomal localization of the remaining IL-1 homologues has not yet been determined.

A schematic representation of all IL-1 members is shown in Fig. 1B. Like all but one of the known IL-1 family members, IL-1ra, the four IL-1Hs do not encode a hydrophobic leader peptide found in most secreted proteins, and hence we would expect these proteins to be released from cells undergoing necrosis or apoptosis. Similar to the intracellular form of IL-1ra, IL-1H1, IL-1H2, and IL-1H3 lack a propeptide sequence, but their entire coding regions align with the mature regions of IL-1α, IL-1β, and IL-1ra (Fig. 1), suggesting that they most likely retain a similar tertiary structure. In contrast, IL-1H4 has a significant propeptide region that, like IL-1α and IL-18, contains a potential proteolytic processing site for caspase 1 (Fig. 1B) that could be necessary for its biological activity. Preliminary data indicate that IL-1H4 is cleaved by caspase 1 at this site.2

To further characterize and compare the properties of these new IL-1 homologues with the known IL-1 members, we initially focused on IL-1H1. Upon expression of an amino-terminal His6-tagged fusion in E. coli, IL-1H1 was found to be

2 S. Kumar, unpublished data.
soluble, and it could be purified through Ni$^{2+}$-nitrilotriacetic acid chromatography. Cleavage at the engineered factor Xa site yielded mature IL-1H1 (data not shown).

Circular Dichroism and Thermal Stability Analyses—To obtain additional evidence that IL-1H1 was structurally similar to other IL-1s, we compared its CD spectrum with that of IL-1ra and IL-1β (Fig. 2A). The shape and amplitude of IL-1ra and IL-1β spectra agree well with previous reports (35, 36). The CD spectrum of IL-1H1 is similar to IL-1ra and consistent with a substantial contribution from β-strands. The $T_m$ for thermal denaturation of IL-1H1 was 61 °C, which is also similar to that for IL-1ra and indicates that the two proteins have a comparable folding energy (Fig. 2B). Interestingly, the spectrum of IL-1β is somewhat distinct, although it also has a similar structure. These data suggest that IL-1H1 is likely to adopt the characteristic β-barrel structure of the IL-1s. The position of 12 β-strands in the IL-1H1 sequence can be predicted from the alignment with IL-1α, IL-1β, and IL-1ra, whose structures are known (see Fig. 1A).

Because it is similar in structure and site of synthesis to other IL-1s, we expected that IL-1H1 might bind to a receptor in the IL-1R family. However, we were unable to demonstrate any binding to the type I IL-1R, IL-18R, or ST2/T1, suggesting that it binds to an as yet unidentified receptor (data not shown).

Expression of Novel IL-1 Homologues—No expression of any of the novel homologues was detected in normal mouse tissues represented on commercially available multiple tissue blots, suggesting that the IL-1 homologues are either expressed at low levels or require suitable stimulation. This is reflected by the rare occurrence of ESTs for the homologues in public or commercial data bases. Thus, human IL-1H1 ESTs were identified only in TNFα- and IFNγ-stimulated keratinocyte and epithelial cell cDNA libraries, while the murine orthologue was found in a placenta library. Similarly, a human IL-1H2 EST was found only in a osteoclastoma cell library; a mouse IL-1H1 EST was found in a 19.5-day embryo library; and human IL-1H4 ESTs were found in a mixed fetal lung, testes, B cell, and colon library. Although we did not detect any expression of murine IL-1H3 in commercially available murine multiple tissue Northern blots, its human orthologue, IL-1HY1, was reported to be expressed in fetal skin and spleen cDNA libraries by polymerase chain reaction analysis (34).

In situ hybridization of the mouse IL-1H1 cDNA to a panel of normal mouse tissue sections revealed expression in only the esophageal squamous mucosa. Low levels of IL-1H1 expression (~100–200 copies/ng of mRNA) were also detected in normal human lung and macrophages by Taqman analysis (data not shown). These data support the lack of widespread expression seen in the human multiple tissue Northern blots. In contrast, IL-1H1 mRNA was found to be induced in TNFα- and IFNγ-treated human keratinocytes with maximal expression when both cytokines were added together (Fig. 3A, lanes 1–4). A similar induction was seen in the epithelial cell line, A431 (Fig. 3A, lanes 5 and 6). Similar to IL-1H1 and consistent with stimulus-dependent expression of these genes, human IL-1H3 (IL-1HY1) was shown to be induced in response to phorbol esters and bacterial lipopolysaccharide (34).

To evaluate changes in IL-1H1 protein levels, polyclonal antibodies were raised to both human and murine IL-1H1. Both immunoprecipitation and Western blot demonstrate that IL-1H1 protein was induced in human keratinocytes by TNFα and IFNγ (Fig. 3B), as seen in the mRNA experiments. However, the protein was only detected in the cell lysates and not in the conditioned media. Intrasacellular expression of IL-1H1 protein was also detected in bacterial lipopolysaccharide-stimulated human monocytes (data not shown). Keratinocytes have been shown to contain IL-1ra and precursor forms of IL-1α and IL-1β that remain in cells and are released into the extracellular environment after damage and lysis of cells (37–39). A similar mechanism may also apply to IL-1H1.

In Vivo Expression of IL-1H1 in Response to Inflammatory Stimuli—To explore the potential pathological role of keratinocyte-expressed IL-1H1, we utilized a murine skin inflammation protocol...
and contact hypersensitivity model (CHS) (30). Mouse ears were irradiated with UVB radiation for 30 min as described (31, 40) or topically treated with oxazolone in an acute or chronic mode as detailed under “Materials and Methods.” UVB irradiation (31) and a single oxazolone sensitization/challenge produced an acute inflammatory response but did not result in significant IL-1H1 induction (Fig. 4A). Mouse ears were also treated in a chronic mode by repeated challenge with oxazolone after an initial sensitization. In this model, exposure to oxazolone leads to a delayed type hypersensitivity reaction. Initially there is a TH1-driven immune response characterized by the presence of elevated TNF-α and IFN-γ and minimal levels of IL-4, but after 4 weeks, the cytokine profile converts to a TH2 immune response as evidenced by increased IL-4 and decreased IFN-γ and TNF-α mRNA (30). Histological analysis of mouse ears chronically exposed to oxazolone revealed hyperplastic epithelium, thickening of subepithelium, and infiltration by neutrophils, eosinophils, and lymphocytes (Ref. 30; data not shown). In contrast to the results seen with acute oxazolone treatment, a 4-week chronic oxazolone treatment led to a significant induction of IL-1H1 RNA (Fig. 4A, lanes 3 and 4).

To confirm that the differences between chronic and acute oxazolone treatment were not due to differences in the kinetics of IL-1H1 induction, we treated mouse ears with oxazolone under acute and chronic conditions and prepared RNA at 3, 6, and 24 h after the last challenge. The expression of IL-1H1 was seen at all time points but only after chronic oxazolone treatment and not under acute treatment (Fig. 4B). These data suggest that the induction of IL-1H1 mRNA reflects underlying changes in the tissue caused by chronic oxazolone treatment and not a response to each application. Consistent with previous reports, the expression of IL-4 mRNA was also induced only under chronic conditions (Fig. 4B and Ref. 30), signaling a switch to a TH2 immune response. These data suggest that the expression of IL-1H1 may be linked to CHS and/or a TH2-driven immune response.

The cellular source of IL-1H1 mRNA in chronically oxazolone-treated mouse ears was determined by in situ hybridization. As shown in Fig. 5, IL-1H1 mRNA was expressed in keratinocytes in the hyperplastic epithelium of ears from mice chronically sensitized with oxazolone and harvested 24 h after the last challenge (Fig. 5A). No signal was observed in control experiments (Fig. 5, B–D).

These data suggest that IL-1H1 may play a role in CHS. Interestingly, IL-1 itself has been implicated in CHS (41–43). However, results from experiments in an iL-1β null mouse and a mouse overexpressing type II IL-1R argue that IL-1a and/or IL-1β are not essential for the oxazolone-induced CHS response, suggesting the involvement of additional factors such as IL-1H1 (44).

Expression of IL-1H1 in Herpes Simplex Virus Infection—

HSV-1 is known to induce a number of cytokines during acute infection, and HSV-1-infected keratinocytes have been implicated as a source of several cytokines and chemokines (45). Given the expression of IL-1H1 in oxazolone-induced CHS, we sought to determine its expression during HSV-1 infection. Mice were infected with HSV-1 (SC-16) following scarification of ear pinnae, and ears were collected daily for analysis of IL-1H1 RNA and protein expression. Ear lesions appeared within 4–5 days following infection and were characterized by degeneration and necrosis of epithelial cells, dermal edema, and neutrophil and mononuclear cell infiltrates and the presence of epithelial intranuclear viral inclusions (data not shown). By Western blot analysis using an anti-murine IL-1H1-specific antibody, a protein of ~18 kDa, the expected size of IL-1H1, was first detected on day 1 and increased substantially in intensity by day 6–7 postinfection (Fig. 6). The expression of viral glycoprotein C was first detected on day 2 and increased in intensity at day 4–6 (Fig. 6). Day 8–10 ear samples were negative for both viral antigen and IL-1H1 (data not shown).

We next performed in situ hybridization studies to localize the source of IL-1H1 in HSV-1-infected mouse ears. Similar to the data obtained with the chronic oxazolone model, IL-1H1 mRNA expression was detected in keratinocytes of the hyperplastic epidermis of HSV-1-infected mouse ears (Fig. 7C). IL-1H1 mRNA was first detected at day 4 (data not shown) and increased to a maximum at day 7. No expression of IL-1H1 mRNA was detected in control uninfected mouse ears (Fig. 7A). Several proinflammatory cytokines and chemokines such as TNF, IL-1, IL-18, IL-6, MIP-2, and KC are expressed during HSV infection of mouse ears. Since viral infection results in significant inflammation and necrosis, it is possible that IL-1H1 expressed and released during this process may contribute to the inflammation. Alternatively, the finding that maximal

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3 R. Tal-Singer, manuscript in preparation.
expression of IL-1H1 occurs in the hyperplastic or regenerative stage epithelium after viral envelope expression or intranuclear viral inclusions have disappeared suggests that IL-1H1 may play a role in viral clearance and/or repair processes.

Conclusions—We have identified four novel homologues of the IL-1 family. While we do not yet know if these new homologues are agonists or antagonists, we speculate that IL-1H1, IL-1H2, and IL-1H3 may be antagonists, since they are most similar to IL-1ra in structure and sequence and similarly lack a propeptide region and a caspase 1 cleavage site, we speculate that it is an agonist.

The finding of four novel members of the IL-1 family was made possible by extensive EST sequencing. With the imminent completion of the sequence of the human genome, we made possible by extensive EST sequencing. With the imminent that it is an agonist.

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