Identification and Characterization of Two Members of a Novel Class of the Interleukin-1 Receptor (IL-1R) Family

DELINÄEEATION OF A NEW CLASS OF IL-1R-RELATED PROTEINS BASED ON SIGNALING

Received for publication, May 12, 2000, and in revised form, July 5, 2000
Published, JBC Papers in Press, July 5, 2000, DOI 10.1074/jbc.M004077200

Teresa L. Born, Dirk E. Smith, Kirsten E. Garka, Blair R. Renshaw, Jeanette S. Bertles, and John E. Sims†

From ImmuneX Corp., Seattle, Washington 98101

Two novel members of the interleukin-1 receptor (IL-1R) family, identified by homology searches of human genomic sequence data bases, are described. The genes have been named according to their structural features: TIGIRR-1 (three immunoglobulin domain-containing IL-1 receptor-related) and TIGIRR-2. TIGIRR-3 has recently been identified as causing mental retardation when mutated (Carrie, A., Jun, L., Bienvenu, T., Vinet, M. C., McDonell, N., Couvert, P., Zemni, R., Cardona, A., Van Buggenhout, G., Frints, S., Hamel, B., Moraine, C., Ropers, H. H., Strom, T., Howell, G. R., Whittaker, A., Ross, M. T., Kahn, A., Fryns, J. P., Beldjord, C., Marynen, P., and Chelly, J. (1999) Nat. Genet. 23, 25–31) and called ILIRAP1, a name we will also use henceforth. Neither receptor alone was able to mediate transcriptional activation of NF-κB in response to IL-1α, IL-1β, or IL-18. In order to begin to elucidate the function of these and other orphan IL-1R family members, we have developed a functional assay utilizing a panel of chimeric receptors containing the extracellular and transmembrane domains of either type I IL-1R or IL-1R accessory protein (AcP) coupled to the cytoplasmic domains of all family members. Coexpression of each IL-1R chimera with each AcP chimera and an NF-κB-responsive reporter demonstrated that the cytoplasmic domains could be classified as IL-1R-like, AcP-like, or neither. Any IL-1R-like cytoplasmic domain could cooperate with any AcP-like cytoplasmic domain. The TIGIRR-1 and ILIRAP1 cytoplasmic domains, however, were unable to signal either IL-1R-like or AcP-like components, suggesting that they function as a new class of receptors within this family.

Interleukin-1 is a proinflammatory cytokine secreted from a wide variety of cell types that acts on a similarly diverse range of cells (reviewed in Ref. 2). Three structurally related cytokines have been identified; IL-1α and IL-1β are agonists that induce identical responses, whereas IL-1 receptor antagonist functions to block IL-1α and IL-1β activity. All known biological functions of IL-1 are mediated through the type I IL-1R (3). All three members of the IL-1R family have been shown to bind the type I IL-1R with high affinity, whereas IL-1β binds the type II IL-1R with high affinity and IL-1α and IL-1 receptor antagonist bind the type II IL-1R with a low affinity. The type II IL-1R contains a severely truncated cytoplasmic domain and acts as a decoy receptor (4). Other IL-1R family members include IL-1Rrp1 (IL-18R), IL-1Rrp2, T1/ST2, IL-1R accessory protein (AcP), AcPL (IL-18RAcP), SIGIRR, and the newly described ILIRAP1 (1, 5–12). AcP by itself has no measurable affinity for IL-1α or IL-1β (hereafter referred to as IL-1 when applicable to both) (8, 13). Upon binding of IL-1 to its receptor, however, a higher affinity binding complex is formed containing both IL-1R and AcP (8). In addition to increasing the affinity of IL-1 for the IL-1R, AcP has been shown to be required for signaling (14, 15). Signaling induced by another IL-1 family cytokine, IL-18, also requires two subunits: IL-18 (IL-1Rrp1) and IL-18RAcP (AcPL) (9).

Many of the downstream signaling events induced by IL-1 have begun to be elucidated. The adapter protein MyD88 (16–18) is recruited to the active IL-1R-AcP complex (19, 20). The IL-1R-associated kinases (IRAK and IRAK-2) are recruited to the activated complex through interaction with MyD88 (19–23). Following their association with the IL-1R complex, IRAK and IRAK-2 have been shown to interact with tumor necrosis factor receptor-associated factor-6 (TRAF-6) (19, 24). In support of a role for these molecules in IL-1 signaling, mice deficient in TRAF6 (25), IRAK-1 (26, 27), or MyD88 (28) are all compromised for IL-1 responses. Downstream of TRAF6 lie at least two independent pathways leading to transcriptional activation. First, TRAF6 has been shown to activate NF-κB through TGF-β-activated kinase-1, NF-κB-inducing kinase, and IκB kinases (29, 30). TRAF-6 also plays a role in IL-1-mediated activation of p38, Jun N-terminal kinase (p54), extracellular signal-regulated kinase (p42/44), and ultimately AP-1 (31–34).

The extracellular portion of IL-1R family members is highly conserved, consisting of three Ig domains. The one exception is SIGIRR, which contains only one putative Ig domain (10). Conservation also exists within the intracellular region of these receptors, with several highly conserved regions having been shown to be critical for signaling (35). Interestingly, these cytoplasmic regions have also been shown to be highly conserved in the Drosophila Toll family of receptors (35–37). This family contains Drosophila proteins, such as Toll and 18-

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† To whom correspondence should be addressed: ImmuneX Corp., 51 University St., Seattle, WA 98101. Tel.: 206-389-4005; Fax: 206-389-9733; E-mail: simsj@immunex.com.

‡ The abbreviations used are: IL, interleukin; IL-1R, interleukin-1 receptor; AcP, IL-1R accessory protein; IRAK, IL-1R-associated kinase; TRAF, tumor necrosis factor receptor-associated factor; TLR, Toll-

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Wheeler, as well as at least six mammalian Toll-related receptors (TLRs) (38–41). Although the cytoplasmic domains and downstream signaling events seem to be largely conserved with those of the IL-1R family, the Toll subfamily extracellular domains are dissimilar from the IL-1R family, containing multiple leucine-rich repeats. Toll itself is critical for the establishment of dorsal-ventral polarity and for antifungal defenses in Drosophila. The mammalian TLRs are similarly implicated in the innate immune response, based on their ability to induce critical immune modulators such as proinflammatory cytokines, effector cytokines, chemokines, and co-stimulatory molecules in response to conserved bacterial and fungal products (42). For example, TLR-4 has been shown to mediate LPS signaling (43, 44). Events implicated in IL-1 signal transduction, such as recruitment of MyD88 and activation of NF-κB have also been shown to be critical for mammalian TLR signaling (38, 45, 46).

We have identified, through examination of human genomic sequence deposited in public data bases, two novel IL-1R family members, which we have called TIGIRR-1 and TIGIRR-2. One of these receptors (TIGIRR-2) has recently been identified by others based on its mutant phenotype and called IL1RAPL (1). Full-length sequence and tissue distribution were determined for both receptors. Initial signaling experiments indicated that neither receptor could mediate NF-κB activation in response to IL-1 or IL-18. Chimeras consisting of the extracellular and transmembrane (extm) domain of the IL-1R and cytoplasmic (cyto) domain of TIGIRR-1 or IL1RAPL were similarly nonresponsive to IL-1. We therefore set out to devise a strategy to characterize more fully the signaling capabilities of novel IL-1R family members. Since both IL-1R and AcP share a high degree of homology, it is possible that any novel receptor may play a role similar to that of IL-1R (i.e. binding and signaling) or to that of AcP (i.e. affinity conversion, signaling). To delineate these possibilities and to verify that the receptor family members could be thus categorized, we constructed a series of chimeric receptors containing the extracellular and transmembrane domain of either IL-1R or AcP and the cytoplasmic domain of each IL-1R family member. Members of the two chimeric series were then coexpressed in every possible combination, along with an NF-κB-driven reporter plasmid, and IL-1-stimulated transcriptional activation was assessed. We were able to characterize all previously reported members of this family functionally as IL-1R-like (IL-1R, IL-18R, IL-1Rp2, T1/ST2) or AcP-like (AcP, IL-18RAcP). TIGIRR-1 and IL1RAPL, however, failed to signal in either capacity, suggesting that they might form a novel IL-1R subfamily.

**EXPERIMENTAL PROCEDURES**

Cloning of TIGIRR-1 and IL1RAPL—Homology searches of GenBank revealed that six different, partly overlapping cosmids derived from the human X chromosome appeared to contain exons 4–12 of a novel IL-1R family member (exon 4 accession number Z74747; exon 5 accession number Z81144; exon 6 accession number Z69721; exon 8 accession number Z68330; exons 9 and 10 accession number Z68908; exons 11 and 12 accession number Z68328; exon numbering is by analogy to type I IL-1R (47)). PCR amplification of human liver first strand cDNA, using primers from within the putative exons 4 and 12, gave a product of the expected size. Sequencing confirmed that the predicted exons had indeed been spliced into the same mRNA. By comparison with other members of the IL-1R family, the portion of the mRNA that we had identified lacked the coding sequence for the initiating methionine and the signal peptide. These were cloned using standard 5′-RACE techniques, with Marathon-Ready™ human liver cDNA (CLONTECH) and a Agt11 human cerebellum library as templates. Once the putative full-length sequence for TIGIRR-1 was obtained, the entire sequence was amplified from human liver cDNA and several independent products were sequenced.

A murine genomic library was screened using the human TIGIRR-1 sequence as a probe. A portion of a resultant clone from this screen was then utilized to screen a murine brain cDNA library, and several overlapping partial sequences were obtained in the clones identified. The murine TIGIRR-1 sequence was further verified by amplification from a murine liver library.

**Northern Blots and Reverse Transcriptase-PCR—** Normal and cancer cell line human multiple tissue Northern blots were purchased from CLONTECH and Biochain Institute, Inc. (San Leandro, CA). The blots were hybridized overnight with a 32P-labeled TIGIRR-1 riboprobe in hybridization buffer containing 50% formamide at 63°C and then washed at 63°C in 1× SSC, 0.1% SDS. Similar blots were probe with IL1RAPL riboprophes under the same conditions, except hybridization was carried out at 68°C and the most stringent wash was in 0.2× SSC, 0.1% SDS at 68°C. After exposure, the blots were rehybridized with a random prime-labeled probe against β-actin for standardization.

An exhaustive panel of human cDNAs (Human Immune System Panel I, Human Panel II, Human Panel III) was purchased from CLONTECH, and both TIGIRR-1-specific and IL1RAPL-specific primers were used for amplification under standard PCR conditions (35 cycles unless otherwise noted).

Expression Plasmids and Protein Purification—Full-length human and murine TIGIRR-1 were generated by PCR and cloned into pDC304 (48). Full-length human IL1RAPL was subcloned into pDC304, a variant of pDC302 (49). The NF-κB-lucerase plasmid has been described previously (50). A panel of chimeric expression vectors was created by first generating IL-1Rextm and AcPextm cassette vectors, both in pDC304. The IL-1Rextm cassette vector contained amino acids 1–382 of murine IL-1R, with a BglII site causing a single amino acid substitution (KVF to KIF) just inside the transmembrane region (36). The AcPextm cassette vector contained amino acids 1–381 of murine AcP with a BstZ17 site created by introducing a silent mutation (see also Table I). The cytoplasmic domains of all known family members were inserted into these cassette vectors by generating the appropriate PCR products. The precise amino acids contained within each construct are presented in Table I.

The human and rat IL-1Rp2 sequences have been reported (6), but not the murine sequence. For the generation of chimeric murine IL-1Rp2 constructs, we cloned the murine IL-1Rp2 sequence by direct PCR amplification and subsequent RACE amplification of murine brain and kidney cDNAs or libraries. At this time, we also cloned the human IL-1Rp2 sequence and found the C terminus of the encoded protein to differ from the GenBank™ entry (U49065) by a frameshift mutation at nucleotide 1767. This frameshift results in a slightly larger protein, with a 18 amino acid deletion of the previous end of the protein that is replaced with the sequence PPQVLQHPTCTRAGPELGSRRKKT-LLTG. We amplified the human IL-1Rp2 mRNA from several different sources and repeatedly isolated this sequence. We have therefore also deposited this revised human IL-1Rp2 sequence. Human TIGIRR-1 and IL1RAPL were used in the chimeras, but we and others (1) report that the identity of human to murine proteins is 94 and 98%, respectively.

Cell Culture and NF-κB Reporter Assays—COS7 (monkey kidney) cells were maintained in Dulbecco’s modified Eagle’s medium/5% fetal bovine serum, and S49.1 (murine T) cells were grown in RPMI 1640/5% bovine serum, and S49.1 (murine T) cells were grown in RPMI 1640/5% fetal bovine serum supplemented with 1 mM sodium pyruvate, 100 μM nonessential amino acids, and 55 μM 2-mercaptoethanol.

COS7 cells were transiently transfected by the DEAE-dextran method as described (51), using 10 ng of each receptor, 50 ng of the reporter plasmid, and enough empty expression vector to yield a total of 1 μg of DNA per 4.5×10⁴ cells. Two days post-transfection, cells were blocked for endogenous IL-1R signaling by incubation for 15 min at 37°C with sheep anti-human IL-1R antisera (36) and then stimulated with 10 ng/ml IL-1α, IL-1β, or IL-18 (PeproTech, Inc., Rocky Hill, NJ) for 4 h (at the dilution used, the antisera blocks the endogenous monkey IL-1R but not the transfected mouse IL-1R). Cells were lysed, and luciferase activity was assessed using Reporter Lysis Buffer and Luciferase Assay Reagent (Promega, Madison WI). All results reported represent at least two independent transfactions.

S49.1 cells (1×10⁶) were electroporated (320 V, 960 microfarads) with 10 ng/ml IL-1 and 10 ng/ml IL-18, and 10 ng/ml TNF-α, or with 10 ng/ml TNF-α and 10 ng/ml IL-18, and then assayed for luciferase activity. All results reported represent at least two independent transfactions.

S49.1 cells (1×10⁶) were electroporated (320 V, 960 microfarads) with 10 ng/ml reporter DNA and 10 μg each of reporter-encoding DNA. It is very likely that the identity of human to murine proteins is 94 and 98%, respectively.
Following electroporation, cells were diluted in fresh medium and aliquoted to five individual wells. After 2 days, cells were stimulated with phosphate-buffered saline (one well), IL-1α (two wells), or IL-1β (two wells), and luciferase levels were measured as described above. All results reported represent at least one electroporation, which was split and assayed in duplicate. Due to the low level of transfection efficiency by electroporation in the S49.1 cells, we were technically unable to demonstrate surface expression of all chimeras in these cells by fluorescence-activated cell sorting analysis. The inability to detect recombinant receptor expression by fluororescence-activated cell sorting is probably due to two factors. The number of cells successfully transfected is quite low, and therefore it is difficult to detect those cells in the background of the nontransfected cells, even when the analysis is done by two-color sorting. Second, the level of receptor expression in the cells that are transfected is probably quite low due to the fact that there is no mechanism for plasmid amplification in these cells. IL-1 signaling is possible with only a small number of receptors per cell, so whereas our expression is sufficient for signaling studies, it is probably insufficient for detection by fluorescence-activated cell sorting analysis (52, 53). We have been able to verify the expression of all chimeric receptors in COS7 cells by radioimmunoprecipitation.

RESULTS

Cloning of TIGIRR-1 and IL1RAPL—Homology searches of sequence data bases revealed two collections of human genomic sequence that appeared to encode genes related to IL-1R. One of these, eventually called TIGIRR-2, was contained in genomic sequence corresponding to chromosome regions Xp11.4–21.3 (exons 4–6) and Xp22–164–166 (exons 10–12). In order to confirm that these predicted exons were indeed spliced together to form a single expressed gene, and to identify the predicted missing exons 7–9, we performed PCR amplification on first strand cDNA obtained from human brain RNA using primers from within the putative exons 5 and 11 (numbering of exons is by analogy to the IL-1R gene structure (47)). A PCR product of the predicted size was obtained, which when sequenced was shown to be formed by joining of the putative exons 5 and 6 and exons 10 and 11, with the inclusion of novel sequence demonstrating homology to exons 7–9 of the IL-1R and the corresponding regions of other known IL-1R family members. In order to obtain the rest of the coding region, 5'-RACE was performed on human testis cDNA. The full-length amino acid sequence, predicted from the cDNA sequence, has been deposited in GenBankTM.

Independently, and subsequent to the completion of the above studies, Carrié et al. published the use of positional cloning to isolate a gene responsible for an X-linked form of "giant cell arteritis". The gene they identified was identical to TIGIRR-2. They gave this gene the name IL1RAPL, and we will use their designation henceforth.

The other putative novel IL-1R family member, called TIGIRR-1, was similarly identified by homology searches of publicly available human X chromosome genomic sequence. As with TIGIRR-2/IL1RAPL, PCR amplification of first strand liver cDNA, followed by 5'-RACE, established that the predicted exons were indeed joined in the mRNA and also supplied the missing exons. The predicted amino acid sequence of human TIGIRR-1 is presented in Fig. 1. Murine TIGIRR-1 cDNA clones have also been isolated. Human and murine TIGIRR-1 are 94.5% identical at the amino acid level.

TIGIRR-1 is most homologous to IL1RAPL (63% amino acid identity), and both novel receptors share between 22 and 48% overall identity to other IL-1R family members. As do other members of this family, both TIGIRR-1 and IL1RAPL contain a signal peptide, three predicted extracellular Ig domains, a single transmembrane domain, and a highly conserved cytoplasmic region. Fig. 2 shows an alignment of the cytoplasmic domains of all members of the IL-1R family. Both TIGIRR-1 and IL1RAPL contain a C-terminal cytoplasmic extension relative to other IL-1R family members, which is in fact reminiscent of the Drosophila (but not human) Toll family cytoplasmic domains and the IL-1R-related protein SIGIRR. Although TIGIRR-1, SIGIRR, IL1RAPL, and Drosophila Toll and 18-Wheeler all have C-terminal tails, only the TIGIRR-1 and IL1RAPL tails show any notable sequence similarity.

A schematic diagram of the TIGIRR-1 genomic structure and the exon/intron junction sequences is presented (Fig. 3, A and B). The TIGIRR-1 introns are placed similarly to those of the IL-1R, both in terms of where they lie within the overall sequence as well as their specific positions with respect to the reading frame. The similarity of intron placement provides confirmation for the conclusion drawn from sequence analysis that the two genes are descended from a common ancestor. Like IL1RAPL, the TIGIRR-1 exons are spread out over a very large segment of genomic DNA (>1500 kb for IL1RAPL (1); >380 kb for TIGIRR-1).

Tissue Distribution of TIGIRR-1 and IL1RAPL—Northern blots were probed with TIGIRR-1 and IL1RAPL riboprobes in order to determine expression patterns. Consistent with the previous report (1), IL1RAPL was expressed as 7.5- and 10.0-kb bands in the brain as well as in heart. An 8.0-kb band was detected in skeletal muscle (Fig. 4). PCR analysis of a human cDNA tissue panel similarly detected IL1RAPL expression in heart, brain, ovary, skin, and to a lesser extent in tonsil, fetal liver, prostate, testis, small intestine, placenta, and colon. Expression was not detected in spleen, lymph node, thymus, bone marrow, leukocytes, lung, liver, skeletal muscle, kidney, or pancreas (data not shown). To determine a possible role for the receptor in carcinogenesis, a tumor tissue blot was also probed with IL1RAPL. Weak expression of an 8.0-kb band was detected in the colorectal adenocarcinoma cell line SW480 (data not shown).

TIGIRR-1 expression overall (by Northern blot or reverse transcriptase-PCR) was generally quite low. Northern blots did not detect any appreciable TIGIRR-1 expression in heart, liver, pancreas, skeletal muscle, testis, spleen, thymus, prostate, ovary, small intestine, colon, PBL, brain, or lung (Fig. 4). PCR analysis of human tissue cDNAs detected TIGIRR-1 expression in skin, with weaker expression in liver, placenta, and fetal brain (data not shown). The human tumor tissue blot was also probed with TIGIRR-1, and no expression was detected in any of the samples (data not shown).

IL-1 Is Unable to Induce Signaling from TIGIRR-1 or IL1RAPL—In order to investigate the potential for activation...
by IL-1, full-length TIGIRR-1 and IL1RAPL were independently overexpressed in COS7 cells together with an NF-
\( \kappa B \)-driven luciferase reporter plasmid. Endogenous responsiveness of the cells was blocked with an inhibitory IL-1R antibody, and then cells were stimulated with IL-1\( \alpha \) or IL-1\( \beta \) and luciferase activity was assessed. We were unable to detect an IL-1-mediated stimulation of NF-
\( \kappa B \) activity upon TIGIRR-1 or IL1RAPL overexpression (Fig. 5A).

IL-18 is structurally related to IL-1, and signaling by IL-18 is mediated by IL-18R and IL-18R{\alpha}C (9, 54, 55), both members of the IL-1R family. It is possible, therefore, that the novel receptors could play a role in IL-18 signaling. To address this, COS7 cells transfected with full-length TIGIRR-1 or IL1RAPL and NF-
\( \kappa B \)-Luc were stimulated with IL-18. Under these conditions, we were unable to demonstrate a role for either receptor in IL-18-mediated signaling (Fig. 5A).

Several orphan members of the IL-1R family have been shown to be able to signal transcriptional activation in response to IL-1 when expressed as a chimeric molecule with IL-1R{\alpha}m, such as IL-18R (5), IL-1R{\beta}p2 (10), and T1/ST2 (36). We therefore investigated if TIGIRR-1 or IL1RAPL could signal when expressed as an IL-1R{\alpha}m chimeric receptor. As shown in Fig. 5B, overexpression of full-length IL-1R or the chimeric IL-1R{\alpha}m-IL-18Rcyto conferred IL-1 responsiveness to these cells, presumably via cooperation with endogenously expressed AcP. Overexpression of IL-1R{\alpha}m-TIGIRR-1{cyto} or IL-1R{\alpha}m-IL1RAPL{cyto} however, did not result in an IL-1-mediated activation of NF-
\( \kappa B \) (Fig. 5B). This result suggests that neither the TIGIRR-1 nor the IL1RAPL cytoplasmic domain is able to transduce a signal in association with AcP, in contrast to the cytoplasmic domains of other members of this family such as IL-18R.

The one striking difference between these two novel receptors and most other members of the IL-1R family is the presence of the C-terminal extension in the cytoplasmic domain. Since the cytoplasmic extension on the Toll receptor may be inhibitory (56), we have removed this tail from the IL-1R{\alpha}m-TIGIRR-1cyto and IL-1R{\alpha}m-IL1RAPLcyto chimeric receptors and assayed transcriptional activation in response to IL-1. Chimeras with the truncated cytoplasmic domain are still non-responsive to IL-1 in these assays (data not shown).

Development of an Alternative Chimeric Receptor Signaling—

The experiments described above address the question of the ability of orphan receptors (such as TIGIRR-1 or IL1RAPL) to act as signaling subunits. It is possible, alternatively, that some of the IL-1R-related orphan receptors function as accessory subunits, similar to AcP. It has been shown that for both IL-1 and IL-18 signaling, two distinct receptor subunits are required for biological response. It is therefore reasonable to assume that other functional receptors in this family are heterodimeric. This being the case, if only one receptor subunit binds the cytokine and elicits a signal, the task of understanding the role of novel receptor family members prior to elucidation of the cognate cytokine or receptor partner is formidable. We set out to devise an experimental strategy to address several questions. 1) Of the known IL-1R orphan family members that signal as chimeric (IL-1R{\alpha}m/orphan receptor{cyto}) receptors, can we determine potential partner or accessory subunits? 2) Of the known IL-1R orphan family members that do not signal in chimeric
form (i.e. IL1RAPL, TIGIRR-1), can we determine if they may function as accessory subunits? If we could functionally classify the known IL-1R family members as IL-1R-like or AcP-like, we could gain a better understanding of those family members functioning as accessory proteins and perhaps gain insight into novel receptor pairs. Additionally, since for both IL-1 and IL-18 receptors, the heterodimeric complex has a higher affinity for the ligand (8, 57), generation of heterodimeric receptors would certainly aid in the elucidation of novel cognate ligands.

As a means of addressing the above issues, we created a series of chimeric receptors. The cytoplasmic domain of each identified prototypical family member was fused to the extracellular and transmembrane domains of both IL-1R and AcP. Thus, we generated a panel of seven IL-1R chimeras and seven AcP chimeras (Table I). We have previously identified a murine T cell lymphoma cell line (S49.1) that does not express mRNA for either IL-1R or AcP and is nonresponsive to IL-1. Transient overexpression of both IL-1R and AcP in these cells was able to confer IL-1 responsiveness (9). We utilized these cells to coexpress the IL-1R and AcP chimeras in all possible combinations. Cells were electroporated with an IL-1R subunit, an AcP subunit, and an NF-κB-driven luciferase reporter plasmid. Two days after electroporation, cells were stimulated with IL-1α or IL-1β for 4 h, and then luciferase activity was assessed. Data from all of the transfections are summarized in Table II. Several conclusions can be drawn from this series of experiments. First, the assay itself was validated in that we observed IL-1-stimulated NF-κB induction when IL-1R and AcP were coexpressed and when IL-1R<sub>extm</sub>-IL-18R<sub>cyto</sub> and AcP<sub>extm</sub>-IL-18RAcP<sub>cyto</sub> were coexpressed. No response was seen in the presence of IL-1R or AcP (full-length or chimeric forms) alone (data not shown). Thus, both known receptor pairs read out in this assay, and both members of the heterodimeric receptor pair are required. Moreover, the extracellular and cytoplasmic domains of these receptors did indeed function as autonomous modules, since we saw NF-κB induction when the cytoplasmic domains of known receptor pairs were interchanged. In other words, IL-1R<sub>extm</sub>-AcP<sub>cyto</sub> and AcP<sub>extm</sub>-IL-1α<sub>cyto</sub> formed a functional receptor pair, as did IL-1R<sub>extm</sub>-IL-18RAcP<sub>cyto</sub> and AcP<sub>extm</sub>-IL-18R<sub>cyto</sub>.

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containing two IL-1R-like cytoplasmic domains (box C) or two AcP-like cytoplasmic domains (box B) did not result in the formation of a functional signaling complex (Table II). It can also be seen (box D) that although many pairs functioned when the cytoplasmic domains were coupled with the “nonnatural” extracellular region, IL-1Rextm or AcPextm, this did not hold true in every case. For example, the pair (IL-1Rextm-AcPcyto and AcPextm-IL-1Rcyto) was able to signal, whereas the pair (IL-1Rextm-AcPcyto and AcPextm-IL-18Rcyto) was unable to signal. In other words, when the extracellular and cytoplasmic domains were mismatched in terms of IL-1R-like or AcP-like, only half of the tested pairs were still able to signal.

It can also be seen in Table II that TIGIRR-1 and IL1RAPL were unable to signal in cooperation with any other members of the family. One could interpret this result as indicating that the appropriate receptor pair for either one of these receptors is not yet known. The bulk of the data, however, suggests that any AcP-like molecule could cooperate in this system with any IL-1R-like molecule, even if they do not represent biologically relevant receptor heterodimers. This is presumably due to the fact that they are being expressed as chimeras, and association is induced by IL-1 and not by the natural ligand. Therefore, we would interpret the lack of any activity seen with both TIGIRR-1 and IL1RAPL as indicative of a novel class of IL-1R family member. The function of this third class of receptor types is not currently known.

Since the C-terminal extension in the cytoplasmic domain of these proteins may be inhibitory, both IL-1Rextm and AcPextm chimeras were created utilizing a truncated form of the TIGIRR-1 or IL1RAPL cytoplasmic domain. These constructs were analyzed for activity against the entire panel of IL-1Rextm or AcPextm chimeras. Removal of the C-terminal cytoplasmic domain extension of both TIGIRR-1 and IL1RAPL still did not render these receptors capable of signaling in these assays (data not shown). In order to verify that the observed lack of function was not due to the lack of expression, we have demonstrated expression of all chimeras by radioimmunoprecipitation (data not shown).

**DISCUSSION**

We describe the identification of two novel IL-1R family members, each of which displays a high degree of sequence conservation in both the extracellular and cytoplasmic domains to existing members of this family. Both receptors also contain a nonhomologous cytoplasmic domain extension, reminiscent of the *Drosophila* IL-1R/Toll family members Toll and 18-Wheeler. It should be noted, however, that sequence comparison within the cytoplasmic domain clearly shows that the new receptors are more similar to the IL-1R family than to the Toll family. Both receptors were identified from publicly available human X chromosome genomic sequence. Notably, these receptors do not lie within the IL-1R family cluster on chromosome 2 (5, 9, 47).

We detected TIGIRR-1 expression predominantly in skin and...
liver, although expression overall was quite low. We detected IL1RAPL expression in the brain, consistent with other findings (1), and in heart and muscle. By reverse transcriptase-PCR, expression was also noted in ovary and testis. Expression patterns of these two novel family members are therefore much more restricted than is IL-1 responsiveness.

We have examined the ability of TIGIRR-1 and IL1RAPL to mediate transcriptional activation in response to either IL-1 or IL-18, either against a full-length receptor or against a chimeric receptor containing the IL-1R extracellular and transmembrane regions. We found no evidence for the involvement of these receptors in signaling by either cytokine. It still could be that these receptors bind IL-1 or IL-18 yet do not elicit an NF-κB-dependent signaling pathway. Preliminary radioimmunoprecipitation experiments, however, show no evidence of binding of TIGIRR-1 or IL1RAPL to IL-1β or to any other IL-1-related ligands (58).

We hypothesized that these or other orphan IL-1R family members may serve an accessory function in IL-1 or IL-1-related cytokine signaling. To address the possibility that these novel receptors could play a role similar to the accessory protein, we created a series of chimeric receptor constructs that

### Table I
Sequences of chimeric receptor expression vectors

| IL-1R transmembrane domain | Receptor | Cytoplasmic domain |
|-----------------------------|----------|---------------------|
| ...YLGDFIILTIVCCVCIY        | IL-1R    | KIFKVDIVLW...*      |
| ...YLGDFIILTIVCCVCIY        | IL-18R   | KIFKVDIVLW...      |
| ...YLGDFIILTIVCCVCIY        | T1/ST2   | KIFKVDIVLW...       |
| ...YLGDFIILTIVCCVCIY        | IL-1Rrp2 | KIFKVDIVLW...       |
| ...YLGDFIILTIVCCVCIY        | AcP      | KIFKVDIVLW...       |
| ...YLGDFIILTIVCCVCIY        | IL-18RAcP| KIFKVDIVLW...       |
| ...YLGDFIILTIVCCVCIY        | TIGIRR-1 | KIFKVDIVLW...       |
| ...YLGDFIILTIVCCVCIY        | hIL-1RAPL| KIFKVDIVLW...       |
| ...YLGDFIILTIVCCVCIY        | hTIGIRR-1A| KIFKVDIVLW...      |
| ...YLGDFIILTIVCCVCIY        | hIL-1RAPL| KIFKVDIVLW...       |

| AcP transmembrane domain | Receptor | Cytoplasmic domain |
|--------------------------|----------|---------------------|
| ...LACGFGATVFLVVLVVYHVY  | IL-1R    | KIFKVDIVLW...       |
| ...LACGFGATVFLVVLVVYHVY  | IL-18R   | KIFKVDIVLW...       |
| ...LACGFGATVFLVVLVVYHVY  | T1/ST2   | KIFKVDIVLW...       |
| ...LACGFGATVFLVVLVVYHVY  | IL-1Rrp2 | KIFKVDIVLW...       |
| ...LACGFGATVFLVVLVVYHVY  | AcP      | KIFKVDIVLW...       |
| ...LACGFGATVFLVVLVVYHVY  | IL-18RAcP| KIFKVDIVLW...       |
| ...LACGFGATVFLVVLVVYHVY  | TIGIRR-1 | KIFKVDIVLW...       |
| ...LACGFGATVFLVVLVVYHVY  | hIL-1RAPL| KIFKVDIVLW...       |
| ...LACGFGATVFLVVLVVYHVY  | hTIGIRR-1A| KIFKVDIVLW...      |
| ...LACGFGATVFLVVLVVYHVY  | hIL-1RAPL| KIFKVDIVLW...       |

* Partial amino acid sequence of all chimeric receptors is presented; those amino acids differing from the native receptor are underscored.

| AcP transmembrane domain | Receptor | Cytoplasmic domain |
|--------------------------|----------|---------------------|
| ...LACGFGATVFLVVLVVYHVY  | IL-1R    | KIFKVDIVLW...       |
| ...LACGFGATVFLVVLVVYHVY  | IL-18R   | KIFKVDIVLW...       |
| ...LACGFGATVFLVVLVVYHVY  | T1/ST2   | KIFKVDIVLW...       |
| ...LACGFGATVFLVVLVVYHVY  | IL-1Rrp2 | KIFKVDIVLW...       |
| ...LACGFGATVFLVVLVVYHVY  | AcP      | KIFKVDIVLW...       |
| ...LACGFGATVFLVVLVVYHVY  | IL-18RAcP| KIFKVDIVLW...       |
| ...LACGFGATVFLVVLVVYHVY  | TIGIRR-1 | KIFKVDIVLW...       |
| ...LACGFGATVFLVVLVVYHVY  | hIL-1RAPL| KIFKVDIVLW...       |
| ...LACGFGATVFLVVLVVYHVY  | hTIGIRR-1A| KIFKVDIVLW...      |
| ...LACGFGATVFLVVLVVYHVY  | hIL-1RAPL| KIFKVDIVLW...       |

* The 5 C-terminal amino acids for the TIGIRR-1 and IL1RAPL constructs are presented, as they are represented in both full-length and truncated (Δ) forms.

* All sequences represent murine forms of the gene, unless indicated as human (h).

### Table II
Assessment of NF-κB activity following cotransfection of the indicated chimeric constructs

|             | IL1R | ILIR-IL18R | ILIR-IL1Rrp2 | ILIR-AcP | ILIR-IL18RAcP | ILIR-TIGIRR-1 | ILIR-hIL1RAPL |
|-------------|------|------------|--------------|----------|---------------|---------------|---------------|
| AcP         | +    | +          | +            | A        | -             | -             | -             |
| AcP-IL18RAcP| +    | +          | +            | +        | -             | -             | -             |
| AcP-IL1R    | -    | -          | -            | -        | -             | -             | -             |
| AcP-IL18R   | -    | -          | -            | -        | -             | -             | -             |
| AcP-ST2     | -    | -          | -            | -        | -             | -             | -             |
| AcP-IL1Rrp2 | -    | -          | -            | -        | -             | -             | -             |
| AcP-TIGIRR-1| -    | -          | -            | -        | -             | -             | -             |
| AcP-hIL1RAPL| -    | -          | -            | -        | -             | -             | -             |

1 Level of NF-κB activity following stimulation with IL-1α or IL-1β in S49.1 cells overexpressing the indicated chimeric receptors and an NF-κB-luciferase reporter plasmid. Activity is characterized as highly induced (+ +), induced (+), or not induced (−). Identical results were obtained with both IL-1α and IL-1β. Data represent results from at least two separate stimulations and luciferase assays from the same electroporation.

2 All sequences are murine except for hIL1RAPL, which is human.
could be systematically tested against one another. Our results indicate that, not surprisingly, the known IL-1R family members do not signal in either of two capacities: IL-1R-like or AcP-like. The already characterized AcP and IL-18RAcP both serve accessory roles in signaling, and in the above described series of experiments they can function with any member of the IL-1R-like class (IL-1R, IL-18R, T1/ST2, IL-1Rrp2) to form a functional signaling complex. Both TIRGIRR-1 and IL1RAPL, however, could not function in either capacity in these assays. Since it has been proposed that perhaps the C-terminal extension in the Toll receptor is inhibitory (56), we have created chimeric receptor constructs containing a truncated cytoplasmic region for both TIRGIRR-1 and IL1RAPL. The truncated ceramicas still did not display any signaling capacity in our assays. These results raise several interesting possible interpretations as to the function of TIRGIRR-1 and IL1RAPL, as well as implications on IL-1R family signaling in general. It may be that TIRGIRR-1 and IL1RAPL function generally similar to the Toll subfamily than to the IL-1R subfamily, despite the fact that a higher amount of sequence conservation exists to the IL-1R subfamily. Since there is currently no evidence that Toll receptors function as obligate heterodimers (as do IL-1Rs), this could explain why TIRGIRR-1 and IL1RAPL failed to signal in the chimeric experiments. Our results, however, do not suggest that these novel receptor functions as homodimers either, in that coexpression of IL-1Ralpha-TIRGIRR-1cytoplasmic and AcPalpha-TIRGIRR-1cytoplasmic did not result in NF-kB signaling. It may also be that different signaling molecules are required by these receptors, which are not expressed in COS7 or S49.1 cells. Alternatively, the secondary structure of the cytoplasmic domain may be disrupted in the chimeric molecules, thus compromising their ability to signal, or perhaps the geometry of the interaction between the two subunits is faulty in the particular chimeras used (59). It is also possible that unclassified receptors such as TIRGIRR-1 and IL1RAPL form a third subunit of IL-1R signaling complexes, and the assays we have undertaken do not address this role. This is unlikely, given that there are many cell types capable of responding to IL-1 yet lacking either TIRGIRR-1 or IL1RAPL expression. Finally, it could be that receptors such as TIRGIRR-1 and IL1RAPL, although bearing high homology to other IL-1R family members, function to signal by a very different mechanism than do the receptors for IL-1 or IL-18 (i.e. they do not result in NF-kB activation).

Another receptor similarly containing an extended cytoplasmic domain is SIGIRR (10). Our studies have not included an exhaustive panel of SIGIRR chimeras because the extracellular region of SIGIRR contains only a single Ig domain, suggesting that its function may be quite different. Preliminary experiments with SIGIRR, however, indicate that it behaves similarly to TIRGIRR-1 and IL1RAPL in the chimera experiments (10). At this time, we clearly do not have a firm understanding of the signaling mechanisms employed by receptors such as SIGIRR, TIRGIRR-1, and IL1RAPL.

The biological roles played by SIGIRR and TIRGIRR-1 are also incompletely understood at this point. The independent identification of IL1RAPL, however, was driven by the search for a gene responsible for X-linked mental retardation (1). This suggests that this novel class of IL-1R-related molecules may indeed have very interesting biological roles, perhaps playing a role in the early development of the organism as does the Drosophila Toll protein. It remains to be seen whether they are also active in immune regulation and host defense as are IL-1R, IL-18R, and TLRs.

Acknowledgments—We thank Gordana Sapina and Deborah Silber for sequence analysis and Gary Carlton for graphical design.
Novel IL-1R Family Members

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