Identification of Two Major Sites in the Type I Interleukin-1 Receptor Cytoplasmic Region Responsible for Coupling to Pro-inflammatory Signaling Pathways

(Received for publication, October 19, 1999, and in revised form, November 29, 1999)

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Type I interleukin-1 receptor is the prototype for a family of proteins, which play a central role in early responses to injury and infection. The similarity of function across the family is reflected in similarity in signaling: all members tested couple to activation of NFκB and stress kinases. The coupling to these pathways is mediated by a 200-residue intracellular domain (the Toll/interleukin-1 receptor domain), in which sequence conservation is primarily confined to three short motifs (boxes 1, 2, and 3) located at amino acid residue positions 10 (box 1), 60 (box 2), and 170 (box 3). We have analyzed the contribution of these motifs to function by alanine scanning mutagenesis of the human interleukin-1 receptor type I. Mutant receptors were tested for expression, ligand binding, activation of receptor-associated kinase(s), NFκB, stress kinases, and transcription. Mutations in all three motifs led to low cell surface expression. Mutants in box 3 were, however, wild type for signaling, whereas mutants in boxes 1 and 2 were defective. We conclude that the conserved motifs box 1 and box 2 mediate the coupling of molecules in the family to inflammation signaling pathways.

Interleukin-1α and interleukin-1β (IL-1α and IL-1β) are two polypeptide cytokines, which play a central role in coordinating immune and inflammatory responses (1). These cytokines and a structurally related antagonist (IL-1ra) bind to a common receptor composed of two type I integral membrane proteins (IL1RI and IL1RαcP) (2, 3) with which several intracellular signaling molecules have been reported to be associated. The two proteins are part of a family of molecules of diverse structure, which share in common an intracellular 200-residue domain (the Toll/Interleukin-1 receptor domain (TIR)): IL1RRP, ST2, IL18R, and IL18RacP (4–6) and have a similar overall structure to IL-1RI (3 Ig domains (extracellular) and a TIR domain (cytoplasmic)). These receptors couple to two major inflammatory signaling systems, NFκB and stress kinases. The family also contains a second subgroup of receptor molecules, type I membrane proteins with a TIR domain and an extracellular region composed of a series of leucine rich repeats, and the prototypic member is the insect protein Toll (4, 7, 8). Toll plays a central role in innate immunity in adult flies, controlling expression of cecropin genes through NFκB-like sites in their promoters (9, 10). Three other TIR insect receptors have been described; mstprox (4), 18 wheeler (11), and Tehao; 18 wheeler also plays a role in host defense (11). Recently, a series of mammalian Tolls have been identified (tlr1 (4, 12), tlr2 (12), tlr3 (12), tlr4 (12), tlr5 (12), and tlr6 (13)). Preliminary evidence suggests that these play a role in innate immunity, most compellingly that the lps mutation maps to the tlr4 locus (14). The family also contains several intracellular proteins, in particular a number of plant proteins, which are implicated in host defense (15). Finally a mammalian family member myD88 (4, 16), composed of an N-terminal death domain and a C-terminal TIR domain, is involved in coupling the IL-1 receptor complex to downstream signaling pathways (17–20). It appears therefore that this family of molecules and the signal pathways they couple to represents an ancient host defense system present throughout the biological world (21). There are also several viral genes, which show significant homology to this family (8 and Fig. 1), and it has been suggested that like the B15K gene of vaccinia (22, 23), a soluble IL-1 receptor, these may play a role in viral subversion of host defenses.

IL-1R family receptors couple to downstream signaling pathways via a number of intermediates. Three pelle homologues have been found in mammals (IRAK-1, IRAK-2, and IRAK-M (19, 24, 25, 27, 32)). IRAK-1 associates with AcP and IRAK-2 with IL-1RI (19). The receptor complex also associates with TRAF-6 (28), myD88 (17), IIP-1 (30), and AcPIP (31). In vitro kinase assays have shown that IL-1 activates a receptor-associated kinase (33, 34). However, recent data suggest that the kinase activity of IRAKs is not necessary for IL-1 signaling (27). TRAF-6 is the upstream regulator of TAK-1 (35) and NFκB-inducing kinase (36) coupling IL-1R to activation of NFκB.

To dissect the structure-function relationships in signal activation for the TIR family we have analyzed the effects of cytoplasmic domain point mutations on IL-1RI activity. The mutations we have generated have been placed into regions of the domain most conserved across the family. In addition we have mutated several other charged residues predicted to be at the protein surface. Several previous reports have described the results of site-directed mutagenesis studies of the IL-1R
domain (34, 37, 38). In general these have focused on the C-terminal conserved region (RFW\textsubscript{X}N, where X is a basic residue); the region first noted as suggesting a relationship between Drosophila Toll and IL-1RI (7). Our data suggest that this region is mainly involved in controlling receptor trafficking and that signaling interactions are mediated through two other conserved regions. Thus the receptor may interact with intracellular proteins through at least three sites, providing a rationale for the pattern of sequence conservation across this family of signaling proteins.

**EXPERIMENTAL PROCEDURES**

**Chimeric Hin-Mext Constructs—**All receptor constructs were cloned into pDC304, a variant of pDC302 (39). To construct the wild type chimeric receptor, we first introduced a \textit{Bgl}II site into the murine IL-1RI 3\textsuperscript{9} of the transmembrane domain. This caused a conservative amino acid sequence change from KVF to KIF (KIF is also the human IL-1RI sequence at this position). The cytoplasmic region of the human IL-1RI was then cloned into the expression construct after \textit{Bgl}II digestion of both receptor cDNAs.

**Mutagenesis—**The result of an alignment of 29 members of the IL-1 receptor family using amino acid sequence fragments spanning the IL-1R homology domain is shown in Fig. 1. The degree of sequence conservation is plotted as the profile score (40). The analysis reveals three short motifs in the human IL-1 receptor that are most conserved across the family members, YDAYILY (box 1), IYGRDDY (box 2), and TRFWKNV (box 3). In addition there is a region between box 2 and box 3 that shows a low but significant level of sequence homology across the family. We performed alanine substitution, selecting charged residues that, by hydrophobicity analysis, are predicted to lie in regions of the protein that are (a) predicted to be on the surface and (b) are conserved across the family. There were five exceptions K428A, R431A/S, E474A, K492A, and W514A, the first two in a predicted protein kinase C site (KKSRR) the third and fourth in poorly conserved regions, and the fifth a bulky hydrophobic residue, because mutants at these sites have been described previously. In addition we mutated Tyr-374 the C-terminal residue in box 1 to F, because this is more conservative than Y → A. In
addition to the point mutants we also generated two triple substitution mutants, in box 1 and box 2 (386AAA: TYDIALY → AAAALY and 411AAA: YGRDDY → YIAADY). We reasoned that this set of mutations would alter potential cytoplasmic binding sites with minimal perturbation of overall protein structure. All mutations were made using site-directed mutagenesis kit (CLONTECH) and the appropriate mutagenic oligonucleotides in the type I receptor construct HinMext (see above). Mutations were confirmed by sequencing. We have shown previously that HinMext with a wild type cytoplasmic domain is functionally indistinguishable from human IL-1RI.

**Transient Transfections and Analysis of Expression of Receptors**—Transfections were done with the DEAE/dextran method, followed by chloroquine treatment, as described, in 293-EBNA cells (human embryonic kidney cells stably expressing the EBNA-1 nuclear antigen (41). 72-h post-transfection, cells were bound with a 125I-labeled rat monoclonal antibody (M5) to the murine IL-1RI or with 125I-labeled human IL-1α. Wells were washed, stripped with sodium hydroxide, and counted, and data were presented as total counts averaged over 2 wells. Controls were done in the presence of 100-fold excess unlabeled antibody or IL-1, and these values were subtracted from the data prior to analysis or presentation. Binding dose-response curves for 125I-labeled human IL-1α were done over a 1000-fold range in IL-1 concentration, and values for affinity constants and site numbers were determined by curve fitting with a simple one site binding model using MLAB for the Macintosh (Civilized Software, Silver Spring, MD).

Analysis of total receptor expression was done by extracting transfected cells with 1% Triton X-100, spotting 1-ml aliquots onto nitrocellulose filters, blocking with binding medium containing 5% (w/v) non-fat dry milk, and incubating with 125I-labeled rat monoclonal antibody (M5) to the murine IL-1RI or with 125I-labeled human IL-1α. Wells were washed, stripped with sodium hydroxide, and counted, and data were presented as total counts averaged over 2 wells. Controls were done in the presence of 100-fold excess unlabeled antibody or IL-1, and these values were subtracted from the data prior to analysis or presentation. Binding dose-response curves for 125I-labeled human IL-1α were done over a 1000-fold range in IL-1 concentration, and values for affinity constants and site numbers were determined by curve fitting with a simple one site binding model using MLAB for the Macintosh (Civilized Software, Silver Spring, MD).

**Signal Transduction Assays**—2 days post-transfection, confluent transfected cells were stimulated with 1.0 ng/ml huIL-1α for 30 min for the NF-κB assay. For the Thr-669 kinase assay (44), stimulation was with 10.0 ng/ml huIL-1 for 15 min. For the IL-8 promoter activation assay, cells were stimulated 1 day post-transfection with 1 ng/ml huIL-1 or left unstimulated. 12–16 h post-stimulation, cells were washed twice with binding medium (RPMI 1640, 20 mM HEPES, pH 7.4, 0.1% (w/v) sodium azide) containing 5% (w/v) non-fat dry milk and blocked with 2 ml of the same medium at room temperature for 30 min. Cells were then incubated at room temperature for 60–90 min with 1:100 dilution of 125I-goat anti-mouse IgG (Sigma) for 60 min at room temperature. Cells were washed four times with the same medium and twice with phosphate-buffered saline, stained with 1 ml of 0.1 M NaOH, and total counts were determined. Results are expressed as total cpm averaged over two wells.

**Confocal Microscopy—**293 EBNA cells (1 × 105 cells/well) cultured in 8-well Lab-Tek Chambered coverglasses were transfected with 1000 ng of plasmid DNA by the DEAE dextran method, cultured for a further 72 h, and fixed with 2% paraformaldehyde (nonpermeabilized) or with 2% paraformaldehyde containing 0.1% Triton X-100 (permeabilized). Immunofluorescence was quenched by treatment with 50 mM NH4Cl for 10 min. For the permeabilized cell analysis, cells were then incubated for 1 h in blocking/permeabilization buffer (phosphate-buffered saline containing 5% normal goat serum and 0.1% Triton X-100). Cells were then incubated with rat monoclonal antibody (M5) to the murine IL-1RI (1 μg/ml in blocking or blocking/permeabilization buffer) for 1 h. Cells were washed three times with blocking/permeabilization buffer and incubated with biotinylated goat anti-rat immunoglobulin (Molecular Probes, 2 μg/ml in blocking/permeabilization buffer) for 1 h. Cells were washed three times with buffer and incubated with a streptavidin Texas Red conjugate (Molecular Probes) at 0.2 μg/ml in buffer for 30 min. Cells were washed three times with buffer and incubated with a streptavidin Texas Red conjugate (Molecular Probes) at 0.2 μg/ml in buffer for 30 min. Cells were washed three times with buffer and incubated with a streptavidin Texas Red conjugate (Molecular Probes) at 0.2 μg/ml in buffer for 30 min. Cells were washed three times with buffer and incubated with streptavidin Texas Red conjugate (Molecular Probes) at 0.2 μg/ml in buffer for 30 min. Cells were washed three times with buffer and incubated with a streptavidin Texas Red conjugate (Molecular Probes) at 0.2 μg/ml in buffer for 30 min. Cells were washed three times with buffer and incubated with a streptavidin Texas Red conjugate (Molecular Probes) at 0.2 μg/ml in buffer for 30 min. Cells were washed three times with buffer and incubated with a streptavidin Texas Red conjugate (Molecular Probes) at 0.2 μg/ml in buffer for 30 min. Cells were washed three times with buffer and incubated with a streptavidin Texas Red conjugate (Molecular Probes) at 0.2 μg/ml in buffer for 30 min. Cells were washed three times with buffer and incubated with a streptavidin Texas Red conjugate (Molecular Probes) at 0.2 μg/ml in buffer for 30 min. Cells were washed three times with buffer and incubated with a streptavidin Texas Red conjugate (Molecular Probes) at 0.2 μg/ml in buffer for 30 min. Cells were washed three times with buffer and incubated with a streptavidin Texas Red conjugate (Molecular Probes) at 0.2 μg/ml in buffer for 30 min. Cells were washed three times with buffer and incubated with a streptavidin Texas Red conjugate (Molecular Probes) at 0.2 μg/ml in buffer for 30 min. Cells were washed three times with buffer and incubated with a streptavidin Texas Red conjugate (Molecular Probes) at 0.2 μg/ml in buffer for 30 min. Cells were washed three times with buffer and incubated with a streptavidin Texas Red conjugate (Molecular Probes) at 0.2 μg/ml in buffer for 30 min. Cells were washed three times with buffer and incubated with a streptavidin Texas Red conjugate (Molecular Probes) at 0.2 μg/ml in buffer for 30 min. Cells were washed three times with buffer and incubated with a streptavidin Texas Red conjugate (Molecular Probes) at 0.2 μg/ml in buffer for 30 min. Cells were washed three times with buffer and incubated with a streptavidin Texas Red conjugate (Molecular Probes) at 0.2 μg/ml in buffer for 30 min. Cells were washed three times with buffer and incubated with a streptavidin Texas Red conjugate (Molecular Probes) at 0.2 μg/ml in buffer for 30 min. Cells were washed three times with buffer and incubated with a streptavidin Texas Red conjugate (Molecular Probes) at 0.2 μg/ml in buffer for 30 min. Cells were washed three times with buffer and incubated with a streptavidin Texas Red conjugate (Molecular Probes) at 0.2 μg/ml in buffer for 30 min. Cells were washed three times with buffer and incubated with a streptavidin Texas Red conjugate (Molecular Probes) at 0.2 μg/ml in buffer for 30 min. Cells were washed three times with buffer and incubated with a streptavidin Texas Red conjugate (Molecular Probes) at 0.2 μg/ml in buffer for 30 min. Cells were washed three times with buffer and incubated with a streptavidin Texas Red conjugate (Molecular Probes) at 0.2 μg/ml in buffer for 30 min. Cells were washed three times with buffer and incubated with a streptavidin Texas Red conjugate (Molecular Probes) at 0.2 μg/ml in buffer for 30 min. Cells were washed three times with buffer and incubated with a streptavidin Texas Red conjugate (Molecular Probes) at 0.2 μg/ml in buffer for 30 min. Cells were washed three times with buffer and incubated with a streptavidin Texas Red conjugate (Molecular Probes) at 0.2 μg/ml in buffer for 30 min.

**RESULTS**

**Analysis of Expression of Receptor Mutants**—All mutants were tested for cell surface expression after transient expression in 293-EBNA cells. Mutations in all three conserved motifs generated a low expression phenotype (Fig. 2). It is not clear

![Image](https://example.com/image.png)

**FIG. 2.** Cell surface expression of cytoplasmic region mutant IL-1 receptor type I chimeras. 293-EBNA cells were transfected with the receptor mutants, wild type IL-1 receptor type I chimera (Hin-Mext), or with murine IL-1 receptor lacking the entire cytoplasmic domain (Mext) at the plasmid doses indicated. Cells were cultured and subsequently tested for cell surface expression at a single dose of 125I-IL-1α, previously established to be saturating for Hin-Mext as described under “Experimental Procedures.” The dotted line indicates the expected expression level for Hin-Mext transfected at 50 ng/culture.
from this experiment whether the apparent depression in expression results from a decrease in affinity for ligand or a decrease in receptor expression. However, we show below that the affinity of all mutants for ligand is indistinguishable from the chimera with a wild type cytoplasmic domain (see Fig. 6).

The effect appears to be clear cut, mutants either fall into a group that are wild type for expression or into a low expression group. Because all three conserved motifs appeared to be involved, subsequent transfection experiments were modified to compensate, for wild type expressers, 50 ng of expression plasmid were used, and for low expressers, 1000 ng, because this is saturating for expression in the system. The expected cell surface expression is indicated by the dotted line in Fig. 2.

To analyze this further, the binding of the rat anti-mouse type I IL-1 receptor monoclonal antibody (M5) was compared with that of IL-1. All transfected cells bound both antibody and IL-1 with 50% as many antibody binding sites as IL-1 binding sites (Fig. 3A), irrespective of the level of IL-1 binding sites present. This is consistent with bivalent binding of the antibody, as shown previously. M5 is a nonblocking antibody and recognizes an epitope outside the IL-1 binding site, thus the constant ratio of ligand to antibody bound suggests that none of the mutant receptors have any gross perturbation of ligand binding. Further, the data show that at the doses established from Fig. 2, the low expresser group express at the cell surface in the same range as the wild type group.

The total level of expression (cell surface and intracellular) of all mutants was determined by M5 and by IL-1 binding in a dot blot assay as described previously (Fig. 3B). Under conditions where there are wild type levels of cell surface expression of the low expressers, extensive accumulation of receptor occurs within the cells. Next, parallel transfected cultures were tested for both cell surface and total expression. The wild type expressers all showed a ratio of cell surface to total expression of
EBNA cells were transfected with 1 μg of plasmid DNA encoding the indicated mutants, incubated in Lab-Tek chambered coverglasses, and tested for binding of mIL1R-M5 by indirect immunofluorescence as described under “Experimental Procedures.”

1 when tested with IL-1; thus all receptors are expressed at the cell surface. By contrast, the low expressers showed low ratios in the range 0.05–0.25. Similar results were obtained with M5, although the ratio for the wild type group is lower, possibly because the antibody binds monovalently to the immobilized receptor and bivalently to intact cells. The data suggest that the wild type group were efficiently transported to the cell surface, whereas the low expressor group share a common defect in transport.

The four low expression point mutants, the wild type receptor, and one wild type expresser (R431A) were transfected into 293-EBNA cells at a high plasmid dose (1000 ng), and the distribution of receptors was examined by confocal microscopy after staining with M5 (Fig. 4) with or without permeabilization. Examination of nonpermeabilized cells showed that both the wild type receptor and R431A were expressed at the cell surface. By contrast little (R411A) or no (D369A, W514A, W514A, K5151A) expression of the low expresser mutants was observed. Examination of permeabilized cells revealed a similar high level of expression for all receptors. Thus at a high plasmid dose, large amounts of protein accumulate in the cells, irrespective of which of the two classes a receptor belongs. Further, there were no obvious differences between the two groups in the pattern of intracellular distribution. Thus the defect in the low expressers does not lead to misfolding and degradation but specifically affects transport.

These findings create a dilemma for functional analysis; if low plasmid doses are used in transfections for the low expressers, little or no expression will occur at the cell surface and they may be spuriously scored as signaling defective. However if high dose transfections are used to compensate, apparent signal defects observed in the face of a large intracellular pool of receptors may be secondary to the transport defect if the intracellular pool, likely inaccessible to ligand, acts as sink for signaling components such as myD88 and TRAF6 by binding and sequestering them.

Coupling of IL-1 Receptor to Signal Transduction Pathways—Mutant receptors were tested for activation of NFκB and stress kinases following IL-1 stimulation. In addition, binding dose-response experiments were done for all mutants; there was no effect of any mutations on the affinity of cell surface receptors for IL-1 (Fig. 6).

Gel shift analysis of nuclear NFκB activity in cells transfected with wild type receptor, a mutant with the entire cytoplasmic region deleted (MEXT), and the cytoplasmic point mutants is summarized in Figs. 5 and 6. In general, with the exception of the triple mutants and the MEXT mutant all the receptors showed at least partial activity. Quantitative analysis of the data (Fig. 6) confirmed that, with the exception of D369A and R411A, which showed a partial reduction in activity, all point mutants were wild type. A critical control aspect of these data is that the trafficking defective box 3 mutants R512A, W514A, and K515A were wild type for activation of NFκB, showing that when a large intracellular pool of receptors accumulates, this does not act as sink for critical signaling components. Thus the NFκB activation phenotype of D369A and R411A is not caused by the trafficking defect.

Fig. 6 also summarizes the capacity of the mutant receptors to couple to the stress kinase pathway; of the point mutants only R411A (box 2) showed any detectable loss of function. Although D369A was wild type, the involvement of box 1 in coupling to this pathway was suggested by the loss of activity observed with the 368AAA mutant. A similar phenotype was also shown by the box 2 triple mutant. Some caution must be exercised in interpreting data from these triple mutants; however, because these may cause structural perturbation of the protein.

We also examined the capacity of the receptors to activate receptor-associated protein kinase(s). Because the data in Figs. 5 and 6 suggest that coupling to the NFκB and stress kinase pathways involves boxes 1 and 2 but not box 3, we focused on these regions of the protein for examination of receptor-associated kinase activity. Given recent findings with IRAKs, suggesting that their kinase activity may not be required for signaling through IL-1 receptors (27, 46), we used the original receptor-associated kinase assay (33). When receptors extracted from cells transfected with HinMext and subsequently treated with IL-1 were immunoprecipitated with M5 and the immunoprecipitates incubated with [γ-32P]ATP and histone H1, there was increased kinase activity relative to samples from cells not treated with IL-1 prior to extraction. A small increase in histone phosphorylation and a large increase in phosphorylation of an endogenous 65K substrate were observed (Fig. 7). Phospho-amino acid analysis showed that all the phosphate incorporated was on serine residues (data not shown). Both D369A (box 1) and R411A (box 2) showed no IL-1-induced receptor-associated kinase activity, whereas the nearby mutants Y374F and R431A were wild type. These data show that there is a correlation between loss of receptor-associated kinase activation and impaired coupling to the NFκB and stress kinase pathways. However complete loss of receptor-associated kinase activity correlates with only partial loss of activation of early downstream signals.

The capacity of mutant receptors to mediate IL-1 induction of gene expression was examined in a model system using a reporter containing the IL-2 receptor α chain under the control of the human IL-8 promoter (−130 to +45 relative to transcrip-
activity after 15 min of IL-1 receptor to activate stress kinases in response to ligand binding. Analysis of the effect of cytoplasmic domain mutations on the capacity of the receptor to activate expression of the reporter is more sensitive to mutations than are activation of the NFκB or stress kinase pathways. Reporter activation, however, correlates well with loss of receptor-associated kinase activation (Fig. 7).

**DISCUSSION**

The receptors and related signal transduction proteins, defined by sharing the IL-1 receptor homology domain, all play a central role in host defense against pathogens. The current availability of sequences from currently over 50 family members allows those regions that are highly conserved among the family members, and thus likely functionally important, to be defined. Most of the significant homology between the family members is confined to three short motifs with consensus sequences: box 1 (FDAPYSY), box 2 (GYKLC—RD—PG), and box 3 (a conserved W surrounded by basic residues). The importance of these sequences for function is underscored by the observation that the P in box 2 is the site of the P^h^ mutation in mtxt1, which gives rise to the Lps5 phenotype (14). This is 4 residues downstream of the conserved R in box 2, which when mutated in huIL1RI leads to both marked depression of cell surface expression and loss of signaling activity. Indeed this is the only point mutation in the set we examined that gave a loss of function phenotype in all assays (Fig. 9).

Several previous reports have examined the effects of mutations in the cytoplasmic region of IL1RI on signaling function. Of these, the first showed that the cytoplasmic region was required for activity; our results with the control MEXT construct are in accord with this finding (47). Later, Kuno et al. (38) mapped the cytoplasmic region of IL1RI by deletion mutagenesis. The structure/function relationships of the intracellular portion of the mouse IL-1 receptor type I were examined with regard to activation of the IL-8 gene in the Jurkat T cell line. Deletion mutations revealed that the boundary for receptor function is localized between 28 and 42 amino acids from the C terminus, spanning box 3. Internal deletion mutants between amino acids 364 and 474 (spanning box 1 and box 2) lost activity. Amino acid substitutions revealed that putative nuclear localization elements (amino acids at 429–433, 523–527, and 507–519) and putative protein kinase C or A acceptor sites (Ser-431, Ser-509, Ser-528) are not critical. Whereas some reservations should be borne in mind with respect to the interpretation of large scale deletion mutations, these data are

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FIG. 7. Effect of box 1 and box 2 mutations on IL-1-induced receptor-associated kinase activity. 293-EBNA cells were transfected with 1 μg of plasmid encoding the indicated mutants or wild type receptors and cultured as in "Experimental Procedures." Cultures were then treated with 1 ng/ml (6 × 10^−11 M) IL-1α and extracted, and receptor complexes were immunoprecipitated with mIL1R1M5, a non-blocking antibody. Receptor-associated kinase activity was assayed by an in vitro kinase assay with histone H1 added as an exogenous substrate. IL-1 induced phosphorylation of H1, and an endogenous 65 K^s^ substrate is observed (arrows) after separation of the reaction mixtures on SDS-polyacrylamide gels (12%) and autoradiography by a PhosphorImager.

**FIG. 6.** Quantitative analysis of mutant IL-1 receptor function. Upper panel, lack of effect of cytoplasmic domain mutations on IL-1α binding activity of the receptor. 293-EBNA cells were transfected and cultured as described under "Experimental Procedures." Subsequently cells were incubated with 125I-IL-1α spanning a 1000-fold range in concentration (10 points). Unlabeled IL-1 was added to determine non-specific binding (2 points) as described previously, and data were corrected by subtraction. Counts were determined by y-counting. Data were converted to molecules ligand bound/cell and free ligand concentration (M) and analyzed by nonlinear least square fitting of a simple onec site binding model using MLAB. The affinity constants are shown with the S.E. derived from the curve fit as the error bar. Middle panel, quantitative analysis of the effect of cytoplasmic domain mutations on the capacity of IL-1 receptor to activate NFκB DNA binding activity in response to ligand binding. Data from electrophoretic mobility shift assay experiments were obtained using a PhosphorImager, and the 16 bit digital images were analyzed using ImageQuant software. The NFκB activity in 293-EBNA cells is detected as two bands, a faster moving band, which is constitutive, and a slower band, which is IL-1-inducible; only the slower band was used in the analysis. Data are expressed as the ratio of band intensity after 30 min of IL-1 treatment with that observed in an untreated cultured transfected with the same receptor construct and cultured in parallel. Lower panel, quantitative analysis of the effect of cytoplasmic domain mutations on the capacity of IL-1 receptor to activate stress kinases in response to ligand binding. Transfected cultures were extracted and analyzed for the capacity to phosphorylate the stress kinase substrate peptide (residues 663–673 of EGFR) as described elsewhere (44). Data are expressed as the ratio of activity after 15 min of IL-1α treatment (10 ng/ml; 6 × 10^−10 M) with that observed in an untreated cultured transfected with the same receptor construct and cultured in parallel. Error bars are from triplicate assays (S.D.).
broadly in agreement with our findings, in particular in showing that deletions affecting all three conserved motifs abolish signaling activity and that mutations outside these regions have no effect on phenotype. Heguy et al. (37) described an analysis of human IL-1RI function using a site-directed mutagenesis strategy based on the homology between IL1RI and Drosophila Toll. Three basic (Arg-431, Lys-515, and Arg-518) and three aromatic (Phe-513, Trp-514, and Tyr-519) amino acids that are conserved in human, murine, and chicken IL-1Rs and lie in regions of similarity to Toll could not be replaced in human IL1RI without abolishing IL-1R-mediated signal transduction. A substitution at another conserved position (Pro-521) reduced significantly the ability of the IL-1R to signal. This report focused primarily on box 3, showing that this was important for signaling activity. We have, however, failed to confirm the observation that mutations in the putative protein kinase C acceptor site at 428–432 affect function, agreeing rather with Kuno et al. (38) in this respect. Also it is now clear that this site is not well conserved across the family. Finally, a recent report analyzed the effects of box 3 mutations on both IRAK activation and coupling of the receptor to activation of NFKB (34). The data were in general agreement with those reported by Kuno et al. (38) showing that deleting residues C-terminal to Box 3 produced little effect, whereas deletion of box 3 led to concurrent loss of both IRAK and NFKB activation by IL-1. In summary previous reports have focused primarily on box 3 and provided strong evidence that it is critical for receptor function. Our data suggest that the primary function of this region is control of subcellular location of the receptor.

The findings of the current report are summarized in Fig. 9. The top left panel shows that mutations in all three conserved motifs cause a marked loss of cell surface expression. We have reported previously that IL-1 receptors are localized to focal adhesions in cells attached to fibronectin and that IL-1 causes rapid phosphorylation of talin and transient changes in cytoskeletal organization (26, 48, 49). Further, recent data from our laboratory show that there is an attachment-mediated recruitment of a cell surface heparan sulfate to the IL-1 receptor complex, correlating with effects on signaling (29). We therefore speculate that box 3 is (part of) a site that mediates binding of IL-1RI to one or more focal adhesion or cytoskeletal components. Disruption of cytoskeletal interactions would be expected to reduce the stability of the cell surface receptor pool in favor of intracellular pools and would provide an explanation for the trafficking properties of the mutants. In support of this view, an EGFP fusion to wild type receptor shows localization to focal adhesions in cells plated on fibronectin, whereas a fusion to W514A does not.2 Box 1 and box 2 mutants are both

FIG. 8. Effect of cytoplasmic domain mutations on the activation of a driven pIL-8 reporter. Reporter 293-EBNA cells were transfected, cultured and stimulated with IL-1α as described under "Experimental Procedures." Data are expressed as the ratio of [125I]-2A3 monoclonal anti-human IL-1Ra chain antibody to cultures transfected and not stimulated; error bars (S.D.) were calculated from duplicate cultures.

FIG. 9. Summary of results showing pairwise correlation between effects of cytoplasmic region mutations on receptor function. Top left panel, data from Fig. 3C replotted with the trafficking defective mutants filled in black in this and all three other panels. Top right panel, correlation between NF-kB and stress kinase (T669) activity data from Fig. 6, B and C. Bottom panels show a correlation between IL-8 reporter activity and the activity of the mutants in the early signal transduction response assays. The straight line drawn in the top left panel is calculated from a simple linear regression analysis for the receptors that have a wild type trafficking phenotype. In the other three panels the lines are hand drawn to emphasize the trends in the data for all receptors (top right) and for the trafficking/reporter activation defective receptors (bottom panels).

2 E. Qwarnstrom, unpublished observations.
trafficking and signaling defective. It is possible that the former is secondary to the latter possibly due to a primary failure to interact with one or more components of the IL-1 signaling complex, destabilizing it and perhaps mimicking the effect of IL-1 binding. This is consistent with the finding that one effect of IL-1 binding to cells is the dissociation of IRAK-1 from the receptor complex with subsequent degradation by proteasomes (32).

When the effects on two early responses (NFκB and stress kinases) are examined (Fig. 9, top right panel) there is an approximately linear relationship, possibly because both pathways couple to the receptor through a common site or at least overlapping sites. The two bottom panels show the correlation plots for the early signaling activities and the activation of the IL-8 promoter. The data show that not only do mutations in all three motifs give rise to a partial defect (box 3) or a complete defect (box 1 and 2), in activation of transcription, but also that there seems to be a correlation between loss of reporter stimulation and loss of activation of early signals. This observation is in one sense unsurprising; the IL-8 promoter contains consensus sites for both AP-1 and NFκB and it would therefore be expected that defects in coupling to NFκB and stress kinase activation would correlate with a defect in activation of this promoter. However, in the box 1 and 2 mutants a partial defect in the activation of these pathways correlates with a complete loss of activity in the reporter assay. Also these mutants show no detectable activity in the receptor-associated kinase assay but still activate NFκB partially. Thus there are likely to be other receptor proximal interactions, which may be other receptor proximal interactions, which can couple IL-1 to but still activate NFkB partially. Thus there are likely to be promoter. However, in the box 1 and 2 mutants a partial defect correlation with a defect in activation of this expected that defects in coupling to NFkB and stress kinase activation would correlate with a defect in activation of this promoter. However, in the box 1 and 2 mutants a partial defect in the activation of these pathways correlates with a complete loss of activity in the reporter assay. Also these mutants show no detectable activity in the receptor-associated kinase assay but still activate NFκB partially. Thus there are likely to be other receptor proximal interactions, which may be other receptor proximal interactions, which can couple IL-1 to such pathways, and other signaling activities, which may be required for full activation of the IL-8 promoter. A recent report has shown that IRAK (−/−) cells reconstituted by transfection with a kinase (−) IRAK mutant respond to IL-1 and that in these cells IRAK is phosphorylated in response to IL-1 (27). Indeed it has been suggested that the kinase activity of IRAK-1 and IRAK-2 is not required for IL-1 signaling (29). These data suggest that there is at least one protein kinase other than IRAK-1 or IRAK-2, possibly IRAK-M, associated with the IL-1 receptor complex, and which by inference participates in IL-1 signaling (46). Disruption of this or other as yet unidentified interactions could account for the loss of reporter activation that we observed.

In summary, the structure function mapping data reported here identify three regions of the human type I IL-1 receptor cytoplasmic region as critical for function. As expected, these map to those regions that show the highest degree of sequence conservation among the ~50 known members of the IL-1 receptor/Toll family. Alanine substitution mutants in several charged residues predicted to be on the surface of the protein but outside these motifs are wild type. The three motifs can be functionally separated into two groups. Whereas mutations in all three lead to a loss of surface expression, only mutations in box 1 and box 2 cause a direct loss of signaling activity. Further, we cannot find any clear cut differences between the effects of box 1 and box 2 mutations in 4 different signaling assays, although box2 mutations appear to have stronger effects, suggesting that these may be part of a single site. We therefore propose, minimally, that two functional sites exist on the IL1R1 cytoplasmic domain, one (box1/2) to which proteins involved in signaling bind and one (box 3) that is primarily involved in directing localization of receptor, possibly through interactions with cytoskeletal elements. Nevertheless, all three conserved motifs play a role in specifying receptor location, consistent with our previous findings that there is an intimate relationship between cell shape and IL-1 signaling.
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Identification of Two Major Sites in the Type I Interleukin-1 Receptor Cytoplasmic Region Responsible for Coupling to Pro-inflammatory Signaling Pathways
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J. Biol. Chem. 2000, 275:4670-4678.
doi: 10.1074/jbc.275.7.4670

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