Recruitment of a Heparan Sulfate Subunit to the Interleukin-1 Receptor Complex

REGULATION BY FIBRONECTIN ATTACHMENT

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In this study, we identified an adhesion-regulated subunit of the interleukin-1 (IL-1) receptor complex. Transfection of fibroblasts with an IL-1 receptor-EGFP construct showed that the fusion protein was located at focal adhesions in cells attaching to fibronectin.

Fibronectin attachment caused enhancement in endogenous IL-1 type I receptor levels from on average 2500 to 4300 receptors/cell. In addition, matrix attachment resulted in a decrease in binding affinity ($K_d$) from $1.0 \times 10^8$ (M$^{-1}$) to $5.6 \times 10^7$ (M$^{-1}$), due to a 2-fold reduction in association rate constant.

The adhesion-mediated effects were reversed by soluble heparin. Cross-linking experiments showed that in cells attached to fibronectin, 50–70% of the radiolabeled IL-1 was associated with a heparinase sensitive, high molecular mass component of about 300 kDa, with a core protein of 80–90 kDa. Formation of the complex was dependent on cell interaction with the heparin binding region in fibronectin and required IL-1/type I IL-1 receptor binding.

This report demonstrates the recruitment of a heparan sulfate to the IL-1 receptor complex, following attachment to fibronectin, which correlates with alterations in receptor function. The data suggest that the heparan sulfate constitutes an attachment regulated component of the IL-1 receptor complex with the role of mediating matrix regulation of IL-1 responses.

Regulation of a number of cytokine and growth factor receptors is mediated in part through accessory proteins, recruited before or following ligand binding. In several instances, such as modulation of fibroblast growth factor-receptor interaction and IL-6 signaling, regulation involves interactions with glycoproteins and proteoglycans (1, 2).

IL-1 responses are regulated through a receptor complex to which an increasing number of components have been found to belong. The receptor uses a well conserved mechanism for regulating immune and inflammatory responses (3–7) but which is still not completely understood. Initiation of the signal occurs through a heterodimeric receptor complex, composed of the interleukin type I receptor (IL-1RI)* (8) and the accessory protein (AcP) (9), both 80–90 kDa glycoproteins. The type I receptor complex is present on T cells and connective tissue derived cells (8, 10) characteristically at low levels (11). The receptor binds both forms of IL-1 (12, 13), with active forms of 17 kDa, and is responsible for IL-1 induced activation of both NF-kB and stress kinase pathways (14–17). In addition, a 67-kDa, nonsignaling receptor (type II) is expressed on B cells, monocytes, and T cells (18–22). The receptor proximal stages of signal activation involve the adapter proteins TRAF 6 (23) and MyD88 (24–26) and two related kinases, IRAK-1 and IRAK-2 (7, 24).

In adherent cells, such as fibroblasts, IL-1 receptors are located at focal adhesions (27, 28), and IL-1 binding to the type I receptor has rapid effects on cell structure (29). IL-1 signal transduction is regulated by cell attachment and spreading (30), and fibronectin attachment is permissive in IL-1 responses in adherent cells (31).

In this report we demonstrate the presence of a novel, attachment-regulated component of the IL-1 receptor complex at focal adhesions that constitutes a heparan sulfate proteoglycan. Recruitment of this component affects receptor function, both in terms of the level of type I IL-1 receptors and their affinity for ligand, and correlates with IL-1-mediated, attachment-regulated signaling and biological responses.

EXPERIMENTAL PROCEDURES

Transfection—Cells grown in 10-cm dishes (3 x 10^6 cells/dish) were transfected with a construct derived from pEGFP-N2 (CLONTECH, Palo Alto, CA), in which the full-length IL-1RI was introduced into the vector mcs upstream of the EGFP sequence. The receptor portion, a chimera (HinMext) containing the mouse extracellular domain linked to the human intracellular domain (32), was excised from the vector pDC406 using XhoI and BsaAI. The vector p-EGFP-N2 was digested with XhoI and Smal, and the IL-1 receptor, lacking the C-terminal 4 residues (VPLG), was inserted in frame in front of a sequence encoding the linker (GIHRPVAT). Transfection was carried out by calcium phosphate precipitation (4 $\mu$g of plasmid/10-cm dish for 4 h at 37 °C, followed by 25% Glycerol shock for 1 min). Twenty-four hours after transfection, cells were detached using EDTA (5 mM), replated on tissue culture treated multwell chambered coverglasses (Labtek, Nunc) coated with fibronectin (10 $\mu$g/ml) as before, and allowed to attach for 4 h.

Immunostaining—Transfected cells, replated on fibronectin for 4 h, as before, were fixed for 10 min in PBS + 4% paraformaldehyde, washed

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§ The abbreviations used are: IL-1RI, interleukin type I receptor; IL, interleukin; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; EGFP, enhanced green fluorescent protein.
three times with PBS, and blocked/permeabilized by incubation in PBS + 5% goat serum + 0.1% Triton X-100. Primary mouse anti-vinculin antibody (V9131, Sigma) (overnight at 4 °C) and secondary biotinylated goat anti-mouse IgG antiserum (Santa Cruz Biotechnology Inc., Santa Cruz, CA) (30 min at room temperature) were applied sequentially at 2 µg/ml. Membrane fractions were analyzed by SDS-PAGE with 10% (w/v) gels or 12% (w/v) gels followed by detection with 4-chloro-1-naphthol (Sigma Chemical Co., St. Louis, MO) for 1-2 h at 4 °C. Following incubation, 60 µl of supernatant was withdrawn to measure free 125I-labeled IL-1 concentrations. The monolayers were washed rapidly (5×) with ice-cold binding medium to remove unbound ligand and harvested following incubation at 37 °C for 15 min in trypsin (0.05%/EDTA) (0.02%). Bound 125I-IL-1 was measured in a gamma or beta counter. Nonspecific binding was determined by buffering with 50 µM Tris. Nonspecific binding was determined by curve fitting using the following equation:

\[
\text{Bound (molecules/cell)} = A \times C,
\]

where A is Avogadros number, B is the specific activity of the radiolabeled IL-1 (cpm/mol), and C is the number of cells determined in duplicate by hemocytometer.

Values for specific binding (molecules/cell) were analyzed by nonlinear least squares fitting using the equation,

\[
\text{Bound (molecules/cell)} = \frac{R \times K \times C}{1 + K \times C}\]  

(Eq. 2)

where K (µM) is the affinity of IL-1 for its receptor, R, is the total receptor concentration (sites/cell), and C (µM) is the IL-1 concentration. Parameter values were estimated by nonlinear least squares fitting of this equation to equilibrium binding data. Association kinetics data were analyzed using a pseudo-first-order treatment as described (27). All calculations were done using MLAB for Macintosh (Civilized Software, Silver Spring, MD). For illustration purposes, in some cases the data were converted to Scatchard format.

Cross-linking and Gel Electrophoresis—For cross-linking experiments, cells were incubated with radiolabeled ligand and rinsed, as above. Prior to harvesting, cultures were incubated with the amine-directed cross-linking agent BS3 (0.1 mg/ml, Pierce) for 30 min at room temperature (36). Following harvesting by EDTA (5 mM) and centrifugation (10,000 rpm for 5 min at 4 °C), the cell pellet was resuspended in extraction buffer (1% Triton X-100, 2 mM phenylmethylsulfonyl fluoride, 1 µM pepstatin, 1 µM leupeptin, 50 µM 106 cells) and incubated for 15 min on ice. Following centrifugation (10,000 rpm for 5 min), supernatant from samples was mixed with an equal volume of 2× sample buffer (0.1 M Tris-HCl, pH 6.8, 1% SDS, 10% glycerol, 0.0025% bromophenol blue, and 0.2% 2-mercaptoethanol) and boiled for 5 min. Extracts were separated by SDS-PAGE (3–12% or 4–12%), using the stacking gel procedure of Laemmli (37). Dried gels were allowed to expose film (X-Omat, Eastman Kodak Co.) with intensifying screens (Hypercassette, Amersham Pharmacia Biotech) for 3–7 days at −70 °C. For quantitation, autoradiograms were scanned with a ScanJet IIcx (Hewlett-Packard, Boise, Idaho) connected to a power Macintosh and analyzed using NIH Image, as above.

Cell Surface Iodination—Cells plated on fibronectin coated plates, as above (six to ten 10-cm plates at 5 × 10⁵ cells/plate) were surface-labeled using the glucosyl oxidase-lactoperoxidase method (38). Briefly, cultures were incubated (15 min at room temperature) with 2.5 µCi/ml of Na125I (NEN Life Science Products, low pH) in iodination buffer (PBS containing lactoperoxidase 0.25 µM, and glucose oxidase 0.25 µM, and glucose 20.00 mM). After washing (0.15 M NaCl in PBS, 1:1), incubation with unlabeled IL-1 (2 × 10⁻⁷ M for 2 h at 4 °C, and cross-linking (BS3, 0.1 mg/ml), cells were detached (EDTA, 5 mM). The cell pellet, obtained by centrifugation (1000 rpm for 5 min) was resuspended in extraction buffer containing 1% Triton X-100 and protease inhibitors, as above, and incubated for 30 min on ice. The supernatant after centrifugation (13,000 rpm, 5 min) was removed and proteins separated using SDS-PAGE, as above.

Heparinase Digestion—After cross-linking, samples (50 µl) were digested with heparinase (0.2 units/ml, 6 h at 37 °C) (EC 4.2.2.7) (Sigma) in a Tris-HCl buffer (50 mM, pH 7.0) containing 0.025% CaCl₂ and in the presence of 0.01% leupeptin and 0.01% pepstatin, with equal amounts of fresh enzyme added at 3 h. Following digestion, samples were analyzed using gel electrophoresis, as above.

Immunoprecipitation—Cells plated on fibronectin were incubated with radiolabeled IL-1 for 2 h, as described previously. In some experiments, cells were instead biosynthetically labeled using [35S]Sulfate (300 µCi/ml, overnight at 37 °C), re-plated on fibronectin following detachment using EDTA, as above, and incubated in the cold (4 °C) with unlabeled IL-1.
Following either protocol, cells were incubated with cross-linking agent, harvested, and extracted, as above.

Following preclearing with secondary antibody (rabbit anti-rat, 290 μg/ml) and protein A-agarose beads (12.5 μg/ml, Santa Cruz Biotechnology Inc.), samples (20 μl/10^6 cells) were subjected to immunoprecipitation by adding a nonblocking anti-human type I IL-1 receptor rat monoclonal antibody (M8, 5 μg/ml, 1 h at 4 °C) (35). Subsequently, secondary antibody (rabbit anti-rat, Capell) (1:20,290 μg/ml) and pre-washed protein A-agarose beads (12.5 μg/ml, Santa Cruz Biotechnology Inc.) were added to each sample, followed by incubation for an additional 1 h at 4 °C.

Samples were spun in a microcentrifuge (10,000 rpm for 1 min at 4 °C) and washed three times with RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 0.5% deoxycholate, 0.5% Nonidet P-40 and 0.1% SDS) and once with HEPES (10 mM, pH 7.5). After the final wash, the pellet was resuspended in electrophoresis sample buffer (50 mM) and analyzed by SDS-PAGE, as above.

RESULTS

Confocal microscopy of human fibroblasts transfected with a 5′-IL-IRI-EGFP 3′ receptor construct showed that 24 h after transfection, the receptor fusion protein was located at the cell membrane. Serial observations of single cells repleted on fibronectin demonstrated a high degree of accumulation of the GFP-tagged fusion protein at extended processes following cell attachment and spreading (Fig. 1a), in agreement with earlier data on localization of the endogenous receptor protein. Immunocytochemical staining of transfected cells showed co-localization of the receptor fusion protein with the transmembrane linkage protein vinculin at these sites, and at occasional sites along the cell membrane (Fig. 1b). Bar, 10 μm.

Additional analyses of binding kinetics at a range of IL-1 concentrations revealed a 2-fold reduction in association rate constant in cells attached to fibronectin (k_{on} = 2.42 × 10^7 M^{-1} min^{-1}) compared with control (k_{on} = 4.77 × 10^7 M^{-1} min^{-1}) (Fig. 3), demonstrating that the effect on the affinity constant shown in Fig. 2a could be accounted for totally by an effect on the on-rate (k_{on}).

The effects of fibronectin attachment on IL-1 receptor binding could be reversed by the addition of heparin. This effect was concentration-dependent and saturable, resulting in about a 40% reduction in IL-1 receptor binding at 10^{-6}–10^{-5} M soluble heparin (data not shown). Thus, in cells plated on fibronectin, cell surface binding of radiolabeled IL-1, measured in the presence of soluble heparin was similar to that measured in cells on bare plastic, whereas addition of chondroitin sulfate had no effect (Fig. 4a). In contrast, in cells plated on bare plastic, addition of soluble heparin had no effect on receptor binding (Fig. 4b).

Chemical cross-linking after incubation with radiolabeled ligand generated a band at 97 kDa, either comprising an IL-1/IL-1 receptor complex or resulting from a ligand/accessory protein (ActP) interaction (9, 11). This was present in cells plated either in the presence or absence of fibronectin. In addition, in cells attached to fibronectin, a broad, high molecular mass complex of 300–350 kDa was present that contained between 50 and 70% of the cross-linked IL-1 (Fig. 6). This complex was barely detectable in cells on tissue culture plastic. The presence of 100-fold excess cold ligand resulted in complete blocking of cross-linking of radiolabeled IL-1 to both the 97-kDa band and to the high molecular mass band, demonstrating specificity of both interactions.

Enzyme digestion of cell extracts from 125I-IL-1 cross-linking experiments showed that incubation with heparinase resulted in loss of the high molecular mass complex, with no effect on the intensity of the ligand/receptor complex at 97 kDa (Fig. 7a). Furthermore, in cells plated on a mutant fibronectin fragment with altered heparin binding sites, no IL-1 cross-linking to the high molecular mass component could be detected. Instead, 100% of the cross-linked IL-1 was present in the 97-kDa band, as in cells on tissue culture plastic (Fig. 7b).

An interaction between the IL-1 receptor and the high molecular mass component was demonstrated by immunoprecipitation, following IL-1 binding and cross-linking. Thus, a non-
blocking monoclonal antibody to the type I IL-1 receptor immunoprecipitated both the 97-kDa and the 300–350-kDa complexes from fibronectin plated cells to which IL-1 had been cross-linked (Fig. 8a). This procedure, after cell surface iodination and cross-linking, followed by heparinase treatment, revealed a core protein of the recruited proteoglycan of 80–90 kDa (Fig. 8b). Furthermore, immunoprecipitation following sulfate labeling of the glycosaminoglycan chains after incubation with or without IL-1 showed that the appearance of the high molecular mass band was dependent on ligand binding to the type I receptor (Fig. 8c).

DISCUSSION

Glycosylated proteins have been shown to play an important role in cytokine and growth factor regulation of biological re-
The increase in type I IL-1 receptor expression induced by fibronectin may reflect an increase in the rate of translocation of receptor protein to the cell surface. It is, however, known that translocation of receptor protein is rapid and that over 95% is normally expressed at the cell surface (46). Alternatively, the increase in steady state surface expression could result from retardation in the basal internalization rate, by stabilization in focal adhesions at the cell surface. Such a mechanism, involving masking or immobilization of the receptor protein, is suggested by the slower association rate of the ligand. In addition, this is consistent with the recruitment of the additional high molecular mass component to the IL-1 receptor complex. Finally, the lack of effect using the RGD peptide demonstrated that integrin mediated signaling was not involved and supports the notion that the changes were induced as a direct consequence of alterations in the ligand or in the receptor complex.

Involvement of heparan sulfate proteoglycan in matrix regulation of IL-1 receptor function was suggested by inhibition of the fibronectin induced effects by soluble heparin and confirmed by digestion of the high molecular mass complex with heparinase. In contrast to heparin regulation of fibroblast growth factor-receptor binding (1), in which the ligand binds the proteoglycan with detectable affinity, no interaction occurs initially between IL-1 and the proteoglycan, as suggested by binding studies on cells attached to bare plastic. The effect involves a cell surface component in close proximity to the IL-1 receptor as demonstrated by cell surface labeling, cross-linking and immunoprecipitation. Furthermore, the heparan sulfate is recruited secondary to IL-1/IL-1 receptor binding, as demonstrated by blocking experiments using a monoclonal antibody or excess cold ligand. The effect involves an increase in a single class of IL-1 binding sites, as judged by affinity constant, again indicating that the IL-1 affinity for the heparan sulfate is low and nondetectable in conventional binding assays. Interfering with IL-1/IL-1 receptor binding required a 10,000-fold higher concentration of the glycosaminoglycan, suggesting an interaction with a $K_a$ in the range of 10^5 M similar to that of integrin/matrix interaction and about 100-fold lower than that of heparan-fibroblast growth factor interaction (1).

The requirement for ligand/IL-1 receptor binding, prior to association of the heparan sulfate suggests a mechanism similar to that in the LIF/oncostatin M system, in which recruitment of gp130 to the receptor complex follows LIF binding (2, 42). Digestion experiments suggested the ligand/heparan sul-
Heparan Sulfate Recruited to IL-1 Receptor Complex

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