THE RECRUITMENT OF THE INTERLEUKIN-1 RECEPTOR-ASSOCIATED KINASE (IRAK) INTO FOCAL ADHESION COMPLEXES IS REQUIRED FOR IL-1β-INDUCED ERK ACTIVATION

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ABSTRACT

The colocalization of IL-1 receptors with focal adhesions has been implicated in the restriction of IL-1 signal transduction to ERK, however the mechanism of this restriction and the requirement of IL-1 receptor-associated proteins have not been characterized. We determined if the interleukin-1 receptor-associated kinase (IRAK) colocalizes with focal adhesions and is required for IL-1-dependent ERK activation. Human gingival fibroblasts were incubated with collagen-coated beads to induce the assembly of focal adhesions at sites of cell-bead contact. Phosphorylated IRAK was transiently detected in focal adhesion complexes isolated from fibroblasts stimulated with IL-1β. Cells forming focal adhesions showed IL-1-induced phosphorylation of ERK, JNK and p38; in contrast, cells plated on poly-L-lysine to prevent focal adhesion formation showed activation only of JNK and p38. ERK activation was partially restored by incubating cells plated on poly-L-lysine with collagen-coated beads prior to IL-1 stimulation. Cells treated with Swinholide A to induce actin filament depolymerization, showed elimination of IL-1-induced ERK activation. Fibroblasts electroporated with an anti-IRAK antibody to block the recruitment of IRAK into FACs failed to activate ERK after IL-1 treatment, indicating that FAC-associated IRAK is required for ERK activation. These data suggest that the integrity of actin filament arrays and the recruitment of IRAK into focal adhesions are involved in the restriction of IL-1 signaling to ERK.

Key Words: Actin, focal adhesions, IL-1 signaling, fibroblasts, IRAK
INTRODUCTION

Interleukin-1 (IL-1) is a potent, multifunctional cytokine that is involved in a host of immune and pro-inflammatory responses (1). The broad spectrum of biological effects attributed to IL-1 results from its ability to induce a wide range of factors which contribute to the inflammatory response. These factors include matrix metalloproteinases (2, 3), nitric oxide synthetase (4), prostaglandin E (5) as well as other cytokines (6, 7). Consequently, IL-1 is able to mediate significant cellular and tissue damage when its expression is upregulated, as seen in the pathogenesis of chronic inflammatory diseases such as rheumatoid arthritis or periodontal diseases (8-11).

Despite extensive research, much remains to be elucidated about the regulation and restriction of IL-1 signals which lead to the multiple biological responses attributed to this cytokine. There are two known membrane-bound IL-1 receptors, IL-1 receptor type I and type II (IL-1R1 and IL-1R1) (12). IL-1R1 alone is capable of generating a signal while IL-1RII acts as a decoy receptor (13-16). The current model for IL-1 signaling suggests that following ligand binding, the IL-1 receptor associated protein (IL-1RAcP) (17) is recruited to IL-1R1 (18), subsequently increasing the avidity of the receptor for its ligand (19). The IL-1 associated kinases (IRAK-1,2) (20, 21) are also recruited to the signaling complex by the adapter protein MyD88 (22, 23) within seconds of IL-1 binding (24, 25). Several studies have indicated that IRAK-1 selectively associates with IL-1RAcP while IRAK-2 associates with IL-1R1, although the biological significance of this difference in affinities is unknown (24). Interestingly, while both kinases are rapidly phosphorylated, the phosphorylation step is not a requirement for signal transduction but, rather, appears to direct degradation through proteolysis (26).
addition, the overexpression of IRAK in the absence of IL-1 leads to its phosphorylation and degradation thereby providing a possible negative feedback mechanism for regulating the IL-1 signaling pathway (27). Following phosphorylation, IRAK dissociates from the complex in order to initiate downstream signaling events (28). Currently, the only downstream IRAK binding partner elucidated has been TRAF-6, a member of the TNF receptor-associated family. TRAF-6 is required to mediate the IL-1 dependent activation of the NFκB and JNK/SAPK signal transduction pathways (29).

IL-1-induced signal transduction is mediated by a number of protein families. One such family is the mitogen activated protein kinase (MAPK) family of threonine-tyrosine phosphorylated signaling molecules (30-33). There are three members of the MAPK family c-Jun NH2-terminal kinases / stress activated protein kinases (JNKs/SAPKs), extracellular signal-regulated kinases (ERKs) and p38MAPK. Many of the upstream and downstream signal transducers are unique to each MAPK, resulting in a cell type-restricted repertoire of responses for JNKs, ERKs and p38 (34-36, 56). The IL-1 dependent activation of these kinases has been implicated in the tissue destruction characteristic of chronic inflammatory diseases, although the kinetics and degree of phosphorylation varies greatly. IL-1 induced phosphorylation of ERK and p38 occurs within 5 minutes while phosphorylated JNK appears after 15 minutes in responsive cell types (34). In HepG2 cells, IL-1β stimulates a 25-fold increase in phospho-p38, a 20-fold increase in phospho-JNK but only a 3-fold increase in phospho-ERK-1,2 (37). Understanding the mechanism of signal restriction that occurs proximal to the IL-1 receptor complex could be a significant step in determining how these MAPK cascades are differentially regulated. Insight
into these events could be important in limiting pharmacologically the action of such a potent pro-inflammatory cytokine.

The cytoskeleton is an important mediator and restriction element in many types of intracellular signaling (38) as it provides a structural framework for the physical association of signaling molecules, including those involved in IL-1 signal transduction. In particular, focal adhesion complexes (FACs) are membrane-associated cytoskeletal structures which have been implicated in many signaling cascades (39, 40). Immunohistochemistry and $^{125}$I labeling experiments with human gingival fibroblasts have established a tight spatial relationship between IL-1 receptor density and FACs, suggesting a potential role for these structures in IL-1 signaling (41-43). When examined in the context of IL-1-induced MAPK phosphorylation, FACs are necessary for IL-1-dependent ERK activation, however, JNK and p38 are not similarly restricted (44).

Although immunoprecipitation studies have shown that IRAK and the IL-1R$_1$ associate in an IL-1 dependent manner (45), IRAK has not been linked to FACs nor has its involvement in ERK activation been investigated. Indeed little is known about the mechanism of IL-1 signal restriction by FACs, specifically with respect to IL-1-dependent ERK activation. The purpose of this study was to examine the role of IRAK, FACs and by extension the cytoskeletal network, in IL-1 signal restriction. We determined: 1) if IRAK associates with the focal adhesion complex; 2) if this association is required for IL-1 induced ERK activation; and 3) whether or not this association is an IL-1 dependent phenomenon. Human gingival fibroblasts were used as a model to study IRAK-FAC association and ERK activation in response to IL-1 stimulation as they constitutively express high levels of the IL-1R$_1$ (43,44).
MATERIALS AND METHODS

MATERIALS

Bovine fibronectin, poly-L-lysine, BSA, aprotinin, leupeptin, mouse monoclonal antibodies to vinculin, talin, β-actin, FITC-conjugated goat anti-mouse, phorbol 12-myristate 13-acetate (PMA), magnetite beads, PMSF, Triton-X 100, Swinholide A and Tween-20 were obtained from Sigma (St. Louis, MO). Rabbit polyclonal antibodies to p38, phospho-p38, ERK1/2, JNK(SAPK), phospho-JNK(SAPK) and mouse monoclonal anti-phospho-ERK1/2 were purchased from New England Biolabs (Beverly, MA). Mouse monoclonal anti-IRAK was purchased from Transduction Laboratories. Horseradish peroxidase conjugated goat anti-mouse(H+L), goat anti-rabbit (H+L) and Latrunculin B were purchased from Cedarlane Laboratories (Homby ON). The ECL Chemiluminescent Kit was purchased from Amersham Life Science (Buckinghamshire, England). Acidified bovine type I collagen (Vitrogen) was purchased from Cohesion Technologies Inc (Palo Alto, CA). A magnetic separation stand was purchased from Promega (Madison, WI). The sheep anti-IL-1R1 antibody was obtained from Dr. S. Simms (Immunex).

CELL CULTURE

Human gingival fibroblasts were grown in minimal essential medium (α-MEM) containing 10% fetal bovine serum and antibiotics (0.17% penicillin V, 0.1% gentamycin sulphate and 0.01% amphotericin) in a humidified atmosphere of 5% CO2 in air. Cells between the 5th and 12th passages were used for all experiments.
COLLAGEN-COATED BEAD PREPARATION

Magnetite beads were added to soluble collagen (100 µg/ml) and vortexed. NaOH was added to a final concentration of 0.1 mM to equilibrate pH to 7.4 and facilitate collagen fibril assembly on the beads. The suspension was incubated at 37°C for 20 minutes. The beads were then washed several times and resuspended in PBS and sonicated for 10 seconds (output setting 3, power 15%).

ISOLATION OF FOCAL ADHESIONS

Cells which had reached 80-90% confluence on 60 mm tissue culture dishes in normal growth medium were used. For each experiment 5 dishes of cultured cells were cooled to 4°C. Collagen-coated magnetite beads were added to each dish. Experiments were conducted in normal growth medium. FACs were isolated from dishes 1 and 2 after 5 and 10 min respectively. Following 10 min at 4°C the temperature of the remaining dishes was increased to 37°C and the FACs were isolated from dishes 3, 4 and 5 after an additional 2, 5 and 10 min respectively.

Focal adhesion complexes were isolated according to the methods of Plopper and Ingber (1993) (52). Cells were gently washed 3 times with ice-cold PBS to remove unbound beads and scraped into ice cold cytoskeleton extraction buffer (CSKB; 5% Triton-X 100, 50 mM NaCl, 300 mM sucrose, 3 mM MgCl₂, 20 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin, 1 mM PMSF, 10 mM PIPES, pH 6.8). The cell bead suspension was sonicated for 10 seconds (output setting 3, power 15% Branson) and the beads were isolated from the lysate using a magnetic separation stand. The beads were resuspended in fresh ice-cold CKSB, homogenized with a Dounce homogenizer (20 strokes) and re-isolated magnetically. The beads were washed
thoroughly in CSKB, pelleted with a microcentrifuge, resuspended in Laemmli sample buffer and placed in a boiling water bath for 10 minutes to allow the collagen-associated complexes to dissociate from the beads. The beads were pelleted and the lysate collected for analysis.

**IMMUNOBLOT ANALYSIS**

The protein concentrations of the cell lysates were determined by a Bradford assay (Biorad, Hercules, CA). Equal amounts of protein were loaded into an SDS-polyacrylamide gel (10% acrylamide), resolved by electrophoresis and transferred to a nitrocellulose membrane. The membrane was incubated overnight at 4°C in a Tris-buffered saline solution with 5% milk to block non-specific binding sites. Membranes were incubated with the primary antibodies for 1-4 hours at room temperature in Tris-buffered saline with 0.1% Tween-20. Horseradish peroxidase secondary antibodies were incubated for 1 hour at room temperature in Tris-buffered saline with 0.1% Tween-20 and 5% milk. Labeled proteins were visualized by chemiluminescence (ECL Chemiluminescent Kit).

**IMMUNOFLUORESCENCE STAINING**

Chamber slides (8-well; Labtek) were coated with fibronectin (10 µg/ml in PBS). Cells were plated and allowed to spread for 24 hours prior to treatment. Following treatment cells were fixed in 3% paraformaldehyde in PBS for ten minutes at room temperature, blocked and permeabilized in PBS with 0.2% Triton-X 100 and 0.2% BSA for 15 minutes at room temperature. Antibodies were diluted in PBS with 0.2% Triton-X 100 and 0.2% BSA. Immunofluorescence staining for vinculin and IRAK was performed with mouse monoclonal anti-vinculin or anti-IRAK antibody (1:50 and 1:20 dilution respectively) for 1 hour at room
temperature or 3 hours at 37°C. Slides were washed with PBS, incubated with goat anti-mouse FITC-conjugated antibody (1:50 dilution) for 60 minutes at 4°C, washed and coverslipped.

**ELECTROPORATION**

Cells were harvested by trypsinization, pelleted and resuspended in serum-free α-MEM buffered with 12.5 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (Hepes). A 30 µl aliquot of cells was placed in a cuvette with 30 µg of mouse monoclonal anti-IRAK antibody in Hepes-buffered α-MEM at 4°C. The cells were electroporated at 100 V/cm and capacitance 960 µF using a Bio-Rad gene pulser with a capacitance expander and gene pulser cuvettes (0.2 cm inter-electrode distance). Cells were incubated at 4°C for 10 minutes and replated in normal growth medium. After 4 hours the medium was aspirated from the cells to remove cellular debris and normal growth medium was added back.
RESULTS

IRAK Recruitment into Nascent FACs Requires IL-1

Focal adhesion complexes (FACs) are actin-rich attachment domains that assemble at sites on the cell membrane where integrin receptors bind to the extracellular matrix (ECM) (46). Magnetite microbeads coated with ECM molecules such as collagen and fibronectin have been used to stimulate the formation as well as to enable the isolation of FACs at the sites of microbead-cell contacts (50). We first confirmed that collagen-coated magnetite microbeads could be used to isolate specific focal adhesion proteins including β-actin, vinculin and talin. Preliminary time-course experiments (at 37°C) revealed that recruitment of these focal adhesion proteins to the sites of cell-bead contact occurred too rapidly to obtain reproducible measurements. Subsequent experiments were conducted at 4°C from 0 to 10 min, followed by an increase in temperature to 37°C from 10 to 20 min. Decreasing the reaction temperature slowed the recruitment of focal adhesion proteins into the bead complex and facilitated accurate protein quantification. Immunoblot analysis of protein bound to an equal number of beads demonstrated the time-dependent recruitment of the focal adhesion proteins β-actin, vinculin and talin into nascent focal adhesions (Fig 1). The amount of bead-bound proteins isolated at 5 min was very low but increased sharply at later incubation times (> 5 min).

Suggested location of Fig. 1

As the collagen coating of the microbeads could have acted as a non-specific trap for cellular proteins, we repeated the experiment with cells which had been pretreated with 1µM Latrunculin B (Lat B) for 30 min. This toxin sequesters actin monomers and promotes the
depolymerization of actin filaments (47). We anticipated that cells pretreated with Lat B would not show focal adhesion protein binding to collagen-coated beads if actin filaments were disrupted prior to incubation of cells with beads. As expected, there was no β-actin, vinculin or talin in protein lysates obtained from these beads (Fig 1), indicating that the focal adhesion proteins isolated as described above were recruited into bead-bound complexes and were not an artifact of non-specific protein absorption to the beads. In addition, beads coated with BSA (1 mg/ml) showed very little binding of β-actin, vinculin or talin (data not shown).

*Suggested location of Fig. 2*

*Suggested location of Fig. 3*

Cultured human gingival fibroblasts express 11,000 +/- 100 IL-1 receptors per cell (42) indicating that this cell type is a useful model system to study IL-1 signaling. Although previous studies have shown that the IL-1 receptors localize to focal contact sites in human fibroblasts and keratinocytes (41-43), and that IRAK and IL-1R₁ associate in IL-1-stimulated cells (45), a physical association between IRAK, IL-1R₁ and focal adhesions has not been established. We first determined by immunoblot analysis that IL-1R₁ could be detected in bead-associated complexes at 10 and 20 min following the addition of collagen-coated microbeads to fibroblasts (Fig 2). To examine the association of IRAK with the focal adhesion complex, collagen bead isolation procedures were carried out on cells which had received either no treatment, IL-1β stimulation alone or Lat B treatment prior to IL-1 stimulation. IRAK is detectable by immunoblotting as an 80 kDa unphosphorylated, inactive form or a 100 kDa phosphorylated, active form (25). We detected bands of approximately 80 kDa and 100 kDa (data not shown) in
immunoblots of cell lysates obtained from cells treated with IL-1β (5 min) and probed with a human anti-IRAK antibody. A 100 kDa band which co-migrated with a phosphorylated IRAK standard (Transduction Laboratories) was detected in FACs isolated from cells which had been treated with IL-1β (20 ng/ml) alone (Fig 3). The IRAK-FAC association was detected after 5 min of IL-1β stimulation (IL-1β was added 5 min after microbeads) but this association was transient: after 7 min of incubation with IL-1β, IRAK had almost completely dissociated from the FAC. Neither the 80 kDa nor the 100 kDa forms of IRAK were detectable in FACs isolated from untreated fibroblasts, nor from cells which had been treated with Lat B (1 µM) 30 min prior to IL-1β stimulation (Fig 3).

*Suggested location of Fig. 4*

Previously, fibronectin-coated microbeads and fluorescence microscopy have been used to show FAC assembly at sites of microbead-cell contact (50, 51). We used a modification of this technique in order to demonstrate the recruitment of IRAK into the FACs of IL-1 stimulated cells. Human gingival fibroblasts were plated on fibronectin-coated (10 µg/ml) glass slides and incubated with collagen-coated latex microbeads for 15 and 30 min at 37°C. Fluorescence microscopy of cells immunostained for the focal adhesion protein vinculin, showed distinct, brightly stained streaks (Fig 4) indicating that gingival fibroblasts were capable of forming typical focal contacts on their ventral surface. In cells incubated for 15 min with beads, only faint vinculin staining was detectable at the periphery of the microbead (Fig 4) while much more intense staining was visible after 30 min (Fig 4), findings that are consistent with the immunoblotting shown in Fig 1. When cells were incubated with collagen-coated microbeads for
30 min there was no IRAK immunostaining around beads, however, after 5 min of IL-1β stimulation at 37°C IRAK staining was localized to the periphery of the microbeads (Fig 4). After 20 min of IL-1 treatment, the staining had decreased to control levels (Fig 4).

*Suggested location of Fig. 5*

Focal Adhesions are Required for IL-1-Induced ERK Activation

As the data above showed that IRAK recruitment into FACs requires IL-1, we next determined if FACs, the actin cytoskeleton and IRAK restrict IL-1-induced MAPK activation. When platted on normal tissue culture plastic, phase contrast microscopy demonstrated the ability of cells to spread and presumably to form focal contacts (Fig 5-A). Indeed, immunofluorescence microscopy of well-spread fibroblasts stained for vinculin revealed discrete, brightly stained sites, indicating the presence of focal adhesions (Fig 5-C). In contrast to cells plated on tissue culture plastic, cells plated on poly-L-lysine cannot form integrin-mediated attachments to the substrate (41) remained rounded and were unable to form FACs (Fig 5-B). Cells plated on poly-L-lysine were unable to activate ERK in response to IL-1β stimulation (Fig 5-G) unlike cells plated on tissue culture plastic which shoed ERK activation (Fig 5-E). However, the presence of focal adhesions did not restrict IL-1 signaling to other MAPK family members: IL-1 stimulation of cells plated on poly-L-lysine resulted in a significant increase in phosphorylated JNK and p38 (Fig 5-G). As plating on poly-L-lysine induced cell rounding and dramatic changes in cell shape, we determined if the apparent focal adhesion restriction of IL-1-induced ERK activation was actually attributable to changes in cell shape. Accordingly, IL-1-induced ERK activation was partially restored if cells plated on poly-L-lysine were pre-incubated
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with collagen-coated microbeads (Fig 5-F), indicating that FACs were indeed critical for the IL-1 restricted signaling.

_Suggested location of Fig. 6_

_Actin Filaments and IL-1 Signaling to ERK_

Focal adhesion complexes are in part comprised of actin filaments. Therefore, to test the requirement for actin filaments in IL-1 signal restriction, fibroblasts were treated with two monomeric actin sequestering drugs, Lat B and Swinholide A (SWA). These agents were used to produce different levels of actin disassembly in treated cells. Short-term Lat B treatment causes loss of stress fibers and the reorganization of actin filaments into fine, branched cellular processes (52) which radiate from the cell body. SWA acts by completely severing existing actin filaments in addition to preventing actin polymerization (47). Fibroblasts were plated on fibronectin-coated (10 µg/ml) glass slides and treated with Lat B (1 µM) for 30 min or SWA (50 ng/ml) overnight and immunostained for vinculin or stained with rhodamine phalloidin to show actin filaments. In Lat B-treated cells, actin filament-enriched extensions, or runners, formed where the cytoskeleton collapsed around previously formed FACs that remained attached to the fibronectin substrate (Fig 6-B). The vinculin in these cells was no longer visible as discrete points, but instead was visible as branch-like formations in the runners (Fig 6-C). These cells retained the ability to phosphorylate ERK in response to IL-1 treatment, however, the level of phosphorylated ERK was markedly reduced (Fig 6-F). SWA treatment resulted in drastically altered cell morphology including rounding of the cell and complete loss of phalloidin staining for actin filaments (Fig 6-D). Immunostaining revealed the retention of some discrete, but very small, vinculin patches (Fig 6-E). SWA-treated cells did not phosphorylate ERK in response to
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IL-1 stimulation (Fig 6-G), indicating that in addition to focal adhesion complexes, the retention of actin filaments is necessary for IL-1 signal transduction.

Suggested location of Fig. 7

IRAK Is Required for IL-1 Dependent ERK Activation

Experiments using cells from Irak knockout mice (53, 63) have established that IRAK is a necessary component of IL-1-induced NF-κB and JNK activation. Since the IL-1 signal transduction pathways for ERK and JNK are differentially restricted in the context of focal adhesions, we investigated the effect of the loss of available IRAK on IL-1 signaling to ERK. Cells were loaded with anti-IRAK antibody to complex IRAK and prevent its association with other receptor complex signaling molecules. Electroporation was used to create transient pores in the cell membrane (48) and was optimized to facilitate the diffusion of large (approximately 150 kDa) protein molecules across the cell membrane (49). We determined using fluorescence microscopy and 150 kDa FITC-conjugated dextran that a field strength of 100 V/cm and a 960 µF capacitor was required to load 95% of cells with the FITC-dextran, a surrogate for mouse monoclonal anti-IRAK antibody. Electroporated cells were plated in normal growth medium (α-MEM / 10% FBS) overnight and stimulated with IL-1β for 5 min. In cells electroporated with an irrelevant isotype control antibody, immunoblots of SDS-PAGE separated lysates showed an increase of IL-1-induced ERK phosphorylation compared with unstimulated cells (Fig 7-A). However, we were unable to detect an increase in phosphorylated-ERK in cells that had been electroporated with the anti-IRAK antibody (Fig 7-A). This indicates that IRAK is required to mediate IL-1-induced activation of ERK. To ensure that electroporation did not prevent the
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formation of focal adhesions or the recruitment of IRAK into nascent FACs, cells electroporated in the absence of antibody or with an irrelevant isotype control antibody were analyzed by immunofluorescence. Bright vinculin and IRAK staining were observed around the perimeter of collagen coated beads (Fig 7 B-E). However, cells electroporated with an anti-IRAK antibody were unable to recruit IRAK into FACs, and presumably the IL-1 receptor complex (Fig 7-F). These results support the finding that IRAK is indeed required for IL-1-induced ERK activation.
DISCUSSION

IL-1 activates multiple signaling cascades which in turn induce strong pro-inflammatory responses in cells expressing as few as 10 receptors per cell (61). These data suggest the existence of specific, regulatory mechanisms to control the activity of this potent cytokine, perhaps at the level of receptor and its interaction with IL-1R1 associated proteins. Our findings that IL-1 receptors are enriched in FACs and that FACs are required for IL-1-induced ERK activation and calcium responses (44, 51) indicate a prominent role for FACs in restricting IL-1 generated signals.

The activation of IL1-R1 by its ligand initiates the recruitment of the interleukin-1 receptor-associated protein (IL-1RACp) and unphosphorylated interleukin-1-associated kinase (IRAK) into a signaling heterocomplex. Although IL-1R1 has been localized to FACs in IL-1 responsive cells (20, 21), little work has been done to examine further this association or to determine if other IL-1R1-associated proteins are present. We detected by immunoblotting both IL-1R1 and phosphorylated IRAK in FACs isolated from IL-1-stimulated human gingival fibroblasts, although IRAK, unlike IL-1R1, was only transiently detected. These results were supported by immunohistochemistry experiments showing the colocalization of IRAK and the focal adhesion protein vinculin in IL-1-treated cells. Our findings suggest that the IL-1 receptor-signaling complex assembles in FACs upon IL-1 stimulation. It has been shown previously that the level of phospho-IRAK peaks 2 to 4 minutes following the recruitment of unphosphorylated IRAK into the ligand-bound receptor complex (26). Phospho-IRAK then found dissociates from the receptor complex and is rapidly degraded, which drastically reduces the total level of IRAK in the cell (26). Interestingly, the amount of IL-1R1 that immunoprecipitates with IRAK remains
almost unchanged, despite the reduction in IRAK, possibly due to the association of multiple IL-1 receptors with a single IRAK molecule (26). In our study, although IRAK could not be detected after 15 min of IL-1β stimulation, it is possible that the number of IRAK molecules present in the isolated FACs were too low to be detected by immunoblotting.

While the precise requirement for FACs in IL-1-induced signaling, and more specifically the functional relationship between FACs and the IL-1 receptor complex remains unclear, we showed previously that FACs are necessary for IL-1-induced ERK activation and calcium responses (44, 51). In this paper we show that it was the lack of FACs and not the altered cell morphology or the presence of poly-L-lysine which inhibited ERK activation: we were able to partially restore IL-1 induced ERK activation in rounded fibroblasts plated on poly-L-lysine by inducing the formation of FACs with collagen-coated beads. A FAC requirement for signaling is not limited to IL-1 as recently, FACs were found to restrict G-protein coupled receptor-induced ERK activation in PC12 rat pheochromacytoma cells and this restriction coincided with the expression of the calcium-regulated focal adhesion kinase Pyk2 (57). Thus FACs may provide a more general mechanism for signal restriction to ERK by participating in multiple signaling cascades originating at the cell membrane. As focal adhesions are not formed by all cell types or at all times, alterations in the abundance of focal adhesions may determine the level of IL-1 responsiveness in a given cell. Indeed, we were able to show that FAC restriction of the IL-1 signal is specific to ERK activation while both JNK and p38 were phosphorylated in the absence of FACs.

We have shown here that actin filaments are important for IL-1-induced ERK activation. A tight, reciprocal association exists between IL-1-induced calcium signaling and the
organization of the actin cytoskeleton (67, 68). IL-1 treatment of cells plated on fibronectin has been shown to cause transient contraction and disruption of actin filaments cell retraction of the cell around focal contacts (60) and a concurrent phosphorylation and reduction in the level of the focal adhesion protein talin (59). The finding that RhoA, a regulator of actin filament and FAC formation, is associated with the ligand-bound IL-1 receptor and is activated by IL-1 (54) provides further evidence of an interdependent relationship between the actin cytoskeleton and IL-1 signaling. It is currently unknown what molecules present in the FAC mediate the exchange of signals between the activated receptor and actin cytoskeleton-related proteins.

In the present study cells treated with the actin monomer sequestering toxin Latrunculin B reorganized of actin filaments into long delicate, processes (47) and showed loss of stress fibers. Despite the retention of focal contacts in the processes, these cells demonstrated reduced IL-1-stimulated ERK activation, indicating that the actin cytoskeleton and FACs are involved in IL-1 signal transduction to ERK. Accordingly, cells treated with SWA, which completely destroyed all actin filaments, were unable to activate ERK in response to IL-1 stimulation. The basis for this restriction is not presently known, however, the actin cytoskeleton may provide a scaffold by which ERK and other signaling molecules are brought together in a non-random fashion. ERK and MEKK1, an upstream activator of JNK, ERK and p38, bind to separate regions on alpha-actinin, a prominent protein in stress fibers (58, 55) and focal adhesions. Alpha-actinin may act as an adapter molecule, linking ERK and MEKK1 to the cytoskeleton, and thereby provide a mechanism by which the two signaling molecules can specifically associate. The depolymerization of actin cytoskeleton by SWA, and to a lesser extent by Lat B, would be
expected to prevent the transport of ERK, MEKK1 and other potential binding partners to specific activation sites and block signal transduction.

Analyses of IRAK-deficient mouse fibroblasts have shown that IRAK is required for IL-1-mediated JNK, p38 and NF-kappaB activation (53, 63). As we have shown that IL-1-induced ERK activity is clearly differentially regulated from the other MAPKs, and as IL-1 is able to phosphorylate FAC-associated proteins (59), we wished to determine if IRAK was a mediator of IL-1 signaling to ERK. Cells electroinjected with an anti-IRAK antibody were unable to undergo IL-1-induced ERK phosphorylation, demonstrating the requirement for IRAK in IL-1-induced ERK activation. Prior to this study, no IL-1 receptor complex member, other than IL-1R had been spatially associated with FACs (41, 43). We determined that IRAK was transiently recruited to the FAC. This recruitment was IL-1 dependent and was necessary for IL-1-induced ERK activation.

Focal adhesions were originally identified simply as actin-dependent cell adhesion structures. It is now thought that these structures are also involved in signaling events that originate at the cell membrane and regulate a variety of cell processes such as proliferation, apoptosis, migration and cell spreading (40, 64). The actin cytoskeleton has also been linked to signal transduction, and may be involved in mediating the interaction of specific signaling molecules in a non-random manner. As the organization and remodeling of the cytoskeleton is required for many cellular processes such as wound healing and inflammatory diseases a improved understanding of how FACs and actin regulate and restrict IL-1 signaling will be essential for understanding the action of this cytokine.
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Our major conclusion here is that the organization of the actin cytoskeleton and associated IL-1 receptor proteins provide one level of signal restriction in IL-1 responsive cells. Focal adhesion complexes not only mediate integrin-receptor attachment to the extracellular matrix, but also provide discrete sites for clustering and interaction of IL-1 signaling molecules.
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FIG. 1 Isolation of FACs with Collagen Coated Magnetic Microbeads. Fibroblasts were plated overnight in normal growth medium (α-MEM / 10% FBS). Cells were incubated with collagen coated magnetic microbeads for 5, 10, 12, 15 and 20 minutes (lanes 1-5). Incubations were at 4°C (from 0 to 10 min) and 37°C (from 10 to 20 min). Focal adhesion complexes were isolated using the rapid isolation method (see Materials and Methods (50)) and the proteins resolved by SDS-PAGE. Blots were probed with mouse monoclonal anti-β-actin, vinculin and talin (Sigma). Cells were either untreated or treated with Latrunculin B (1 µM) for 30 min prior to the addition of collagen-coated beads. Whole cell human gingival fibroblast lysate was used as a positive control.

FIG. 2 Identification of IL-1 Receptor in Isolated FACs. Fibroblasts were incubated with collagen coated magnetic microbeads for 1, 10 and 20 min (lanes 1-3). Focal adhesion complexes were isolated as described in Figure 1 legend. FAC proteins were resolved by SDS-PAGE and blot probed with sheep anti-human IL-1R<sub>1</sub> antibody (Simms, Immunex).

FIG. 3 Recruitment of IRAK into Nascent Focal Adhesion Complexes in IL-1β-Stimulated Human Gingival Fibroblasts. Fibroblasts were plated overnight in normal growth medium (α-MEM / 10% FBS). Cells were incubated with collagen coated magnetic microbeads for 5, 10, 12, 15 and 20 minutes (lanes 1-5). Focal adhesion complexes were isolated as described in Figure 1 legend. FAC proteins were resolved by SDS-PAGE and blots were probed with mouse monoclonal anti-IRAK (Transduction Laboratories). Cells were either untreated, treated...
with IL-1β (20 ng/ml) commencing 5 min after microbead addition (n=3) or treated with Latrunculin B (1 µM) for 30 min prior to microbead and IL-1β addition.

FIG. 4 **IRAK is Transiently Recruited into FACs Following IL-1β Stimulation.** Immunofluorescence micrographs of human gingival fibroblasts plated on fibronectin (10 µg/ml) for 48 hours in normal growth medium (α-MEM / FBS). Fibroblasts were incubated with collagen coated latex microbeads for 15 min (A) and 30 min (B) and immunostained for vinculin. Note discrete FAC (arrow). Fibroblasts were incubated for 30 min with collagen coated latex microbeads to induce FAC formation, then stimulated with IL-1β (20 ng/ml) for 0 min (C), 5 min (D) or 20 min (E) and immunostained for IRAK.

FIG. 5 **Focal Adhesion Complexes are Required for IL-1β Activation of ERK.** Phase contrast micrographs of human gingival fibroblasts plated on tissue culture plastic (A) or poly-L-lysine (1 mg/ml) (B) in normal growth medium (α-MEM / 10% FBS) for 6 hours. Immunofluorescence micrograph of fibroblasts plated on fibronectin (10 µg/ml) for 48 hours (C) or poly-L-lysine (1 mg/ml) for 6 hours (D) and immunostained for vinculin. Cells plated on fibronectin-coated glass slides were fixed after 48 hours to optimize the formation of focal contacts. Note the bright, discrete staining of focal adhesion complexes in cells grown on fibronectin. Immunoblot analysis of cells which were plated on tissue culture plastic in normal growth medium (α-MEM / 10% FBS) for 6 hours and stimulated with IL-1β (20 ng/ml) for 0, 5 and 20 min (lanes 1, 2 and 3). ERK activity was assessed by immunoblotting (E). Note the marked increase of the level of phosphorylated ERK over basal levels. Human gingival fibroblasts were grown on poly-L-lysine (1 mg/ml) for 6 hours and treated with collagen coated
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microbeads 3 hours prior to IL-1 stimulation (F). Cells plated on poly-L-lysine were stimulated with IL-1β (20 ng/ml) for 0, 5 and 20 min (lanes 1, 2 and 3) (G). ERK1,2, p38 and JNK1,2 activities were assessed by separating lysates via SDS-PAGE, and probing blots with mouse monoclonal anti-phospho-ERK1,2 or rabbit polyclonal anti-phospho-p38 or anti-phospho-JNK1,2. Total ERK1,2, p38 and JNK1,2 was assessed by reprobing blots with rabbit polyclonal anti-ERK1,2, anti-p38 or anti-JNK1,2 (New England Biolabs). PMA (10 ng/ml), or UV-treated cells (5 and 20 min) were used as a positive control for ERK1,2, p38 and JNK1,2 activities respectively.

FIG. 6. Actin Stress Fibers Are Involved in IL-1-Induced ERK Activation. Immunofluorescence micrographs of human gingival fibroblasts plated on fibronectin (10 µg/ml) for 48 hours and left untreated (A), treated with Latrunculin B (1 µM) for 30 minutes (B, C) or treated with Swinholide A (50 nM) overnight (D, E). Fixed cells were stained with rhodamine phalloidin (A-C) to show actin stress fibers or with anti-vinculin (D, E) to show focal contacts. For immunoblots (F, G) human gingival fibroblasts were plated on tissue culture plastic in normal growth medium (α-MEM / 10% FBS). Cells were incubated with IL-1β (20 ng/ml, 37°C) for 0, 5 and 20 minutes (lanes 1, 2 and 3). Cells were pretreated with Latrunculin B (1 µM) for 30 minutes prior to IL-1 treatment (F). Cells were treated with Swinholide A (50 nM) for 24 hours prior to incubation with IL-1β (G). ERK1,2 activity was assessed by separating lysates via SDS-PAGE and probing blots with mouse monoclonal anti-phospho-ERK1,2. Total ERK1,2 was assessed by stripping and reprobing blots with rabbit polyclonal anti-ERK1,2 (both New
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England Biolabs). PMA (10ng/ml) treated cells were used as a positive control for ERK1,2 activity.

FIG. 7. IRAK Is Required for IL-1β-Induced ERK Activation. Human gingival fibroblasts were electroinjected with goat-anti-mouse IgG or mouse-monoclonal anti-IRAK antibody 24 hours prior to treatment. Fibroblasts electroporated with no antibody, with an irrelevant isotype control antibody or with anti-IRAK were plated on fibronectin (10 µg/ml, overnight), incubated for 30 min with collagen-coated microbeads and immunostained for vinculin (A-E). Fibroblasts which had been electroporated with the control antibody (D) or with the anti-IRAK antibody (E) were stimulated with IL-1β (20 ng/ml) for 5 min and immunostained for IRAK. ERK1,2 activity was assessed by separating lysates via SDS-PAGE and probing resulting blots with mouse monoclonal anti-phospho-ERK1,2. Total ERK1,2 was assessed by reprobing blots with rabbit polyclonal anti-ERK1,2 (both New England Biolabs) (F).
FIGURE 1

![Image of a gel showing protein bands for β-Actin, Vinculin, and Talin under untreated and Latrunculin B treated conditions.]

- Untreated
  - β-Actin
  - Vinculin
  - Talin

- Latrunculin B Treated
  - Positive Control
FIGURE 2
FIGURE 3

Untreated

+ 20 ng/ml IL-1β

+ 20 ng/ml IL-1β + 1uM Latrunculin B

Positive Control
FIGURE 5

[Images of cellular assays showing Phospho-ERK1,2, ERK1,2, Phospho-JNK1,2, Phospho-p38, and a positive control in panels A, B, C, and D.]

Panel E and F show the staining intensity of Phospho-ERK1,2 and ERK1,2, respectively, with positive controls indicated.

Panel G presents a summary of the staining results for Phospho-ERK1,2, Phospho-p38, and Phospho-JNK1,2, with positive controls marked.
FIGURE 7

| Control | Nonsense Antibody | Anti-IRAK Antibody |
|---------|-------------------|--------------------|
| Phospho-ERK1,2 | ![Control](image1) | ![Nonsense](image2) |
| ERK1,2 | ![Control](image3) | ![Anti-IRAK](image4) |
The recruitment of the interleukin-1 receptor-associated kinase (IRAK) into focal adhesion complexes is required for IL-1β-induced ERK activation
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