Requirements of Focal Adhesions and Calcium Fluxes for Interleukin-1-induced ERK Kinase Activation and c-fos Expression in Fibroblasts*

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Interleukin-1 (IL-1) is an important inflammatory mediator and plays a central role in the destruction of connective tissue matrices in diseases such as arthritis and periodontitis. It is well established that IL-1 activation of the mitogen-activated protein (MAP) kinase pathway and induction of c-fos expression is a required step in the induction of matrix metalloproteinase expression involved in tissue degradation. Previous studies in our laboratory showed that IL-1-induced calcium flux is dependent on focal adhesion formation, suggesting a matrix-dependent restriction system for IL-1 signaling. Therefore, in the present study, we examined the consequences of this restriction on IL-1-mediated activation of the MAP kinase family and on c-fos expression. Treatment of human gingival fibroblasts with IL-1-activated extracellular signal-regulated kinases (ERK), c-Jun N-terminal kinase (JNK), and p38 kinase activity and induced c-fos expression in a dose- and time-dependent fashion. Plating cells on poly-l-lysine prevented focal adhesion formation, eliminated IL-1-induced calcium influx, abolished ERK stimulation, and blocked c-fos expression. Cells in suspension and hence with no suitable substratum for focal adhesion formation also showed no ERK activation or enhanced c-fos expression in response to IL-1. In contrast, eliminating focal adhesion formation or calcium depletion in cells plated on fibronectin had no effect on IL-1 stimulation of JNK and p38 kinases, demonstrating that their activation was mediated through pathways independent of focal adhesions and calcium. Calcium depletion abolished IL-1-induced calcium uptake, ERK activation, and c-fos expression. The focal adhesion dependence of IL-1-induced ERK activation and c-fos expression could be circumvented in cells plated on poly-l-lysine by simultaneous incubation with IL-1 and the calcium ionophore ionomycin. In transfection studies, IL-1 stimulation of serum responsive element (SRE) transcriptional activity was dependent on the presence of extracellular calcium. This is consistent with a requirement for calcium in the activation of ERKs and their involvement in the induction of c-fos expression through the SRE site on the 5′ promoter of the c-fos gene. Our results demonstrate that in cells attached to substrates by focal adhesions, IL-1-mediated calcium flux is required for ERK activation and c-fos expression but not for JNK or p38 activation. We conclude that cellular interactions with the extracellular matrix play an important role in restricting ERK and c-fos-dependent processes.

Interleukin-1 (IL-1) is an important mediator of inflammatory responses and is released from a variety of hematopoietic and stromal cell types (1). The uncompensated actions of such a potent cytokine can have deleterious consequences, and indeed IL-1 has been implicated in a wide range of pathological processes including sepsis, septic shock, and inflammatory diseases such as arthritis and periodontitis (2–4). The cellular responses to IL-1 stimulation are well documented and include increased production of matrix metalloproteinases (5, 6), prostaglandin E2 (7), and reactive oxygen species (8). However the detailed mechanisms leading to these biological activities after IL-1 binding to its cognate receptor are poorly defined. Many signaling systems (e.g. protein kinases A and C, phospholipase C, and G-proteins) have been reported to be utilized in IL-1-induced responses (9, 10). Small molecules such as ceramide (11) and reactive oxygen species (8, 12) may also act as second messengers for cytokines, including tumor necrosis factor-α and IL-1.

Recent advances in IL-1 signaling strongly suggest that protein kinases are involved in transducing signals elicited by IL-1 binding. An IL-1 receptor activated kinase has recently been identified and cloned (13). Cytokines such as IL-1 and tumor necrosis factor-α can also exert their effects via MAP kinase cascades (14). Members of this kinase family are classified into three subgroups (14, 15); the extracellular signal-regulated kinases (ERKs), the stress-activated protein kinases or c-Jun N-terminal kinases (JNKs), and p38 kinase. The MAP kinases are strongly activated by growth factors and stress-related signals including cytokine stimulation, ultraviolet light radiation, and osmotic shock. IL-1 activation of the MAP kinase cascade is necessary for the induction of the transcription factors, c-fos and c-jun, as well as the activation of the c-fos/c-Jun heterodimer, the activating protein-1 (AP-1). Since cytokine stimulation of AP-1 transcriptional activity is involved in a number of pathophysiological processes including cellular pro-

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¶ The abbreviations used are: IL-1, interleukin-1; MAP, mitogen-activated protein; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; fura 2/AM, fura 2 and fura 2 acetoxymethyl ester; PMA, phorbol 12-myristate 13-acetate; BAPTA/AM, 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid; [Ca2+]i, intracellular calcium concentration; MBP, myelin basic protein, GST, glutathione S-transferase; SRE, serum responsive element; TCF, ternary complex factor; PL, poly-l-lysine; BSA, bovine serum albumin; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline.

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liferation and production of matrix metalloproteinases, the signals modulating the MAP kinase pathway may play an important role in regulating cellular responses in disease.

Although the mechanism of IL-1 signaling is an area of intensive research, much less effort has been devoted to studying the regulation of IL-1 receptor signaling by the intracellular matrix. In *vitro*, many types of stromal cells form contacts with their substrata through focal adhesions, which are discrete macromolecular complexes that function in cell attachment (16). In fibroblasts, IL-1 receptors are enriched at these focal adhesion sites (17, 18). This localization may play an important role in regulating the signals induced following IL-1 binding to the receptor, possibly through the utilization of tyrosine kinases and phosphorylation of cytoskeletal proteins enriched at focal adhesions sites (19). For example, we have previously shown that cell attachment reciprocally modulates IL-1 signaling since focal adhesions are required for IL-1 induction of intracellular calcium flux (18, 20). In this study, we examined the requirement of focal adhesions and calcium uptake on IL-1 induced activation of the three MAP kinase subgroups and on c-fos expression.

**EXPERIMENTAL PROCEDURES**

**Materials**—Recombinant human IL-1β and biotinylated IL-1β were obtained from R & D Systems (Minneapolis, MN). The protein was purified by sequential chromatography (to >97% purity), and the endotoxin level was determined to be 0.1 ng/μg of IL-1 by the manufacturer. Ionomycin was purchased from Calbiochem (La Jolla, CA). Fura 2 and fura 2 acetoxyethyl ester (fura 2/AM) were from Molecular Probes (Eugene, OR). Bovine fibronectin, poly-l-lysine, BSA, mouse monoclonal antibody to vinculin, FITC-conjugated antibodies, EGTA, phorbol 12-myristate 13-acetate (PMA), 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid, acetoxyethyl ester (BAPTA/AM) were obtained from Sigma. Myelin basic protein was from Upstate Biotechnology Inc. (Lake Placid, NY). Anti-ERK2 and anti-p38 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

**Cell Culture**—Human gingival fibroblasts were grown in minimal essential medium (α-MEM) containing 15% fetal bovine serum and antibiotics (0.17% penicillin V, 0.1% gentamicin sulfate, and 0.01 μg/ml amphotericin) at 37 °C in a humidified atmosphere of 5% CO₂ in air. Cells between the 4th and 12th passages were used in all experiments. Prior to experiments with IL-1, cells were detached by incubation for 15 min at 37 °C with 0.5 mM EDTA after washing with phosphate-buffered saline (PBS). Fibroblasts were plated on coverslips, on 100-mm culture dishes, or in chamber slides. Cells were plated 1 day before experiments. Cells between the 4th and 12th passages were used in all experiments.

\[ [Ca^{2+}]_i \] *Measurement*—Intracellular calcium concentration ([Ca^{2+}]_i) was measured as described (20). The calcium response was studied in cells plated on coverslips coated with fibronectin or poly-l-lysine or in suspended cells. In experiments with Ca^{2+}-free medium, cells were washed with medium containing 0.5 mM EGTA without Ca^{2+} to chelate free Ca^{2+} ions and preincubated with BAPTA/AM in Ca^{2+}-free medium before adding IL-1β.

**Immunofluorescence Staining**—Multichannel glass slides were coated with fibronectin (10 μg/ml) or poly-l-lysine (200 μg/ml). Cells were then plated for 6 h, fixed in 2% paraformaldehyde for 30 min at room temperature, permeabilized with 0.3% Triton X-100, and thoroughly rinsed with PBS. Immunofluorescence staining for vinculin was performed with a mouse anti-vinculin antibody (1:20 dilution) for 1 h at 37 °C followed by a FITC-conjugated goat anti-mouse antibody (1:50 dilution) for 1 h at 37 °C. Nonspecific control staining was performed on the same slide by using secondary antibody only. The spatial distribution of staining for vinculin in focal adhesions was examined by confocal microscopy (Leica CLSM). For FITC-labeled antibodies, laser excitation. Emission signals were obtained with a 590/30 nm band pass filter. Ten thousand cells were analyzed in each sample, and data were collected in list mode using logarithmic amplifiers. To eliminate signals due to cellular debris, particles with forward light scatter comparable with previously established threshold values for fibroblasts were assessed.

**RESULTS**

**Requirement of Focal Adhesions for IL-1 Activation of MAP Kinases**—We have demonstrated previously that IL-1 stimulation of human fibroblasts and bovine chondrocytes leads to elevation of intracellular calcium ion concentration (18, 20), a response that requires the formation of focal adhesions. Consequently we investigated the relationship between this adhesion-dependent calcium flux and the downstream signaling events activated by IL-1. Since members of the MAP kinase family are potential targets in the IL-1 signaling pathway, we first assessed the activity of members of the MAP kinase family under conditions that permit or prevent focal adhesion formation. Fibroblasts were plated on fibronectin-coated (10 μg/ml) coverslips to allow integrin-dependent attachment and spreading. As shown earlier (18), immunolocalization of the focal adhesion protein vinculin by fluorescence microscopy showed bright, punctate staining in presumptive focal adhesions of receptor cells (Fig. 1A). In contrast, cells plated on poly-l-lysine (200 μg/ml), which mediates nonspecific, integrin-independent attachment, failed to spread and remained rounded. Immunofluorescence detection of vinculin in cells plated on poly-l-lysine showed only diffuse staining with no discrete, brightly staining sites (Fig. 1A).

**Calcium-dependent ERK Activation and c-fos Expression**—Cells were lysed in 800 μl of lysis buffer containing 50 mM HEPES at pH 7.5, 150 mM NaCl, 2 mM EDTA at pH 8, 1% Nonidet P-40, 10 mM sodium fluoride, 0.2 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 10 mM sodium pyro-phosphate, 1 μg/ml leupeptin, 2 μg/ml aprotinin. ERK or p38 kinase activity was determined to be incubating 200–300 μg of cellular extracts with 1 μM of anti-ERK2 or anti-p38 antibody, and protein A-agarose for 3 h at 4 °C. The protein A beads were then washed thoroughly before the addition of 20 μl of kinase buffer (20 mM HEPES at pH 7.5, 10 mM MgCl₂, 2 mM MnCl₂, 2 mM dithiothreitol, and 25 mM ATP) containing 2 μCi [γ-32P]ATP and 10 μg of substrate protein (MBP for ERK and GST-ATF2 for p38 kinase). After incubation at 30 °C for 30 min, the reactions were stopped with ice cold buffer, analyzed by SDS-PAGE, dried, and exposed to x-ray films.

**Transfection and Luciferase Assay**—Human fibroblasts were propagated overnight on 35-mm plates. Transfections were done with 10–15 μg of the reporter plasmid SRE-luc and 5 μg of lacZ DNA by the calcium phosphate technique according to a standard protocol (22). After 48 h, cells were harvested in lysis buffer (1% Triton X-100, 0.1 mM EDTA, FO4 at pH 7.8, and 1 mM dithiothreitol), and luciferase activity was determined as described by Xie et al. (23). Results were measured in a Lumat luminometer (Berthold) and expressed as relative light units.

**Northern Blot Analysis**—Total RNA was isolated by the acidified guanidine isothiocyanate method (12) and subjected to electrophoresis on a denaturing gel. Denatured RNA samples (10–15 μg) were analyzed by gel electrophoresis in a 1% denaturing agarose gel, transferred to a nylon membrane (Bio-Rad), cross-linked with an ultraviolet cross-linker (Stratagene UV Stratalinker 1800), and hybridized with a 32P-labeled c-fos cDNA probe. The blots were subsequently stripped and re-probed with 32P-labeled glyceraldehyde phosphate dehydrogenase (GAPDH) cDNA as an internal standard to ensure roughly equal loading.

**Flow Cytometry**—Assessment of IL-1 receptors was conducted by flow cytometry and affinity labeling (R & D Systems). Single cell suspensions were prepared by treating cell cultures with 0.5 mM EDTA and washing twice with saline buffer. The cells were subsequently stained with biotinylated IL-1β and then with streptavidin FITC. Measurements of fluorescence intensity for individual cells due to specific binding of IL-1β were obtained by comparing cells stained with IL-1β and streptavidin-FITC, or cells stained with streptavidin-FITC only, or with cells incubated with IL-1β in the presence of a blocking antibody to IL-1 (polyclonal goat IgG against human IL-1β). These control groups were used to determine fluorescence channel thresholds for specific staining. Samples were analyzed on a FACSTAR Plus flow cytometer (Becton-Dickinson, Mississauga, Ontario, Canada) at 200 cells/sec with 488 nm laser excitation. Emission signals were obtained with a 530/30 nm band pass filter. Ten thousand cells were analyzed in each sample, and data were collected in list mode using logarithmic amplifiers. To eliminate signals due to cellular debris, particles with forward light scatter comparable with previously established threshold values for fibroblasts were assessed.
We examined ERK, p38, and JNK activities after IL-1 treatment of cells plated on fibronectin or poly-L-lysine. Fibronectin-adherent cells stimulated with IL-1 showed ERK activation; however, no significant activation occurred in cells attached to poly-L-lysine (Fig. 1B). In contrast, both JNK and p38 kinase activities were very strongly activated by IL-1 in cells plated on either fibronectin or poly-L-lysine. In a separate assessment of the importance of cellular matrix attachment on IL-1-mediated ERK activation, the same treatment protocol was modified, and suspended cells were used instead of poly-L-lysine-attached cells. Again, significant increases of ERK activation above controls were observed only for cells adherent to fibronectin but not for suspended cells while JNK and p38 were activated independent of cell attachment (Fig. 1C). Hence, in contrast to ERK, focal adhesion formation does not appear to be required for IL-1 activation of the JNK or p38 kinase pathways.

Requirement of Calcium for IL-1 Stimulation of MAP Kinases—Since one effect of IL-1 stimulation, namely an increase in intracellular calcium, is a focal adhesion-dependent process (18, 20), we asked if this calcium response could be the restricting factor leading to the differential effects of IL-1 on ERK activation. In cells plated on fibronectin, there was about a 2-fold increase in [Ca\textsuperscript{2+}] (209 ± 30 nM above baseline, n = 3; Fig. 2A) upon IL-1 stimulation. Cell shape itself evidently did not play a role in the effects described above since poly-L-lysine-attached cells incubated with fibronectin but not BSA-coated beads also exhibited IL-1-induced calcium flux (Fig. 2A, right upper inset). Under these conditions, integrins are recruited to the bead-attachment sites, and this results in the formation of focal adhesions on the ventral surface of cells which have not

![Image](http://www.jbc.org/)

**FIG. 1.** Effect of focal adhesions on IL-1 stimulation of ERK activity. A, immunofluorescence micrographs of human fibroblasts stained for vinculin. Cells were plated on either fibronectin (FN; 10 μg/ml) or poly-L-lysine (PL; 200 μg/ml) in normal medium for 6 h. Note the bright, punctate staining of focal adhesions in cells on fibronectin. B, ERK, JNK, and p38 kinase activities in IL-1-stimulated fibroblasts plated on fibronectin (FN) or poly-L-lysine (PL). Cells were plated on either one of the two substrata in Ca\textsuperscript{2+}-containing medium for 6 h before the addition of 20 ng/ml of IL-1. After 15 min of incubation, kinase activity was examined as described under “Materials and Methods.” ERK activity was detected by immunoprecipitation with an ERK antibody and immunocomplex kinase assays using myelin basic protein (MBP) as substrate. JNK activity was assayed by solid phase assay using GST-c-Jun as substrate. p38 activity was assayed using GST-AFT2 as substrate. C, ERK, JNK, and p38 kinase activities in attached or suspended cells (SP) after IL-1 treatment. Fibroblasts were either grown on fibronectin or detached by using 0.5 mM EGTA in Ca\textsuperscript{2+}-free PBS. After the cells were washed with medium, 20 ng/ml of IL-1 was added for 15 min. Kinase activities were detected as described in panel B.

**FIG. 2.** Requirement of focal adhesions and calcium for IL-1 activation of ERK but not JNK or p38 kinase. A, intracellular calcium concentration in cells incubated in normal medium (Normal) or pre-incubated with BAPTA/AM in Ca\textsuperscript{2+}-deficient medium (Ca\textsuperscript{2+}-free). Cells were deprived of calcium by washing thoroughly with 0.5 mM EGTA and pre-incubated with 3 μM BAPTA/AM in Ca\textsuperscript{2+}-free medium for 30 min prior to IL-1 (20 ng/ml) treatment. Note that the normal calcium trace has been offset vertically 90 nM of [Ca\textsuperscript{2+}], to facilitate display of the traces. Right inset: cells plated on poly-L-lysine were loaded with fura-2 and pre-incubated with fibronectin-coated or BSA-coated beads and then stimulated with IL-1. Left inset: cells were plated on fibronectin and stimulated with ionomycin. B, effects of calcium depletion on IL-1 activation of ERK, JNK, and p38 kinase. Fibroblasts were either pre-incubated in normal or Ca\textsuperscript{2+}-free medium with 3 μM BAPTA/AM for 30 min before the addition of IL-1 (20 ng/ml). Substrates for various kinases are as described in Fig. 1B. C, restoration of ERK activation in cells plated on poly-L-lysine by simultaneous addition of ionomycin and IL-1. Fibroblasts were plated on fibronectin or poly-L-lysine and treated with either IL-1 (20 ng/ml) for 15 min, with ionomycin (Iom; 2 μM) for 15 min, or with IL-1 (20 ng/ml) and ionomycin (2 μM) together for 15 min. ERK activity was assessed.
Calcium-dependent ERK Activation and c-fos Expression

spreads and remained round (data not shown; see Ref. 20). As expected, calcium depletion (Ca²⁺-free medium plus BAPTA/AM) led to a nearly complete reduction in the amplitudes of IL-1-induced calcium transients (Fig. 2A). Cells pre-incubated in Ca²⁺-free medium but without BAPTA/AM also showed similar reductions of calcium transients, indicating that the rise in intracellular calcium was primarily due to influx through the plasma membrane (data not shown; see Ref. 18).

When ERK activity was measured in cells plated on fibronectin, only cells incubated in calcium-containing buffer exhibited greatly increased IL-1-mediated activation of ERK than cells in calcium-free buffer (Fig. 2B), demonstrating that the availability of calcium in the bathing buffer was important for stimulation of ERK by IL-1. As IL-1-induced ERK stimulation was evidently calcium-dependent, we examined if this requirement also applied to the other two MAP kinases. As measured by solid phase and immunocomplex kinase assays, respectively, neither JNK or p38 activation by IL-1 showed the calcium dependence that was required by ERK (Fig. 2B). This common characteristic of calcium-independence is consistent with the notion that both JNK and p38 kinase activation are adhesion-independent and also share many properties including activation by the same stresses and cytokines (15, 24–27).

In the next series of experiments, we asked if we could circumvent the apparent focal adhesion dependence for IL-1-induced ERK activation in cells by inducing calcium entry with the ionophore ionomycin. Cells plated on fibronectin or poly-L-lysine were treated with and without IL-1 and ionomycin. Preliminary dose-response experiments were conducted with ionomycin to determine the optimal dose for stimulation of calcium flux, and this was found to be 2 μM. Treatment with ionomycin alone led to a significant calcium influx in cells plated on fibronectin (Fig. 2A, left upper inset) or poly-L-lysine (not shown) but did not activate ERK in cells on fibronectin or poly-L-lysine (Fig. 2C), suggesting that calcium fluxes alone are necessary but not sufficient for ERK activation. Ionomycin and IL-1 induced ERK activation at a similar or greater level than that of IL-1 alone in fibronectin-attached cells (Fig. 2C, lanes 2 and 4). Notably, in cells plated on poly-L-lysine, the combination of ionomycin and IL-1 was able to strongly stimulate ERK activation, whereas IL-1 or ionomycin alone had little or no significant effect. These data indicate that artificially induced calcium entry by ionomycin fulfill the requirements provided by focal adhesions in IL-1-induced activation of ERK even when cells are plated on poly-L-lysine and focal adhesions are absent.

Requirement of Focal Adhesions and Calcium on Serum Response Element (SRE) Activation and c-fos Expression—Since IL-1 activation of ERKs is thought to be a required step in the activation of the SRE and c-fos expression, we examined the effect of ERK inhibition by depriving cells of calcium and then measuring SRE transcriptional activity and c-fos expression in response to IL-1 (Fig. 3). The SRE is bound and regulated by the serum response factor (SRF) along with the ternary complex factor (TCF/Elk-1; Ref. 28). TCF/Elk-1 is a major substrate for ERK, which upon activation translocates to the nucleus. Thus ERK activation indirectly enhances transcriptional activity of SRE-dependent genes. To establish whether intracellular calcium increases are required for SRE activity, fibroblasts transfected with a luciferase reporter plasmid under the control of a SRE were treated with IL-1 in the presence and absence of extracellular calcium. In the presence of calcium, luciferase activity was stimulated 5-fold by IL-1 (Fig. 3). However, this stimulation did not occur if calcium was not available in the bathing buffer. These data demonstrate the dependence of IL-1 stimulation of SRE activity on calcium fluxes. However, the inability of ionomycin to stimulate luciferase activity in transfected fibroblasts (data not shown) suggests that artificially induced increases in intracellular calcium are not sufficient to induce SRE transcriptional activity.

The c-fos gene is an immediate early response gene that is rapidly and transiently induced upon stimulation of quiescent cells with cytokines, growth factors, and serum. Serum growth factors and cytokines that activate MAP kinases stimulate c-fos expression through the SRE (29). Thus, agents or conditions that alter SRE activity will modulate c-fos expression. In cells plated on fibronectin, IL-1 (20 ng/ml; continuous exposure) induced a transient increase of c-fos mRNA that peaked at 30 min (Fig. 4A, top). Expression of GAPDH mRNA was used as a loading control and was unaffected by IL-1 treatment. IL-1 induced a dose-dependent increase of c-fos mRNA that peaked at 20 ng/ml IL-1β when cells were examined at 30 min after treatment (Fig. 4A, middle). Induction of c-fos by IL-1 was dependent on the formation of focal adhesions since cells plated on poly-L-lysine showed only a minimal response to IL-1 (Fig. 4A, bottom), and cells in suspension showed no detectable response (data not shown).

To investigate if the IL-1-induced calcium increase was required for the expression of c-fos, we examined the effect of IL-1β on [Ca²⁺], in the presence (1.8 ms) and in the absence of extracellular calcium (Ca²⁺-free medium with 0.5 mM EGTA for 15 min). The amplitudes of IL-1-induced calcium transients above baseline after incubation in EGTA without Ca²⁺ were significantly reduced (16 ± 4 ms above baseline; p < 0.05; n = 4). Cells pre-incubated with the intracellular calcium chelator BAPTA/AM showed no further reduction of calcium transients, indicating that entry of calcium from the external buffer was critical for generation of transients, whereas intracellular release was less important. Indeed preincubation of cells with thapsigargin (1 μM; 15 min in calcium containing buffer) before IL-1 treatment showed that depletion of thapsigargin-sensitive calcium stores reduced the IL-1-induced calcium flux by only ~37%. In cells treated with normal or calcium-depleted buffer and probed for c-fos expression 30 min after IL-1 treatment,
calcium was due to internalization of IL-1 receptors. Cells were incubated with biotinylated IL-1β in the presence, and absence of calcium and surface receptor staining was measured by flow cytometry. Fluorescence due to labeled receptor was not reduced after calcium depletion (background fluorescence = 3 ± 0.1; cells in normal medium = 18 ± 0.2; cells in calcium-depleted medium = 22 ± 0.2). These data indicate that receptor availability was not limited by calcium depletion and could not explain the inhibition of IL-1-induced responses in the absence of focal adhesions and calcium.

**DISCUSSION**

Cell proliferation and matrix metalloproteinase production are critical processes in tissue destruction of inflammatory diseases (3). Although it is well established that increased levels of cytokines such as IL-1 can stimulate these processes in vivo (1), little is known about the cellular mechanisms that regulate the type and intensity of cytokine-induced responses in tissue destruction. The elevation of extracellular levels of cytokines may be insufficient to induce the cellular responses that lead to irreversible degradation of tissues. Other restrictions such as cell shape, differentiation state, and cell-matrix interactions may also be involved in determining the types of responses induced by cytokines such as IL-1. Recently, we have found that cell-matrix interactions can restrict IL-1-induced calcium flux in chondrocytes (20) and gingival fibroblasts (18), indicating that cell-matrix interactions play an important role in regulating the type of cellular responses induced by IL-1. Since calcium has been shown to act as a second messenger in a number of critical signaling events in inflammation including the induction of c-fos (30), we considered the possibility that cell-matrix interactions mediated by focal adhesions may be involved in regulating IL-1 induction of genes associated with these processes. As cytokine induction of c-fos is an important regulatory step in the stimulation of AP-1-dependent gene expression and is required for the expression of matrix metalloproteinases and cell proliferation, we examined whether cell-matrix interactions mediated by focal adhesions modulate IL-1 induction of c-fos expression. The principal finding of this study
Calcium-dependent ERK Activation and c-fos Expression

is that IL-1 stimulation of c-fos expression requires focal adhesions. We propose that in the absence of focal adhesions or their in vivo counterparts, cells are not capable of responding to extracellular IL-1, either by the induction of c-fos expression or subsequently by the induction of AP-1-dependent processes including proliferation and expression of matrix metalloproteinases. This restriction may serve to limit inflammatory tissue destruction in vivo when there are transient increases in the levels of cytokines after injury.

In this study, we identified some of the potential upstream mechanisms by which fibroblasts restrict IL-1 induction of c-fos. Although it is evident that all three MAP kinase pathways are targets for IL-1, the cellular and biochemical determinants that dictate their responses to cytokines are different. Unlike p38 kinase and JNK, focal adhesion formation and calcium flux were required for IL-1-induced ERK activation in human gingival fibroblasts. These data indicate that the formation of focal adhesions can specifically alter ERK kinase-dependent processes such as c-fos expression, without affecting JNK and p38 kinase-dependent responses. IL-1 stimulation of ERK kinases is involved in the phosphorylation of TCF/ELK-1, which is a required step in the activation of SRE response elements and induction of c-fos expression (29). The requirement of focal adhesions for IL-1 stimulation of SRE transcriptional activity and c-fos expression is consistent with their dependence on ERK kinase activity. Our previous data have shown that IL-1-induced calcium uptake is dependent on focal adhesions (18, 20); in turn, calcium uptake is required for the activation of ERK kinase and SRE activation (29). These processes eventually lead to the induction of c-fos expression and AP-1-dependent processes. The exact details of the mechanism by which focal adhesions and intracellular calcium regulate these signaling events are currently being investigated in our laboratory.

Currently it is unknown if focal adhesion restriction of c-fos expression is cell type- or receptor-specific. We have shown that focal adhesions are required for calcium uptake in both bovine chondrocyte and human gingival fibroblast cultures (20, 18). Furthermore, calcium uptake is required for the induction of c-fos in a number of cell types (30), indicating that the requirement of focal adhesions and calcium for cytokine induction of c-fos expression may be widely distributed among other cell types. However, this restriction may not apply to all types of cytokine receptors and their downstream pathways. For example, carbachol stimulation of muscarinic acetylcholine receptors in rat 1a fibroblasts results in the stimulation of ERKs and JNKs (31). However, in this cell type, calcium chelation totally abolishes stimulation of JNK but has no effect on ERK activity. More recently, we have data demonstrating that PDGF induction of c-fos expression is not restricted by the absence of focal adhesions, indicating that PDGF receptors may utilize separate signaling pathways to stimulate c-fos expression which do not require focal adhesions. Thus it seems likely that different receptors offer distinct modes of regulation of the same kinase pathways and c-fos expression; but conceivably, focal adhesion restriction of c-fos induction and AP-1-dependent genes is specific to IL-1 and potentially other cytokine receptors.

Our data provide strong evidence that cell signaling can be regulated by cell-matrix interactions. The enrichment of IL-1 receptors in focal adhesions (17, 18) and the interactions of receptors with extracellular matrix components can endow cells with a restricted spectrum of responses to external stimuli. Focal adhesion sites are enriched in signaling molecules that are utilized by integrins to transduce signals from the extracellular surface to the nucleus. Perhaps, IL-1 binding to its receptor also triggers activation of integrin-associated signaling components within the same focal adhesion complex. Indeed, integrin-fibronectin interactions have been shown to activate MAP kinases and mediate calcium flux (32, 33). Localization of IL-1 receptors and integrin-associated signaling molecules at focal adhesions may be required to enhance the sensitivity to IL-1 binding and generation of calcium fluxes; this sensitization could induce signaling levels that are sufficient for the activation of ERK kinases and c-fos expression. While the mechanism of regulation of ERK activity by calcium is not known, recent studies have suggested that a calcium/calmodulin-dependent kinase can activate MAP kinases (34). One intriguing possibility is that IL-1 may induce reciprocal interactions between IL-1 receptors and integrins; thus the effect of IL-1 on calcium and MAP kinase activation may be mediated through the c-fos integrin, an abundant fibronectin receptor in gingival fibroblasts. On the other hand, IL-1 can also modulate cell-matrix interactions by inducing phosphorylation of the focal adhesion protein talin (19). Hence, the colocalization of IL-1 receptors in focal adhesions facilitates reciprocal interactions between matrix proteins, adhesion complexes, and signaling receptors.

The c-fos protein forms heterodimers with c-Jun to form the AP-1, which is required for cellular proliferation and the expression of AP-1-dependent genes such as collagenase and stromelysin in response to cytokines such as IL-1. Thus, mechanisms regulating the expression of c-fos expression are of interest in a number of diseases such as cancer, arthritis, and periodontitis. We have shown previously that focal adhesion formation is required for IL-1-induced calcium influxes (18, 20) but the importance of this restriction in the context of early gene responses to IL-1 was unknown. As c-fos is a calcium-dependent gene (30, 35), it is not surprising that calcium uptake is a limiting second messenger in c-fos expression and AP-1 activation. Ionomycin-induced calcium entry promoted only very low levels of c-fos expression that were independent of IL-1 and of focal adhesions. This finding indicates that a high amplitude calcium flux alone may not be enough to stimulate robust c-fos expression. Thus increases in intracellular calcium induced by ionomycin were evidently not sufficient to induce maximal levels of c-fos expression compared with IL-1. The apparently additive effects of ionomycin and IL-1 on c-fos expression by fibroblasts plated on poly-l-lysine suggests that other signals induced by IL-1 are also important in the induction of c-fos expression, independent of calcium. Further, treatment of cells with ionomycin (2 μM) is not a pathophysiological stimulus and may induce gene responses because it is a major cell stressor. Notably, when cells were plated in calcium-depleted buffer or on a substrate that prevents focal adhesion formation, the generation of IL-1-induced calcium signals and c-fos expression was effectively blocked. We conclude that focal adhesions and intracellular calcium fluxes are important restriction elements in c-fos expression.

The concentration of IL-1 receptors in focal adhesions described previously (18, 36) is an example of how cytokine signaling and matrix proteins can interact reciprocally to modulate cellular responses (37). Integrin clustering in response to binding to collagen or fibronectin regulates the assembly of cytoplasmic plaques and stress fibers (38) which in turn will regulate the clustering of IL-1 receptors, a point that is emphasized by the lack of IL-1 receptor clustering in cells that are plated on poly-l-lysine (18). IL-1 can regulate matrix proteins by inducing metalloproteinase expression (39), and degrada-

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2 Y. Y. C. Lo, unpublished observations.
3 T. Cruz and E. Turley, unpublished data.
Calcium-dependent ERK Activation and c-fos Expression

Ca2+-dependent matrix proteins in turn is likely to affect focal adhesion formation since the conformation of critical motifs in matrix proteins are required for integrin binding (40) and the assembly and maintenance of focal adhesions (41). We have shown that the generation of IL-1 signals and early gene responses is very dependent on the clustering of IL-1 receptors to focal adhesions. While focal adhesions are principally an in vitro phenomenon, their in vivo counterparts are rarely seen in normal connective tissues (41). Conceivably, the absence of focal adhesions may restrict the expression of AP-1-dependent genes such as matrix metalloproteinases which are rarely induced in healthy tissues. In contrast, there are marked alterations in the nature of adhesive contacts between cells and matrix proteins in inflamed and regenerating tissues (42). These changes could markedly affect fibroblast-matrix interactions, including the clustering of IL-1 receptors into focal adhesions and resultant downstream gene responses (36).

We propose that these alterations would lead to increased cytokine-induced gene responses that are dependent on calcium and AP-1 activation. Notably, pharmacological inhibition of AP-1 activity in vivo or in vitro markedly attenuates chronic inflammatory disease processes including cellular proliferation and production of matrix-degrading proteases. We conclude that focal adhesions and calcium fluxes regulate IL-1 induction of AP-1-dependent processes that are important in the pathogenesis of chronic inflammatory diseases.

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