Tyrosine Phosphatase SHP-2 Regulates IL-1 Signaling in Fibroblasts Through Focal Adhesions

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Interleukin-1β (IL-1β) mediates destruction of matrix collagens in diverse inflammatory diseases including arthritis, periodontitis, and pulmonary fibrosis by activating fibroblasts, cells that interact with matrix proteins through integrin-based adhesions. In vitro, IL-1β signaling is modulated by focal adhesions, supramolecular protein complexes that are enriched with tyrosine kinases and phosphatases. We assessed the importance of tyrosine phosphatases in regulating cell–matrix interactions and IL-1β signaling. In human gingival fibroblasts plated on fibronectin, IL-1β enhanced the maturation of focal adhesions as defined by morphology and enrichment with paxillin and α2-integrin. IL-1β also induced activation of ERK and recruitment of phospho-ERK to focal complexes/adhesions. Treatment with the potent tyrosine phosphatase inhibitor pervanadate, in the absence of IL-1β, recapitulated many of these responses indicating the importance of tyrosine phosphatases. Immunoblotting of collagen bead-associated complexes revealed that the tyrosine phosphatase, SHP-2, was also enriched in focal complexes/adhesions. Depletion of SHP-2 by siRNA or by homologous recombination markedly altered IL-1β-induced ERK activation and maturation of focal adhesions. IL-1β-induced tyrosine phosphorylation of SHP-2 on residue Y542 promoted focal adhesion maturation. Association of Gab1 with SHP-2 in focal adhesions correlated temporally with activation of ERK and was abrogated in cells expressing mutant (Y542F) SHP-2. We conclude that IL-1β-mediated maturation of focal adhesions is dependent on tyrosine phosphorylation of SHP-2 at Y542, leading to recruitment of Gab1, a process that may influence the downstream activation of ERK. J. Cell. Physiol. 207: 132–143, 2006.

The cytokine interleukin-1 (IL-1β) is a critical mediator in the inflammatory destruction of extracellular matrices in diverse diseases including rheumatoid arthritis, pulmonary fibrosis, and periodontitis (Kline et al., 1993; Dayer, 2003; Dunne and O’Neill, 2003; Graves and Cochrane, 2003). IL-1β promotes degradation of extracellular matrix (ECM) proteins, in part by enhancing the synthesis and secretion of proteases and other mediators (Boyle et al., 1997; van den Berg, 1999). Matrix-degrading proteases such as matrix metalloproteinases (MMPs) are strongly expressed by fibroblasts (Tolboom et al., 2002). Conversely, fibroblasts synthesize ECM proteins such as collagen that are required for tissue repair, but, if laid down in an abnormal manner or excessive amount, contribute to organ dysfunction (reviewed in Goldring, 2003). Thus elucidation of the factors that determine the balance between matrix degradation and synthesis is critical for an improved understanding of tissue fibrosis and repair.

Currently, the intracellular signaling pathways that regulate IL-1-dependent responses in fibroblasts are incompletely understood. Activation of MMPs in fibroblasts is critically dependent on IL-1-induced activation of ERK (Reunanen et al., 1998). Importantly, this pathway is modulated by cellular interactions with the substratum through focal complexes or adhesions (MacGillivray et al., 2000, 2003), adhesive domains that comprise the termini of actin stress fibers in physical association with specific actin-binding proteins (Burridge and Chrzanowska-Wodnicka, 1996). These structures mediate cell adhesion to the substratum and also regulate signal transduction pathways triggered by growth factors and cytokines. Notably, IL-1 receptors in human fibroblasts are concentrated at focal adhesions (Qwarnstrom et al., 1988; Arora and McCulloch, 1994) and stimulation of these cells with IL-1β leads to dynamic remodeling of actin filaments and focal adhesions (Qwarnstrom et al., 1991) by which the cytokine rapidly modulates cell–matrix interactions during inflammation and wound healing.

Nascent focal adhesions contain a number of signaling molecules in addition to growth factor and cytokine receptors (Miyamoto et al., 1996; Garrington and Johnson, 1999). Reversible tyrosine phosphorylation, dependent on the coordinated actions of protein tyrosine kinases and phosphatases (PTPs), regulates transmission of IL-1 signals from the receptor into the cytosol and

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nucleus (Arora et al., 1995; Rzymkiewicz et al., 1995; Yamada et al., 2001). Importantly, PTPs including PTPζ, SHP-2, and low molecular weight PTP are associated with focal adhesions and modulate their function (Chiarugi et al., 1998; Manes et al., 1999; Rigacci et al., 2002; Webb et al., 2004; Zhang et al., 2004). SHP-2, for example, is particularly important in modulating focal adhesions (Yu et al., 1998; Zhang et al., 2004). How PTPs regulate these processes is not well defined.

Here, we examined the role of PTP in IL-1β signaling and cell–matrix interactions. We demonstrate that IL-

1β induces dynamic alterations in focal adhesions that are critical in ERK activation, a response that is dependent on tyrosine phosphorylation of SHP-2 on residue Y542 and activation of its adaptor function. These observations have important implications for the potential therapeutic modulation matrix degradation and repair in inflammatory diseases.

MATERIALS AND METHODS

Materials

Bovine fibronectin, poly-L-lysine, BSA, aprogin, leupeptin, PMSF, PAO, sodium orthovanadate, Triton-X 100, Tween-20, mouse monoclonal antibodies to vinculin, z-smooth muscle actin, b-actin, z-actin and phallolidin-FITC, and ferro-
magnetic beads were obtained from Sigma-Aldrich (St. Louis, MO). Rabbit polyclonal antibodies to p38, phospho-p38, JNK (SAPK), phospho-JNK (SAPK), ERK1/2, phospho-ERK1/2, phospho-MEK1/2, Gab1 and Gab2, phospho-Gab1 and Gab2, phospho-tyrosine100 and phospho-SHP-2 (Tyrs580 and Tyr542) were from Cell Signaling (Beverly, MA). Rabbit polyclonal antibodies to SHP-2 (COOH-terminus), mouse monoclonal antibodies to SHP-2 (COOH-terminus), and the protein A/B-agarose were from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase conjugated goat anti-mouse (H+L), goat anti-rabbit were from Cedarlane Biotechnology (Oakville, ON). The ECL Chemiluminescence Kit was from Perkin Elmer Life Science (Boston, MA). FITC-labeled rabbit polyclonal anti-mouse antibodies and Texas Red-labeled goat anti-mouse antibodies were from Jackson Immuno Research Laboratories (West Grove, PA). Acidified bovine type I collagen (Vitrogen) was from Cohesion Technologies, Inc. (Palo Alto, CA). A magnetic separation stand was obtained from Promega (Madison, WI). IL-1b and platelet derived growth factor (PDGF) BB were from R&D (Minneapolis, MN). Parsenbus A and bis (N,N-Dimethylhydroxamido)hy-
droxooxovanadate (DMHV) were from Calbiochem (La Jolla, CA). DAPI was from Molecular Probes (Eugene OR). Glu-
tathione sepharose was from Amersham Biosciences (Piscata-
away, NJ). siRNAs were from Ambion (Austin, TX).

Cell culture and bead preparations

Human gingival fibroblasts between the 5th and 12th passages were grown in minimal essential medium (MEM), as previously described (MacGillivray et al., 2003). 3T3 immortalized fibroblasts SHP-2 (46–110) derived from exon 3-deficient mouse, and SHP-2 (46–110) expressing wild-type human SHP-2 (reconstituted) and Y542F mutants (provided by Dr. Benjamin G. Neel, Harvard Medical School, Boston, MA) (Zhang et al., 2002) were grown in high glucose DMEM. Bead-associated proteins were eluted in boiling Laemli sample buffer, separated by SDS–PAGE, and analyzed by Western blotting. Beads bound to the cells were counted electronically (Coulter Counter) to normalize bead-associated proteins.

Isolation of focal complexes/adhesions

Focal complexes/adhesions were isolated from fibroblasts as previously described (MacGillivray et al., 2000, 2003). Bead-associated proteins were eluted in boiling Laemli sample buffer, separated by SDS–PAGE, and analyzed by Western blotting. Beads bound to the cells were counted electronically (Coulter Counter) to normalize bead-associated proteins.

Adhesion assay with collagen coated beads

Cells were incubated with collagen-coated green fluorescent latex beads (2 µm; Polysciences, Warrington, PA) for 60 min. Single cell suspensions were prepared by trypsinization, a procedure that removed loosely bound beads. Cell suspensions were analyzed by flow cytometry. The percentage of cells with attached beads was computed.

SDS–PAGE and immunoblotting

SDS–PAGE and immunoblotting were conducted as previously described in detail (MacGillivray et al., 2000, 2003) ensuring that equal amounts of protein were loaded into each lane. Labeled proteins were visualized by chemiluminescence according to the manufacturer's instructions (Amersham, Oakville, ON). Densitometry values of blots were normalized so that one represents the densitometry value of control samples. Blots that are shown are representative of 3–4 independent experiments.

Immunofluorescence

Fibroblasts grown on chamber slides (Labtek) were coated with fibronectin (10 µg/ml in PBS), fixed, permeabilized, and stained with antibodies to z-actin, vinculin and paxillin, as previously described (MacGillivray et al., 2003). Staining for actin filaments was performed with FITC-phalloidin. Slides were viewed with a Zeiss LSM510 confocal microscope or a Q-

Imaging camera mounted on a Leica DMRA2 inverted fluorescence microscope. Digital images were processed with OpenLab (Improvision 4). The projected surface area of focal complexes/adhesions was estimated by measurement of pax-

illin staining in adherent cells using the same imaging software. Focal complexes and adhesions are defined as integrin-based discrete adhesive domains that comprise the termini of actin stress fibers and are identified by the presence of characteristic actin-binding proteins including vinculin, talin, paxillin, and z-actinin (Burridge and Chrzanowska-Wodnicka, 1996). Focal complexes are defined as small (~1 µm²) adhesion sites that contain paxillin. Focal adhesions are defined as larger (several µm²) and more elongated structures near the periphery of the cells that contain paxillin and z-actinin. Super-mature focal adhesions are defined as those that are larger (15–20 µm²) that also contain z-actinin (Dugina et al., 2001).

Short interfering RNA (siRNA)

Specific inhibition of SHP-2 was conducted with siRNA (sequence: ag-cccaaaaagauauauucc-gtt, (1080–1100 of the SHP-2 sequence). Human gingival fibroblasts were transfected with 50 nM of SHP-2 or GFP-siRNA (control) for 72 h using Oligofectamine according to the manufacturer’s specifications.

Immunoprecipitation

Proteins were purified by immunoprecipitation and ana-
lyzed by SDS–PAGE and western analysis, as previously described (MacGillivray et al., 2003). To assess the complexity of the SHP-2 immunoprecipitates, silver staining of the nitrocellulose membranes was performed. For these studies, a monoclonal anti-SHP-2 antibody (B-2 clone, Santa Cruz Biotechnology) bound to Protein A/G Plus (Santa Cruz Biotechnology) was used. These studies revealed a predomi-

nant band of Mr ~68 kDa band corresponding to SHP-2 and several less abundant bands of Mr ~30, 110, and 130 kDa (not illustrated).

Data analysis

For continuous variables, means, and standard errors of means were computed. Data were analyzed by ANOVA with correction for multiple comparisons (Dunnett or Sheffe) or by paired or unpaired Student’s t-test, as indicated. For analysis of the data in Figure 5D, because the absolute values of the areas for SHP-2wt and SHP-2Δcells varied by 3–4 fold, we conducted a log(t) transformation of the data followed by ANOVA. Statistical significance was considered for P-values of <0.05.
RESULTS

IL-1-induced super-maturation of focal adhesions and role of protein tyrosine phosphatases

When cells contact ECM ligands such as fibronectin or laminin, they attach, spread, and eventually develop mature focal adhesions, a dynamic process requiring many hours to days, depending on the cell type (Arora et al., 1995; Geiger et al., 2001; Laukaitis et al., 2001; Geiger and Bershadsky, 2002). We characterized the effects of IL-1β on attachment to the ECM by monitoring human gingival fibroblasts plated on fibronectin substrates in the presence or absence of this cytokine (Fig. 1A). Compared to untreated controls, IL-1β treatment induced the accumulation of paxillin and then later, α-actinin, into larger elongated adhesive structures at the periphery of the cell (Fig. 1A, parts a–h).

The incorporation of α-actinin into focal adhesions was associated with the formation of more mature forms of these adhesive structures (Dugina et al., 2001; von Wichert et al., 2003). This maturation process was accompanied by cell spreading and membrane ruffling in a high proportion of cells and was associated with enhanced tyrosine phosphorylation of multiple cellular proteins, as indicated by immunoblotting of whole cell lysates (not illustrated).

As protein tyrosine phosphatases (PTP) regulate tyrosine phosphorylation events and are known to be associated with focal complexes/adhesions (Retta et al., 1996; Petrone and Sap, 2000; MacGillivray et al., 2003; von Wichert et al., 2003), we investigated the specific mechanisms by which they influence the development and maturation of these adhesive domains. Initially, we treated cells with pervanadate (PV), a potent PTP

![Figure 1](https://example.com/figure1.png)

Fig. 1. IL-1β and pervanadate promote maturation of focal adhesions. A: Human gingival fibroblasts plated on fibronectin for 16 h in normal growth medium were treated with IL-1β (20 ng/ml) fixed and immunostained for paxillin and α-actinin. Note the more peripheral distribution and enlargement of focal adhesions after IL-1 treatment. Arrows denote supermature focal adhesions defined by inclusion of α-actinin. B: Length of focal adhesions measured on paxillin-stained vehicle control (C), IL-1β or pervanadate (PV)-treated cells. Data are mean ± SD of 30 independent measurements. Data were analyzed by an unpaired Student’s t-test (P < 0.01).
inhibitor (Trudel et al., 1990), and monitored alterations in cell morphology and cell-substrate attachment. Similar to IL-1β, PV treatment induced rapid recruitment of tyrosine phosphorylated proteins such as paxillin to small adhesion sites on the ventral surface of the cells (not illustrated). This was followed by enlargement and elongation of these structures and recruitment of α-actinin. Morphometric analysis of these focal-adhesion-like structures induced by IL-1β and PV revealed that they were larger than those that formed spontaneously during attachment to a fibronectin substrate (Fig. 1B), an appearance that has been termed 'super-mature' (Dugina et al., 2001).

To quantify these dynamic events at the biochemical level, we employed a previously described model system (Arora et al., 2000; MacGillivray et al., 2003) in which ferro-magnetic beads coated with integrin ligands are added to the dorsal surface of fibroblasts attached to a substrate such as a fibronectin. Structures that are analogous to bone fide focal complexes/adhesions form at the site of bead-cell contact on the dorsal cell surface. In these experiments, the beads were coated with collagen so that there would not be 'competition' between fibronectin receptors (ζ[2]β3 and ζ[1]β3) sequenced on the basal surface and those recruited to the developing focal complexes/adhesions on the dorsal surface (ζ[1]β3 and ζ[2]β3). Purification of bead-associated proteins using magnetic separation allows quantitative, kinetic analyses of proteins associated with these model focal complexes/adhesions (MacGillivray et al., 2000). With this approach, immunoblotting of bead-associated proteins revealed that both IL-1β and PV induced increased recruitment of α-actinin to sites of bead-cell contacts (Fig. 2A,B). This result is consistent with the maturation we observed by immunofluorescence of proteins associated with authentic focal complexes/adhesions at the sites of cell-substrate contact on ventral cell surfaces (c.f. Fig. 1A). To ensure that the observed binding of focal adhesion proteins to the beads was specific, collagen-coated beads were mixed directly with fibroblast lysates (Fig. 2A; control beads). Under these conditions, the beads bound much less focal adhesion proteins compared to beads added on the dorsal surfaces of intact cells, indicating that recruitment of focal adhesion proteins to beads involves active cellular processes and is not attributable to non-specific binding to the beads.

**Inhibition of PTP induces focal complex/adhesion-dependent ERK activation**

We and others have previously reported that ERK, a MAP kinase family member required for IL-1-induced pro-inflammatory responses, is associated with, and is modulated by, signaling events in focal complexes/adhesions (Arora et al., 2000; MacGillivray et al., 2003; Webb et al., 2004), including a focal adhesion requirement for IL-1β-induced ERK activation (Arora et al., 1995; MacGillivray et al., 2000, 2003). Our goal here was to define the specific roles of IL-1β and PTP in dynamic alterations in ERK during substrate attachment. We first examined the effects of IL-1β and PV on the subcellular distribution and activity of ERK in fibroblasts during attachment to fibronectin. IL-1β treatment of fibroblasts rapidly induced ERK activation and recruitment to nascent focal complexes/adhesions, as shown by co-localization of phospho-ERK with paxillin (Fig. 2C) and its presence in focal adhesion-associated proteins in a complex with α-actinin (Fig. 2A). Similar responses were observed after PV treatment in the absence of other agonists (Fig. 2B).

To verify the dependence of ERK activation on focal adhesions under these experimental conditions (MacGillivray et al., 2000, 2003), we found that when focal adhesion formation is promoted by adhesion to fibronectin, both IL-1β and PV induced robust activation of ERK (Fig. 3A). Conversely, when formation of focal complexes/adhesions is prevented by attachment of cells to a poly-L-lysine coated substrate, both IL-1β and PV treatment failed to induce ERK activation (Fig. 3A). To ensure that the effects of PV were attributable to modulation of PTP, we measured ERK activation in response to two other chemically distinct PTP inhibitors, phenylarsine oxide (PAO) and bis(N,N-Dimethylhydroxamido)hydroxooxovanadate (DMHV); these PTP inhibitors also induced comparable activation of ERK1/2 (Fig. 3A). The response to phorbol myristate acetate (PMA) is included as a positive control for ERK activation. PV-induced ERK activation was sustained for at least 240 min and this prolonged activation of ERK was also focal adhesion dependent (Fig. 3B). Further, ERK activation after treatment with IL-1β or PTP inhibitors was restored in cells attached to poly-L-lysine when focal complexes/adhesions were induced on the dorsal surfaces of cells using collagen-coated beads (Fig. 3C). The similarity in the cellular responses to IL-1β and to PTP inhibitors with respect to maturation of focal adhesions and the common requirement for focal adhesion formation in ERK activation suggested the involvement of one or more PTPs in IL-1β-induced signaling at focal adhesions.

To determine the importance of substrate attachment for ERK activation in response to IL-1β and PV, we developed an experimental system in which the degree of focal adhesion-mediated attachment to the substrate was varied. Cells were allowed to adhere to glass cover slips, either uncoated or coated with fibronectin. On fibronectin-coated cover slips, cells develop well-formed focal complexes and adhesions (see above) whereas on uncoated glass, cells secrete small amounts of endogenous fibronectin and form only rudimentary focal complexes (not illustrated). Under these latter conditions, both IL-1β and PV-induced ERK activation were roughly proportional to the degree of focal adhesion formation (Fig. 3D). Importantly, IL-1β induced ERK activation was potentiated by PV, providing evidence that the signaling pathways activated by these two agonists converge on ERK.

**SHP-2 Y542 phosphorylation is required to prolong ERK activation induced by IL-1**

The data above implicate PTP in the maturation of focal complexes and in activation of the ERK pathway, but do not identify the individual PTP(s) involved or the mechanism by which PTP modulate this important pathway. Several PTP including SHP-2, PTPα, and LMW-PTP are known to be associated with focal adhesions or with signaling proteins that are present in focal adhesions and thus are potential candidates (Feng, 1999; Petrone and Sap, 2000; MacGillivray et al., 2003; von Wichert et al., 2003). We focused our attention on SHP-2, a non-receptor type PTP that has been implicated in regulation of growth factor and cytokine signaling and in modulation of cell-substrate adhesion (Yu et al., 1998; MacGillivray et al., 2003). Importantly, SHP-2 itself undergoes phosphorylation on several key tyrosine residues including Y-580 and Y-542, events
that are known to modulate its catalytic and adaptor functions (Lu et al., 2001). As we reported previously (MacGillivray et al., 2003), IL-1β treatment of fibroblasts induces phosphorylation of SHP-2 on Y-542 (Fig. 4A). By comparison, exposure to PV also induced phosphorylation of SHP-2 on Y-542 including the fraction of SHP-2 localized to focal adhesions (Fig. 4B).

To provide more direct evidence for the role of SHP-2 in cellular responses to IL-1 and to define the mechanisms involved, we compared ERK activation in SHP-2 expressing cells with responses in cells deficient in SHP-2, obtained either by RNA interference (RNAi) in primary human gingival fibroblasts or by homologous recombination in murine embryonic fibroblasts. In primary human fibroblasts, depletion of SHP-2 by RNAi substantially diminished IL-1β (Fig. 4C) and PV (not illustrated)-induced ERK activation. Consistent with these observations, IL-1β-induced ERK activation was also substantially diminished in SHP-2-deficient murine embryonic fibroblasts derived from a SHP-2 exon 3-deficient mouse (SHP-2Δ) (Zhang et al., 2002) (Fig. 4D, SHP-2Δ, middle part). Additionally, in these cells, phosphorylated ERK failed to concentrate in focal complexes/adhesions in response to IL-1β treatment in sharp contrast to reconstituted cells expressing wild-type SHP-2 (Fig. 5A). Given that IL-1β increases SHP-2 phosphorylation on residue Y-542 (Fig. 4A), we reasoned that the mechanism of IL-1β-induced signaling leading to ERK activation might involve modulation of the adaptor function of SHP-2 in these events.

To provide direct evidence for the importance of phosphorylation of tyrosine 542 of SHP-2 in IL-1β...
induced ERK activation, we compared responses in three fibroblast cell lines: (i) SHP-2 murine embryonic fibroblasts, (ii) SHP-2 fibroblasts reconstituted with wild-type SHP-2 (SHP-2 wt), and (iii) SHP-2 fibroblasts expressing a SHP-2-Y542F mutant protein (SHP-2 Y542F). As illustrated in Figure 4D, IL-1β-induced ERK phosphorylation, although still detectable at early times (10 min) in SHP-2Δ and SHP-2 Y542F cells, was markedly diminished compared to SHP-2 wt cells indicating that phosphorylation of SHP-2 on tyrosine 542 is required for sustained activation of ERK in response to IL-1β.

SHP-2 is required for the maturation of the focal adhesions induced by IL-1

We also noted that the absence of functional SHP-2 resulted in alterations in focal adhesions, consistent with previous reports (Yu et al., 1998). Specifically, in murine embryonic fibroblasts expressing wild-type SHP-2 (SHP-2 wt), IL-1β promoted the maturation of focal complexes to focal adhesions (Fig. 5A, part f). In response to IL-1β, active ERK localized to focal adhesions (Fig. 5A, part b). By comparison, in SHP-2-deficient murine embryonic fibroblasts (SHP-2Δ), while
Fig. 4. SHP-2 phosphorylation at Tyrosine 542 is required for IL-1β to induce prolonged ERK activation. A: Human gingival fibroblasts grown on fibronectin were stimulated with IL-1β (20 min) or vehicle (control) as indicated. SHP-2 was purified by immunoprecipitation and associated proteins were separated by SDS–PAGE, transferred to nitrocellulose, and blotted with antibodies to phospho-SHP-2 Y542 and total SHP-2. Note that the ‘control beads’ sample represents proteins adherent to beads alone incubated with cell lysates (i.e., no SHP-2 primary antibody). B: Human gingival fibroblasts plated on fibronectin in normal growth medium for 16 h were incubated with collagen-coated beads for 20 min at 37°C to induce focal adhesions. Cells were treated with the vehicle (control) or with pervanadate (50 μM) and incubated at 37°C for 15 min. Focal adhesion-associated proteins were isolated from equivalent numbers of beads, separated by SDS–PAGE, and probed for phospho-SHP-2 Y542 and total SHP-2. C: Cells plated on fibronectin in normal growth medium for 16 h were transfected with SHP-2-siRNA (50 nM) or with control-siRNA (50 nM; control) for 72 h. Cells were treated with IL-1β (20 ng/ml) for 15 min. Lysates were separated by SDS–PAGE and immunoblotted for SHP-2, phospho-ERK, and total ERK. The densitometric data is presented graphically and represents mean ± SD of three independent experiments. Data was analyzed by ANOVA and * denotes significance at P < 0.05. D: Mutant SHP-2 murine embryonic fibroblasts (SHP-2Δ), or the same cells expressing the wild-type protein (SHP-2 wt) or mutant SHP-2Y542 (SHP-2 Y542F) were plated on fibronectin (10 μg/ml) in normal growth medium for 16 h. FAC-dependent ERK activation was induced by IL-1β for 10, 20, and 40 min. Lysates were separated by SDS–PAGE, transferred to nitrocellulose membranes and probed for phospho-ERK, phospho-SHP-2 Y542, and total-ERK1/2. Densitometric analysis of phospho-ERK blots was performed and normalized so that one represents the densitometry value of control samples. All experiments were performed four times with similar results.
paxillin-stained focal complexes were more numerous than in the wild-type cells, they were smaller, scattered throughout the cell, and failed to redistribute to the cell borders in response to IL-1β (Fig. 5B, part h). SHP-2wt murine embryonic fibroblasts spread in response to IL-1β as assessed by an increase in projected cell area whereas SHP-2Δ cells, although larger in surface area at baseline, failed to spread in response to IL-1β (Fig. 5B). Thus, SHP-2 appears to be involved in the maturation of focal adhesions and in cell spreading induced by IL-1β, an event that correlates with ERK activation.

We next explored the specific role of SHP-2 Y542 in the assembly of mature focal adhesions induced by IL-1β. SHP-2wt, SHP-2Δ, and SHP-2 Y542F cells were treated with vehicle or IL-1β and stained for paxillin, α-actinin, and α-smooth muscle actin (SMA). The incorporation of α-SMA into stress fibers is known to accelerate and stabilize focal adhesion maturation in myofibroblasts (Hinz et al., 2003). IL-1β induced cell polarization and directional elongation of focal adhesions (super-maturation) in SHP-2wt cells (Fig. 6B, part g). As shown above (Fig. 1A), IL-1β-induced maturation of focal adhesions correlated with the assembly of α-actinin into focal adhesions (Fig. 6A, part j), and with the formation of cell extensions containing α-SMA (Fig 6B, part a). Interestingly, in SHP-2Δ and SHP-2 Y542F cells, focal adhesions failed to become super-mature in response to IL-1β (Fig. 6A, parts h, i, k, and l). IL-1β treated SHP-2Δ and SHP-2 Y542F mutant fibroblasts also failed to form elongated cell extensions (Fig. 6B, parts b and c) and maintained a more symmetrical shape.

**Importance of the adaptor function of SHP-2 in signaling to the ERK pathway**

IL-1β activates the Ras-ERK pathway (Grumbles et al., 1997; O’Neill, 2000), but the proteins that integrate IL-1β signals with ERK activation at focal adhesions are undefined. To delineate the signaling pathway by reading the text and understanding the context.
consequences of IL-1β-induced SHP-2 phosphorylation on Y542 in the context of focal adhesions, we focused on the adaptor protein Gab1 because of its known association with SHP-2 and involvement in signaling pathways leading to ERK activation (Wong and Johnson, 1996; Ali, 2000; Shi et al., 2000; Cunnick et al., 2002). We first determined that treatment with IL-1β enhances SHP-2 interaction with the focal adhesion protein paxillin, and with the tyrosine phosphorylated adaptor protein Gab1, by co-immunoprecipitation (Fig. 7A). Immunoblotting of collagen bead-associated proteins demonstrated that IL-1β enhanced association of Gab1 with focal adhesions.

Fig. 6. SHP-2 phosphorylation at Y542 is required for IL-1β-induced maturation of focal adhesions. A, B: Mutant SHP-2 murine embryonic fibroblasts (SHP-2Δ) expressing the wild-type protein (SHP-2 wt) or mutant SHP-2Y542 (SHP-2 Y542F) were plated on fibronectin (10 μg/ml) in normal growth medium for 16 h, and treated with vehicle control or IL-1β (20 ng/ml, 40 min), fixed and immunostained for paxillin, α-actinin and α-smooth muscle actin. Scale bars represent 20 μm. Arrows in (A) parts g and j denote supermature focal adhesions defined by directional elongation of paxillin and α-actinin contained structures. Arrows (B) a denote cell extensions that contain α-smooth muscle actin (SMA).
TYROSINE PHOSPHATASES MODULATE IL-1 SIGNALING

A

 WB: SHP2

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WB: paxillin

IP: paxillin

WB: P-Gab1

WB: Gab1

WB: SHP2

IP: SHP2

B

IL-1 control

0 15 min

Focal adhesion proteins

WB: Gab1

C

SHP2 wt SHP2 Δ SHP2 Y542F

0 15 0 15 min IL-1

WB: Gab1

Fig. 7. IL-1 induces Gab1 recruitment to focal adhesions that is dependent on SHP-2 Y542 phosphorylation. A: Human gingival fibroblasts were plated on fibronectin in normal growth medium for 16 h. Cells were untreated or treated with IL-1β (20 ng/ml). Paxillin and SHP-2 were purified by immunoprecipitation as described (see Experimental Procedures). Resultant proteins were separated by SDS–PAGE, transferred to nitrocellulose and blotted with the indicated antibodies. Note that the sample labeled 'heads' represents precipitation of beads alone incubated with cell lysates (i.e., no SHP-2 primary antibody). B: Human gingival fibroblasts grown on fibronectin were stimulated with IL-1β for 15 min. Focal adhesion proteins were purified from collagen bead associated complexes and separated by SDS–PAGE, transferred to nitrocellulose and blotted with antibodies to Gab1. C: Mutant SHP-2 murine embryonic fibroblasts (SHP-2A) expressing the wild-type protein (SHP-2 wt) or mutant SHP-2 Y542F (SHP-2 Y542F) were stimulated with IL-1β (20 ng/ml, 15 min). Focal adhesion proteins were purified from collagen bead associated complexes and separated by SDS–PAGE, transferred to nitrocellulose and blotted with antibodies to Gab1. All experiments were performed four times with similar results.

(See Fig. 7B). Importantly, IL-1β-induced association of Gab1 with focal adhesions was abolished in SHP-2Δ and SHP-2 Y542F cells (Fig. 7C), indicating that SHP-2 and specifically phosphorylation of tyrosine 542 induced by IL-1β, regulates Gab1 recruitment to focal adhesions.

DISCUSSION

Our major finding is that PTP, and specifically SHP-2, play pivotal roles in IL-1β-induced maturation of focal adhesions in fibroblasts and consequently in modulating the signaling pathways leading to ERK activation. We have defined phosphorylation of Y542 of SHP-2 as a crucial event in IL-1β-induced recruitment of α-actinin to focal adhesions, an important step in focal adhesion maturation (Hinz et al., 2003). We have also shown a requirement for SHP-2-in IL-1β-induced association of focal adhesions with Gab1, an adaptor protein implicated in ERK activation (Cunnick et al., 2001, 2002; Ren et al., 2004). Our data indicate that Y542 of SHP-2 modulates the assembly and maturation of focal adhesions and the formation of cell extensions, possibly by increasing cytoskeletal tension at cell-matrix contacts. This regulatory system could mediate IL-1β-induced alterations of cellular interactions with connective tissue matrices in inflamed sites in vivo.

By immunofluorescence microscopy and biochemical isolation, we demonstrated that IL-1β and PV induce super-maturation of focal adhesions as defined by morphology and incorporation of α-actinin (Zaidel-Bar et al., 2003). The mechanism of this process is not defined but given the similarities between the cellular responses to IL-1β and to PTP inhibitors, alterations in focal complexes/adhesions may be related to direct modulation of the state of tyrosine phosphorylation of one or more structural proteins, such as paxillin (Turner; 2000; Laukkaitis et al., 2001), α-actinin (Lewis et al., 1996; Lin et al., 2004), signaling molecules such as FAK, Src, Csk (Webb et al., 2003, 2004), or integrin-associated protein (IAP; CD47)(Brown et al., 1990; Maile et al., 2003). Alternatively, super-maturation may be an indirect, downstream consequence of tyrosine phosphorylation-dependent events such as Rho-dependent alterations in the actin cytoskeleton (Schoenwaelder et al., 2000), or alterations of integrin affinity or avidity (Takagi and Saito, 1995), perhaps involving SHPS-1 (Manes et al., 1999; Inagaki et al., 2000).

We observed that treatment of cells with IL-1β enhanced phosphorylation of SHP-2 at Y542, implying activation of one (or more) tyrosine kinases responsible for direct phosphorylation of SHP-2. Currently the identity of these putative tyrosine kinase(s) is unknown but some hints are available from the literature. Since IL-1R1 has no intrinsic kinase activity, it is likely that other receptor or non-receptor tyrosine kinases are responsible for SHP-2 phosphorylation. The interleukin-1 receptor associated kinase (IRAK-1) associates with the IL-1 receptor and participates in transmission of downstream signals (Cao et al., 1996), including signal transduction through focal adhesions (MacGillivray et al., 2000). Nonetheless, IRAK-1 is a serine/threonine specific protein kinase and could not account for direct phosphorylation of SHP-2 on Y542. Importantly, SHP-2 can undergo auto-dephosphorylation (Lechleider et al., 1993; Feng, 1999); in our system, treatment with PV or other PTP inhibitors would be expected to prevent this reaction by inhibition of SHP-2 catalytic activity, thereby enhancing and prolonging tyrosine kinase-mediated phosphorylation. In view of the signal-enhancing effects of SHP-2 on the ERK pathway (Shi et al., 2000; MacGillivray et al., 2003), the prolonged SHP-2 phosphorylation observed in our experiments is consistent with the protracted and enhanced ERK activation observed in cells treated with IL-1β and PV.

Our studies highlight the adaptor function of SHP-2 in promoting prolonged ERK activation via a mechanism that may involve recruitment to focal adhesions of SHP-2 binding partners such as Gab1. Phosphorylation of Y542 on SHP-2 correlates with enhanced binding of intermediary molecules to SHP-2, indicating augmentation of its adaptor function (Bennett et al., 1994; Lu et al., 2001, 2003 Araki et al., 2003). Notably, these pathways...
are spatially localized to developing focal adhesions. The notion that tyrosine phosphorylation of SHP-2 triggers ERK activation is supported by observations in other systems. For example, in fibroblasts treated with fibroblast growth factor or platelet-derived growth factor, phosphorylation of Tyr-542 of SHP-2 leads to binding of Grb2 and facilitates ERK activation (Araki et al., 2003). Association of SHP-2 with Gab1 is also important in growth factor-induced ERK activation (Cunnick et al., 2002; Ren et al., 2004). In this context, SHP-2 is activated by interacting with juxta-membrane Gab1, leading to dissociation of Csk from the paxillin-Src complex, activation of Src, and downstream activation of ERK (Cunnick et al., 2001, 2002; Ren et al., 2004). Interaction of SHP-2 with Gab1 inhibits Ras-GAP binding, which in turn leads to Ras activation (Montagner et al., 2004). Although, we observed that IL-1β-induced phosphorylation of SHP-2 on Y542 is required for increased Gab1 association with focal adhesions, which likely promotes SHP-2 activation, further experiments are needed to determine if Gab1—SHP-2 interactions mediate IL-1-induced ERK activation.

Recent studies implicate ERK in modulation of focal adhesions during cell migration (Liu et al., 2002; Brahmbhatt and Klemke, 2003; Mitsushima et al., 2004; Webb et al., 2004). Notably, our immunofluorescence experiments showed recruitment of activated ERK to maturing focal adhesions. In previous studies, pharmacological inhibition of ERK prevented the association of paxillin with FAK that is required for spreading and adhesion induced by hepatocyte growth factor or platelet-derived growth factor receptor beta to Rac. Proc Natl Acad Sci USA 91(7):7335–7339. 


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