SHP-2 Modulates Interleukin-1-induced Ca\(^{2+}\) Flux and ERK Activation via Phosphorylation of Phospholipase C\(\gamma_1\)*

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Interleukin-1 (IL-1) signaling is dependent on focal adhesions, structures that are enriched with tyrosine kinases and phosphatases. Because the non-receptor tyrosine phosphatase Src homology 2 domain-containing protein tyrosine phosphatase-2 (SHP-2) is enriched in focal adhesions and IL-1-induced ERK activation requires increased Ca\(^{2+}\), we determined whether SHP-2 modulates IL-1-induced Ca\(^{2+}\) signaling. In SHP-2-deficient fibroblasts, IL-1-induced Ca\(^{2+}\) signaling and ERK activation were markedly diminished compared with cells expressing SHP-2. IL-1-induced Ca\(^{2+}\) release from the endoplasmic reticulum occurred in the vicinity of focal adhesions and was strongly inhibited by the blockade of phospholipase C (PLC) catalytic activity. Immunoprecipitation and immunostaining showed that SHP-2, the endoplasmic reticulum-specific protein calnexin, and PLC\(\gamma_1\) were associated with focal adhesions; however, these associations and IL-1-induced ERK activation dissipated after cells were plated on non-integrin substrates. IL-1-promoted phosphorylation of SHP-2 and PLC\(\gamma_1\). IL-1-induced phosphorylation of PLC\(\gamma_1\) was diminished in SHP-2-deficient cells but was restored by stable transfection with SHP-2. BAPTA/AM (1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis(acetoxyethyl ester)) blocked IL-1-induced phosphorylation of SHP-2 and PLC\(\gamma_1\), indicating mutually dependent interactive roles for Ca\(^{2+}\), SHP-2, and PLC\(\gamma_1\) in IL-1 signaling. We conclude that SHP-2 is critical for IL-1-induced phosphorylation of PLC\(\gamma_1\) and thereby enhances IL-1-induced Ca\(^{2+}\) release and ERK activation. Focal adhesions co-localizing with the endoplasmic reticulum may provide molecular staging sites required for ERK activation.

Interleukin-1 (IL-1)* is a crucially important, proinflammatory cytokine associated strongly with the destruction of extracellular matrices in several highly prevalent diseases, including rheumatoid arthritis, periodontitis, and cancer (1–4). IL-1 mediates the degradation of extracellular matrix proteins by enhancing the synthesis and secretion of proteases that are expressed by connective tissue cells including tissue macrophages and fibroblasts. The expression of matrix-degrading genes in these cells is regulated by the amplitude and duration of IL-1-induced signals (5, 6), particularly those signals that lead to the activation of mitogen-activated protein kinases (7, 8). Previous studies (9) of IL-1 signaling pathways in cultured fibroblasts have shown that the IL-1-induced activation of the mitogen-activated protein kinase ERK requires the formation of focal adhesions, suggesting that focal adhesion-associated proteins such as integrins and focal adhesion kinase (FAK) (10) may play critical, modulatory roles in the generation and regulation of IL-1-induced signals. Although IL-1 type 1 (signaling) receptors are confined largely to focal adhesions in cultured fibroblasts (10–12) and thus may be responsible for the focal adhesion restriction of IL-1 signaling, it is likely that other focal adhesion proteins with intrinsic capacities for post-translational modification also contribute to this phenomenon. Many signaling systems in cells involve protein phosphorylation, which in turn is controlled by the net relative activities of protein kinases and protein phosphatases. Notably, although tyrosine phosphatases may play a role in the termination of various receptor tyrosine kinase-induced signals, they often play positive roles in signaling as well. For example, several receptor tyrosine kinase-signaling pathways that lead to ERK activation utilize protein tyrosine phosphatases such as SHP-2 to help sustain ERK activity (13), possibly by modulating signals from integrins or integrin-associated proteins. SHP-2 also has an impact on IL-1-induced ERK activation, and this activation is tightly linked to integrin clustering in focal adhesions (11). Previous work (14) has established that SHP-2 is required for integrin-mediated ERK activation, possibly by Src family-related kinase phosphorylation of SHPS-1 and the recruitment of SHP-2 (13). Furthermore, SHP-2 regulates cell spreading on fibronectin and focal adhesion formation (15). However, the mechanisms and the protein-protein interactions that are required for integrins and SHP-2 to mediate the IL-1 activation of ERK are not defined.

Recent studies (16) have demonstrated that SHP-2 regulates dione; U-73343, 1-(6-[N\(\delta\)-tetraacetic acid tetrakis (acetoxymethyl ester).
the activation of Src family kinases, and ultimately the activation of ERK, by controlling the recruitment of C-terminal Src kinase. The activation of ERK through the Src family kinase system is thought to involve the phosphorylation of PLCγ1, a step in the signaling pathway required for the release of calcium from internal stores and the subsequent activation of the Ras-Raf-MEK-ERK (where MEK is mitogen-activated protein kinase/extracellular signal-regulated kinase kinase) cascade. Ras activation may in turn rely on its anchorage to endoplasmic reticulum membranes (17) as well as its recruitment of SHP-2 to plasma and endoplasmic reticulum membranes (16). However, the mechanisms whereby the various participants in this signaling pathway are marshaled to specific sites within the cell are not known. As noted earlier, IL-1-induced ERK activation is focal adhesion-restricted (9), requires the release of calcium from the endoplasmic reticulum (18), and may depend on the sequestration of signaling molecules such as Ras to endoplasmic reticulum membranes (17). Accordingly, we considered that focal adhesions and the endoplasmic reticulum may provide spatially discrete staging sites (19) that enable the SHP-2-dependent activation of ERK by IL-1.

EXPERIMENTAL PROCEDURES

Materials—Bovine fibronec, poly-l-lysine, BSA, sodium orthovanadate, mouse monoclonal antibodies to vinculin, and goat anti-rabbit antibodies coupled to fluorescein isothiocyanate were obtained from Sigma. Rabbit polyclonal antibodies to ERK1/2 and mouse monoclonal anti-phospho-ERK1/2 were purchased from New England Biolabs (Beverly, MA). Phospho-SHP-2 ( Tyr-542), PLCγ2, PLCγ1, and phospho-PLCγ1 (Tyr-783) antibodies were purchased from Cell Signaling (Beverly, MA). Rabbit polyclonal antibodies to SHP-2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal anti-calnexin was obtained from BD Biosciences. Horseradish peroxidase-conjugated goat anti-mouse and -anti-rabbit antibodies were obtained from Calbiochem. Fetal bovine serum (25) was purchased from Lipid Associates, Natick, MA. Human IL-1β was obtained from R&D Systems (Minneapolis, MN). Pansorbin A was purchased from Calbiochem. 2-Aminoethoxydiphenyl borate (2-APB), exostosin C, PLC inhibitor 1-6-[17β-3-methoxestra-1,3,5(10)trin-17-yl)amino]hexyl)-1H-pyrrole-2,5-dione (U-73122) and its inactive congeners 1,6-[17β-3-methoxestra-1,3,5(10)trin-17-yl)amino]hexyl)-1H-2,5-pyrrolidine-dione (U-73433) and swinholide A were obtained from Calbiochem.

Immunofluorescence—Chamber slides (8-well, Labtek) were coated with poly-l-lysine (100 μg/ml in PBS). Cells were plated for 4–6 h prior to incubation with fibronec-coated latex microbeads for 30 min at 37 °C. Cells were fixed, permeabilized with saponin, and incubated with 2 μM mag-fura-2/AM for 20 min at 37 °C, and re-isolated magnetically. The beads were washed in CSKB, sedimented with a microcentrifuge, resuspended in Laemmli sample buffer, and placed in a boiling water bath for 10 min to allow the collagen-associated complexes to dissociate from the beads. The beads were sedimented and lysates were collected for analysis.

Calcium Measurement—For measuring whole cell [Ca2+]i, cells on coverslips were loaded with 3 μM fura-2/AM for 20 min at 37 °C, and cells were measured by ratio fluorometry as described previously (23). For estimating the Ca2+ concentration near the plasma membrane (juxtamembrane concentration), cells attached to coverslips were briefly permeabilized with saponin and incubated with 2 μM fura-C18, pentapotassium salt at 25 °C for 20 min followed by washing with PBS (Ca2+-free) containing mag-fura-2/AM (4 μM) for 150 min at 37 °C in minimum essential medium containing 10% fetal bovine serum (25). The nominally calcium-free buffer consisted of a bicarbonate-free medium containing 150 mM NaCl, 5 mM KCl, 10 mM d-glucose, 1 mM MgSO4, 1 mM Na2HPO4, and 20 mM HEPES at pH 7.4, with an osmolality of 291. For experiments requiring external Ca2+, 2 mM CaCl2 was added to the buffer; for experiments requiring internal Ca2+, 1 mM EGTA was added to the incubation with fura-2/AM, an examination of the cells by fluorescence microscopy demonstrated no vesicular compartmentalization of fura-2, suggesting that the dye loading method permitted measurement of cytosolic Ca2+. A visual inspection of fura-C18-loaded cells and mag-fura-2-loaded cells showed fluorescent labeling of the plasma membrane and intracellular organelles, respectively. Whole cell and plasma membranes were isolated (22) from [(3H)H]ER-labeling (Beverly, MA). Rabbit polyclonal antibodies to SHP-2 were purchased from Cell Signaling Technology (Beverly, MA). Murine embryonic fibroblasts (SHP-2 (H9252), murine embryonic fibroblasts (H9262) were generated by transfection with wild-type murine SHP-2 and the same cells reconstituted with wild-type murine SHP-2. The activity of Src family kinases, and ultimately the activation of PLCγ1 (4), is thought to involve the phosphorylation of PLCγ1, a step in the signaling pathway required for the release of calcium from internal stores and the subsequent activation of the Ras-Raf-MEK-ERK (where MEK is mitogen-activated protein kinase/extracellular signal-regulated kinase kinase) cascade. Ras activation may in turn rely on its anchorage to endoplasmic reticulum membranes (17), as well as its recruitment of SHP-2 to plasma and endoplasmic reticulum membranes (16). However, the mechanisms whereby the various participants in this signaling pathway are marshaled to specific sites within the cell are not known. As noted earlier, IL-1-induced ERK activation is focal adhesion-restricted (9), requires the release of calcium from the endoplasmic reticulum (18), and may depend on the sequestration of signaling molecules such as Ras to endoplasmic reticulum membranes (17). Accordingly, we considered that focal adhesions and the endoplasmic reticulum may provide spatially discrete staging sites (19) that enable the SHP-2-dependent activation of ERK by IL-1.

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Immunoprecipitation—Cells at 80–90% confluence on 100-mm tissue culture dishes in normal growth medium were treated with IL-1 and washed three times in ice-cold PBS. Triton X-100 lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, 20 μg/ml leupeptin, 20 μg/ml aprotinin, 50 mM NaF, 10 mM sodium PY, 1 mM Na3VO4) was added to each well. The cell lysates were scraped into a microcentrifuge tube and rotated for 20 min at 4 °C. Pansorbin A was used to block nonspecific binding in the lysates (1 h at 4 °C), and after centrifugation for 15 min at 4 °C, the supernatants were transferred to new tubes. The lysates were incubated overnight with 50 μl of agaroose-conjugated rabbit polyclonal SHP-2 (5 μl of antibody, 50 μl of agaroose beads) at 4 °C on a rotating wheel. The pellet of beads was washed three times with Triton X-100 lysis buffer, resuspended in 2× SDS-PAGE sample buffer, and boiled for 10 min. Equal amounts of the eluted
proteins were analyzed by SDS-PAGE followed by immunoblotting using anti-SHP-2, anti-phospho-SHP-2, anti-PLCγ1, and anti-phospho-PLCγ1 antibodies.

**Short Interfering RNA**—Specific inhibition of SHP-2 was conducted with siRNA (sequence AG-CCCAAAAGAGUACAUUGGC-DTT, residues 1080–1100 of the SHP-2 sequence). Human gingival fibroblasts grown in 24-well plates were transfected with 100 nM of SHP-2 siRNA or GFP-siRNA (control) using Oligofectamine according to the manufacturer’s specifications. Gene silencing was conducted for 72 h. Cells were washed in PBS, lysed with Laemmli buffer, and immunoblotted to assess the efficacy of SHP-2 knockdown.

**Data Analysis**—Means and S.E. were calculated for [Ca$^{2+}$]i measures.

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**Fig. 1.** IL-1-induced Ca$^{2+}$ fluxes occur locally around fibronectin-coated beads. A, human gingival fibroblasts plated on poly-L-lysine were loaded with fura-2/AM, preincubated with fibronectin-coated (FN) or BSA-coated latex microbeads, and then treated with IL-1 (20 ng/ml). Intracellular [Ca$^{2+}$]i was measured in 5-μm zones around the periphery of attached beads (Beads) and in similarly sized, randomly chosen zones within the projected area of the cell but at least 10 μm from the attached beads (Remote). Single measurements of bead-associated and remote sites each from 5 cells were conducted for each group in each experiment. Bead-associated calcium increases were significantly larger than those in the remote sites (summary data are mean ± S.E. from n = 3–5 experiments; p < 0.001) for fibronectin- but not BSA-coated beads. B, fura-C18-loaded cells attached to poly-L-lysine were incubated with fibronectin- or BSA-coated beads and then stimulated with IL-1 (20 ng/ml). The fura-C18 ratio was measured by ratio fluorometry in zones as described in A. Summary data from n = 5 cells are shown from three experiments; for fibronectin-coated beads, the difference between beads versus remote sites is p < 0.001. C, mag-fura-2 ratio data are shown for the estimation of [Ca$^{2+}$]ER. Cells plated on poly-L-lysine were loaded with mag-fura-2/AM and preincubated with fibronectin- or BSA-coated beads. The mag-fura-2 ratio was measured as described in A after stimulation with IL-1 (20 ng/ml). Traces represent three independent experiments each with n = 3–5 cells. Summary data are mean ± S.E. Data show an increased [Ca$^{2+}$], or fura-C18 ratio above base line or a decreased mag-fura-2 ratio below base line (p < 0.001) in zones close to or remote from beads. D, human gingival fibroblasts plated on poly-L-lysine were incubated with fibronectin- or BSA-coated latex microbeads for 30 min at 37 °C and immunostained for calnexin. The microscope was focused on the dorsal surface of the cell to image bead-associated proteins. DIC, differential interference contrast.
FIG. 2. SHP-2 is required for IL-1-induced cytosolic Ca\textsuperscript{2+} flux, Ca\textsuperscript{2+} release from the ER, and ERK activation. A, reduced amplitude and elevated [Ca\textsuperscript{2+}]i, and Ca\textsuperscript{2+} release from the ER are shown in cells lacking functional SHP-2. HGFs or SHP-2 (mutant Δ46–110) murine embryonic fibroblasts stably transfected with wild-type SHP-2 (Rescue) and SHP-2 (mutant Δ46–110) murine embryonic fibroblasts (SHP-2/−/) were plated on fibronectin-coated coverslips and loaded with fura-2/AM or mag-fura-2/AM. The intracellular [Ca\textsuperscript{2+}]i and mag-fura-2 ratios were measured after treatment with IL-1 (20 ng/ml) (arrow). Traces are representative of at least 3 separate cells in three independent experiments. Summary data (mean ± S.E.) from 3 cells in each experiment and n = 4–6 experiments. Data show increased [Ca\textsuperscript{2+}]i above base line (p < 0.001) or decreased mag-fura-2 ratio below base line (p < 0.01) in HGFs, rescue and SHP-2/−/− cells. B, HGFs, rescue, and SHP-2/−/− cells were loaded with fura-2/AM and stimulated with 1 μM prostaglandin E\textsubscript{2} (PGE\textsubscript{2}) as control. Summary data (5 separate cells) show no significant difference in peak [Ca\textsuperscript{2+}]i, above base line (p > 0.2). C, reduced activation of ERK is shown in cells without functional SHP-2. HGFs, rescue, and SHP-2/−/− cells were plated overnight on fibronectin-coated dishes. IL-1 (20 ng/ml) was added to cells in the three left lanes. Vehicle was added to HGFs in the extreme right lane (−). Phospho-ERK (P-ERK) and ERK were evaluated by immunoblot analysis using specific antibodies against phospho-ERK and ERK. Right panels, rescued and SHP-2/−/− cells were plated on fibronectin (1 μg/ml) in normal growth medium for 16 h, treated with IL-1, and then immunostained with paxillin to show focal adhesions. D, human gingival fibroblasts were transfected with 100 nM SHP-2-siRNA or GFP-siRNA (control) for 72 h, as described under "Experimental Procedures," plated on fibronectin-coated coverslips in normal growth medium for 16 h, and then loaded with fura-2/AM or mag-fura-2/AM. Intracellular [Ca\textsuperscript{2+}]i, and mag-fura-2 ratios were measured after treatment with IL-1 (20 ng/ml). Traces are representative of 3 cells from three independent experiments. Summary data (mean ± S.E.) are 3 cells from each of n = 3 experiments. The data show an increased [Ca\textsuperscript{2+}]i, above base line (p < 0.01) or a decreased mag-fura-2 ratio below base line (p < 0.02) in HGFs.
ments, including the base line [Ca^{2+}], and the net change of [Ca^{2+}], above base line. For all continuous variables, means and S.E. were computed, and, when appropriate, comparisons were made between two groups with the paired Student’s t test or, for multiple samples, with analysis of variance. Post hoc testing was carried out with Tukey’s test. Statistical significance was set at \( p < 0.05 \). For all experiments, \( n \geq 3 \) independent cultures were used. For calcium measurements, only a single cell in each culture was measured.

RESULTS

IL-1-induced Ca^{2+} Influx and ER Release Occur Locally around Fibronectin-coated Beads—IL-1-induced Ca^{2+} signals are dependent on focal adhesions in cultured cells (9, 18), but the spatial relationships between focal adhesions and sites of IL-1-induced Ca^{2+} transients have not been described. We first determined whether IL-1-induced Ca^{2+} signaling is restricted to focal adhesion sites. Focal adhesions were induced by applying fibronectin-coated beads to the dorsal surfaces of cells plated on poly-L-lysine (i.e. with no focal adhesions on their ventral surfaces). Cells plated on poly-L-lysine were loaded with fura-2/AM (to measure cytosolic Ca^{2+}), fura-C18 (to measure juxtaembranous Ca^{2+}), or mag-fura-2/AM (to measure ER Ca^{2+}). Cells were then preincubated with fibronectin- or BSA-coated beads (2-μm diameter) and treated with IL-1. Ratios of [Ca^{2+}] (fura-C18), fura-C18, and mag-fura-2 were measured by ratio imaging for the whole cell in circumscribed 5-μm zones around the periphery of attached beads and in similarly sized, randomly chosen zones within the projected area of the cell but at least 10 μm from the attached beads (Fig. 1, A–C). IL-1-induced localized increases of intracellular Ca^{2+} (fura-2/AM), Ca^{2+} transients subjacent to the cell membrane (fura-C18), and Ca^{2+} release from the ER (mag-fura-2/AM), but these changes were found only when cells were incubated with fibronectin-coated beads and only in the vicinity of fibronectin beads. No significant changes in Ca^{2+} were detected at sites remote from the beads, and no evidence for IL-1-induced Ca^{2+} signaling was found in cells plated on poly-L-lysine and incubated with BSA-coated beads. Therefore, as shown previously (10, 12), integrin-dependent focal adhesion formation is essential for IL-1-induced Ca^{2+} signals. The novel aspect of the current observations is that Ca^{2+} fluxes induced by IL-1 originate from and are strictly localized to the vicinity of focal adhesions.

Because there was very tight co-localization of mag-fura-2 Ca^{2+} signals with fibronectin-coated beads, we asked whether the focal adhesions were co-distributed with the endoplasmic reticulum. Fibronectin or BSA-coated latex beads were added to the dorsal surfaces of cells attached previously to poly-L-lysine to induce focal adhesion assembly at sites of cell-bead contacts. Immunostaining for calnexin, an endoplasmic reticulum-specific protein, showed that within 30 min after adding fibronectin or BSA-beads, calnexin accumulated at sites adjacent to the fibronectin beads but not BSA beads (Fig. 1D), indicating that the endoplasmic reticulum is spatially associated with nascent focal adhesions. These experiments confirmed that the localized generation of focal adhesions on rounded cells was sufficient to restore responsiveness to IL-1 without inducing cell spreading (26) and showed that IL-1-induced Ca^{2+} release from the endoplasmic reticulum is spatially associated with nascent focal adhesions.

**SHP-2 Is Required for IL-1-induced Cytosolic Ca^{2+} Flux, ER Ca^{2+} Release, and ERK Activation**—A general role for protein tyrosine phosphorylation in the regulation of Ca^{2+} fluxes has been reported (27, 28), and we demonstrated earlier that the protein tyrosine phosphatase SHP-2 is enriched in the focal adhesions of fibroblasts and can modulate IL-1-induced ERK activation (20). Although several protein tyrosine phosphatases, including protein tyrosine phosphatase-a and low molecular weight protein tyrosine phosphatase, are known to associate with focal adhesions (20, 29–31), we focused on SHP-2, a non-receptor type of protein tyrosine phosphatase that has been implicated in the regulation of cytokine, growth factor, and integrin signaling (15, 20). Importantly, SHP-2 itself undergoes phosphorylation on several key tyrosine residues, including Tyr-580 and Tyr-542, which are modifications that are known to alter its catalytic and adaptor functions (20). We utilized a cell model system that allowed direct analysis of the role of SHP-2 in IL-1-induced Ca^{2+} fluxes and ERK activation through focal adhesions. SHP-2−/− (Δ46–110, designated here as SHP-2−/−) murine embryonic fibroblasts cells express a SHP-2 protein with a deletion of 65 amino acids in the N-terminal SH2 domain (21), which prohibits receptor-mediated ERK activation in several different signaling systems (32). These cells have also been stably transfected with wild-type SHP-2 to reconstitute SHP-2 function (designated as rescue). In a comparison with human gingival fibroblasts (HGFs) or with cells rescued with stably transfected wild-type SHP-2, we found that IL-1-induced cytosolic Ca^{2+} flux, Ca^{2+} release from the endoplasmic reticulum, and ERK activation were reduced in the SHP-2−/− fibroblasts (Fig. 2, A and B). Moreover, as assessed by paxillin staining, the SHP-2−/− cells did not exhibit maturation of focal adhesions (e.g. elongation and condensation) in response to IL-1 (Fig. 2C). Furthermore, in SHP-2−/− cells, phosphorylated ERK did not concentrate in focal complexes/adhesions in response to IL-1 (data not shown). Collectively, these observations provide direct evidence that SHP-2 is involved in the IL-1-induced Ca^{2+} signaling that leads to ERK activation.

We compared IL-1-induced cytosolic Ca^{2+} flux, Ca^{2+} release from the endoplasmic reticulum, and ERK activation in human gingival fibroblasts transfected with irrelevant siRNA (to GFP) and in the same cells transfected with siRNA to SHP-2. Consistent with the observations for the SHP-2 embryonic fibroblasts, the knockdown of SHP-2 by siRNA (as confirmed by immunoblotting; Fig. 2E) substantially diminished IL-1-induced cytosolic Ca^{2+} flux, Ca^{2+} release from the endoplasmic reticulum, and ERK activation (Fig. 2, D and E). These observations provide direct evidence that SHP-2 is involved in the regulation of IL-1-induced Ca^{2+} signaling and ERK activation in gingival fibroblasts.

**Involvement of InsP3 Receptor and PLC in IL-1-induced Ca^{2+} Fluxes**—The cell-permeant InsP3 receptor antagonists 2-APB and xestospongin C abrogated IL-1-induced Ca^{2+} release in human gingival fibroblasts transfected with irrelevant siRNA and zeste-spongin C, which are thought to interfere with the function of InsP3 receptors located on the endoplasmic reticulum, were used to ascertain whether IL-1-induced Ca^{2+} release was dependent on InsP3 receptors. To minimize cell-rounding effects that might have an impact on calcium signaling, the regular Ca^{2+}-containing buffer (2 mM Ca^{2+}) was changed to Ca^{2+}-free buffer immediately prior to adding 2-APB (75 μM) or xestospongin C (100 μM). Pretreatment of cells with 2-APB or xestospongin C abrogated IL-1-induced Ca^{2+} transients in human gingival fibroblasts and SHP-2 rescue cells (Fig. 3, A and B, \( n = 4–6 \)), indicating that IL-1-induced Ca^{2+} release is
dependent on functional InsP₃ receptors on intracellular stores. Because these data and IL-1-induced Ca²⁺ release from the endoplasmic reticulum suggested the potential involvement of PLC in IL-1-induced Ca²⁺ mobilization, we examined the impact of the PLC antagonist U-73122 on IL-1-induced Ca²⁺ fluxes. U-73122 totally blocked IL-1-induced intracellular Ca²⁺ fluxes, whereas the inactive analog (U-73343) had almost no effect (Fig. 3, C and D). These results indicated that IL-1-induced Ca²⁺ release from endoplasmic reticulum stores is a PLC-mediated and IP₃-dependent process.

SHP-2 Regulation of IL-1 Signaling

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**FIG. 3.** The InsP₃ receptor antagonists 2-APB and xestospongin C reduce IL-1-evoked Ca²⁺ fluxes. A, experiments were conducted in cells that had been rapidly switched from full medium to Ca²⁺-free medium. HGFs, SHP-2-reconstituted murine embryonic fibroblasts (Rescue) and SHP-2 (mutant Δ46–110) murine embryonic fibroblasts (SHP-2⁻/⁻) were preincubated for 30 min at 25 °C in the presence of either vehicle (Control), 75 μM 2-APB, or 100 μM xestospongin C. Cells were stimulated with IL-1 (20 ng/ml) (arrow). [Ca²⁺], was measured in fura-2-loaded cells. B, summary data (mean ± S.E.) are shown from single cells obtained in n = 4–6 separate experiments. Data show increased [Ca²⁺] above base line after stimulation with IL-1 in HGFs and rescue cells (p < 0.02) but not in SHP-2⁻/⁻ cells (p > 0.2). Pretreatment with 2-APB and xestospongin blocked IL-1-induced calcium release. C, the PLC antagonist U-73122 inhibited IL-1-induced Ca²⁺ fluxes. Cells were pretreated for 10 min at 25 °C with vehicle (control), U-73122 (3 μM), or the inactive U-73122 analog U-73343 (3 μM), respectively, and then stimulated with IL-1 (20 ng/ml). [Ca²⁺], was measured in fura-2-loaded cells. D, summary data (mean ± S.E.) are shown from 3 cells in each of n = 4–6 experiments. The data show increased [Ca²⁺] above base line in HGFs and rescue cells after stimulation with IL-1 (p < 0.01) but not in SHP-2⁻/⁻ cells or cells treated with U-73122.
relationships among SHP-2, the endoplasmic reticulum, and PLCγ1 with a model system described previously (20, 33) that assesses proteins isolated from focal adhesions. Immunoblotting collagen bead-associated proteins showed that vinculin, SHP-2, the endoplasmic reticulum-specific protein calnexin, and PLCγ1 were recruited to sites of bead contact with cells (Fig. 4A). We ensured that the collagen-coated beads were able to specifically recruit focal adhesion proteins by repeating the experiment with cells that had been pretreated with swinholide A (50 nM). This toxin severs actin filaments and prevents actin polymerization, thereby blocking the formation of focal adhesions (34). Under these conditions, much less vinculin, SHP-2, calnexin, and PLCγ1 were recruited to the beads (Fig. 4A). BSA-coated beads (Fig. 4A) and poly-L-lysine-coated beads (data not shown) also failed to recruit significant amounts of these proteins, indicating a collagen receptor specificity. These immunoblotting data were consistent with immunofluorescence studies of SHP-2, calnexin, and PLCγ1 showing that these...
proteins co-localized with authentic focal adhesions around fibronectin-coated latex beads on dorsal cell surfaces (Fig. 4B).

**SHP-2 and PLCγ1 Are Phosphorylated in Response to IL-1—**

SHP-2 immunoprecipitates were prepared from whole cell lysates (Fig. 4C) or from proteins isolated from focal adhesions (Fig. 4D) obtained from cells treated with vehicle (control) or IL-1. Immunoprecipitates were immunoblotted with antibodies to phospho-SHP-2, total SHP-2, phospho-PLCγ1, or total PLCγ1. Purification of SHP-2 by direct immunoprecipitation from either cell lysates or isolated focal adhesions showed that PLCγ1 was present in the immune complexes, indicating a close association with SHP-2. These experiments also demonstrated that SHP-2 and PLCγ1 are phosphorylated in response to IL-1 and that a significant fraction of these modifications occurs in protein complexes associated with focal adhesions.

**Defective IL-1-induced Phosphorylation of PLCγ1 in SHP-2-deficient Cells**—To determine whether IL-1-induced phosphorylation of PLCγ1 required SHP-2 catalytic function, HGfs, SHP-2-reconstituted murine embryonic fibroblasts (rescue), and SHP-2−/− cells were stimulated with IL-1 or vehicle (control). Cell lysates were immunoblotted with antibodies against phospho-SHP-2, total SHP-2, phospho-PLCγ1, and total PLCγ1 (Fig. 5). IL-1 promoted phosphorylation of SHP-2 and PLCγ1 in both human gingival fibroblasts and SHP-2 rescue fibroblasts. In contrast, phosphorylation of SHP-2 and PLCγ1 was markedly decreased in the SHP-2−/− cells. Thus, in IL-1 signaling, SHP-2 is required for IL-1-induced phosphorylation of PLCγ1. We also asked whether the PLC catalytic function would have an impact on IL-1-induced phosphorylation in gingival fibroblasts. U-73122 totally blocked IL-1-induced phosphorylation of PLCγ1, whereas the inactive analog (U-73343) had no effect (Fig. 6A). These results indicated that IL-1-induced phosphorylation of PLCγ1 requires PLC catalytic function.

**Role of Ca2+ in IL-1-induced Phosphorylation of SHP-2 and ERK**—Previous data have shown that ERK phosphorylation in response to IL-1 requires calcium signaling (9). When we challenged intracellular Ca2+ by loading cells with BAPTA/AM prior to IL-1 treatment, the phosphorylation of SHP-2 and ERK was blocked (Fig. 6B), suggesting a role for intracellular Ca2+ in regulating SHP-2 and ERK functions. This result was not caused by cell shrinkage or the loss of cell adhesions because immunostaining showed no loss of paxillin staining in focal adhesions or a change of projected cell area (data not shown). We also considered that ionophore-induced increases of [Ca2+]i might be able to replicate increases of phosphorylation of

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**FIG. 5. Decreased IL-1-induced phospho-PLCγ1 in SHP-2-deficient cells.** HGfs, SHP-2-reconstituted murine embryonic fibroblasts (Rescue), and SHP-2−/− murine embryonic fibroblasts (SHP-2−/−) were plated on fibronectin (1 μg/ml) overnight. Cells were treated with either PBS (vehicle control) (−) or IL-1 (20 ng/ml) (+) at 37 °C for 20 min. Lysates were separated by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted for phospho-SHP-2 (P-SHP-2), total SHP-2, phospho-PLCγ1 (P-PLCγ1), and total PLCγ1. Each experiment was repeated at least three times. The proportions below each blot indicate the blot density compared with the maximum blot density in each group. Note that the very faint band in lane 6 in the top row and lane 5 in the second row of the SHP-2−/− cells represents the mutant, non-functional protein (mutant Δ46−110).

**FIG. 6. Roles of PLC and Ca2+ in IL-1 signaling.** A, the role of PLC catalytic activity in IL-1-induced phosphorylation of PLCγ1 is shown. Human gingival fibroblasts were pretreated for 10 min at 25 °C with Control (U-73122 3 μM), the inactive U-73122 analog U-73343 (3 μM), respectively, and then stimulated with IL-1 (20 ng/ml) (+). Cell lysates were immunoblotted for phospho-PLCγ1 (P-PLCγ1) and total PLCγ1. The proportions below each blot indicate the blot density compared with the maximum blot density in each group. B, the role of Ca2+ in IL-1-induced phosphorylation of SHP-2 and ERK is shown. Human gingival fibroblasts were preincubated in normal Ca2+-free medium with 5 μM BAPTA/AM for 20 min or in 2 μM ionomycin for 15 min before the addition of IL-1 (20 ng/ml) (+). Phosphorylation of SHP-2 and ERK was analyzed using phospho-SHP-2 and phospho-ERK antibodies. Each experiment was repeated at least three times.

SHP-2 and ERK, independently of IL-1. However, the treatment of cells with the calcium ionophore ionomycin in the absence of IL-1 was unable to replicate IL-1-induced SHP-2 phosphorylation, indicating that Ca2+ entry alone was not sufficient to promote SHP-2 signals without other critical elements in the signaling pathway mediated by IL-1.

**DISCUSSION**

The major finding of this study is that the close approximation of focal adhesions with the endoplasmic reticulum combined with the association of SHP-2 with PLCγ1 to focal adhesions is an important determinant of IL-1-induced Ca2+ release and ERK activation. Because Ras signaling to ERK involves the sequestration of Ras to endoplasmic reticulum membranes (17), the co-localization of focal adhesions with the endoplasmic reticulum provides an attractive mechanism for optimizing signal transduction. We suggest that the spatial approximation of these organelles creates sites at which the multiple molecules required for IL-1 signal transduction are appropriately marshaled in time and space. These molecular staging sites facilitate an efficient conversion of upstream IL-1 signals into the activation of the protein kinase pathways that lead ultimately to ERK activation. Protein kinases and phosphatases do not interact with their substrates by simple diffusion but are targeted frequently to specific subcellular sites that facilitate effective molecular interactions (19). For complex signaling systems like IL-1, multiple protein interactions are involved that may require the anchorage of several signaling proteins to specific organelles or organelar membranes. We hypothesize that a specific cellular property for effective signal transduction is the spatial approximation of organelles that brings together groups of potentially interacting molecules. The focal adhesion restriction of IL-1 signaling in fibroblasts allows the examination of this hypothesis.

Focal adhesions are enriched with the IL-1 signaling recep-
SHP-2 Regulation of IL-1 Signaling

SHP-2, a protein tyrosine phosphatase that is enriched in focal adhesions (20), associates with the IL-1 signaling receptor (36), regulates Ras and ERK activation (16) and cell adhesion (15), and is thought to integrate signals among discrete cytoplasmic pathways (30). An important novel finding reported here is that SHP-2, by virtue of its interactions with PLCγ1 and its enrichment in focal adhesions, may provide an essential adaptor function by which calcium signaling is mediated. Indeed, in cells treated with siRNA specific to SHP-2 or in cells devoid of normal SHP-2 expression, we found that the IL-1-induced calcium signal was not generated. Because IL-1-induced calcium fluxes are required for ERK activation (9), SHP-2 may restrict IL-1 signaling at the level of the generation of the calcium signal.

We sought to analyze the possibility of a more direct connection between SHP-2 and the IL-1-induced calcium signal. As expected, we found that IL-1-induced calcium release required PLC function (by blocking experiments with U-73122). In SHP-2 immunoprecipitates prepared from focal adhesion-associated proteins, there were significant amounts of PLCγ1 immunoprecipitates prepared from focal adhesion-associated proteins, there were significant amounts of PLCγ1 that were constitutively present. Notably, IL-1 also strongly increased the phosphorylation of PLCγ1 in these preparations. Recently, the phosphorylation of PLCγ1 in whole cell lysates has been shown to be an important step in both fibroblast and epidermal growth factor-stimulated activation of Ras/ERK (16). Our finding of PLCγ1 phosphorylation in focal adhesions and its functional linkage to the release of calcium from the endoplasmic reticulum provides important information on the discrete subcellular localization of this process in IL-1 signaling.

We found that SHP-2 was essential for mediating the phosphorylation of PLCγ1 because this process did not occur in cells devoid of functional SHP-2. As reported recently (16), SHP-2 may activate Src family kinases by dephosphorylating their inhibitory tyrosines; this process may enhance the phosphorylation of PLCγ1 and then later the release of calcium. The deletion of SHP-2 by siRNA or by homologous recombination showed that SHP-2 is indeed required for both processes because IL-1-induced PLCγ1 phosphorylation and calcium release were almost completely blocked. We also tested whether calcium release, which is downstream of IL-1-induced SHP-2 and PLC phosphorylation, might affect these events, possibly by a negative feedback system involving the formation and functional attributes of focal adhesions (18). This notion was examined in cells that had been preincubated with BAPTA/AM to reduce IL-1-induced alterations of [Ca2+]i. Under these conditions, IL-1 was unable to induce increased phosphorylation of SHP-2 or PLCγ1. Thus, whereas the alterations in [Ca2+]i are clearly downstream of PLCγ1, Ca2+ can also control steps that are evidently upstream of both SHP-2 and PLCγ1 phosphorylation. The locus of this control is not known, but it is not likely controlled by a feedback system of the structure of focal adhesions (which were morphologically normal by Paxillin staining). Instead, the upstream regulatory role of calcium could be attributed to a feedback system in which the failure to generate early IL-1-induced calcium signals (i.e., within 30 s of IL-1 treatment (18)) does not promote the approximation of focal adhesions and endoplasmic reticulum, which we suggest is critical for optimizing the protein kinase cascades that lead to SHP-2, PLCγ1, and ultimately ERK activation.

A previous report (36) showed that SHP-2 is not required for IL-1α-induced ERK activation. Different observations between that study and those reported here may be related to differences in the use of the IL-1 isoforms (i.e., α versus β, used here) and the result in the previous study showing that the level of phosphorylated ERK in untreated control cells was very high. Conceivably, it may have been difficult to induce any further increase of ERK phosphorylation after IL-1 treatment. We are confident however that SHP-2 is indeed required for IL-1-induced phosphorylation of ERK, because we also found a consistent block of IL-1-induced ERK activation in human gingival fibroblasts treated with siRNA for SHP-2.

In conclusion, SHP-2 and its enrichment in focal adhesions provide critical regulatory functions that are needed for IL-1 signal generation. Our finding that focal adhesions co-distribute with the endoplasmic reticulum is consistent with an earlier report showing that integrin-based adhesion complexes sequester the endoplasmic reticulum proteins kinectin, Rap, and calreticulin (37). Accordingly, we suggest that SHP-2-enriched focal adhesions and their relationship with the endoplasmic reticulum create spatially discrete staging sites that facilitate activation of ERK by IL-1.

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SHP-2 Modulates Interleukin-1-induced Ca\(^{2+}\) Flux and ERK Activation via Phosphorylation of Phospholipase C \(\gamma_1\)

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