Phosphorylation of SHP-2 Regulates Interactions between the Endoplasmic Reticulum and Focal Adhesions to Restrict Interleukin-1-induced Ca\textsuperscript{2+} Signaling*

Received for publication, July 5, 2006 Published, JBC Papers in Press, August 11, 2006, DOI 10.1074/jbc.M606392200

Qin Wang\textsuperscript{1}, Maria Teresa Herrera Abreu\textsuperscript{1,2}, Katherine Siminovitch\textsuperscript{1}, Gregory P. Downey\textsuperscript{1,\dagger,§}, and Christopher A. McCulloch\textsuperscript{1,2}

From the \textsuperscript{1}Canadian Institutes of Health Research Group in Matrix Dynamics, University of Toronto, Toronto, Ontario M5S 3E2, the \textsuperscript{2}Division of Respirology, Department of Medicine, University of Toronto, Toronto, Ontario M5G 2M9, the \textsuperscript{3}Samuel Lunenfeld Institute, Mount Sinai Hospital, Toronto, Ontario M5G 1X5, and the \textsuperscript{4}Toronto General Hospital Research Institute, University Health Network, Toronto, Ontario M5G 2M9, Canada

Interleukin-1 (IL-1)-induced Ca\textsuperscript{2+} signaling in fibroblasts is constrained by focal adhesions. This process involves the protein-tyrosine phosphatase SHP-2, which is critical for IL-1-induced phosphorylation of phospholipase C\textgamma, thereby enhancing IL-1-induced Ca\textsuperscript{2+} release and ERK activation. Currently, the mechanisms by which SHP-2 modulates Ca\textsuperscript{2+} release from the endoplasmic reticulum are not defined. We used immunoprecipitation and fluorescence protein-tagged SHP-2 or endoplasmic reticulum (ER)-protein expression vectors, and an ER-specific calcium indicator, to examine the functional relationships between SHP-2, focal adhesions, and IL-1-induced Ca\textsuperscript{2+} release from the ER. By total internal reflection fluorescence microscopy to image subplasma membrane compartments, SHP-2 co-localized with the ER-associated proteins calnexin and calreticulin at sites of focal adhesion formation in fibroblasts. IL-1\textbeta promoted time-dependent recruitment of SHP-2 and ER proteins to focal adhesions; this process was blocked in cells treated with small interfering RNA for SHP-2 and in cells expressing a Y542F SHP-2 mutant. IL-1-stimulated inositol 1,4,5-trisphosphate receptor-mediated Ca\textsuperscript{2+} release from the ER subjacent to the plasma membrane that was tightly localized around fibronectin-coated beads and was reduced 4-fold in cells expressing Tyr-542 SHP-2 mutant. In subcellular fractions enriched for ER proteins, immunoprecipitation demonstrated that IL-1-enhanced association of SHP-2 with the type 1 inositol 1,4,5-trisphosphate receptor was dependent on Tyr-542 of SHP-2. We conclude that Tyr-542 of SHP-2 modulates IL-1-induced Ca\textsuperscript{2+} signals and association of the ER with focal adhesions.

IL-1\textbeta\textsuperscript{3} is a key pro-inflammatory cytokine that mediates degradation of the extracellular matrix in several common dis-eases, including rheumatoid arthritis, pulmonary fibrosis, periodontitis, and cancer (1–4). IL-1 enhances destruction of extracellular matrices by promoting increased expression of proteases from 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) and most notably of signals that activate mitogen-activated protein kinases (7, 8). In cultured fibroblasts IL-1-induced activation of the mitogen-activated protein kinase ERK is dependent on maturation of focal adhesions (9). Conceivably, focal adhesion-associated proteins such as integrins and the focal adhesion kinase (10) may modulate the generation of IL-1-induced signals. Although IL-1 type 1 (signaling) receptors are restricted largely to focal adhesions in cultured fibroblasts (10–12) and thus may account for the dependence of IL-1 signaling on these adhesive domains, other proteins associated with focal adhesions that are post-translationally modified may also contribute to this phenomenon.

Many signaling systems in cells utilize protein phosphorylation, a post-translational modification that is controlled in turn by the net relative activities of protein-tyrosine kinases and protein-tyrosine phosphatases. Although protein-tyrosine phosphatases can terminate various receptor tyrosine kinase-induced signals, they can also activate signaling cascades. Protein-tyrosine phosphatases such as SHP-2 form part of the signaling cascade triggered in various protein kinase-linked receptors that lead to ERK activation. For example, SHP-2 helps to sustain ERK activity in response to platelet-derived growth factor (13), possibly by modulating signals arising from integrin-associated proteins (14). SHP-2 can also enhance IL-1-induced ERK activation; this process is tightly linked to integrin clustering in focal adhesions (15, 16) and is mediated possibly by Src family-related kinase phosphorylation of SHPS-1 and recruitment of SHP-2 (13), or by interaction of SHP-2 with focal adhesion proteins such as Paxillin and Gab1 (16). Further, SHP-2 regulates cell spreading on fibronectin, focal adhesion formation (17), and RhoA activity (18). Currently, the mechanisms and the protein-protein interactions that are required for

\*This work was supported in part by operating grants from the Canadian Institutes of Health Research (CIHR) (to C. A. M. and G. P. D.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

\textsuperscript{1}Recipient of a Tier 1 Canada Research Chair.

\textsuperscript{2}To whom correspondence should be addressed: CIHR Group in Matrix Dynamics, University of Toronto, Rm. 244, Fitzgerald Bldg., 150 College St., Toronto M5S 3E2, Ontario, Canada. Tel.: 416-978-1258; Fax: 416-978-5956; E-mail: christopher.mcculloch@utoronto.ca

\textsuperscript{3}The abbreviations used are: IL-1, interleukin-1; IP\textsubscript{3}, inositol 1,4,5-trisphosphate; IP\textsubscript{3}, receptor; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate; 2-APB, 2-aminoethoxydiphenylborate; ERK, extracellular signal-regulated kinase; ER, endoplasmic reticulum; BSA, bovine serum albumin; siRNA, small interfering RNA; PBS, phosphate-buffered saline; GFP, green fluorescent protein; YFP, yellow fluorescent protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TIRF, total internal reflection fluorescence.
IL-1 Signaling through the Endoplasmic Reticulum

integrins and SHP-2 to mediate IL-1 activation of ERK are not defined.

SHP-2 regulates the activity of Src family kinases, and ultimately the activation of ERK, by controlling the recruitment of Csk (14). The activation of ERK through Src family kinases is thought to involve phosphorylation of phospholipase Cγ1, a step in the signaling pathway that is required for calcium release from internal stores and the subsequent activation of the Ras-Raf-Mek-ERK cascade (14). Ras activation may in turn rely on its anchorage to ER membranes (19) as well as recruitment of SHP-2 to plasma and ER membranes (14). Currently, the mechanisms by which the various participants in this signaling pathway are marshaled to specific sites within the cell are not known.

For many complex signaling systems, including IL-1, anchorage of signaling proteins to specific organelles or organelar membranes is required for effective signal transduction (20). Because IL-1-induced ERK activation requires the release of Ca^{2+} from the ER (21), we considered that focal adhesions and the ER may provide spatially discrete staging sites (22), enabling SHP-2-dependent activation of ERK by IL-1. Notably, the ER proteins kinectin (23) and the IP_3 receptor (24) co-localize with focal adhesions. We have shown earlier that the ER-associated protein calnexin co-localizes with focal adhesions (25) and that SHP-2 is critical for IL-1-induced focal adhesion maturation (16), in part by regulating phosphorylation of phospholipase Cγ1. Accordingly, we sought to determine which molecular determinants of SHP-2 are responsible for mediating IL-1 promotion of interactions between focal adhesions and the ER.

EXPERIMENTAL PROCEDURES

**Materials**—Fibronectin, poly-l-lysine, BSA, puromycin, antisomycin, and mouse monoclonal antibodies to vinculin were obtained from Sigma. Rabbit polyclonal and mouse monoclonal anti-SHP-2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antibody to phospho-SHP-2 (tyrosine 542) was from Cell Signaling (Beverly, MA). Mouse monoclonal anti-calnexin and anti-Bip/GRP78 were obtained from BD Biosciences (Mississauga, Ontario, Canada). Rabbit anti-calnexin was obtained from Stressgen (Victoria, British Columbia, Canada). Rabbit polyclonal anti-IP_3R-1 was obtained from Affinity BioReagents (Golden, CO). Goat anti-integrin α5β1 was purchased from Chemicon (Temecula, CA). FuGENE 6 transfection reagent and X-tremeGENE siRNA tranfection reagent was purchased from Roche Applied Science. Acidified bovine type I collagen (Invitrogen) was purchased from Cohesion Technologies Inc. (Palo Alto, CA). Recombinant human IL-1β was obtained from R&D Systems (Minneapolis, MN). Fluoro-4/AM and mag-fluo-4/AM were obtained from Molecular Probes (Eugene, OR). 2-APB, xestospongin C, and swinholide A were obtained from Calbiochem.

**Cell Culture and Bead Preparations**—Human gingival fibroblasts were grown in minimal essential medium containing 10% fetal bovine serum. Rat2 cells were maintained in Dulbecco’s modified Eagle’s medium containing 5% fetal bovine serum and antibiotics (100 units/ml penicillin, 100 units/ml streptomycin, and 2 μg/ml puromycin). Cells were used between the 5th and 12th passages as previously described (25, 26). Latex microbeads were coated with fibronectin (10 μg/ml) or BSA (2 mg/ml). For collagen coating, 3 mg/ml acidified bovine type I collagen (Celltrix, Palo Alto, CA) was used. NaOH was added to the solution to a final concentration of 0.1 M to equilibrate the pH to 7.4 and thereby facilitate collagen fibril assembly on the beads. Bead suspensions were incubated at 37 °C for 20 min. Beads were washed three times, re-suspended in PBS, and sonicated for 10 s (output setting 3 and power 15%, Branson).

**Isolation of Focal Adhesions**—Cells were grown to 80–90% confluence on 60-mm tissue culture dishes and cooled to 4 °C prior to the addition of collagen-, BSA-, or anti-integrin α5β1-coated magnetite beads. Focal adhesion complexes were isolated from cells after specific incubation time periods as described previously (27). In brief, cells were washed three times with ice-cold PBS to remove unbound beads and scraped into ice-cold cytoskeleton extraction buffer (CSKB, 0.5% Triton X-100, 50 mM NaCl, 300 mM sucrose, 3 mM MgCl_2, 20 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM phenylmethylsulfonyl fluoride, 10 mM PIPES, pH 6.8). The cell-bead suspension was sonicated for 10 s (output setting 3 and power 15%, Branson), and the beads were isolated from the lysate using a magnetic separation stand. The left lysates were used for non-focal adhesion fraction. The beads were re-suspended in fresh ice-cold CSKB, homogenized with a Dounce homogenizer (20 strokes), and re-isolated magnetically. The beads were washed in CSKB, sedimented with a microcentrifuge, re-suspended in Laemmli sample buffer, and placed in a boiling water bath for 5 min to allow the collagen-associated complexes to dissociate from the beads. The beads were sedimented, and lysates were collected for analysis.

**Subcellular Fractionation**—Cells were harvested, resuspended in an ice isotonic buffer (10 mM Tris, pH 7.6, 100 mM CaCl_2, 200 mM sucrose), and disrupted by Dounce homogenization followed by 20 strokes. The homogenate was spun at 800 × g for 10 min, and the supernatant was recovered and further centrifuged for 10 min at 8,000 × g. The resulting supernatant was further centrifuged for 1.5 h at 28,000 × g. The resulting pellet constituted the microsomal ER fraction (28). The authenticity of the ER fraction was confirmed by immunoblotting for the ER-specific protein calnexin.

**Immunoblotting**—The protein concentrations of cell lysates were determined by Bradford assay (Bio-Rad, Hercules, CA). Equal amounts of protein were loaded onto SDS-polyacrylamide gels (10% acrylamide), resolved by electrophoresis, and transferred to nitrocellulose membranes. Membranes were incubated for 1 h at room temperature in Tris-buffered saline solution with 5% milk or 0.2% BSA to block nonspecific binding sites. Membranes were incubated with the primary antibodies overnight at 4 °C in Tris-buffered saline with 0.1% Tween 20. Horseradish peroxidase secondary antibodies were incubated for 1 h at room temperature in Tris-buffered saline with 0.1% Tween 20 and 5% milk or 0.2% BSA. Labeled proteins were
IL-1 Signaling through the Endoplasmic Reticulum

visualized by chemiluminescence as per the manufacturer’s instructions (Amersham Biosciences).

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 apro tinin, 50 mM NaF, 10 mM NaPp, 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 with 50 μl agarose-conjugated rabbit polyclonal SHP-2 (5 μl of antibody/50 μl of agarose beads) overnight at 4 °C on a rotating wheel. The pellet of beads was washed three times with Triton X-100 lysis buffer, re-suspended 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.

Before immunoprecipitation, ER fractions were solubilized in 1% CHAPS-containing buffer (5 mM sodium phosphate, pH 7.4, 2.5 mM EDTA, 100 mM NaCl, 1 mM NaF, 1 mM sodium orthovanadate) and incubated with anti-SHP-2 antibody bound to protein A/G-agarose beads (Santa Cruz Biotechnology). Protein complexes were separated by SDS-PAGE, transferred onto nitrocellulose membranes, and probed for the indicated proteins.

Transfection—Cells were seeded in 6-well plates at a density of 1 × 105/well 24 h before transfection to yield a 30–40% confluent culture on the day of transfection.Transient transfections were performed using FuGENE 6 transfection reagent (Roche Applied Science), according to the manufacturer’s protocol. Briefly, cells were incubated with DNA-FuGENE 6 reagent (1:3) complexes for 5–7 h. Within 48 h after transfection, cells were subjected to further experiments.

Short Interfering RNA—Specific inhibition of SHP2 was conducted with siRNA (sequence: ag-cccaaaaagaguuacauugcc-dtt (1080–1100 of the SHP-2 sequence). Rat2 cells grown in 6-well wells were transfected with SHP-2-siRNA or GFP-siRNA (control) using X-tremeGENE siRNA transfection reagent (Roche Applied Science) according to the manufacturer’s specification. Measurement of the gene-knockdown was performed in 24–72 h. Within 48 h after transfection, cells were subjected to further experiments.

Fluorescence Microscopy—Chamber slides (2- and 4-well, Lab-Tek) were coated with poly-L-lysine (100 μg/ml in PBS) and fibronectin- or BSA-coated latex microbeads. Cells were plated for 3–4 h at 37 °C. Prior to immunostaining, cells were stimulated with IL-1 (20 ng/ml for 20 min), fixed (4.0% formaldehyde in PBS for 10 min at room temperature), blocked, and permeabilized in PBS with 0.2% Triton X-100 for 15 min at room temperature. Antibodies were diluted in PBS with 1% BSA. Immunofluorescence staining was performed with rabbit anti-SHP-2 antibody (1:100) and mouse anti-calnexin (1:150) for 1 h at room temperature. Slides were washed with PBS, incubated with goat anti-rabbit fluorescein isothiocyanate-conjugated antibody or goat anti-mouse Texas Red conjugate antibody for 1 h, washed, and sealed with coverslips. The slides were viewed by total internal reflection microscopy.

Total Internal Reflection Microscopy—We examined the spatial relationships between SHP-2, the ER, and focal adhesions following IL-1 treatment using green or yellow fluorescent protein-tagged SHP-2 and ER-protein expression vectors combined with total internal reflection microscopy (also called evanescent wave microscopy). TIRF generates fluorescence excitation within a narrow zone (100–150 nm) from the coverslip, which excites fluorescent proteins only on the ventral cell surface and immediately below the plasma membrane. Light from an argon laser (488/543 nm) was introduced into an inverted microscope through a single mode fiber and two illumination lenses (Nikon). The light was focused at the back focal plane of a high aperture objective lens (×60, numerical aperture 1.65, oil immersion). To observe green fluorescent protein fluorescence, we used a 488 nm laser line for excitation and a 515/15 nm bandpass filter. For fluorescence from orange/yellow fluorescent proteins (YFP), we used 543 nm excitation and a long-pass 590 nm filter. Comparisons of GFP- and YFP-transfected cells showed very low levels of cross-over emission into the yellow and green channels, respectively. To monitor the localization of the single SHP-2 molecular or ER organelle, the GFP-tagged SHP-2 (GFP-SHP-2) or ER-specific protein (YFP-calnexin, GFP-calreticulin, and YFP-KDEL/calreticulin)-transfected cells were cultured in chamber slides previously coated with poly-L-lysine and fibronectin or BSA beads. Cells were incubated for 3–4 h at 37 °C in normal medium and then stimulated with IL-1. Nikon immersion oil (refractive index = 1.515) was used to establish optical contact between the objective lens and the coverslip. Using fluorescence calibration beads, the measured penetration depth of excitation light was ~150 nm.

Electron Microscopy—Rat2 cells plated on poly-L-lysine (200 ng/ml)-coated coverslips for 3–4 h were incubated with fibronectin-coated latex microbeads for 1 h and then treated with IL-1 (20 ng/ml) at 37 °C for 30 min. Cells were fixed with 2% glutaraldehyde, 1% tannic acid, 0.1 M sodium cacodylate at pH 7.4 for 2 h at room temperature, washed three times in 0.1 M sodium cacodylate, pH 7.4, and then post-fixed in 1% OsO4 at room temperature in the same buffer. All samples were washed and dehydrated by stepwise exposure to increasing concentrations of ethanol (25, 50, 75, 95, and 100%, v/v) before embedding in Spurrs resin. Sections (80 nm thick) were cut on a Reichert Ultracut E microtome and stained with uranyl acetate and sodium bismuth. The sections were examined with a Hitachi 7000 scanning transmission electron microscope. Electron micrographs are representative of data obtained in two experiments.

Imaging of Ca2+ in Sub-membrane Cytosolic and ER Stores—Cells were incubated with 3–4 μM fluo-4/AM and 0.01% pluronic acid for 30–40 min, or 4–5 μM mag-fluo-4/AM, and 0.01% pluronic acid for 30–40 min at 37 °C. 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 osmolarity of 291 mosM. For experiments requiring external Ca2+, 2 mM CaCl2 was added to the buffer; for experiments
IL-1 Signaling through the Endoplasmic Reticulum

A

|                  | FN-coated beads | FN-coated beads | BSA-coated beads |
|------------------|-----------------|-----------------|-----------------|
|                  | - IL-1          | + IL-1          | + IL-1          |
| GFP-SHPI         | DIC             | DIC             | DIC             |
| YFP-Calnexin     | DIC             | DIC             | DIC             |
| GFP-Calreticulin | DIC             | DIC             | DIC             |

B

|                  | Control/IL-1   | Collagen-beads | BSA-beads/SWA | Integrin-Ab-beads |
|------------------|-----------------|----------------|---------------|-------------------|
| Vinculin         |                 |                |               |                   |
| SHP-2            |                 |                |               |                   |
| Calnexin         |                 |                |               |                   |
| BIP/GRP78        |                 |                |               |                   |
| GAPDH            |                 |                |               |                   |

C

|                  | FN-beads | BSA-beads | Integrin-Ab-beads |
|------------------|----------|-----------|-------------------|
| Vinculin         |          |           |                   |
| DIC              |          |           |                   |

D

Attached vs. Unattached

E

Vinculin, Calnexin, Merge
requiring chelation of external Ca\(^{2+}\), 1 mM EGTA was added. Imaging and fluorescence intensity of sub-membrane cytosolic and ER calcium were obtained with TIRF microscopy using C-MAGING SYSTEMS-Simple PCI software (Compix, Inc., Cranberry Twp., PA) with an excitation wavelength of 488 nm and an emission bandpass filter of 515/15 nm.

Data Analysis—Means ± S.E. were calculated for calcium measurements, including baseline fluo-4/mag-fluo-4 fluorescence intensity and the net change of fluorescence intensity above baseline. For all continuous variables, means ± S.E. were computed and, when appropriate, comparisons between two groups were made with the unpaired Student’s t test or, for multiple samples, with analysis of variance. Post hoc testing was done 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

Sub-membrane Co-localization of SHP-2 to Focal Adhesions and the ER—To examine the spatial relationships between SHP2, focal adhesions, and the ER, Rat2 fibroblasts were co-transfected with one of either GFP-SHP2, YFP-calnexin, GFP-calreticulin, or YFP-calreticulin fusions. Transfected cells were re-plated on poly-L-lysine-coated coverslips on which either fibronectin or BSA-coated microbeads had been previously attached. Cells were incubated with IL-1 or vehicle. TIRF imaging was used to assess protein recruitment into nascent focal adhesion-like structures that were induced by the matrix ligand-coated beads attached to the coverslip (Fig. 1A). Under these conditions, SHP-2, calnexin, and calreticulin co-localized with fibronectin-coated but not with BSA-coated beads. Staining intensity around fibronectin bead-associated focal adhesions for each of these proteins was further enhanced by IL-1 treatment.

Fluorescence microscopic studies of transfected proteins were complemented by examination of endogenous focal adhesion and ER proteins that were associated with collagen-coated beads (Fig. 1B). In these experiments collagen magnetite beads were bound to the dorsal surfaces of cells as previously described (29) and, following IL-1 or vehicle control treatments, the bead-associated proteins were assessed by immunoblot analysis. For the focal adhesion protein vinculin, as well as SHP-2 and the ER-associated proteins calnexin and BiP, IL-1 treatment increased the relative abundance of proteins associated with collagen-coated beads but not with BSA-coated beads. For those proteins that remained in the lysis buffer after preparation of focal adhesions (i.e. the non-focal adhesion fraction), there was no IL-1-induced enhancement of vinculin, SHP-2, calnexin, or BiP. For all experiments, equal amounts of proteins were loaded in each lane. GAPDH, a control, non-ER and non-focal adhesion cytoplasmic protein did not associate with beads, and its relative abundance in focal adhesion preparations was not enhanced by IL-1 treatment. In cells treated with swinholide, an actin severing toxin that disrupts focal adhesions (30), there were no significant amounts of ER or focal adhesion proteins detected in the pool of bead-associated proteins.

We next sought to determine if beads coated with antibody to the fibronectin receptor could induce focal adhesions and if focal adhesion proteins were recruited by IL-1. Magnetite or latex beads were coated with antibody to \( \alpha_5 \beta_1 \), integrin and then incubated with cells (Fig. 1, B and C). Under these conditions, IL-1 did not induce recruitment of vinculin, SHP-2, calnexin, or BiP to beads indicating that clustering of integrins without concomitant activation was not sufficient to induce mature focal adhesions.

As analyses of bead-associated proteins indicated associations between focal adhesions around beads and the endoplasmic reticulum, we incubated fibronectin-coated beads with cells and examined them by electron microscopy (Fig. 1D). This analysis showed that, in beads attached to cells, endoplasmic reticulum cisternae were immediately subjacent to beads while in cells without contacting beads, there was no obvious recruitment of endoplasmic reticulum to the cell membrane. We also examined co-localization of endogenous vinculin and calnexin in conventional focal adhesions that formed on the ventral surface of cells and the bead-adherent fibronectin-coated glass (Fig. 1E). Similar to the co-localization of endoplasmic reticulum proteins around fibronectin-coated beads, vinculin and calnexin exhibited co-localization on ventral cell surfaces.

We compared the location of endogenous and transfected SHP-2 and calnexin by immunostaining and transfection with fluorescent fusion proteins (Fig. 2, A and B). There was close co-localization of SHP-2 and the ER protein calnexin with...
FIGURE 2. SHP-2 co-localized with ER around fibronectin-coated latex beads on dorsal surface near plasma membrane in IL-1-stimulated cells. A, Rat2 cells are grown on poly-l-lysine (200 ng/ml) and fibronectin- or BSA-latex microbeads coated coverslips for 3–4 h. After cells are stimulated with IL-1 (20 ng/ml) at 37 °C for 30 min, they are fixed, co-immunostained for SHP-2 and calnexin, and viewed by TIRF microscopy. B, Rat2 cells were co-transfected with GFP-SHP2 and YFP-calnexin. After 24–48 h of incubation, the transfected cells were re-plated on poly-l-lysine (200 ng/ml) and fibronectin- or BSA-latex microbead-coated coverslips for 3–4 h in normal growth medium, then stimulated with IL-1 (20 ng/ml) for 30 min at 37 °C. The images of fluorescence-labeled proteins localization in Rat2 cells were captured by TIRF microscopy after addition of IL-1 (20 ng/ml) at 1, 15, and 30 min, respectively.
A. Sub-membrane cytosolic Ca$^{2+}$

Fibronectin coated beads

| DIC | - IL-1 | + IL-1 3 min | + IL-1 6 min |
|-----|--------|-------------|-------------|
| Beads | 2 min | 320 | 320 | 320 |
| Remote | 2 min | 240 | 240 | 240 |

B. Sub-membrane ER Ca$^{2+}$

FN-coated beads

| DIC | + IL-1 | + IL-1+thapsigargin |
|-----|-------|---------------------|
| Beads | 2 min | 320 | 320 |
| Remote | 2 min | 240 | 240 |

BSA-coated beads

| DIC | + IL-1 | + IL-1+thapsigargin |
|-----|-------|---------------------|
| Beads | 2 min | 320 | 320 |
| Remote | 2 min | 240 | 240 |

FIGURE 3. IL-1-induced Ca$^{2+}$ signals are highly localized around fibronectin-coated beads. A, human gingival fibroblasts were plated on poly-L-lysine (200 ng/ml) and fibronectin- or BSA-latex microbead-coated coverslips for 3–4 h in normal growth medium, then loaded with fluo-4. The fluo-4 fluorescence in cells was captured by TIRF microscopy after addition of vehicle (0 min) or after addition of IL-1 (20 ng/ml) at 3 and 6 min, respectively. The Ca$^{2+}$-dependent fluorescence intensity was measured in 5-μm zones around the periphery of attached beads (Beads, red color-coded circles) and in similarly sized, randomly chosen zones within the projected area of the cell but at least 10 μm from the attached beads (Remote, blue color-coded circles). Single measurements of bead-associated and remote sites each from five cells were conducted for each group in each experiment. Bead-associated calcium increase was 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. Inset, IL-1 cannot promote an increase of sub-membrane [Ca$^{2+}$] around anti-integrin α5β1-coated beads. B, mag-fuo-4 fluorescence is shown for the estimation of ER Ca$^{2+}$. Cells were plated on poly-L-lysine (200 ng/ml) and fibronectin- or BSA-latex microbead-coated coverslips for 3–4 h in normal growth medium, then loaded with mag-fuo-4. The Ca$^{2+}$-dependent fluorescence intensity was measured after addition of IL-1 as described in A. The upper panel inset shows cells pre-treated with thapsigargin. The lower panel inset demonstrates that IL-1 cannot promote a transient reduction of juxtamembrane [Ca$^{2+}$]$_{ER}$ around anti-integrin antibody α5β1-coated beads. Summary data are mean ± S.E. from n = 3–5 experiments. Data show a decreased mag-fuo-4 fluorescence intensity below baseline (p < 0.001) in zones close to or remote from beads.

fibronectin-coated beads but not with BSA-coated beads after IL-1 treatment. Further, when cells were co-transfected with GFP-SHP-2 and YFP-calnexin, there was very strong co-localization around the fibronectin but not BSA-coated beads in live cell imaging (Fig. 2B). Collectively these data demonstrate that IL-1 enhances the association of SHP-2 and ER proteins with substrates to encourage focal adhesion formation only around beads. Cells were then loaded with Ca$^{2+}$-sensitive dyes to enable TIRF imaging of sub-membrane [Ca$^{2+}$] and juxtamembrane [Ca$^{2+}$]$_{ER}$. Changes in fluorescence intensity were measured in sampling regions adjacent to the bead and at regions of the cell that were remote from the bead. IL-1 promoted a rapid focal adhesions and that transfected and endogenous proteins exhibit similar behavior.

We examined further the association of SHP-2 with ER proteins and with focal adhesions. Whole cell lysates and focal adhesion-associated proteins were immunoprecipitated for SHP-2, and the immunoprecipitates were immunoblotted for the ER chaperone protein BiP, for calnexin, for the focal adhesion protein vinculin, and for GAPDH (Fig. 2C). In both whole cell lysates and focal adhesion-associated proteins, IL-1 enhanced interactions between SHP-2 and the ER proteins or vinculin, but there were no detectable interactions with GAPDH, and, as expected, there were no changes in levels of proteins in whole cell lysates after IL-1 treatment. The effect of IL-1 in promoting associations of SHP-2, focal adhesions, and the ER was assessed in a time series in which Rat2 cells were co-transfected with SHP-2 and calnexin or SHP-2 and a KDEL fusion construct, followed by treatment with IL-1. By TIRF microscopy, there were marked increases of staining intensity adjacent to beads and immediately subjacent to the cell membrane for SHP-2, calnexin, and the KDEL/calreticulin fusion construct that were particularly notable at early times between 1 and 15 min (Fig. 2D).

Focal Adhesion and ER-restricted Calcium Signaling Induced by IL-1—If the spatial co-localization of focal adhesions with the ER is functionally significant, we asked if IL-1-induced Ca$^{2+}$ signaling and Ca$^{2+}$ release from the ER were restricted to focal adhesions. Human gingival fibroblasts, cells that express high levels of type 1 IL-1 signaling receptors (12) and exhibit focal adhesion-restricted IL-1 calcium signaling (10), were plated on poly-L-lysine and fibronectin or BSA-coated bead.
increase of sub-membrane $[Ca^{2+}]$ and a transient reduction of juxtamembrane $[Ca^{2+}]_{ER}$ around fibronectin-coated beads but not in remote parts of the cell or around BSA-coated beads (Fig. 3, A and B, $p < 0.001$). Cells plated on $\alpha_5\beta_1$ integrin antibody-coated beads showed no IL-1-induced $[Ca^{2+}]$ increases or release from the ER (Fig. 3, A and B, insets). Depletion of ER stores by thapsigargin pre-treatment blocked IL-1-induced release of $Ca^{2+}$ from the ER as expected. These data indicated that the IL-1-induced $Ca^{2+}$ signal is tightly localized to sub-membrane sites in the cell where focal adhesions and the ER are in close proximity.

**Impact of SHP-2 on IL-1-induced Ca$^{2+}$ Signaling**—Because SHP-2 is important in mediating IL-1-induced focal adhesion maturation (16) and IL-1-induced Ca$^{2+}$ signaling (25), we determined if a specific tyrosine-phosphorylated SHP-2 residue that is important for IL-1-induced focal adhesion formation (Tyr-542) (16), would impact IL-1-induced Ca$^{2+}$ signaling and Ca$^{2+}$ release from the ER. In cells expressing wild-type SHP-2, IL-1-induced increases of cytoplasmic Ca$^{2+}$ signaling and Ca$^{2+}$ release from the ER were robust (Fig. 6, A and B). In the same cells in which a mutant SHP-2 was present (Y542F), cytoplasmic Ca$^{2+}$ signaling and Ca$^{2+}$ release from the ER were significantly reduced compared with the wild-type cells ($p < 0.02$). In addition, SHP-2 cells with the Tyr-542 SHP-2 mutant exhibited no increase in the number of well developed focal adhesions after IL-1 treatment, whereas cells expressing wild-type SHP-2 exhibited a 3-fold increase of well developed focal adhesions ($p < 0.001$, Fig. 4C).

We next considered if Tyr-542 of SHP-2 was important in mediating focal adhesion association with the ER. SHP-2 wild-type cells or cells in which the Y542F mutant was expressed, were transfected with either YFP-calnexin or GFP-calreticulin, plated on fibronectin or BSA-coated beads and incubated with IL-1. Cells expressing wild-type SHP-2 showed co-localization of ER proteins with fibronectin-coated beads, whereas these same cells were incapable of recruiting ER proteins around BSA-coated beads as were the cells expressing the SHP-2 Tyr-542 mutant (Fig. 5A). In a parallel biochemical analysis, identical experimental designs were conducted except that collagen-coated beads were added to the dorsal surface of cells, and the bead-asso-
Associated proteins were purified and characterized by immunoblot analysis. Consistent with the fluorescence microscopy data, the SHP-2 rescue cells (but not the SHP-2 Tyr-542 cells) exhibited IL-1-induced recruitment of calreticulin, calnexin, and BiP to fibronectin or collagen-coated beads (Fig. 5B). There was no detectable GAPDH in these preparations. This same experimental design was applied in which immunoprecipitation of SHP-2 from whole cell lysates was followed by immunoblotting with antibodies to SHP-2, calnexin, BiP/GRP78, and GAPDH. 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.

**FIGURE 5.** SHP-2 is required for ER aggregation with focal adhesions during IL-1 stimulation. A, SHP-2-reconstituted murine embryonic fibroblasts (SHP-2) and SHP-2 Tyr-542 cells were transfected with YFP-calnexin or GFP-calreticulin. After 24–48 h of incubation, transfected cells were re-plated on poly-L-lysine (200 ng/ml) and fibronectin- or BSA-latex microbead-coated coverslips for 3–4 h in normal growth medium. TIRF was used to image fluorescence-labeled protein localization in Rat2 cells without IL-1 or following stimulation with IL-1 (20 ng/ml) for 30 min. B, cells were plated overnight, incubated with collagen-coated magnetic microbeads for 30 min at 37 °C to induce focal adhesion formation, and were untreated (vehicle control) or treated with 20 ng/ml IL-1 at 37 °C for 30 min. Focal adhesion complexes were isolated, and protein content was measured by Bio-Rad assay. Equivalent amounts of protein were loaded into each lane of an SDS-polyacrylamide gel, separated, transferred, and immunoblotted with antibodies to SHP-2, calnexin, BiP/GRP78, and GAPDH. SHP-2 cells with and without IL-1 stimulation were lysed and immunoblotted with the antibodies above. The proportions below each blot indicate the blot density compared with the maximum blot density in each group. C, SHP-2 and SHP-2 Tyr-542 cells grown on fibronectin were stimulated with IL-1 (20 ng/ml for 30 min) or vehicle control as indicated. SHP-2 was purified by immunoprecipitation (IP) from whole cell lysates, separated by SDS-PAGE, transferred to nitrocellulose, and blotted (WB) with antibodies to SHP-2, calnexin, BiP/GRP78, vinculin, and GAPDH. 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. D, Rat2 cells were transfected with SHP2-siRNA and GFP-siRNA (control) as described under “Experimental Procedures,” then treated with IL-1 (20 ng/ml for 30 min) or vehicle control. Whole cell lysates and focal adhesion proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes, and probed with antibodies to SHP-2, calnexin, and BiP/GRP78.
noprecipitated proteins. The wild-type SHP-2 cells but not the
SHP-2-Tyr-542 mutant cells exhibited IL-1-enhanced association
of calnexin or BiP with SHP-2. Notably, IL-1 also increased the
association of SHP-2 with the focal adhesion protein vinculin (Fig.
5C). Knockdown of SHP-2 by siRNA was used to determine the
importance of SHP-2 on IL-1-induced recruitment of endogenous
calnexin and BiP to focal adhesions. Consistent with the data
shown above, knockdown of SHP-2 by siRNA also eliminated
IL-1-induced recruitment of calnexin and BiP to focal adhesions
(Fig. 5D).

If co-localization of ER with focal adhesions requires IL-1-
induced Ca\(^{2+}\) release from the ER, we asked if inhibition of
Ca\(^{2+}\) release would block ER-focal adhesion apposition. The
antibiotic puromycin is a translation inhibitor that purges ER
translocon pores of nascent polypeptides (31) and specifically
reduces [Ca\(^{2+}\)]\(_{ER}\) through its impact on the translocon (32).
The inhibitory mechanism is independent of ER Ca\(^{2+}\) pumps
and of IP\(_3\) or ryanodine receptors (33). In transfected Rat2 cells,
puromycin treatment (200 \(\mu\)M) blocked IL-1-induced co-local-
zation of YFP-calnexin or GFP-calreticulin with fibronectin-
coated beads (Fig. 6A). Because puromycin is a translation
inhibitor, there was a possibility that simply inhibiting protein
synthesis mediated dissipation of the co-localization of cal-
nexin and calreticulin with focal adhesions. Accordingly, we
performed the same experiment with the protein synthesis
inhibitor anisomycin, a bacterial antibiotic that binds to 60 S
ribosomal subunits and blocks the peptidyl transferase and
peptide bond formation (34) but does not affect Ca\(^{2+}\)\(_{ER}\) (32). Ani-
somycin treatment at a concentration (200 \(\mu\)M) that is known to
inhibit protein synthesis in Rat2 cells did not affect IL-1-in-
duced recruitment of ER proteins to fibronectin-coated beads (Fig. 6A). To verify that puromycin was directly affecting the ER translocon complex as previously reported (32, 33), we loaded cells with mag-fluo-4 and estimated \([\text{Ca}^{2+}]_{\text{ER}}\) after IL-1 treatment. Cells that were preincubated with puromycin showed greatly reduced IL-1-induced release of \([\text{Ca}^{2+}]_{\text{ER}}\), whereas cells that were pre-treated with anisomycin exhibited IL-1-induced release of \([\text{Ca}^{2+}]_{\text{ER}}\) that was indistinguishable from controls (Fig. 6B).

The experiments described above indicate that IL-1-induced release of \([\text{Ca}^{2+}]_{\text{ER}}\) occurs in cells in which focal adhesion-associated SHP-2 co-localizes with the ER (Fig. 4). These data suggested the possibility that the IP₃ release channel in the ER (i.e. the IP₃ receptor) may be impacted by ER membrane proteins indirectly, or is perhaps directly involved in mediating ER-focal adhesion interactions. We first determined if the SHP-2 cell lines used here (wild-type and Y542F mutant) were sensitive to inhibition of the IP₃ receptor and uncoupling of the ER from plasma membrane channels. Cells were loaded with fluo-4, and, immediately prior to IL-1 treatment, the medium was depleted of \([\text{Ca}^{2+}]_{\text{ER}}\) so that we could measure IL-1-induced release of \([\text{Ca}^{2+}]_{\text{ER}}\) into the cytoplasm. As expected, the SHP-2 wild-type cells exhibited robust IL-1-induced release of \([\text{Ca}^{2+}]_{\text{ER}}\), whereas release in the Y542F mutant cells was <30% of wild-type cells (Fig. 7A).

In wild-type and Y542F mutants, 2-aminoethoxydiphenyl borate (2-APB), an inhibitor of functional coupling between IP₃-sensitive ER calcium stores and plasma membrane channels in the generation of \([\text{Ca}^{2+}]_{\text{ER}}\) signals (35, 36), and xestospongin C (a reversible and membrane-permeable blocker of IP₃-mediated \([\text{Ca}^{2+}]_{\text{ER}}\) release that does not interact with the IP₃-binding site), both blocked IL-1-induced release of \([\text{Ca}^{2+}]_{\text{ER}}\) (<25% of control values, Fig. 7A). These data indicated that the IP₃ receptor and/or functional coupling of the ER with plasma membrane channels are important components for IL-1-induced release of \([\text{Ca}^{2+}]_{\text{ER}}\).
To determine if the IP₃ receptor physically associated with SHP-2, we prepared purified ER fractions from wild-type and Y542F SHP-2 cells after IL-1 treatment. The relative purity of the ER fractions from the different cell types was evaluated by immunoblotting equal amounts of protein for the ER lectin calnexin; these data showed equivalent loading of calnexin on the gels (Fig. 7B). SHP-2 and IP₃ receptor type 1 immunoprecipitates of the ER fractions were immunoblotted for IP₃ receptor type 1 protein and SHP-2, respectively. We were unable to detect IP₃ receptor protein 2 in these cells (data not shown). IL-1 induced increased association of SHP-2 with IP₃ receptor protein 1 (but not the IP₃ receptor type 3, data not shown) in immunoprecipitates prepared from the SHP-2 wild-type cells but not in the Y542F mutant cells. Conversely, IL-1 enhanced the association of the IP₃ receptor protein 1 (but not the IP₃ receptor protein 3, data not shown) with SHP-2 in wild-type cells but not in the Y542F cells.

We considered that phosphorylation of SHP-2 at Tyr-542 is important for mediating associations between focal adhesions and the ER. Accordingly, we immunoblotted cells for phosphorylated SHP-2 (Tyr-542) and found as shown earlier (15) that IL-1 increased SHP-2 phosphorylation (Fig. 7C). Notably, in cells incubated with IL-1, immunoprecipitates of IP₃ receptor type 1 showed higher amounts of bound phosphorylated SHP-2 than cells treated with vehicle (Fig. 7D).

**DISCUSSION**

Our major finding is that the protein-tyrosine phosphatase SHP-2 mediates associations between focal adhesions and the ER. These associations are enhanced by IL-1-induced Ca²⁺ release from the ER, a process that is in turn dependent on phosphorylation of residue Tyr-542 of SHP-2 in focal adhesions. Although previous studies have demonstrated co-localization of focal adhesions and the ER (23–25), this is the first study to demonstrate functional associations between these two organelles that impact on signal transduction. The functional dependence of IL-1 signaling on SHP-2 and ER-focal adhesion interactions suggests a novel paradigm for regulating the signal generating activity of a powerful pro-inflammatory cytokine, IL-1. In this pathway, SHP-2 enhances interactions between focal adhesions and the ER, critical sites for IL-1-induced release of Ca²⁺ (21). Because generation of calcium signals in response to IL-1 is essential for activation of the mitogen-activated protein kinase ERK (9), release of Ca²⁺ from the ER is an important determinant of ERK-dependent signaling pathways that includes the production of matrix protein degrading proteases (5, 6).

Focal adhesions are critical, IL-1 signal-restricting membrane domains (10) enriched with multiple signaling proteins (37), including SHP-2 (25, 38, 39), and provide molecular staging sites for focusing of signals leading to several pathways, including ERK (40, 41). We have provided strong evidence for ER-focal adhesion associations using both immunoprecipitation and TIRF, an experimental approach indicating that these signaling pathways occur immediately below the plasma membrane in the vicinity of focal adhesions. Several highly abundant, ER-associated proteins were linked with focal adhesions, including BiP, calnexin, calreticulin, and IP₃ receptor type 1. Although calreticulin and the IP₃ receptor can also localize to the plasma membrane and other organelles (42, 43), it is unlikely that all four proteins would randomly co-localize with focal adhesions by chance alone. Indeed, our use of TIRF microscopy and a model system in which cells are attached to poly-L-lysine (which prevents focal adhesion formation) and isolated fibronectin-coated beads (which stimulates focal adhesion formation immediately adjacent to the bead), provide a high resolution system for imaging only those transfected or endogenous molecules that are immediately subjacent to the cell membrane. The functional importance of plasma membrane-proximal, IL-1-induced signaling events was demonstrated by imaging of Ca²⁺ fluxes in the cytoplasm, and release from the ER as estimated by fluo-4 and mag-fluo-4 imaging, respectively.

One of the novel features of this current report is that focal adhesions, in addition to being enriched with IL-1 receptors (12), also provide a locus by which SHP-2 brings into apposition the signaling molecules and the organelles that are required for efficient IL-1 signal transduction. Previous reports have indicated that SHP-2, in addition to mediating Ras/ERK activation because of its adaptor function (through Grb2/SOS (44)) and its phosphatase activity (13), is also important in integrin signaling (17, 45, 46). Although the negative or positive impact of SHP-2 on integrin-mediated Src and FAK phosphorylation is not well defined (17, 45–47), in focal adhesion-dependent IL-1 signaling in fibroblasts, phosphorylation of SHP-2 at Tyr-542 is evidently a critical step for IL-1-induced ERK activation (16, 36). Further, Tyr-542 is the major in vivo site of SHP-2 tyrosine phosphorylation (44) and is sufficient to activate the mitogen-activated protein kinase pathway (48). In view of these data we transfected cells that constitutively express only a SHP-2, SH2 domain deletion mutant (SHP-2⁻/⁻), with either wild-type SHP-2 or with a Y542F SHP-2 mutant. The data from these experiments thus allow direct comparisons of cells with only one residue difference in SHP-2 (Y542F). Evidently, Tyr-542 of SHP-2 is required for IL-1-induced Ca²⁺ release from the ER and for IL-1-induced association of the ER with focal adhesions.

The temporal sequence of these events indicated that SHP-2-dependent focal adhesion maturation is a pre-condition for IL-1-induced Ca²⁺ release and that, in turn, Ca²⁺ release precedes the association of ER proteins with focal adhesions. The critical role for Tyr-542 in this pathway appears to involve focal adhesion maturation, as defined by recruitment of α-actinin and increased focal adhesion size (16). As shown in the current report, it is also apparent that block of Tyr-542 phosphorylation prevents IL-1-induced recruitment of the ER to focal adhesions. This step may rely on ER Ca²⁺ release, because pharmacological inhibition of ER Ca²⁺ release by puromycin (32) prevents the recruitment of ER proteins to focal adhesions. This result was not due to block of protein synthesis, because anisomycin (34) did not affect ER protein recruitment.

Ca²⁺ from the ER release is a central regulatory step in the IL-1-signaling process that leads to ERK activation (9–11, 21). As anticipated, we found that pharmacological interference with the IP₃ receptor by xestospongin C or interference of ER coupling with plasma membranes channels with 2-APB blocked IL-1-induced ER Ca²⁺ release. Because IP₃ receptors co-localize and co-immunoprecipitate (24) with talin and vinculin, prominent focal adhesion proteins, we considered that the IP₃ receptor interacts with...
SHP-2. Although we did not find a detectable interaction with the type 2 IP$_3$ receptor or any significant changes with the type 3 IP$_3$ receptor, there was marked association with the type IP$_3$ receptor, and IL-1 treatment enhanced the association of SHP-2 with the type 1 receptor. Notably, this IL-1 enhanced association also required Tyr-542 of SHP-2. The IP$_3$ receptor is a large molecule, but only 5% comprises the ion conduction pore; the remainder contains regulatory and binding domains for the wide variety of proteins and small molecules with which the IP$_3$ receptor interacts (43). On the basis of these findings we suggest that the dependence of Tyr-542 of SHP-2 for IL-1-induced ER Ca$^{2+}$ release involves direct association with the IP$_3$ receptor and possibly an alteration of the gating function of the receptor to control ER Ca$^{2+}$ release. The nature of this association remains to be determined.

In summary, the data indicate that: 1) Tyr-542 of SHP-2 is important for focal adhesion formation, 2) focal adhesions in turn are required for IL-1-induced Ca$^{2+}$ release from the ER in the vicinity of ER-focal adhesion appositions, and 3) the formation of ER-focal adhesion appositions requires IL-1-induced Ca$^{2+}$ release from the ER. These appositions are dependent upon SHP-2-IP$_3$ receptor interactions that rely on Tyr-542 of SHP-2.

Acknowledgments—We thank Laura Silver for assistance in preparation of the manuscript, G. S. Feng (Burnham Institute) for the SHP-2 mutant cells and the SHP-2 bacterial expression system, B. G. Neel (Harvard) for the Tyr-542 SHP-2 mutant cells, and M. Michalak (University of Alberta) and M. Opas (University of Toronto) for YFP-calnexin, GFP-calreticulin, and YFP-KDEL/calreticulin.

REFERENCES
1. Dinarello, C. A. (1996) Blood 87, 2095–2147
2. Honig, J., Rordorf-Adam, C., Siegmund, C., Wiedemann, W., and Erard, F. (1998) J. Periodontal. Res. 24, 362–367
3. O’Neill, L. A., and Dinarello, C. A. (2000) ImmunoL Today 21, 206–209
4. van den Berg, W. B. (1999) J. Rheumatol. 26, 136–141
5. Boyle, D. L., Han, Z., Rutter, J. L., Brinckerhoff, C. E., and Firestein, G. S. (1999) Arthritis Rheum. 40, 1772–1779
6. Bhat-Nakshatri, P., Newton, T. R., Goulet, R., Jr., and Nakshatri, H. (1998) Trends Biochem. Sci. 23, 571–578
7. Carragher, N. O., and Frame, M. C. (2004) FASEB J. 18, 241–249
8. Oh, E. S., Gu, H., Saxton, T. M., Timms, J. F., Hausdorff, S., Frevert, E. U., Yamada, K. M. (2002) J. Biol. Chem. 277, 4098–4106
9. Tsuda, M., Matozaki, T., Fukunaga, K., Fujioka, Y., Imamoto, A., Noguchi, Z., Ichihashi, M., and Kasuga, M. (2000) J. Biol. Chem. 275, 33259–33264
10. Bennett, A. M., Tang, T. L., Sugimoto, S., Walsh, C. T., and Neel, B. G. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 7335–7339
11. Inagaki, K., Noguchi, T., Matozaki, T., Horikawa, K., Fukunaga, K., Tsuda, M., Ichihashi, M., and Kasuga, M. (2000) Oncogene 19, 75–84
12. Oh, E. S., Gu, H., Saxton, T. M., Timms, J. F., Hausdorff, S., Frevert, E. U., Kahn, B. B., Pawson, T., Neel, B. G., and Thomas, S. M. (1999) Mol. Cell. Biol. 19, 3205–3215
13. Manes, S., Mira, E., Gomez-Mouton, C., Zhao, Z., J. L., Lalcea, R. A., and Martinez, A. C. (1999) Mol. Cell. Biol. 19, 3125–3135
14. Araki, T., Mohi, M. G., Ismat, F. A., Bronson, R. T., Williams, I. R., Kutok, J. L., Wang, W., Pao, L. I., Gilliland, D. G., Epstein, J. A., and Neel, B. (2004) Nat. Med. 10, 849–857
15. Wachtel, D., and Wonderlin, W. F. (2001) Curr. Opin. Genet. Dev. 11, 343–350
16. Schoenwaelder, S. M., Petch, L. A., Williamson, D., Shen, R., Feng, G. S., and Burridge, K. (2000) Curr. Biol. 10, 1523–1526
17. Yu, D. H., Qu, C. K., Henegarri, O., Lu, X., and Feng, G. S. (1998) J. Biol.
18. Yu, D. H., Qu, C. K., Henegarri, O., Lu, X., and Feng, G. S. (1998) J. Biol.
19. Arora, P. D., Fan, L., Sodek, J., Kapus, A., and McCulloch, C. A. (2003) Exp. Cell Res. 286, 366–380
20. Arora, P. D., Fan, L., Sodek, J., Kapus, A., and McCulloch, C. A. (2003) Exp. Cell Res. 286, 366–380
21. Liu, Y. Y., Luo, L., McCulloch, C. A., and Cruz, T. F. (1998) J. Biol. Chem. 273, 7059–7065
22. Arora, P. D., Fan, L., Sodek, J., Kapus, A., and McCulloch, C. A. (2003) Exp. Cell Res. 286, 366–380
23. Schoenwaelder, S. M., Petch, L. A., Williamson, D., Shen, R., Feng, G. S., and Burridge, K. (2000) Curr. Biol. 10, 1523–1526
24. Yu, D. H., Qu, C. K., Henegarri, O., Lu, X., and Feng, G. S. (1998) J. Biol.