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Ras-guanine nucleotide releasing factors 1 and 2 interact with PLC γ at focal adhesions to enable IL-1-induced Ca²⁺ signaling, ERK activation and MMP-3 expression

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Abbreviations Footnote

BAPTA/AM: O,O Ebis(2-aminophenyl)ethyleneglycol-N,N,N',N'-tetraacetic acid, tetra-acetoxymethyl ester
 BSA: bovine serum albumin
 CKSB: cytoskeleton extraction buffer
 ER: endoplasmic reticulum
 ERK: extracellular signal regulated kinase
 FA: focal adhesion
 GAPDH: glyceraldehyde 3-phosphate dehydrogenase
 GDP: guanosine diphosphate
 GTP: guanosine triphosphate
 GRF: guanine nucleotide releasing factor
 HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
 IL-1: interleukin-1
 IP3: inositol tri-phosphate
 IP3R: inositol tri-phosphate receptor
 IPTG: isopropyl- β -D-thio-galactoside
 IQ domain: an amino acid sequence motif containing isoleucine and glutamine as the first two amino acids
 MAPK: mitogen activated protein kinase
 MEK: mitogen activated protein kinase kinase
 MMP: matrix metalloproteinase
 PBS: phosphate buffered saline
 PIPES: piperazine-N,N'-bis(2-ethanesulfonic acid)
 PLC: phospholipase C
 PMSF: phenylmethylsulfonyl fluoride
 RBD: Ras binding domain
 SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis
 siRNA: short interfering RNA
 2-APB: 2-aminoethoxydiphenylborate,

Author Contributions

Q. Wang contributed to the experimental design, conducted the experiments, composed the figures and wrote the first draft of the paper. K. Siminovitch contributed to the second draft of the paper. G. Downey contributed to the second-fourth drafts of the paper and helped to design the experiments. C. McCulloch was responsible for the overall concept, as well as drafting and revision of the manuscript at all stages.

SYNOPSIS

Interleukin-1 (IL-1) signaling in anchorage-dependent cells involves focal adhesion-restricted and Ca^{2+} -dependent Ras and extracellular signal-related kinase (ERK) activation that lead to MMP release and extracellular matrix remodeling. Ras activity is regulated in part by the Ca^{2+} -responsive Ras guanine-nucleotide-releasing factors 1 and 2 (Ras-GRF1/2), but the mechanisms that link and localize IL-1-induced Ca^{2+} signaling to focal adhesions are not defined. Herein we characterized the role of Ras-GRF1/2 in Ca^{2+} and Ras→ERK signaling after IL-1 stimulation. By immunoprecipitation we found that Ras-GRF1/2 associates with PLC γ 1. This association enables PLC γ 1 recruitment to focal adhesions and is required for Ras signaling, ERK activation and MMP-3 release downstream of IL-1 stimulation. Depletion of PLC γ 1 by siRNA abolished IL-1-induced Ras activation and MMP-3 expression. Buffering of cytosolic Ca^{2+} reduced Ras interactions with Ras-GRF1/2 and blocked MMP-3 release. Our results show that in addition to their functions as Ras exchange factors, Ras-GRF1 and GRF2 may act as adaptors that bind PLC γ 1 and restrict Ca^{2+} -signaling to the vicinity of focal adhesions, indicating a new role for these GRFs that is required for IL-1 induction of the Ras→ERK pathway and MMP-3 expression.

Key Words: Fibroblasts, proteases, PLC γ , IP $_3$

INTRODUCTION

IL-1 is a critical, pro-inflammatory cytokine that promotes degradation, remodeling, and disorganized deposition of excessive amounts of extracellular matrix proteins in many common diseases such as rheumatoid arthritis, pulmonary fibrosis, periodontitis, and cancer [1-3]. IL-1 enhances extracellular matrix remodeling by augmenting protease expression and release from tissue macrophages and fibroblasts. The expression of matrix-degrading genes in these cells is regulated in part by the amplitude and duration of IL-1-induced signals [4, 5], which act through the Ras pathway to induce mitogen-activated protein kinases such as ERK [6, 7] and increased expression and secretion of matrix metalloproteinases (MMPs) [8].

The Ras family of small GTPases, including H, N and K-Ras, are key regulators of many signal transduction pathways [9-11]. In their active forms Ras proteins can activate a variety of downstream targets, including the Raf/MEK/ERK cascade [12]. Ras proteins are activated by guanine-nucleotide exchange factors that promote the release of GDP bound to Ras and exchange for the activating GTP. Ras guanine nucleotide-releasing factors (GRF) 1 and 2 promote GTP loading of H, N and K-Ras proteins [13-17]. Ras-GRFs contribute to the activation of Ras proteins in response to upstream signals such as increased Ca^{2+} levels, which trigger calmodulin binding to the Ras-GRF IQ domain [18]. Ras-GRFs can thus couple Ras activation to a number of signal transduction processes mediated by calcium/calmodulin/calmodulin kinase as well as many other signaling pathways [18-21].

Ca^{2+} release from the endoplasmic reticulum (ER) is an important regulatory step in the coupling of IL-1-signaling to ERK activation [22]. Previously we reported on the rapid and focal adhesion-dependent release of Ca^{2+} from the ER in response to IL-1 [23, 24], suggesting a functional relationship between these different organelles, as well as spatial sequestration of signaling molecules involved in Ca^{2+} signaling [25]. Notably, focal adhesions are critical protein aggregates [26] that provide molecular staging sites [27] for transduction of IL-1 signaling [28, 29]. Consistent with this signaling model, focal adhesions are enriched with the canonical Ras and GRF1/2, proteins required for IL-1-induced ERK activation [30].

Currently the mechanisms by which focal adhesion-restricted, IL-1-induced Ca^{2+} signaling mediates ERK activation and MMP-3 release are not defined. As Ras-GRF1/2 are

enriched in focal adhesions [30] and are important for MMP-3 production in rheumatoid arthritis synovial tissue [31], we hypothesized that these proteins may functionally and spatially integrate Ca^{2+} signaling with Ras activation at focal adhesions to enable MMP-3 release. Here we provide evidence that Ras-GRF1/2 bind phospholipase $\text{C}\gamma 1$ ($\text{PLC}\gamma 1$) and may serve as adaptors for sequestering Ca^{2+} signaling to focal adhesions, thereby facilitating IL-1-driven Ras $\rightarrow$ ERK activation and MMP-3 expression.

EXPERIMENTAL

Reagents, antibodies and plasmids

Poly-L-lysine, BSA, human plasma thrombin, ionomycin and mouse monoclonal antibody to vinculin were obtained from Sigma-Aldrich (Oakville, ON). The inhibitors of IP_3 -mediated Ca^{2+} release (xestospongine C and 2-aminoethoxydiphenylborate, 2-APB) were obtained from Calbiochem (La Jolla, CA). Mouse monoclonal antibodies to H-Ras (F-235) and N-Ras (F-155); rabbit polyclonal antibodies to K-Ras (C-19), Ras-GRF1 (C-18), Ras-GRF1/2 (H-223); goat polyclonal antibody to Ras-GRF2 (C-20); siRNA control, siRNA Ras-GRF1 and siRNA Ras-GRF2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antibody against p44/42 MAPK, mouse monoclonal against phospho-p44/42 MAPK, rabbit polyclonal antibody against $\text{PLC}\gamma 1$, rabbit antibody against phospho- $\text{PLC}\gamma 1$ (Tyr783) and siRNA for $\text{PLC}\gamma 1$ were purchased from Cell Signaling (Beverly, MA). Goat antibody that recognizes the mouse integrin $\alpha 5\beta 1$ was purchased from Chemicon (Temecula, CA). DharmaFECT 1 Transfection Reagent was purchased from Thermo Fisher Scientific (Mississauga, ON). Glutathione Sepharose 4B was purchased from GE Healthcare (Baie D'Urfe, Quebec). Acidified, pepsin-digested bovine type I collagen was purchased from Cohesion Technologies Inc. (Palo Alto, CA). Recombinant human IL-1 β , TNF- α , PDGF and mouse monoclonal antibody to mouse MMP-3 were obtained from R&D Systems (Minneapolis, MN). BAPTA/AM and mag-fura-2/AM were purchased from Invitrogen (Mississauga, ON). A bacterially expressed Ras-GTP-binding domain of Raf1 (RBD) fused to GST was a gift from D. Rotin (Hospital for Sick Children, Toronto, Canada). Recombinant human $\text{PLC}\gamma 1$ (amino acids 1192-1291; 37 kDa; GST tag at the N-terminal) and human RasGRF1 (amino acids 1-110; 38 kDa; GST at the N-terminal) were obtained from Abnova (Taipei, Taiwan).

Cell culture and siRNA transfection

NIH 3T3 fibroblasts were grown in DMEM containing 10% calf serum. The cell culture media contained 0.17% w/v penicillin V and 0.01 g/mL amphotericin B; siRNA transfections were conducted with DharmaFECT 1 (Thermo Fisher Scientific).

Isolation of focal adhesions

Cells were grown to 80-90 % confluence on 60 mm tissue culture dishes and were cooled to 4°C prior to the addition of collagen, fibronectin or BSA-coated magnetite beads. Focal adhesion complexes were isolated from cells after specific incubation time periods as described [32]. In brief, cells were washed three times with ice-cold PBS to remove unbound beads and scraped into ice-cold cytoskeleton extraction buffer (CKSB; 0.5% Triton X-100, 50 mM NaCl, 300 mM sucrose, 3 mM MgCl_2 , 20 $\mu\text{g}/\text{ml}$ aprotinin, 1 $\mu\text{g}/\text{ml}$ leupeptin, 1 $\mu\text{g}/\text{ml}$ pepstatin, 1 mM PMSF, 10 mM PIPES, pH 6.8). The cell-bead suspension was sonicated for 10 s (output setting 3, power 15% Branson), and the beads were isolated from the lysate using a magnetic separation stand. The beads were re-suspended in fresh ice-cold CKSB, dispersed with a Dounce homogenizer (20 strokes) and re-isolated magnetically. The beads were washed in CKSB, sedimented with a microcentrifuge, re-suspended 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. Beads bound to the cells were counted electronically to normalize the relative abundance of bead-associated proteins.

Immunoblotting, immunoprecipitation and protein binding

Protein concentrations of cell lysates were determined by Bradford assay (BioRad, Hercules, CA). Equal amounts of protein were loaded onto SDS-PAGE gels, resolved by electrophoresis and transferred to nitrocellulose membranes. Membranes were incubated for 1 hour at room temperature in Tris-buffered saline solution with 5% milk or 0.2% BSA to block non-specific binding sites. Membranes were incubated with the primary antibodies overnight at 4°C in Tris-buffered saline with 0.1% Tween-20. Horseradish peroxidase-conjugated secondary antibodies were incubated for 1 hour at room temperature in Tris-buffered saline with 0.1% Tween-20 and 5% milk or 0.2% BSA. Proteins were visualized by chemiluminescence (GE Healthcare, Oakville, ON). For immunoprecipitations, cells were lysed in RIPA buffer (50 mM HEPES, pH 7.4, 1% deoxycholate, 1% Triton X-100, 0.1% SDS, 150 mM NaCl, 1 mM EDTA, 1 mM Na₃VO₄) containing 1 mM PMSF, 10 µg/ml leupeptin, and 10 µg/ml aprotinin. Equal amounts of protein from cleared extracts were immunoprecipitated and immunoblotted. For assessments of associations of RasGRF1 with PLCγ1, GST-tagged recombinant proteins attached to solid supports were incubated with cell lysates and bound proteins were assessed by immunoblotting. For study of direct interactions between these proteins, immunoblotting for the GST tag of the protein that bound to the interacting protein on a solid support was performed.

Ras activation

Affinity precipitation of Ras-GTP was performed as described [30]. Briefly, GST-RBD was induced in the BL21 *E. coli* strain using 1 mM IPTG for 3 h at 37°C. After expression and pelleting, bacteria were re-suspended in lysis buffer (for 500 ml lysis buffer: 10 ml 1 M Tris pH 8.0, 15 ml 5 M NaCl, 1 ml 0.5 M EDTA). The lysate was sonicated on ice 6 times for 1 min and the supernatant was cleared by centrifugation. The lysate was supplemented with 0.5% NP-40 and incubated with glutathione-agarose beads at room temperature for 1 h at 4°C overnight with rocking. The beads were isolated by centrifugation and washed 2-3 times with RIPA buffer and re-suspended in sample buffer (10% glycerol, 60 mM Tris, pH 6.8, 2% SDS, 300 mM β-mercaptoethanol). Protein samples were analyzed by immunoblotting and Ras isoforms were detected with Ras antibodies.

Quantitative Real-time PCR

Total RNA was isolated from cells using the RNeasy mini kit (Qiagen) following the manufacturer's instructions. The integrity of isolated RNA was confirmed by an Agilent Bioanalyzer 2100 (Santa Clara, CA) prior to analysis. Total RNA samples (1 µg) were reverse transcribed using iScript™ cDNA synthesis kit (Bio-Rad, Hercules, CA) according to the manufacturer's protocol. Real-time quantitative PCR was performed using the primers and methods described earlier. Relative quantification was done using the ΔΔCt method, in which the target gene (MMP-3) was normalized to a reference gene (GAPDH). The % differences were calculated relative to controls.

Measurement of Ca²⁺ in ER stores

For estimating [Ca²⁺]_{ER}, cells were incubated with mag-fura-2/AM (4 µM) for 150 min at 37°C in culture medium and measured by ratio fluorimetry as described [33]. Visual inspection of mag-fura-2-loaded cells showed fluorescent labeling of intracellular organelles that most likely represented dye sequestration to the ER. Fluorescence emission (520 nm) was measured in single, magfura-2-loaded cells upon excitation with alternating 346/380 nm light (C-IMAGING SYSTEMS; Compix, Cranberry, PA).

Experimental design and analysis

All experiments were repeated at least 3 times on different days using different batches of cells. The data shown are representative examples from these experiments. For numerical data, means and standard errors of the mean were calculated and, when appropriate, Student's *t*-test was used to compare two groups or ANOVA for three or more experimental groups. Statistical significance was inferred with the assumption of a type I error rate of $p < 0.05$.

RESULTS

Specificity of IL-1 signaling to Ras through focal adhesions

IL-1 activation of Ras is dependent on the *a priori* formation of focal adhesions in cultured cells [30]. We determined whether separate and distinct, pro-inflammatory Ras activation agonists required focal adhesions for signal transduction. NIH 3T3 fibroblasts were plated on collagen to promote focal adhesion formation or on poly-L-lysine to prevent focal adhesion formation as described earlier [24]. Cells were stimulated with IL-1 (20 ng/ml; 30 min) or thrombin (2 NIH unit/ml; 30 min) or PDGF (20 ng/ml; 15 min) or TNF- α (10 ng/ml; 30 min); Ras activation was then measured. Under these conditions all of these agonists strongly stimulated H, K and N-Ras activation in cells that formed focal adhesions. However, IL-1-induced activation of Ras was reduced in cells that were plated on poly-L-lysine (no focal adhesions) while the other agonists robustly activated Ras when plated on poly-L-lysine, independent of focal adhesion formation (Fig. 1). These data underscore the selective importance of focal adhesions in IL-1 signaling to Ras.

IL-1 activation of Ras and ERK requires PLC- γ

As Ras activation is required for IL-1-induced ERK phosphorylation [30], we examined NIH 3T3 cells after PLC γ 1 knockdown to assess whether Ras activation in response to IL-1 depends on PLC γ 1. Silencing of PLC γ 1 in these cells was associated with >80% diminution in IL-1-induced H- and K-Ras activation and ~60% reduction of N-Ras activation (Fig. 2A).

IL-1 stimulates Ca²⁺ release from the ER [33], indicating a potential role for PLC γ in IL-1-induced Ca²⁺ mobilization. The PLC γ antagonist U-73122 [34] completely blocked IL-1-induced ER-Ca²⁺ release (Fig. 2B) and reduced IL-1-induced ERK phosphorylation to controls levels (Fig. 2C). In cells treated with U-73122, ionomycin overcame the effect of the PLC γ antagonist to enable ERK activation in cells treated with IL-1.

We complemented these inhibitor studies with PLC γ 1 knockdown using siRNA treatment of NIH3T3 cells to determine the role of PLC γ 1 in IL-1-induced ERK activation (Fig. 2D). Knockdown of PLC γ 1 markedly reduced IL-1-induced ERK activation (by >60%). Collectively these results demonstrate that IL-1-induced Ras and ERK activation involve PLC γ 1.

Ras-GRF1 and Ras-GRF2 associate with focal adhesions after IL-1 stimulation

As previous studies showed that Ras-GRF1 is enriched in the particulate fraction of cells [19], we examined whether sequestration of Ras-GRF1 and Ras-GRF2 to the detergent-insoluble (cytoskeletal) fraction of cells is influenced by the ability of cells to form focal adhesions. Cells were plated on substrates that promote (collagen or fibronectin) or block (poly-L-lysine) focal adhesion formation, stimulated with IL-1 and then the levels of endogenous Ras-GRF1 and Ras-GRF2 in the Triton-insoluble cytoskeletal fractions were assessed [35]. While IL-1 did not induce changes in Ras-GRF1 or GRF2 levels in the cytoskeletal fractions of cells plated on collagen (or fibronectin, data not shown), Ras-GRF1 and Ras-GRF2 were depleted in cytoskeletal fractions of cells plated on poly-L-lysine, either before or after IL-1 treatment (Fig. 3A). Notably, cells plated on collagen (or fibronectin, data not shown) form abundant, vinculin-containing focal adhesions that were not observed in cells plated on poly-L-lysine (Fig. 3A).

As Ras can be recruited to the ER and plasma membrane and then initiate signals at the ER or plasma membrane after stimulation [36, 37], we examined the effects of IL-1 on accumulation of Ras-GRF1 and Ras-GRF2 in ER fractions prepared as described earlier [24] and in focal adhesion fractions [30]. Ras GRF-1 and GRF-2 were abundant in both ER and focal adhesion fractions derived from NIH3T3 cells (Fig. 3B). These data do not reflect contamination from other cellular compartments as these fractions expressed respectively, the ER lectin calnexin and the focal adhesion protein $\alpha 5 \beta 1$ integrin at levels that were similar in IL-1-treated and untreated cells. The effects of IL-1 on the spatial co-localization of Ras-GRF1 and Ras-GRF2 with focal adhesions were explored by TIRF microscopy [30]. This analysis revealed marked up-regulation (by >2-fold; $p < 0.05$; Fig. 3C) of Ras-GRF1/2 co-localization with the focal adhesion protein vinculin after IL-1 stimulation.

PLC γ 1 associates with Ras-GRFs

IL-1-induced Ca^{2+} release from the ER is sequestered at sites of apposition of the ER with focal adhesions and requires PLC γ 1 [24]. As Ras-GRFs are also enriched in focal adhesions [30], we determined whether Ras-GRF1 or Ras-GRF2 associate with PLC γ 1 by immunoprecipitation analyses. These assays showed an association of PLC γ 1 with Ras-GRF1 and with Ras-GRF2 that was increased by IL-1 (>40% increase for Ras-GRF1; >70% increase for Ras-GRF2; Fig. 4A, left panels). These associations were reduced to levels near that of vehicle controls by depletion of cytosolic Ca^{2+} with BAPTA (Fig. 4A, middle panels). The associations were strongly enhanced by the calcium ionophore, ionomycin (Fig. 4A, right panels). Immunoprecipitations conducted with pre-immune serum or with buffer (and no immunoprecipitating antibody) showed no associations between Ras-GRFs and PLC γ 1 (Fig. 4A). Immunoprecipitations using PLC γ 1 as the immunoprecipitating antibody yielded similar results as assays conducted with RasGRFs as the immunoprecipitating antibody (Fig. 4B). Thus PLC γ 1 associates with Ras-GRF1 and Ras-GRF2 and IL-1-induced enhancement of these associations is influenced by Ca^{2+} .

We examined whether Ras-GRF1 associates directly with PLC γ 1 in assays that used recombinant proteins with incorporated GST-tags. We first incubated cell lysates with GST-PLC γ 1 bound to beads and immunoblotted bound proteins for Ras-GRF1. Under these conditions Ras-GRF1 bound to GST-PLC γ 1 (Fig. 5A). We performed the reverse experiment in which GST-Ras-GRF1 bound to beads was incubated with cell lysates and immunoblotted for PLC γ 1, an approach that also demonstrated binding. Finally, after cleavage of the GST tag from recombinant GST-PLC γ 1, we incubated PLC γ 1 with GST-Ras-GRF1 bound to beads, and found that PLC γ 1 bound to Ras-GRF1 (Fig. 5B).

Ras-GRF1/2 regulate PLC γ 1 recruitment to focal adhesions, PLC γ 1 phosphorylation and Ca^{2+} release

As Ras-GRF1 and Ras-GRF2 associate with PLC γ 1, we assessed whether these effectors regulate recruitment of PLC γ 1 to focal adhesions. We measured the relative abundance of PLC γ 1 in focal adhesions of cells that had been treated with siRNA knockdown for either Ras-GRF1 or Ras-GRF2. In focal adhesion fractions, knockdown of Ras-GRF1 or Ras-GRF2 reduced PLC γ 1 by 30% and by 60% respectively (Fig. 6A).

Feedback and feed-forward behaviors are important features of finely tuned Ras signaling pathways [38]. As Ras-GRF1/2 may modulate PLC γ 1 function and therefore affect Ca^{2+} signaling, we examined the effects of Ras-GRF1 or Ras-GRF2 knockdown on IL-1-induced PLC γ 1 phosphorylation and ER- Ca^{2+} release. In control siRNA-treated cells, IL-1 increased PLC γ 1 phosphorylation by >75% (Fig. 6B). Knockdown of Ras-GRF1 reduced IL-1-induced PLC γ 1 phosphorylation to below levels of vehicle controls (Fig. 6B). Knockdown of Ras-GRF2 reduced

IL-1-induced PLC γ 1 phosphorylation by >50%. Consistent with these findings, knockdown of Ras-GRF1 or Ras-GRF2 reduced IL-1-stimulated ER-Ca²⁺ release by >50% (Fig. 6C). Therefore Ras-GRFs not only recruit PLC γ 1 to focal adhesions but also regulate upstream Ca²⁺ signaling in response to IL-1.

Ras-GRFs are required for IL-1-induced Ras and ERK activation

We examined the extent of Ras-GRF1 and 2 modulation of IL-1 induced Ras-GTPase activity and ERK activation. In cells in which Ras-GRF1 or Ras-GRF2 was knocked down, IL-1-stimulated GTP loading on H- and K-Ras was reduced by >90% and of N-Ras by >30% (Fig. 7A). Knockdown of both Ras-GRF1 and Ras-GRF2 reduced IL-1-induced N-Ras activation by >50%. Downstream of Ras we found that knockdown of Ras-GRF1 or Ras-GRF2 reduced IL-1-induced ERK activation to less than that of levels measured in vehicle controls (Fig. 7B). Thus Ras-GRF1 and Ras-GRF2 appear to play critical roles in IL-1-evoked Ras and ERK activation in fibroblasts.

PLC γ 1 and Ras-GRFs are involved in MMP-3 expression

We examined whether PLC γ 1 is required for IL-1-induced MMP-3 release, which was measured by immunoblotting the concentrated medium of cultured cells. In cells with PLC γ 1 knockdown, IL-1-induced MMP-3 release was reduced by >60% (Fig. 8A).

We next determined whether IL-1 induction of MMP-3 expression depends on IP₃ receptor function and ER-Ca²⁺ release. This process was examined in IL-1-stimulated cells pre-treated with 2-APB (75 μ M), which is an inhibitor of functional coupling between IP₃-sensitive ER-Ca²⁺ stores and plasma membrane channels in the generation of Ca²⁺ signals [39]. In related experiments, cells were treated with xestospongin C (100 μ M), a reversible and membrane-permeable blocker of IP₃-mediated Ca²⁺ release [40] that does not interact with the IP₃-binding site. These inhibitors were used at concentrations that we have optimized earlier and are effective for blockade of IP₃-mediated Ca²⁺ release [24, 33]. As expected, both inhibitors blocked IL-1-induced release of ER-Ca²⁺ (Fig. 9A) and IL-1-induced ERK activation ($p < 0.001$; Fig. 9B). Pre-treatment of cells with 2-APB or xestospongin C reduced IL-1-driven expression of MMP-3 mRNA (Fig. 8B) and MMP-3 protein in the culture media to control levels (Fig. 8C), suggesting that Ca²⁺ regulated processes impact MMP-3 expression.

We examined whether Ras-GRFs impact MMP-3 expression in response to IL-1. Control siRNA, Ras-GRF1 or Ras-GRF2 knockdown cells were treated with vehicle or IL-1; MMP-3 protein in the culture medium was measured by immunoblotting. Knockdown of Ras-GRF1 or Ras-GRF2 reduced IL-1-induced increases of MMP-3 by >85% (Fig. 8D). Taken together with their association with PLC γ 1 (Fig. 4A,B) and enrichment in focal adhesions (Fig. 6A), these data indicate that Ras-GRF1/2 enhance Ca²⁺-signal transduction to enable IL-1-induced activation of ERK that leads to MMP-3 expression.

DISCUSSION

IL-1 promotes degradation and increased remodeling of the extracellular matrix by augmenting the expression of endoproteinases by tissue macrophages, chondrocytes, synoviocytes and fibroblasts [1-3]. These effects reflect, at least in part, the impact of IL-1 on expression of matrix-degrading genes such as MMPs [8]. IL-1 regulates MMPs through the Ras pathway and downstream induction of mitogen-activated protein kinases [6, 7] and transcription factors such as Fos [22]. The spatially and temporally appropriate involvement of Ras proteins in enabling IL-1 signal transduction requires induction of Ras-activating GRFs, but the specific roles for Ras GRFs in IL-1 signaling have not been defined. Here we show that in fibroblasts, Ras-GRF1 and GRF2 act as both Ras exchange factors and as PLC γ 1 binding adaptors at focal adhesions that

promote Ca^{2+} -signaling downstream of IL-1 stimulation. Our data demonstrate that these exchange factors are necessary for IL-1 activation of the Ras→ERK pathway and MMP-3 expression. Consistent with these findings, Ras-GRF1 has been shown to be important for promoting MMP-3 production in rheumatoid arthritis synovial tissue [31]. Thus Ras-GRFs may play integral roles in the regulation of matrix remodeling in IL-1-related human diseases.

Although earlier reports suggest that Ras-GRF1 mediates activation of H-Ras, N-Ras and K-Ras [41], more recent data suggest that of the three Ras species, only H-Ras is activated by Ras-GRF1 [42]. Our data reveal that knockdown of Ras-GRF1 or Ras-GRF-2 is associated with very large reductions of IL-1-induced H- and K-Ras activation and to a lesser extent, N-Ras activation. The combined knockdown of these exchange factors substantially reduced activation of all three Ras isoforms. Further, knockdown of Ras-GRF1 and Ras-GRF2 strongly inhibited IL-1-induced recruitment of PLC γ 1 to focal adhesions. Thus Ras-GRF1 and Ras-GRF2 appear to play important roles in both mediating GTP exchange and in sub-cellular targeting of critical Ca^{2+} signaling proteins that are involved in IL-1 signal transduction.

Ras proteins are sequestered and function in a broad range of internal membrane environments such as the ER, plasma membrane and Golgi [36, 43-45]. Spatial sequestration provides additional modes of regulation that enable increased specificity of signal outputs [37]. We showed earlier that IL-1-induced responses in fibroblasts depend on focal adhesion sequestration of Ras isoforms [30]. Here we show that Ras-GRF1 and Ras-GRF2 localize to the focal adhesion fraction following IL-1 stimulation and that this localization is important for Ca^{2+} signaling, as well as Ras and ERK activation. Earlier reports have also shown that Ras-GRF1 movement to the plasma membrane [46, 47] and ER [48] is important for its guanine nucleotide exchange activity and that such localization may involve binding of its PH1 domain to membrane phospholipid moieties [49-51].

As Ca^{2+} regulates Ras signaling [22] through the functions of Ca^{2+} -regulated Ras-GRFs, we also explored the mechanisms whereby Ras-GRFs might modulate Ca^{2+} -dependent Ras signaling to ERK downstream of IL-1. Our data reveal that in addition to their roles as exchange factors, Ras-GRF1 and GRF2 act as adaptors that bind PLC γ 1, thereby sequestering Ca^{2+} -signaling to focal adhesions. This process is evidently required for IL-1 activation of the Ras→ERK pathway and MMP-3 expression. Notably, in addition to the Ca^{2+} -sensitive Ras-GRF regulation of Ras, CAPRI [52] and DAGK may negatively impact Ras activation in a Ca^{2+} -sensitive manner, underlining the complexity of Ca^{2+} inputs in Ras signaling [21].

We conclude that Ras-GRFs are enriched in the focal adhesions of fibroblasts after IL-1 stimulation. This enrichment facilitates sequestration of critical components of Ca^{2+} -signaling, which in turn are needed for Ras and ERK activation and for expression of matrix-degrading endoproteinases such as MMP-3.

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FIGURE LEGENDS

Figure 1. Specificity of focal adhesion-dependent IL-1 signaling. NIH 3T3 cells were plated on collagen or poly-L-lysine and then stimulated with the following reagents: A- IL-1 (20 ng/ml; 30 min); B- thrombin (2 NIH unit/ml; 30 mins); C- PDGF (20 ng/ml; 15 min) or D- TNF- α (10 ng/ml; 30 min). Ras activation was assessed as described in “Experimental”. Briefly, cells were lysed and incubated with immobilized GST-Raf1-RBD (Raf-RBD) to precipitate active (GTP-bound) Ras, which was detected with H-, K- or N-Ras antibodies, respectively, as shown in each top panel. The lower panels show the amounts of total Ras in each reaction.

Figure 2. PLC γ 1 knockdown inhibits IL-1-induced Ras and ERK activation.

A. NIH 3T3 fibroblasts were transfected with control (irrelevant) siRNA or PLC γ 1-siRNA as described in “Experimental”, stimulated without or with IL-1 (20 ng/ml) for 30 min, followed by Ras-GTP pull-down and immunoblotting analysis. Inset- lysates of control-siRNA- and PLC γ 1-siRNA-transfected cells were immunoblotted for PLC γ 1 to assess the knockdown of PLC γ 1. Densitometry of blots was conducted and means $\pm$ SE of % active Ras are shown. Data from three independent experiments were analyzed and the mean $\pm$ SE ($n=3$ replicates) are shown.

B. PLC antagonist U-73122 blocks IL-1-induced ER-Ca $^{2+}$ release. Cells were pre-treated for 10 min at 25°C with vehicle (control) or with U-73122 (3 μ M), respectively, and then stimulated with IL-1 (20 ng/ml). ER-Ca $^{2+}$ was measured in mag-fura-2-loaded cells. Mag-fura-2 ratios drop temporarily below baseline levels after stimulation with IL-1 indicating Ca $^{2+}$ efflux from the ER. Summary data (mean $\pm$ S.E.) from $n = 4-6$ experiments.

C. NIH 3T3 fibroblasts plated on tissue culture plastic in normal growth medium were pre-treated with either vehicle, or U-73122 (3 μ M) for 20 min at 25°C, respectively, before treatment with vehicle (-) or with (+) ionomycin (2 μ M) for 20 min at 25°C. Cells were then stimulated with vehicle (-) or with (+) IL-1 (20 ng/ml) for 30 min. Cell lysates were separated by SDS-PAGE and immunoblotted with phospho-ERK and total ERK antibodies. The % of phosphorylated ERK (P-ERK) was calculated from the blot densities of phospho-ERK and total-ERK. Results of three independent experiments were averaged; mean density $\pm$ SE ($n = 3$).

D. NIH 3T3 fibroblasts were treated with control or PLC γ 1 siRNA and incubated with vehicle or IL-1 (20 ng/ml) for 30 mins. Cells were immunoblotted for total and phospho-ERK and blot densities were measured as described in Experimental Procedures. Data from three independent experiments were analyzed and the mean % of densities $\pm$ SE ($n=3$ replicates) are shown.

Figure 3. A. Expression of RasGRF1 and RasGRF2 in IL-1-stimulated NIH 3T3 fibroblasts plated on collagen or poly-L-lysine. Cells were plated on collagen or poly-L-lysine-coated dishes for 5-6 h before addition of IL-1 (20 ng/ml) for 30 min. Triton-insoluble pellets were immunoblotted with antibodies to RasGRF1 or RasGRF2. Lysates from the same experiment were immunoblotted for GAPDH as loading control. The numbers beneath the blots are the mean $\pm$ SE ratios of RasGRF1 and RasGRF2 to the GAPDH controls, respectively. In the panel to the right, cells were plated on either collagen or poly-L-lysine in normal cell culture medium for 5 h and immunostained for vinculin to show dissipation of focal adhesions in cells plated on poly-L-lysine.

B. Whole cell lysates, ER fractions and focal adhesion proteins prepared from NIH 3T3 fibroblasts were immunoblotted for endogenous RasGRF1 or RasGRF2 after treatment with vehicle (-) or with (+) IL-1 (20 ng/ml) for 30 min. For loading controls, whole cell lysates, ER

fractions and focal adhesion proteins were immunoblotted for GAPDH, calnexin and $\alpha 5\beta 1$ integrin, respectively. The numbers beneath the blots represent the mean $\pm$ SE ratios of RasGRF1 and RasGRF2 to the GAPDH, calnexin and $\alpha 5\beta 1$ integrin controls, respectively.

C. NIH 3T3 fibroblasts were stimulated with vehicle or with IL-1 (20 ng/ml) for 30 min and co-immunostained for vinculin and RasGRF1 or RasGRF2 antibodies and imaged by TIRF microscopy. Histograms of Pearson's correlation coefficient (*Rr*) for vinculin and RasGRF1 or RasGRF2 co-localization were obtained by ImageJ analysis of TIRF images. Values are means $\pm$ SE from 20-30 cells in 3 independent experiments.

Figure 4. Ca^{2+} regulates PLC γ 1 association with RasGRFs.

A. NIH 3T3 fibroblasts were pre-treated for 20 min at 25°C with vehicle (Control), BAPTA/AM (5 μ M) or ionomycin (2 μ M), respectively, then stimulated with vehicle (-) or with (+) IL-1 (20 ng/ml) for 30 min. RasGRF1 or RasGRF2 were immunoprecipitated (IP) from whole-cell lysates (WCL). Immunoprecipitates were separated by SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted with antibodies for PLC γ 1, RasGRF1 or RasGRF2. Whole-cell lysates and immunoprecipitates were also immunoblotted with GAPDH antibody. Inset- negative controls for non-specific binding, purified IgG was used for immunoprecipitation from whole-cell lysates; Ras-GRF1 or RasGRF2 were immunoprecipitated from lysate buffer. WCL and immunoprecipitates were separated by SDS-PAGE and immunoblotted with antibodies for PLC γ 1, RasGRF1 or RasGRF2. The histograms show the mean $\pm$ SE % of the ratios of densities of PLC γ 1 to RasGRF1 or RasGRF2, respectively. Data from three independent experiments were analyzed and the mean ratios $\pm$ SE (*n*=3 replicates) are shown.

B. Reverse IP with anti-PLC γ 1 was done in the same conditions as A. PLC γ 1 was immunoprecipitated (IP) from whole-cell lysates of cells treated with IL-1 and with vehicle, BAPTA/AM or ionomycin. Immunoprecipitates were separated by SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted with antibodies for Ras-GRF1, RasGRF2 or PLC γ 1.

Figure 5. In vitro pull-down and binding assay of PLC γ 1 and RasGRF1.

A. Association between PLC γ 1 and RasGRF1 was assessed by pull-down assays using Glutathione-Sepharose bead-bound fusion proteins: GST-PLC γ 1 or GST-RasGRF1. GST-PLC γ 1, GST-RasGRF1 or GST-control was incubated with whole-cell lysates (WCL), respectively. Proteins bound to beads and WCL were immunoblotted for RasGRF1 and PLC γ 1. As controls, GST-PLC γ 1, GST-RasGRF1, GST-control and whole cell lysates were probed with antibodies to PLC γ 1 or RasGRF1.

B. The GST tag was removed from the GST-PLC γ 1 fusion protein by enzymatic cleavage. GST-control or GST-RasGRF1 beads were incubated with purified PLC γ 1. After centrifugation, the supernatant (S) and bead-associated proteins (in the pellet, P) were analyzed by immunoblotting for GST.

Figure 6. A. RasGRFs are important for PLC γ 1 sequestration to focal adhesions. NIH 3T3 fibroblasts were transfected with control-siRNA, or with RasGRF1 or Ras-GRF2 siRNAs, or with both RasGRF siRNAs and treated with vehicle (-) or with (+) IL-1 (20 ng/ml for 30 min). Focal adhesion proteins were prepared, separated by SDS-PAGE, transferred to nitrocellulose membranes, and probed with antibodies to PLC γ 1, RasGRF1 or RasGRF2. Focal adhesion proteins were also immunoblotted for $\alpha 5\beta 1$ integrin to assess equality of loading. Lysates of

control-siRNA- and RasGRF-siRNA-transfected cells were immunoblotted for RasGRF1 or RasGRF2 to confirm the knockdowns. Data from three independent experiments were obtained. The mean % ratios of densities $\pm$ SE ($n=3$) replicates are shown.

B. IL-1-induced PLC γ 1 phosphorylation is reduced by knockdown of RasGRF1 or RasGRF2. NIH 3T3 fibroblasts were transfected with control-siRNA, RasGRF1-siRNA or RasGRF2-siRNA and stimulated with vehicle (-) or with (+) IL-1 (20 ng/ml) for 30 min. Phospho-PLC γ 1 activity was assessed by separating lysates with SDS-PAGE and probing blots with antibody to phospho-PLC γ 1 (Tyr783). Total PLC γ 1 was assessed by probing separate blots with rabbit polyclonal antibody to PLC γ 1. The activation of PLC γ 1 was quantified by computing the % of phosphorylated PLC γ 1 (P-PLC γ 1) as a ratio of total PLC γ 1. Data from three independent experiments were averaged and the mean ratio of densities $\pm$ SE ($n=3$ replicates) are shown.

C. ER-Ca²⁺ release induced by IL-1 is inhibited by knockdown of RasGRF1 or RasGRF2. NIH 3T3 fibroblasts were transfected with control-siRNA, RasGRF1-siRNA or RasGRF2-siRNA, loaded with mag-fura-2 and stimulated with IL-1. Data in histograms are mean $\pm$ SE of mag-fura-2 ratios from 3 replicates obtained from 4 independent experiments.

Figure 7. A. NIH 3T3 fibroblasts were transfected with either control-siRNA, RasGRF1-siRNA or with Ras-GRF2-siRNA, respectively, or co- transfected with both RasGRF1-siRNA and Ras-GRF2-siRNA. Cells were stimulated with vehicle (-) or with IL-1 (+) (20 ng/ml) for 30 min, followed by Ras-GTP pull-down and immunoblotting analysis as described in Fig.1. Lysates were immunoblotted for RasGRF1 or RasGRF2 to confirm the efficacy of knockdown of RasGRF1 or RasGRF2 or both. Data in the histograms are mean $\pm$ SE of % ratios of active to total Ras, which were computed from 3 replicates obtained from 4 separate experiments.

B. RasGRF1 and RasGRF2 are required for IL-1-induced ERK activation. NIH3T3 fibroblasts were transfected with control-siRNA, RasGRF1-siRNA or RasGRF2-siRNA and stimulated with vehicle (-) or with (+)IL-1 (20 ng/ml) for 30 min. Phospho-ERK activity was assessed by immunoblotting. Ratios of phospho-ERK to total ERK were quantified by densitometry and shown as % mean ratios of densities $\pm$ SE of 4 replicates from 3 separate experiments.

Figure 8. A. MMP-3 release in response to IL-1 requires PLC γ 1 expression. NIH 3T3 fibroblasts were transfected with control (irrelevant) siRNA or with PLC γ -siRNA as described in "Experimental", stimulated without or with IL-1 (20 ng/ml) for 30 min. The conditioned media were collected, concentrated 10 times, separated by SDS-PAGE and immunoblotted for MMP-3. Whole cell lysates prepared from same experiment were immunoblotted for GAPDH. Quantification by densitometry of immunoblots are shown as histograms of mean % ratios of densities $\pm$ SE ($n=4$ replicates from 3 separate experiments). Efficacy of PLC γ 1 knockdown is shown in immunoblot for PLC γ 1 from equal amounts of cell lysates.

B. ER-Ca²⁺ release is required for IL-1-induced MMP-3 protein production. NIH 3T3 fibroblasts plated on fibronectin-coated tissue culture plastic dishes in normal growth medium were pre-treated with either vehicle (control), 75 μ M 2-APB or 100 μ M xestospongine C for 30 min at 25°C, respectively before stimulation with vehicle (-) or with (+) IL-1. Cells were analyzed by quantitative RT-PCR as described in Experimental Procedures. Data are mean $\pm$ SE of % differences relative to MMP-3 mRNA levels normalized to GAPDH mRNA levels. Data were analyzed from 3 replicates obtained from 4 independent experiments.

C. Same treatments as in panel 6B but conditioned media from cell culture were processed as described in panel 6A. Quantification by densitometry of immunoblots are shown as histograms of mean ratio of densities $\pm$ SE ($n=3$ replicates from 4 independent experiments).

D. NIH 3T3 fibroblasts were transfected with control-siRNA, RasGRF1-siRNA or RasGRF2-siRNA, respectively and then stimulated with vehicle (-) or with (+) IL-1 (20 ng/ml) for 30 min. Conditioned media from the cell cultures were analyzed for MMP-3 protein as described above. Whole cell lysates prepared from same experiment were immunoblotted for GAPDH. Quantification by densitometry of immunoblots are shown as histograms of mean ratios of densities $\pm$ SE of MMP-3 to GAPDH ($n=4$ replicates from 3 independent experiments). Immunoblots (inset) show efficacy of Ras-GRF knockdown.

Figure 9.A. IP₃ receptor antagonists, 2-APB and xestospongine C inhibit IL-1-induced ER-Ca²⁺ release. Experiments were conducted in Ca²⁺-free buffer. NIH 3T3 fibroblasts were pre-incubated for 30 min at 25°C in the presence of either vehicle (control), 75 μ M 2-APB or 100 μ M xestospongine C. Cells were stimulated with IL-1 (20 ng/ml). ER-Ca²⁺ was measured in mag-fura-2-loaded cells. Summary data (mean $\pm$ S.E.) are from $n = 4-6$ experiments.

B. IL-1-induced ERK activation is inhibited in cells pre-treated with 2-APB or xestospongine C. NIH 3T3 fibroblasts plated on tissue culture plastic in normal growth medium were pre-treated with vehicle, 75 μ M 2-APB or 100 μ M xestospongine C for 30 min at 25°C, respectively, before stimulation with vehicle (-) or with (+) IL-1 (20 ng/ml) for 30 min. The % of phospho-ERK was quantified as in Figure 7B. Results of three independent experiments were averaged; mean density $\pm$ SE ($n = 3$).

References

- 1 van den Berg, W. B. (1999) The role of cytokines and growth factors in cartilage destruction in osteoarthritis and rheumatoid arthritis. *Z. Rheumatol.* **58**, 136-141
- 2 Dinarello, C. A. (1996) Biologic basis for interleukin-1 in disease. *Blood.* **87**, 2095-2147
- 3 O'Neill, L. (2000) The Toll/interleukin-1 receptor domain: a molecular switch for inflammation and host defence. *Biochem. Soc. Trans.* **28**, 557-563
- 4 Boyle, D. L., Han, Z., Rutter, J. L., Brinckerhoff, C. E. and Firestein, G. S. (1997) Posttranscriptional regulation of collagenase-1 gene expression in synoviocytes by adenosine receptor stimulation. *Arthritis Rheum.* **40**, 1772-1779
- 5 Bhat-Nakshatri, P., Newton, T. R., Goulet, R., Jr. and Nakshatri, H. (1998) NF-kappaB activation and interleukin 6 production in fibroblasts by estrogen receptor-negative breast cancer cell-derived interleukin 1alpha. *Proc. Natl. Acad. Sci. U. S. A.* **95**, 6971-6976
- 6 Brauchle, M., Gluck, D., Di Padova, F., Han, J. and Gram, H. (2000) Independent role of p38 and ERK1/2 mitogen-activated kinases in the upregulation of matrix metalloproteinase-1. *Exp. Cell Res.* **258**, 135-144
- 7 Reunanen, N., Westermarck, J., Hakkinen, L., Holmstrom, T. H., Elo, I., Eriksson, J. E. and Kahari, V. M. (1998) Enhancement of fibroblast collagenase (matrix metalloproteinase-1) gene expression by ceramide is mediated by extracellular signal-regulated and stress-activated protein kinase pathways. *J. Biol. Chem.* **273**, 5137-5145
- 8 Wang, Q., Rajshankar, D., Laschinger, C., Talior-Volodarsky, I., Wang, Y., Downey, G. P. and McCulloch, C. A. (2010) Importance of protein-tyrosine phosphatase-alpha catalytic domains for interactions with SHP-2 and interleukin-1-induced matrix metalloproteinase-3 expression. *J. Biol. Chem.* **285**, 22308-22317
- 9 Downward, J. (1998) Ras signalling and apoptosis. *Curr. Opin. Genet. Dev.* **8**, 49-54
- 10 Campbell, S. L., Khosravi-Far, R., Rossman, K. L., Clark, G. J. and Der, C. J. (1998) Increasing complexity of Ras signaling. *Oncogene.* **17**, 1395-1413
- 11 Rebollo, A. and Martinez, A. C. (1999) Ras proteins: recent advances and new functions. *Blood.* **94**, 2971-2980
- 12 Takai, Y., Sasaki, T. and Matozaki, T. (2001) Small GTP-binding proteins. *Physiol. Rev.* **81**, 153-208
- 13 Wolfman, A. and Macara, I. G. (1990) A cytosolic protein catalyzes the release of GDP from p21ras. *Science.* **248**, 67-69
- 14 Martegani, E., Vanoni, M., Zippel, R., Coccetti, P., Brambilla, R., Ferrari, C., Sturani, E. and Alberghina, L. (1992) Cloning by functional complementation of a mouse cDNA encoding a homologue of CDC25, a *Saccharomyces cerevisiae* RAS activator. *EMBO J.* **11**, 2151-2157
- 15 Shou, C., Farnsworth, C. L., Neel, B. G. and Feig, L. A. (1992) Molecular cloning of cDNAs encoding a guanine-nucleotide-releasing factor for Ras p21. *Nature.* **358**, 351-354
- 16 Wei, W., Mosteller, R. D., Sanyal, P., Gonzales, E., McKinney, D., Dasgupta, C., Li, P., Liu, B. X. and Broek, D. (1992) Identification of a mammalian gene structurally and functionally related to the CDC25 gene of *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci U S A.* **89**, 7100-7104
- 17 Cen, H., Papageorge, A. G., Zippel, R., Lowy, D. R. and Zhang, K. (1992) Isolation of multiple mouse cDNAs with coding homology to *Saccharomyces cerevisiae* CDC25: identification of a region related to Bcr, Vav, Dbl and CDC24. *EMBO J.* **11**, 4007-4015
- 18 Farnsworth, C. L., Freshney, N. W., Rosen, L. B., Ghosh, A., Greenberg, M. E. and Feig, L. A. (1995) Calcium activation of Ras mediated by neuronal exchange factor Ras-GRF. *Nature.* **376**, 524-527

- 19 Buchsbaum, R., Telliez, J. B., Goonesekera, S. and Feig, L. A. (1996) The N-terminal pleckstrin, coiled-coil, and IQ domains of the exchange factor Ras-GRF act cooperatively to facilitate activation by calcium. *Mol. Cell. Biol.* **16**, 4888-4896
- 20 Freshney, N. W., Goonesekera, S. D. and Feig, L. A. (1997) Activation of the exchange factor Ras-GRF by calcium requires an intact Dbl homology domain. *FEBS Lett.* **407**, 111-115
- 21 Cullen, P. J. and Lockyer, P. J. (2002) Integration of calcium and Ras signalling. *Nat. Rev. Mol. Cell Biol.* **3**, 339-348
- 22 Lo, Y. Y., Luo, L., McCulloch, C. A. and Cruz, T. F. (1998) Requirements of focal adhesions and calcium fluxes for interleukin-1-induced ERK kinase activation and c-fos expression in fibroblasts. *J. Biol. Chem.* **273**, 7059-7065
- 23 Wang, Q., Downey, G. P., Herrera-Abreu, M. T., Kapus, A. and McCulloch, C. A. (2005) SHP-2 modulates interleukin-1-induced Ca²⁺ flux and ERK activation via phosphorylation of phospholipase Cγ1. *J. Biol. Chem.* **280**, 8397-8406
- 24 Wang, Q., Herrera Abreu, M. T., Siminovitch, K., Downey, G. P. and McCulloch, C. A. (2006) Phosphorylation of SHP-2 regulates interactions between the endoplasmic reticulum and focal adhesions to restrict interleukin-1-induced Ca²⁺ signaling. *J. Biol. Chem.* **281**, 31093-31105
- 25 Luo, L., Cruz, T. and McCulloch, C. (1997) Interleukin 1-induced calcium signalling in chondrocytes requires focal adhesions. *Biochem. J.* **324** (Pt 2), 653-658
- 26 Arora, P. D., Ma, J., Min, W., Cruz, T. and McCulloch, C. A. (1995) Interleukin-1-induced calcium flux in human fibroblasts is mediated through focal adhesions. *J. Biol. Chem.* **270**, 6042-6049
- 27 Carragher, N. O. and Frame, M. C. (2004) Focal adhesion and actin dynamics: a place where kinases and proteases meet to promote invasion. *Trends Cell Biol.* **14**, 241-249
- 28 Alahari, S. K., Reddig, P. J. and Juliano, R. L. (2002) Biological aspects of signal transduction by cell adhesion receptors. *Int. Rev. Cytol.* **220**, 145-184
- 29 Howe, A. K., Aplin, A. E. and Juliano, R. L. (2002) Anchorage-dependent ERK signaling--mechanisms and consequences. *Curr. Opin. Genet. Dev.* **12**, 30-35
- 30 Wang, Q., Downey, G. P. and McCulloch, C. A. (2011) Focal adhesions and Ras are functionally and spatially integrated to mediate IL-1 activation of ERK. *FASEB J.* **25**, 3448-3464
- 31 Abreu, J. R., de Launay, D., Sanders, M. E., Grabiec, A. M., van de Sande, M. G., Tak, P. P. and Reedquist, K. A. (2009) The Ras guanine nucleotide exchange factor RasGRF1 promotes matrix metalloproteinase-3 production in rheumatoid arthritis synovial tissue. *Arthritis Research & Therapy.* **11**, R121
- 32 Wang, Q., Rajshankar, D., Branch, D. R., Siminovitch, K. A., Herrera Abreu, M. T., Downey, G. P. and McCulloch, C. A. (2009) Protein-tyrosine phosphatase-α and Src functionally link focal adhesions to the endoplasmic reticulum to mediate interleukin-1-induced Ca²⁺ signaling. *J. Biol. Chem.* **284**, 20763-20772
- 33 Wang, Q., Downey, G. P., Choi, C., Kapus, A. and McCulloch, C. A. (2003) IL-1 induced release of Ca²⁺ from internal stores is dependent on cell-matrix interactions and regulates ERK activation. *FASEB J.* **17**, 1898-1900
- 34 Yule, D. I. and Williams, J. A. (1992) U73122 inhibits Ca²⁺ oscillations in response to cholecystokinin and carbachol but not to JMV-180 in rat pancreatic acinar cells. *J. Biol. Chem.* **267**, 13830-13835
- 35 Kam, Y. and Exton, J. H. (2001) Phospholipase D activity is required for actin stress fiber formation in fibroblasts. *Mol. Cell Biol.* **21**, 4055-4066

- 36 Chiu, V. K., Bivona, T., Hach, A., Sajous, J. B., Silletti, J., Wiener, H., Johnson, R. L., 2nd, Cox, A. D. and Philips, M. R. (2002) Ras signalling on the endoplasmic reticulum and the Golgi. *Nat. Cell Biol.* **4**, 343-350
- 37 Hancock, J. F. (2003) Ras proteins: different signals from different locations. *Nat. Rev. Mol. Cell Biol.* **4**, 373-384
- 38 Harding, A. and Hancock, J. F. (2008) Ras nanoclusters: combining digital and analog signaling. *Cell Cycle*. **7**, 127-134
- 39 Ma, H. T., Venkatachalam, K., Li, H. S., Montell, C., Kurosaki, T., Patterson, R. L. and Gill, D. L. (2001) Assessment of the role of the inositol 1,4,5-trisphosphate receptor in the activation of transient receptor potential channels and store-operated Ca^{2+} entry channels. *J. Biol. Chem.* **276**, 18888-18896
- 40 Hu, Q., Deshpande, S., Irani, K. and Ziegelstein, R. C. (1999) $[\text{Ca}^{2+}]_i$ oscillation frequency regulates agonist-stimulated NF- κ B transcriptional activity. *J. Biol. Chem.* **274**, 33995-33998
- 41 Coccetti, P., Mauri, I., Alberghina, L., Martegani, E. and Parmeggiani, A. (1995) The minimal active domain of the mouse ras exchange factor CDC25Mm. *Biochem. Biophys. Res. Commun.* **206**, 253-259
- 42 Jacquet, E., Baouz, S. and Parmeggiani, A. (1995) Characterization of mammalian C-CDC25Mm exchange factor and kinetic properties of the exchange reaction intermediate p21.C-CDC25Mm. *Biochemistry*. **34**, 12347-12354
- 43 Roy, S., Wyse, B. and Hancock, J. F. (2002) H-Ras signaling and K-Ras signaling are differentially dependent on endocytosis. *Mol. Cell Biol.* **22**, 5128-5140
- 44 Perez de Castro, I., Bivona, T. G., Philips, M. R. and Pellicer, A. (2004) Ras activation in Jurkat T cells following low-grade stimulation of the T-cell receptor is specific to N-Ras and occurs only on the Golgi apparatus. *Mol. Cell Biol.* **24**, 3485-3496
- 45 Matallanas, D., Sanz-Moreno, V., Arozarena, I., Calvo, F., Agudo-Ibanez, L., Santos, E., Berciano, M. T. and Crespo, P. (2006) Distinct utilization of effectors and biological outcomes resulting from site-specific Ras activation: Ras functions in lipid rafts and Golgi complex are dispensable for proliferation and transformation. *Mol. Cell Biol.* **26**, 100-116
- 46 Cen, H., Papageorge, A. G., Vass, W. C., Zhang, K. E. and Lowy, D. R. (1993) Regulated and constitutive activity by CDC25Mm (GRF), a Ras-specific exchange factor. *Mol. Cell Biol.* **13**, 7718-7724
- 47 Quilliam, L. A., Huff, S. Y., Rabun, K. M., Wei, W., Park, W., Broek, D. and Der, C. J. (1994) Membrane-targeting potentiates guanine nucleotide exchange factor CDC25 and SOS1 activation of Ras transforming activity. *Proc. Natl. Acad. Sci. U S A.* **91**, 8512-8516
- 48 Arozarena, I., Matallanas, D., Berciano, M. T., Sanz-Moreno, V., Calvo, F., Munoz, M. T., Egea, G., Lafarga, M. and Crespo, P. (2004) Activation of H-Ras in the endoplasmic reticulum by the RasGRF family guanine nucleotide exchange factors. *Mol. Cell Biol.* **24**, 1516-1530
- 49 Lemmon, M. A. and Ferguson, K. M. (2000) Signal-dependent membrane targeting by pleckstrin homology (PH) domains. *Biochem. J.* **350 Pt 1**, 1-18
- 50 Harlan, J. E., Hajduk, P. J., Yoon, H. S. and Fesik, S. W. (1994) Pleckstrin homology domains bind to phosphatidylinositol-4,5-bisphosphate. *Nature*. **371**, 168-170
- 51 Maffucci, T. and Falasca, M. (2001) Specificity in pleckstrin homology (PH) domain membrane targeting: a role for a phosphoinositide-protein co-operative mechanism. *FEBS Lett.* **506**, 173-179

52 Liu, Q., Walker, S. A., Gao, D., Taylor, J. A., Dai, Y. F., Arkell, R. S., Bootman, M. D., Roderick, H. L., Cullen, P. J. and Lockyer, P. J. (2005) CAPRI and RASAL impose different modes of information processing on Ras due to contrasting temporal filtering of Ca^{2+} . *J. Cell Biol.* **170**, 183-190

Figure 1

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