Novel Role for p21-activated Kinase 2 in Thrombin-induced Monocyte Migration*

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Background: The major goal of this study is to test the hypothesis that thrombin plays a role in inflammation.

Results: Thrombin stimulates monocyte F-actin cytoskeletal remodeling and migration by PAR1, Gα12, Pyk2, Gab1, Rac1, and RhoA-dependent Pak2 activation.

Conclusion: Pak2 mediates thrombin-PAR1-induced monocyte/macrophage migration.

Significance: PAR1 could be a potential target for the development of anti-inflammatory drugs.

To understand the role of thrombin in inflammation, we tested its effects on migration of THP-1 cells, a human monocyte cell line. Thrombin induced THP-1 cell migration in a dose-dependent manner. Thrombin induced tyrosine phosphorylation of Pyk2, Gab1, and p115 RhoGEF, leading to Rac1- and RhoA-dependent Pak2 activation. Downstream to Pyk2, Gab1 formed a complex with p115 RhoGEF involving their pleckstrin homology domains. Furthermore, inhibition or depletion of Pyk2, Gab1, p115 RhoGEF, Rac1, RhoA, or Pak2 levels substantially attenuated thrombin-induced THP-1 cell F-actin cytoskeletal remodeling and migration. Inhibition or depletion of PAR1 also blocked thrombin-induced activation of Pyk2, Gab1, p115 RhoGEF, Rac1, RhoA, and Pak2, resulting in diminished THP-1 cell F-actin cytoskeletal remodeling and migration. Similarly, depletion of Gα12 negated thrombin-induced Pyk2, Gab1, p115 RhoGEF, Rac1, RhoA, and Pak2 activation, leading to attenuation of THP-1 cell F-actin cytoskeletal remodeling and migration. These novel observations reveal that thrombin induces monocyte/macrophage migration via PAR1-Gα12-dependent Pyk2-mediated Gab1 and p115 RhoGEF interactions, leading to Rac1- and RhoA-targeted Pak2 activation. Thus, these findings provide mechanistic evidence for the role of thrombin and its receptor PAR1 in inflammation.

Atherosclerosis is the major cause of mortality and morbidity in the world (1). Inflammation appears to be the major factor underlying the atherogenesis, and it may be initiated with endothelial cell dysfunction, leading to leukocyte adhesion, ROS production, and low density lipoprotein (LDL) oxidation (2, 3). Several studies have demonstrated that the recruitment of leukocytes and monocytes/macrophages to the site of vascular injury is dependent on the production of cytokines and adhesion molecules by the dysfunctional endothelium, resulting in their adherence to the endothelium and their subsequent trans-endothelialization (4, 5). In addition, the injured/dysfunctional endothelium may expose the underlying vascular matrix to the flowing blood, which can lead to activation of platelets and production of thrombin (6, 7). Thrombin, which is an extracellular protease, plays an important role in blood clotting by converting fibrinogen to fibrin (7). A large amount of literature suggests that thrombin, besides its pivotal role in clotting, acts as a mitogen and chemotactic factor to a variety of cell types, including smooth muscle cells (8, 9). Thrombin mediates its effects via a family of G protein-coupled receptors (GPCRs),2 namely, protease-activated receptors (PARs) (10, 11). PARs are activated by enzymatic cleavage of their extracellular N-terminal end, exposing a new N terminus, which acts as a tethered ligand. The tethered ligand then activates the serine protease receptor by folding back onto the extracellular second loop. Among the four receptors identified to date, thrombin cleaves PAR1, PAR3, and PAR4, with the highest affinity for PAR1 (10–12). PARs mediate blood clotting and mitogenic and chemotactic effects of thrombin via coupling to trimeric G proteins, mostly Gα12, Gα13, and Gα12/13 (13, 14). Furthermore, the activation of different G proteins leads to stimulation of various phospholipases, particularly phospholipases Cβ, resulting in the hydrolysis of phosphatidylinositol 4,5-bisphosphate and the production of inositol 1,4,5-trisphosphate and diacylglycerol, which then causes Ca2+ mobilization and PKC activation (15, 16). Recently, we have reported that thrombin stimulates Pyk2, a Ca2+-dependent proline-rich tyrosine kinase, in mediating human aortic smooth muscle cell (HASMC) migration and proliferation and that the activation of Pyk2 is essential for balloon injury-induced neointima formation (17). A role for Pyk2 in atherosclerosis has also been reported previously (18). Because thrombin is produced at the site of vascular injury (6, 7) and it causes endothelial cell barrier disruption (19) and inflammation (20), we asked the question of whether it also influences the migration of monocytes/macrophages and, if so, whether it requires Pyk2 activation. In the present study, we report that

2 The abbreviations used are: GPCR, G protein-coupled receptor; PAR, protease-activated receptor; HASMC, human aortic smooth muscle cell; SH2 and SH3, Src homology 2 and 3, respectively; PKC, protein kinase C; SH2 and SH3, Src homology 2 and 3, respectively; PH, pleckstrin homology domain; BM, binding motif; ASO, antisense oligonucleotide; TRITC, tetramethylrhodamine isothiocyanate; GEF, guanine nucleotide exchange factor; IB, immunoblot; IP, immunoprecipitation.
thrombin induces THP-1 cell migration, and this effect requires PAR1-Gø12,15-mediated Pyk2-dependent Gab1-p115 RhoGEF interactions, leading to Rac1- and RhoA-targeted Pak2 activation.

**MATERIALS AND METHODS**

**Reagents**—Aprotinin, dithiothreitol, HEPES, hydroxyurea, gelatin, leupeptin, pertussis toxin, phenylmethylsulfonyl fluoride (PMSF), sodium orthovanadate, and sodium deoxycholate were obtained from Sigma. Growth factor-reduced Matrigel (catalog no. 354230) was purchased from BD Biosciences. Recombinant human thrombin (catalog no. 194083) was bought from MP Biomedicals Inc. (Solon, OH), EHT1864 (catalog no. 3872) and SCH79797 (catalog no. 1592) were purchased from Tocris Bioscience (Ellisville, MO), and Pyk2 inhibitor (PF431396) was obtained from Chiyoda-ku, Japan. Anti-phospho-Pak (Thr-423) (ab2477) and anti-Pyk2 (ab32571) antibodies were bought from Abcam Inc. (Cambridge, MA). Anti-Park2 (2608S) and anti-phospho-Pyk2 (Tyr-724) were amplified using the following primers: WT Gab1 (5'-GATTCATCCTCTGCTACTCTACC-3') and antisense (5'-GCTGGAATGTTGTCGT-3') and antisense (5'-GGAGCGGTGGTGAAGT-3') and antisense (5'-GGCGGUTCTGCGTACG-3'). Huh7 cells were grown in DMEM/F-12 medium supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, and 5% (v/v) fetal bovine serum, and RAW264.7 cells were grown in DMEM/F-12 medium supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% (v/v) fetal bovine serum (Invitrogen). DNA Constructs—Wild type (WT) Gab1 encoding amino acids 1–724; Gab1 ΔPH encoding amino acids 152–724; Gab1 pleckstrin homology (PH) domain encoding amino acids 1–151; the SH2-binding motif (SH2-BM) encoding amino acids 1–912; p115 RhoGEF encoded amino acids 1–350; DH/Dbl domain encoding amino acids 351–625; and the SH3-binding motif (SH3-BM) encoding amino acids 463–462; p115 RhoGEF codons 1–724; Gab1 (23). To clone p115 RhoGEF domains, total RNA extracted from HAsMCs using TRIzol reagent was reverse transcribed using the GeneAmp® RNA PCR kit (Applied Biosystems). The cDNA was then used to amplify WT p115 RhoGEF encoding amino acids 1–912; p115 RhoGEF ΔPH encoding amino acids 1–625; p115 RhoGEF RGS domain encoding amino acids 1–350; DH/Dbl domain encoding amino acids 351–625; and PH domain encoding amino acids 626–762 (24) using the following primers: WT p115 RhoGEF (NM_001992.3), ASO1 (5'-GCCUU-CGACAGGTTTTCATUGAGCA-3'), ASO2 (5'-GCCUU-CGACAGGTTTTCATUGAGCA-3') and antisense (5'-TGGTACCTCAAGTGCAGCCAGG-3') and antisense (5'-TGGTACCTCAAGTGCAGCCAGG-3') and antisense (5'-ATGGTACCTCAAGTGCAGCCAGG-3') and antisense (5'-ATGGTACCTCAAGTGCAGCCAGG-3'). All of the sense strand primer sequences are shown in the boldface text. All amplifications were performed using High Fidelity Taq polymerase (Clontech) as per the manufacturer’s instructions. The purified PCR products were digested with EcoRI and Kpn1 and ligated with EcoRI and Kpn1-digested HA-tagged (Gab1 constructs) or Myc-tagged (p115 RhoGEF constructs) pCMV. The cloned inserts of Gab1 and p115 RhoGEF were verified by sequencing using vector primers.
002067.2), ASO1 (5′-GCGUCUTGCTCTCCATCGCGU-GUU-3′), ASO2 (5′-UCACCTTCTCGCTCGAC-3′); hGNA12 (NM_007353.2), ASO1 (5′-GGUUGGAAATGTTGTTAAGAGAGUGG-3′), ASO2 (5′-GGCAAACTCTGCTTCAGCGU-3′); hPyk2 (NM_004103), ASO1 (5′-GUUCUGTTACTTAGGTCCGCGGGGC-3′), ASO2 (5′-CCUGTTCCATAGCTGACAGUCCC-3′), ASO3 (5′-GGUCUCCACATC-TGCCUCU-3′); hGab1 (NM_207123), ASO1 (5′-GUUCGCT-TCTCACCATCACTUUCCU-3′), ASO2 (5′-GGUCUGTTAC-TTAGGTCCGCGGGGC-3′), ASO3 (5′-CATGCTAAACGC-TTCACT-3′); hRhoGEF (NM_199002), ASO1 (5′-UUCUCCATCACTCCATCCCA-3′), ASO2 (5′-CCUUGCCTCTTAGTCAATCCGC-3′), ASO3 (5′-GCUUACCTGGCTCTUGGGC-3′); hRac1 (NM_006908), ASO1 (5′-UCCGUCTCCACACGACACAUU-3′), ASO2 (5′-UUCUCCATCTCTCTCCUACU-3′); hRhoA (NM_001664), ASO1 (5′-ACUCU-ATCTGCTTCATCCACACUCCU-3′), ASO2 (5′-UGGUTGTCAGGTGGGAGUGG-3′), ASO3 (5′-ACUCUTCTCATTCC-CUCCUCCU-3′); hPak2 (NM_002577), ASO1 (5′-UCAUCATCATC-ATCTCTCCUCUGUCG-3′), ASO2 (5′-GGUUGGGGGCTGTTCCUUGGC-3′), ASO3 (5′-GTCACCAATCTCTCCTCC-3′). All of the ASOs have two terminal phosphodiester bonds replaced with phosphorothioate bonds during synthesis to extend their half-life inside the cells. The THP-1 cells were transfected with ASOs at 2 nmol/ml concentration using Lipofectin reagent obtained from Invitrogen following the manufacturer’s protocol. Plasmids at 5 μg of DNA/5 × 10^5 cells were used for transfections. After a 6-h incubation period with the oligonucleotides or plasmid DNAs, cells were replaced with fresh RPMI medium containing FBS and maintained for 24 h, after which the cells were growth-arrested in serum-free RPMI medium overnight.

Cell Migration—THP-1 and RAW 264.7 cell migration was measured using a modified Boyden chamber migration assay using Transwell inserts with 8-μm porous membrane (Nalgene Nunc International, Rochester, NY) as described by Park et al. (25). The outer surface of the membrane was coated with growth factor-reduced Matrigel (70%), and the inserts were placed in a 24-well tissue culture plate (coated surface of the membrane facing toward the outer chamber). Quiescent cells were seeded into the inner chamber (onto the non-coated inner membrane facing toward the outer chamber). Quiescent cells were placed in a 24-well tissue culture plate (coated surface of the membrane) at 1–5 × 10^5 cells/well. Vehicle or agonists were added to the outer chamber at the indicated concentrations. Hydroxyurea (5 mM) was added to the medium overnight. After a 6-h incubation period with the indicated treatments, cells on the inner side of the membrane were washed with PBS, and the membrane was fixed in 3.7% formaldehyde in PBS for 20 min, permeabilized in 0.2% Triton X-100 for 5 min, and blocked with 1% bovine serum albumin in PBS. Cells were stained with rhodamine-conjugated phalloidin (Biotium, Hayward, CA) for 30 min. Fluorescence was observed under a Zeiss inverted fluorescent microscope (Zeiss AxioObserver.Z1) via a ×40, numerical aperture 0.6 objective, and pictures were captured using AxioCam MRm camera.

Immunoprecipitation—After rinsing with cold phosphate-buffered saline (PBS), cells were lysed in 250 μl of lysis buffer (PBS, 1% Nonidet P40, 0.5% sodium deoxycholate, 0.1% SDS, 100 μg/ml PMSF, 100 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 mM sodium orthovanadate) for 20 min on ice. The cell extracts were cleared by centrifugation at 12,000 rpm for 20 min at 4 °C. The cleared cell extracts containing an equal amount of protein from control and the indicated treatments were incubated with the indicated antibodies overnight at 4 °C, followed by incubation with protein A/G-Sepharose CL4B beads for 2 h with gentle rocking. The beads were collected by centrifugation at 4000 rpm for 1 min at 4 °C and washed four times with lysis buffer and once with PBS. The immunocomplexes were released by heating the beads in 40 μl of Laemmli sample buffer and analyzed by Western blotting for the indicated molecules using their specific antibodies.

Pak1/2 Activities—Cell extracts consisting of equal amounts of protein from control and each treatment were analyzed for Pak1/2 activities as described previously (28). The 32P-labeled MBP band was visualized by autoradiography, and the band intensities were quantified using ImageJ (National Institutes of Health).

Pull-down Assay—Equal amounts of protein from control and treatments were incubated with GST-Pak or GST-rho-tek-in-conjugated glutathione-Sepharose 4B beads for 45 min at 4°C. The beads were collected by centrifugation, washed in lysis buffer, and heated in Laemmli sample buffer for 5 min, and the released proteins were resolved on 10% SDS-12% PAGE and immunoblotted with anti-Rac1 or anti-RhoA antibodies.

Western Blotting—An equal amount of protein from control and treatment samples was separated by SDS-PAGE and immunoblotted for the indicated molecules using their specific antibodies. The antigen-antibody complexes were detected by enhanced chemiluminescence Western blotting detection reagents (GE Healthcare).

Statistics—All of the experiments were repeated three times, and the data are presented as means ± S.D. Student’s t test was used to perform statistical analysis, and p values of <0.05 were considered statistically significant.

RESULTS

Thrombin Stimulates THP-1 Cell Migration via Gab1—To understand the potential mechanisms of monocyte/macrophage recruitment to the sites of endothelial injury, we studied DNA Synthesis—DNA synthesis was measured by [3H]thymidine incorporation as described previously and expressed as counts/min/dish (27).

Phalloidin Staining—THP-1 cells were grown on glass cover-slips coated with 1× attachment factor, containing 0.1% gelatin (Cascade Biologics, Carlsbad, CA). After appropriate treatments, cells were fixed in 3.7% formaldehyde in PBS for 20 min, permeabilized in 0.2% Triton X-100 for 5 min, and blocked with 1% bovine serum albumin in PBS. Cells were then stained with 20 μm rhodamine-conjugated phalloidin (Biotium, Hayward, CA) for 30 min. Fluorescence was observed using a Zeiss inverted fluorescent microscope (Zeiss AxioObserver.Z1) via a ×40, numerical aperture 0.6 objective, and pictures were captured using AxioCam MRm camera.
the role of thrombin. Thrombin induced a human monocytic cell line, THP-1, cell migration in a dose-dependent manner with maximum effect at 0.5 unit/ml (Fig. 1A, left). In addition, the time course experiment shows that nearly maximal effect of thrombin on THP-1 cell migration occurs at 8 h (Fig. 1A, right). In order to examine the potency of thrombin in the stimulation of monocyte/macrophage migration, we compared its effects with monocyte chemotactic protein-1 (MCP-1), a potent monocyte chemoattractant, and platelet-derived growth factor-BB (PDGF-BB), a receptor tyrosine kinase agonist, on THP-1 cell migration. Thrombin, although found to be as potent as that of PDGF-BB, exhibited 80% of the capacity of MCP-1 on THP-1 cell migration (Fig. 1B, left). To find whether thrombin exerts chemotactic effects on monocytes/macrophages of other species, we tested its effects on a murine macrophage cell line, RAW 264.7, cell migration. Consistent with its effects on THP-1 cell migration, thrombin induced RAW 264.7 cell migration as well (Fig. 1B, middle). In order to find whether thrombin influences monocyte/macrophage migration by stimulating their proliferation, we next tested its effects on THP-1 cell DNA synthesis. Thrombin, although inducing DNA synthesis in HASMCs, failed to trigger a similar response in THP-1 cells, suggesting that it does not influence monocyte/macrophage proliferation (Fig. 1B, right). Because hydroxyurea was used to prevent replicative DNA synthesis in the migration assay, we also examined whether it has any influence on THP-1 cell migration. The presence of hydroxyurea in the medium did not affect thrombin-induced THP-1 cell migration as compared with the assay condition without hydroxyurea in the medium, suggesting that hydroxyurea by itself does not influ-

FIGURE 1. Gab1 mediates thrombin-induced THP-1 cell migration. A, left, quiescent THP-1 cells were treated with vehicle or the indicated doses of thrombin for 8 h, and their migration was measured by the modified Boyden chamber method. Right, quiescent THP-1 cells were subjected to the indicated time periods of thrombin (0.5 unit/ml)-induced migration. B, left, quiescent THP-1 cells were subjected to thrombin (0.5 unit/ml)-, MCP-1 (50 ng/ml)-, or PDGF-BB (20 ng/ml)-induced migration. Middle, quiescent RAW264.7 cells were subjected to thrombin (0.5 unit/ml)-induced migration. Right, quiescent THP-1 cells or HASMCs were treated with vehicle or thrombin (0.5 unit/ml) for 36 h, and DNA synthesis was measured by "Hthymidine incorporation. C, left, thrombin (0.5 unit/ml)-induced THP-1 cell migration was conducted in the presence and absence of hydroxyurea (5 mM) in the medium. Right, thrombin (0.5 unit/ml)-induced THP-1 cell migration was conducted using the membrane coated with growth factor-reduced Matrigel (70%) or 0.1% gelatin on its outer surface. D, equal amounts of protein from quiescent control and thrombin (0.5 unit/ml)-treated THP-1 cells were immunoprecipitated with anti-Gab1 or anti-PY20 antibodies, and the immunocomplexes were analyzed by Western blotting using anti-PY20 and anti-Gab1 antibodies, respectively. The anti-Gab1 antibody immunoprecipitated blot was normalized for the total Gab1 levels. E, 48 h after transfection with control or the indicated Gab1 ASOs, cell extracts were prepared, and an equal amount of protein from control and each treatment was analyzed by Western blotting for Gab1, CDK4, and ß-tubulin levels using their specific antibodies to show the effect of the ASOs on their target and off-target molecules. F, THP-1 cells that were transfected with control or the indicated Gab1 ASO and quiesced were subjected to thrombin (0.5 unit/ml)-induced migration. The numbers of cells used for the migration assay in A–C and F were 2 × 10^5 and 1 × 10^5, respectively. The bar graphs represent the mean ± S.D. (error bars) of three independent experiments. *, p < 0.01 versus control or control ASO; **, p < 0.01 versus thrombin or control ASO + thrombin. HU, hydroxyurea; GFRM, growth factor-reduced Matrigel.
ence THP-1 cell migration (Fig. 1C, left). Similarly, when the outer surface of the membrane was coated with growth factor-reduced Matrigel or gelatin to trap the migrated cells, no differences were found in thrombin-induced THP-1 cell migration, indicating that Matrigel does not influence the migration of these cells (Fig. 1C, right). Together, these observations suggest that thrombin is a potent modulator of monocyte/macrophage migration.

Many studies have shown that several signaling molecules are recruited to growth factor- and cytokine-activated receptors via scaffolding/adapter proteins, such as Gab1 (Grb2-associated binder-1), Gab2, and Gab3 (29–32). It was also reported that Gab1 possesses a PH domain that mediates its interactions with specific membrane lipids (33). Thus, Gab1 has been described as a critical adaptor molecule in the mediation of agonist-induced PI3K/Akt and SHP2/ERK1/2 signaling in many cell types and has been shown to play critical roles in several biological processes, including cell survival, differentiation, and morphogenesis, that are influenced by both receptor tyrosine kinase and GPCR agonists (28–33). Therefore, we wanted to test the role of Gab1 in thrombin-induced THP-1 cell migration. Thrombin induced Gab1 tyrosine phosphorylation in a time-dependent manner (Fig. 1D). In addition, down-regulation of Gab1 inhibited thrombin-induced THP-1 cell migration (Fig. 1, E and F). To understand the mechanisms by which Gab1 mediates thrombin-induced THP-1 cell migration, we tested its role in F-actin cytoskeleton formation. Thrombin induced F-actin cytoskeleton remodeling, as observed by lamellipodia and filopodia formation, in a time-dependent manner (Fig. 1G). Down-regulation of Gab1 levels by its antisense oligonucleotides inhibited the THP-1 cell F-actin cytoskeleton formation (Fig. 1H).

**Thrombin-induced THP-1 Cell Migration Requires Gab1-dependent p115 RhoGEF Activation**—Guanine nucleotide exchange factors (GEFs) play an important role in agonist-induced activation of small GTPases, such as the Rho family of GTPases (34, 35), which in turn, by their involvement in cytoskeletal reorganization, play a role in cell migration (36, 37). To test whether any guanine nucleotide exchange factors (GEFs) are activated by thrombin in THP-1 cells, we first tested its effects on RhoGEFs, namely LARG, PDZ RhoGEF, and p115 RhoGEF. Thrombin had no significant effect on the tyrosine phosphorylation of LARG; however, it induced the tyrosine phosphorylation of p115 RhoGEF and PDZ RhoGEF in a time-dependent manner with maximum effects at 30 min (Fig. 2A). Because p115 RhoGEF tyrosine phosphorylation is more intense than that of PDZ RhoGEF, we next examined its role in thrombin-induced THP-1 cell F-actin cytoskeleton formation and migration. Down-regulation of p115 RhoGEF substantially

![Figure 2. Thrombin-induced THP-1 cell migration requires p115 RhoGEF activation. A, equal amounts of protein from control and the indicated time periods of thrombin (0.5 unit/ml)-treated THP-1 cells were immunoprecipitated with anti-PY20 antibodies, and the immunocomplexes were analyzed by Western blotting for p115 RhoGEF, PDZ RhoGEF, and LARG using their specific antibodies. B, an equal amount of protein from control and the indicated p115 ASO-transfected THP-1 cells was analyzed by Western blotting for p115 RhoGEF, CDK4, and β-tubulin levels using their specific antibodies to show the effect of the ASOs on their target and off-target molecules. C and D, THP-1 cells that were transfected with control or the indicated p115 RhoGEF ASO and quiesced were subjected to thrombin (0.5 unit/ml)-induced F-actin cytoskeleton formation (C) or migration (D). The number of cells used for the migration assay in D was 5 × 10^5. The bar graphs represent the mean ± S.D. (error bars) of three independent experiments. *p < 0.01 versus control or control ASO; **p < 0.01 versus thrombin or control ASO + thrombin.

![Image 1](https://example.com/image1.png)

![Image 2](https://example.com/image2.png)

![Image 3](https://example.com/image3.png)
PAR1 Mediates THP-1 Cell Migration

A

B

C2

C3

C4

C5

D

Control ASO
Gab1 ASO2
Thrombin

Control ASO
Gab1 ASO2

Control ASO
Gab1 ASO2

PAR1 Mediates THP-1 Cell Migration
suppressed THP-1 cell F-actin cytoskeleton formation and migration (Fig. 2, B–D). In order to find whether there is any link between Gab1 and p115 Rhogef, we performed coimmunoprecipitation experiments. Coimmunoprecipitation assays revealed that Gab1 forms a complex with p115 Rhogef in a time-dependent manner in response to thrombin (Fig. 3A). Furthermore, cloning and transfection of expression vectors for WT, PH deletion mutants (ΔPH), or various domains of Gab1 and p115 Rhogef in a combinatorial manner followed by coimmunoprecipitation assays revealed that Gab1 interaction with p115 Rhogef depends on their PH domains (Fig. 3, B and C1–C5). Specifically, whereas forced transfection of Gab1 and p115 Rhogef ΔPH mutants failed to facilitate their interactions, forced transfection of their PH domains restored these interactions (Fig. 3, C1–C5). Down-regulation of Gab1 by its antisense oligonucleotides inhibited thrombin-induced p115 Rhogef tyrosine phosphorylation (Fig. 3D).

**Thrombin-induced THP-1 Cell Migration Depends on Gab1 and p115 Rhogef-mediated Rac1 and RhoA Activation**—The Rhö family GTPases play a crucial role in the regulation of cytoskeletal reorganization and cell migration (36, 37). Specifically, it was demonstrated that whereas Rac1 plays a role in lamellipodium/filopodium formation, RhoA was shown to be important for the development of focal adhesions (37). Because Gab1 forms a complex with p115 Rhogef and facilitates its tyrosine phosphorylation in response to thrombin, we wanted to find whether these interactions lead to the activation of small Rho GTPases. Thrombin induced the activation of both Rac1 and RhoA in a time-dependent manner with maximum effect at 30 min (Fig. 4A). Down-regulation of Rac1 or RhoA by their respective antisense oligonucleotides inhibited thrombin-induced THP-1 cell F-actin cytoskeleton formation and migration (Fig. 4, B–D). In addition, depletion of p115 Rhogef and Gab1 levels by their respective antisense oligonucleotides inhibited thrombin-induced Rac1 and RhoA activation (Fig. 4, E and F). Some reports indicated that Rac1 and RhoA reciprocate in the mediation of cellular responses, such as cell migration (38). Because down-regulation of either p115 Rhogef or Gab1 inhibited thrombin-induced activation of both Rac1 and RhoA, we were intrigued to find whether there is any interaction between these Rho GTPases. Down-regulation of Rac1 by its specific antisense oligonucleotides inhibited thrombin-induced Rac1 and RhoA activation. In contrast, down-regulation of RhoA by its specific antisense oligonucleotides had no effect on Rac1 activation (Fig. 4, G and H). To obtain an additional line of evidence for the role of Rac1 in thrombin-induced RhoA activation, we also used a pharmacological approach. EHT1864, a selective inhibitor of Rac1 (39), substantially blocked thrombin-induced RhoA activation (Fig. 4I). These results imply that the immediate downstream effector of p115 Rhogef is Rac1 but not RhoA and that Rac1 mediates thrombin-induced RhoA activation.

**Thrombin-induced THP-1 Cell Migration Requires Gab1, p115 Rhogef-, Rac1-, and RhoA-dependent Activation of Pak2—Pak1 is an effector of Rac1 and plays an important role in cell migration (40, 41). Therefore, having found that thrombin activates both Rac1 and RhoA downstream to p115 Rhogef, we wanted to identify their effector molecule(s) in mediating thrombin-induced THP-1 cell migration. As measured by both phosphorylation and kinase activities, thrombin activated Pak2 but not Pak1 in THP-1 cells in a time-dependent manner (Fig. 5A). As a positive control, we showed that thrombin at 0.5 unit/ml induces Pak1 activity in HASMCs. This result reinforces the observation that thrombin activates only Pak2 but not Pak1 in THP-1 cells. In addition, down-regulation of Pak2 levels by its antisense oligonucleotides inhibited thrombin-induced THP-1 cell F-actin cytoskeleton formation and migration (Fig. 5, B–D). The Pak2 activation was also significantly inhibited by depletion of RhoA, Rac1, p115 Rhogef, or Gab1 levels using their respective antisense oligonucleotides (Fig. 5, E–H). Because Pak2 are the effectors of Rac1 (40, 41), but our results revealed that RhoA is downstream to Rac1 and mediates thrombin-induced Pak2 activation, we applied a pharmacological approach to confirm these unusual observations. EHT1864, a selective inhibitor of Rac1 (39), and Rho inhibitor I, a selective inhibitor of RhoA (42), also suppressed thrombin-induced Pak2 activity (Fig. 5J). These findings suggest that thrombin-induced stimulation of Pak2 requires Gab1, p115 Rhogef, Rac1, and RhoA activation.

**Pyk2 Mediates Thrombin-induced Gab1-p115 Rhogef-Rac1-RhoA-Pak2 Activation and THP-1 Cell Migration—**Gab1, an adapter molecule, is tyrosine-phosphorylated mostly by receptor tyrosine kinases, such as EGF receptor, creating the binding sites for SH2 domain-containing signaling molecules (29–32).
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It also possesses proline-rich regions for binding to SH3 domain-containing molecules (43). Because Gab1 forms a complex with and facilitates the tyrosine phosphorylation of p115 RhoGEF, we asked what modulates these effects. We found that thrombin, although having no major effect on tyrosine phosphorylation of Src and Syk, time-dependently induced the tyrosine phosphorylation of Pyk2 with maximum effect at 10 min (Fig. 6A). In addition, pharmacological blockade of Pyk2 by its selective inhibitor, PF431396 (44), attenuated thrombin-induced THP-1 cell F-actin cytoskeleton formation and migration (Fig. 6, B and C). PF431396 also inhibited thrombin-induced tyrosine phosphorylation of Gab1 and p115 RhoGEF (Fig. 6, D and E). Consistent with these effects, thrombin-induced Rac1, RhoA, and Pak2 activation were also inhibited by PF431396 (Fig. 6, F and G). To confirm the role of Pyk2 in thrombin-induced Gab1-p115 RhoGEF-Rac1-RhoA-Pak2 activation, we used an antisense oligonucleotide approach. Down-regulation of Pyk2 by its specific antisense oligonucleotides...
PAR1 Mediates THP-1 Cell Migration

FIGURE 5. Thrombin activates Pak2 via a Gab1-, p115 RhoGEF-, Rac1-, and RhoA-dependent manner in mediating THP-1 cell migration. A, equal amounts of protein from control and the indicated time periods of thrombin (0.5 unit/ml)-treated THP-1 cells were analyzed by Western blotting for phospho-Pak1 and phospho-Pak2, and the blots were normalized for total Pak1 or Pak2 levels. Equal amounts of protein from the same cell extracts were immunoprecipitated with anti-Pak1 or anti-Pak2 antibodies, and the immunocomplexes were subjected to an immunocomplex kinase assay using MBP and [γ-32P]ATP as substrates. The number of phosphorylated MBP was measured in a scintillation spectrometer, and the number of phosphorylated MBP was normalized for total Pak1 or Pak2 levels. B, equal amounts of protein from control or the indicated time periods of thrombin (0.5 unit/ml)-treated THP-1 cells were analyzed by Western blotting for Pak2 and pPak2 levels and Pak2 activity.

PAR1 Mediates Thrombin-induced Pyk2-Gab1-p115 RhoGEF-Rac1-RhoA-Pak2 Stimulation and THP-1 Cell Migration—It is known that thrombin mediates its effects via PARs in various cell types (11, 12). To delineate the far upstream mechanisms by which thrombin mediates THP-1 cell migration, we tested the role of PAR1 using a pharmacological approach. SCH79797, a selective antagonist of PAR1 (45), inhibited thrombin-induced THP-1 cell F-actin cytoskeleton formation and migration as well as Gab1 and p115 RhoGEF tyrosine phosphorylation and Rac1, RhoA, and Pak2 activation (Fig. 7, A–F).

blocked thrombin-induced THP-1 cell F-actin cytoskeleton formation and migration as well as Gab1 and p115 RhoGEF tyrosine phosphorylation and Rac1, RhoA, and Pak2 activation (Fig. 7, A–F).

PAR1 Mediates Thrombin-induced Pyk2-Gab1-p115 RhoGEF-Rac1-RhoA-Pak2 Stimulation and THP-1 Cell Migration—It is known that thrombin mediates its effects via PARs in various cell types (11, 12). To delineate the far upstream mechanisms by
PAR1 Mediates THP-1 Cell Migration

![Image](Image78x683 to 150x692)

**Figure 6. Pyk2 mediates thrombin-induced Gab1, p115 RhoGEF, Rac1, RhoA, and Pak2 activation in the modulation of THP-1 cell migration.** A, equal amounts of protein from control and the indicated time periods of thrombin (0.5 unit/ml)-treated THP-1 cells were analyzed by Western blotting for phospho-Src (pSrc), phospho-Syk (pSyk), and phospho-Pyk2 (pPyk2) levels. B, and C, quiescent THP-1 cells were treated with and without thrombin (0.5 unit/ml) in the presence and absence of PF431396 (5 μM) for 30 min and subjected to F-actin cytoskeleton formation (B) or migration (C). D and E, conditions were the same as in C except that after treatment with and without thrombin, equal amounts of protein from each were immunoprecipitated with anti-Gab1 or anti-p115 RhoGEF antibodies; the immunocomplexes were analyzed by Western blotting using anti-PY20 antibodies; and the blots were reprobed for total Gab1 and p115 RhoGEF levels. F, conditions were the same as in C except that samples were analyzed by a pull-down assay for Rac1 and RhoA activation. The same cell extracts were also analyzed by Western blotting for total Rac1, total RhoA, and β-tubulin levels using their specific antibodies. G, conditions were the same as in C except that after treatment with thrombin, equal amounts of protein from control and thrombin-treated THP-1 cells were analyzed by Western blotting using anti-phospho-Pak antibodies, and the same blot was sequentially reprobed for Pak2 and β-tubulin levels. Equal amounts of proteins from the same cell extracts were used to measure Pak2 activity as described in the legend to Fig. 5A. The number of cells used for the migration assay in C was $3 \times 10^5$. The bar graphs represent the mean ± S.D. (error bars) of three independent experiments. *, p < 0.01 versus control; **, p < 0.01 versus thrombin.

and THP-1 cell F-actin cytoskeleton formation and migration, we also used an antisense oligonucleotide approach. Depletion of PAR1 by its ASOs inhibited thrombin-induced THP-1 cell F-actin cytoskeleton formation and migration as well as Pyk2, Gab1, p115 RhoGEF, Rac1, RhoA, and Pak2 activation (Fig. 9, A–G).

**Gα_{12} Mediates Thrombin-induced PAR1-dependent Pyk2-p115 RhoGEF-Rac1-RhoA-Pak2 Activation and THP-1 Cell Migration**—Previous studies have reported that Gα_{12} G protein plays a role in monocyte/macrophage migration in response to MCP-1 (46). Because PARs are coupled to G proteins (47, 48), we wanted to find which G protein is involved in thrombin-induced Pyk2, p115 RhoGEF, Rac1, RhoA, and Pak2 activation in THP-1 cells facilitating their migration. In this quest, we tested the effect of pertussis toxin, a potent and specific inhibitor of Gα_{12} G protein signaling (49), on thrombin and MCP-1-induced THP-1 cell migration. It was interesting to note that pertussis toxin, although blocking the effects of MCP-1, failed to inhibit thrombin-induced THP-1 cell migration (Fig. 10A). This result indicates that thrombin-induced THP-1 cell migration does not involve Gα_{12} G protein activation. Next, we investigated the role of other G proteins. Among the other G proteins tested, treatment with thrombin led to a time-dependent dissociation of Gα_{12} but not Gα_{q} or Gα_{11} from PAR1, suggesting that thrombin activates Gα_{12}-coupled PAR1 receptors in THP-1 cells (Fig. 10B). In addition, depletion of only Gα_{12} and not Gα_{q} or Gα_{11} inhibited thrombin-induced THP-1 cell F-actin cytoskeleton remodeling and migration (Fig. 10, C–E). To test the specificity of PAR1-Gα_{12} signaling in thrombin-induced THP-1 cell migration, we also examined their role in MCP-1-induced THP-1 cell migration. Depletion of either PAR1 or Gα_{12} had no effect on MCP-1-induced THP-1 cell migration, suggesting that their effects on thrombin-induced THP-1 cell migration are specific (Fig. 10F). Based on these
observed, we tested the role of Go12 in thrombin-induced Pyk2, p115 RhoGEF, Rac1, RhoA, and Pak2 activation. ASO-mediated down-regulation of Go12 levels attenuated thrombin-induced Pyk2, Gab1, p115 RhoGEF, Rac1, RhoA, and Pak2 activation (Fig. 10, G–K).

**DISCUSSION**

Several studies have shown that upon ligand binding and tyrosine phosphorylation, receptor tyrosine kinase receptors, such as c-Met, EGF receptor, and VEGF receptor, recruit and phosphorylate an adaptor protein, Gab1 (29–32). Upon tyrosine phosphorylation, Gab1 acts as a docking site for the assembly of multiprotein complexes with the receptor tyrosine kinase receptors. Gab1 contains multiple tyrosyl residues and proline-rich regions that allow its interactions with SH2 and SH3 domain-containing signaling molecules, such as Grb2, PI3K, phospholipase Cγ, SHP2, and Pak4, in the mediation of cellular responses (29–32, 43, 50). In addition, it has been reported that Gab1 PH domain is required for its membrane translocation and binding to specific membrane lipid molecules, such as phosphatidylinositol 3,4,5-triphosphate (33). In this study, we present evidence that Gab1 is activated by thrombin, a GPCR agonist, in THP-1 cells, mediating their F-actin cytoskeleton reorganization and migration. In addition, the effect of Gab1 on thrombin-induced THP-1 cell F-actin cytoskeleton reorganization and migration appears to be mediated via its interactions with p115 RhoGEF, because both Gab1 and p115 RhoGEF formed a complex with each other involving their PH domains in response to thrombin, and the down-regulation of either one attenuated thrombin-induced THP-1 cell F-actin cytoskeleton formation and migration. GEFs catalyze the exchange of GDP for GTP and mediate activation of small GTPases, such as the Rho family of GTPases (34, 35). Because RhoGEFs modulate small RhoGTPases activation, we envisioned that Gab1 via p115 RhoGEF might be modulating the stimulation of RhoGTPases, such as Rac1 and RhoA, in the mediation of thrombin-induced THP-1 cell migration. In accordance with this view, we found that down-regulation of either Gab1 or p115 RhoGEF levels attenuated thrombin-induced Rac1 and RhoA activation, resulting in diminished THP-1 cell F-actin cytoskeleton formation and migration. Although our findings demonstrate that the interactions between Gab1 and p115 Rho-
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FIGURE 8. PAR1 mediates thrombin-induced Pyk2, Gab1, p115 RhoGEF, Rac1, RhoA, and Pak2 activation in the modulation of THP-1 cell migration. A and B, quiescent THP-1 cells were treated with or without thrombin (0.5 unit/ml) in the presence and absence of SCH79797 (2 μM), and F-actin cytoskeleton formation (A) and migration (B) were measured as described in the legend to Fig. 1, G and A, respectively. C–G, conditions were the same as in A except that after treatment with and without thrombin (0.5 units/ml) for 30 min, cell extracts were prepared, and equal amounts of protein from each condition were analyzed for phospho-Pyk2 (pPyk2), phospho-Gab1 (pGab1), phospho-p115 (pp115) RhoGEF, GTP-Rac1, GTP-RhoA, and phospho-Pak2 (pPak2) levels as described in the legends to Figs. 6A, 1D, 6E, 4A, and 5A, respectively. Wherever appropriate, the phosphoblots were reprobed with their respective total antibodies or anti-β-tubulin antibodies for normalization. The same cell extracts that were used for Rac1 and RhoA activation were also analyzed by Western blotting for total Rac1 and RhoA levels using their respective antibodies. Wherever phospho-Pak2 levels were measured, the same cell extracts were also analyzed for Pak2 activity. The number of cells used for the migration assay in B was 3 × 10⁶. The bar graphs represent the mean ± S.D. (error bars) of three independent experiments. *, p < 0.01 versus control; **, p < 0.01 versus thrombin. IB, Western blot; IP, immunoprecipitation.

GEF require their PH domains, it is also possible that, as in the case of Gab1, p115 RhoGEF membrane translocation may also depend on its PH domain, and this may facilitate its interaction with Gab1. Indeed, a previous study from another laboratory (24) showed that the membrane translocation of p115 RhoGEF requires its PH domain. It was reported that Gab1 PH domain is required for its role in c-Met-mediated epithelial cell-cell contacts (33). The Rho GTPases play an important role in mediating cell-cell contacts by enhancing focal adhesions (36, 37). As Gab1 via recruiting RhoGEFs, such as p115 RhoGEF, mediates Rho GTPases activation, there is the possibility that Gab1-induced cell-cell contacts downstream to c-Met may be mediated by Rac1 or RhoA. Some reports showed that both Rac1 and RhoA reciprocate in the stimulus-induced modulation of cell migration and invasion (38). However, in the present study, we showed that Rac1 and RhoA cooperate in the mediation of thrombin-induced F-actin cytoskeleton formation and migration. Other studies have also reported that Rac1 and Cdc42 act upstream to RhoA in the mediation of endothelial cell migration and neutrophil polarization (51, 52). Because a similar cascade of signaling events was induced by thrombin in the modulation of HASMC migration, it may be suggested that Gab1-p115 RhoGEF-Rac1-RhoA may be a unifying mechanism in the modulation of cell migration, at least in response to this agonist.

The Pak family consists of six members, including Pak1 and Pak2. Although the expression of Pak1 appears to be tissue-specific, Pak2 is expressed ubiquitously (40). Studies have shown that Pak proteins participate in the regulation of actin cytoskeleton reorganization, focal adhesion formation, and cell motility (53). A large body of evidence also suggests that Rac1 mediates its effects on actin cytoskeleton remodeling via Pak1 (40, 41, 53). In this aspect, we have previously reported that Gab1-p115 RhoGEF–Rac1–RhoA signaling leads to Pak1 activation in thrombin-induced HASMC migration (17). However, in THP-1 cells, in response to thrombin, we found that only Pak2 and not Pak1 gets activated and mediates THP-1 cell...
F-actin cytoskeleton formation and migration. Furthermore, Pak2 activation was found to be dependent on activation of Gab1, p115 RhoGEF, Rac1, and RhoA. Therefore, based on our previous and present findings, it may be speculated that the Gab1, p115 RhoGEF, Rac1, and RhoA signaling may be diverging-in at Pak1/2 levels in the modulation of HASMC versus THP-1 cell migration in response to thrombin (17). A role for Pak2 in macrophage migration has also been demonstrated previously (54). Furthermore, the activation of Pak2 by heterotrimeric G proteins and the adaptor protein NCK may indicate that several signaling events influence Pak2 in the mediation of cell migration (55).

Previously, we showed that Pyk2 mediates Gab1 activation in the modulation of thrombin-induced HASMC migration (17). Consistent with these observations, thrombin also activated Pyk2 in THP-1 cells mediating their migration. Interestingly, knockdown of Pyk2 levels inhibited thrombin-induced Gab1, p115 RhoGEF, Rac1, RhoA, and Pak2 phosphorylation or activation, suggesting that Pyk2 acts upstream to all of these signaling events in response to thrombin in THP-1 cells. Previously, we have also shown that Pyk2 plays a role in vascular wall remodeling in response to injury (17). Other studies have reported that Pyk2 is involved in the pathogenesis of atherosclerosis (18). Although our previous study demonstrated that Pyk2-mediated neointimal hyperplasia requires activation of Gab1, p115 RhoGEF, Rac1, RhoA, and Pak1, the mechanisms by which Pyk2 mediates atherosclerosis were not clear. Because macrophage migration and foam cell formation are crucial

### FIGURE 9. Depletion of PAR1 inhibits thrombin-induced Pyk2, Gab1, p115 RhoGEF, Rac1, RhoA, and Pak2 activation and THP-1 cell migration.

A, THP-1 cells were transfected with control or the indicated PAR1 ASOs, and 48 h later, cell extracts were prepared, and equal amounts of proteins from each were analyzed by Western blotting for PAR1 levels using their specific antibodies. The blot was reprobed for CDK4 and β-tubulin levels using their specific antibodies to show the effect of ASOs on their off-target molecules. B, cells that were transfected with control or the indicated PAR1 ASOs and quiesced were subjected to thrombin-induced F-actin cytoskeleton formation, as described in the legend to Fig. 1G. C, all of the conditions were the same as in B except that cells were subjected to thrombin-induced migration. D–H, conditions were the same as in B except that after treatment with and without thrombin (0.5 unit/ml) for 30 min, cell extracts were prepared, and equal amounts of protein from control and each treatment were analyzed for phospho-Pyk2 (pPyk2), phospho-Gab1 (pGab1), phospho-p115 (p(p115) RhoGEF, GTP-Rac1, GTP-RhoA, and phospho-Pak2 (pPak2) levels, as described in the legends to Figs. 6A, 1D, 6E, 4A, and 5A, respectively. Wherever appropriate, the phosphoblots were reprobed with their respective total antibodies or anti-β-tubulin antibodies for normalization. The same cell extracts that were used for Rac1 and RhoA activation were also analyzed by Western blotting for total Rac1 and RhoA levels using their respective antibodies. Wherever phospho-Pak2 levels were measured, the same cell extracts were also analyzed for Pak2 activity. The number of cells used for the migration assay in C was $3 \times 10^4$. The bar graphs represent the mean ± S.D. (error bars) of three independent experiments. *, $p < 0.01$ versus control; **, $p < 0.01$ versus thrombin.

| A | Control ASO | PAR1 ASO1 | PAR1 ASO2 | PAR1 ASO3 |
|---|-------------|-----------|-----------|-----------|
| 61 | CON | PAR1 | + | – | – |
| 43 | PAR1 | – | + | – | – |
| 55 | β Tubulin | – | – | – | – |

| B | Control ASO | PAR1 ASO2 | PAR1 ASO2 + Thrombin |
|---|-------------|-----------|----------------------|
| 116 | Pyk2 | – | + | + |
| 61 | Pyk2 | + | – | + |
| 55 | β Tubulin | – | – | + |

| C | Control ASO | PAR1 ASO2 | PAR1 ASO2 + Thrombin |
|---|-------------|-----------|----------------------|
| 27 | Gab1 | – | + | + |
| 21 | Gab1 | + | – | + |
| 55 | β Tubulin | – | – | + |

| D | Control ASO | PAR1 ASO2 | PAR1 ASO2 + Thrombin |
|---|-------------|-----------|----------------------|
| 116 | pPak2 | – | + | + |
| 55 | β Tubulin | – | – | + |

| E | Control ASO | PAR1 ASO2 | PAR1 ASO2 + Thrombin |
|---|-------------|-----------|----------------------|
| 116 | GTP-Rac1 | – | + | + |
| 27 | GTP-Rac1 | + | – | + |
| 55 | β Tubulin | – | – | + |

| F | Control ASO | PAR1 ASO2 | PAR1 ASO2 + Thrombin |
|---|-------------|-----------|----------------------|
| 116 | GTP-RhoA | – | + | + |
| 27 | GTP-RhoA | + | – | + |
| 55 | β Tubulin | – | – | + |

| G | Control ASO | PAR1 ASO2 | PAR1 ASO2 + Thrombin |
|---|-------------|-----------|----------------------|
| 27 | pPak2 | – | + | + |
| 55 | β Tubulin | – | – | + |

| H | Control ASO | PAR1 ASO2 | PAR1 ASO2 + Thrombin |
|---|-------------|-----------|----------------------|
| 116 | pPak2 | – | + | + |
| 55 | β Tubulin | – | – | + |
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events in the pathogenesis of atherosclerosis (2, 3) and Pyk2 mediates the migration of THP-1 cells, it may be speculated that Pyk2 via facilitating the recruitment of monocytes/macrophages to the sites of endothelial injury may be aiding the disease pathogenesis. However, it remains to be explored whether thrombin stimulates foam cell formation and if Pyk2 has a role in such effects. Macrophages demonstrate an abundance of actin filaments and actin-associated proteins in the cortical cytoplasm. Cortical actin polymerization and the subsequent extension of pseudopodia are important components of the
host-defense mechanisms of these cells (56). Based on these observations, one may also anticipate that thrombin via activation of Pyk2-Gab1-p115 RhoGEF-Rac1-RhoA-Pak2 signaling and enhancing actin polymerization may also be involved in the host defense mechanisms of macrophages.

Thrombin, which is generated at the site of vascular injury, acts through PARs to initiate its coagulant, mitogenic, and/or chemotactic effects. A large body of evidence suggests that thrombin and its receptors (PARs), particularly PAR1, play a role in endothelial barrier disruption, promoting the sticking of leukocytes and monocytes/macrophages to the endothelium and their infiltration into the subendothelial space (4, 6, 20, 21), which are the hallmarks of atherogenesis (57). An enhanced expression of PAR1 in the regions of inflammation associated with macrophage influx, smooth muscle cell proliferation, and mesenchymal-like intimal cells further supports a role for its involvement in atherosclerosis (58–60). In line with this assumption, in the present study, we demonstrate that SCH79797, a selective antagonist of PAR1 or PAR1 depletion, inhibits thrombin-induced Pyk2, Gab1, p115 RhoGEF, Rac1, RhoA, and Pak2 activation, leading to diminished THP-1 cell F-actin cytoskeleton formation and migration. This observation infers that PAR1 may be involved in monocyte/macrophage migration to the sites of endothelial injury and perhaps, via disruption of endothelial cell barrier function, may also be playing a role in the transendothelialization of these cells. Many reports have shown that thrombin influences its cellular effects via its PARs coupled to various G proteins, including G\textsubscript{q}, G\textsubscript{11}, G\textsubscript{12/13}, and G\textsubscript{q/11} (47, 48). In this aspect, a recent report showed that thrombin induces embryonic fibroblast migration via G\textsubscript{q/11}-coupled GPCRs that target RhoA activation through LARG, PDZ-RhoGEF, and p115 RhoGEF (61). On the other hand, some studies reported that activation of G\textsubscript{q/11} inhibits thrombin-induced epithelial cell migration (62). However, the present findings indicate that thrombin-induced monocyte/macrophage migration requires the activation of G\textsubscript{q/11} but not G\textsubscript{q/11}. Together, these observations reveal that thrombin mediates cell migration via its PARs that are coupled to various G proteins in different cell types. Some studies have reported that G\textsubscript{q/11} associates with RhoGEFs, such as p115 RhoGEF, involving their RH and DH domains, and enhances their GEF activity (63). It was also reported that the enhancement of the RhoGEF activity by G\textsubscript{q/11} was greater if the RhoGEF is tyrosine-phosphorylated as compared with its non-phosphorylated form (64). In this context, in addition to the present study, other reports have also shown that G\textsubscript{q/11} mediates Pyk2 activation (65). In addition, we show that Gab1 interacts with p115 RhoGEF, involving their PH domains, and this interaction requires Gab1-Pyk2 activation. Based on all of these observations, it may be speculated that PAR1, upon activation, triggers the formation of a multimeric protein complex comprising G\textsubscript{q/11}, Pyk2, Gab1, and p115 RhoGEF that leads to activation of Rac1-RhoA-Pak2 signaling in the modulation of monocyte/macrophage migration. A large body of literature suggests that Rac1 and Cdc42 bind and activate Pakks (40, 41). However, the present observations reveal that RhoA acts upstream to Pak2 activation. Because RhoA does not bind to Pak2 directly, a possible explanation is that it indirectly mediates Pak2 activation. Indeed, a previous study showed that RhoA in concert with Raf1 mediates Pak1 activation in the modulation of fibroblast transformation (66). In summary, as

![Figure 10. Depletion of G\textsubscript{q/11} inhibits thrombin-induced Pyk2, Gab1, p115 RhoGEF, Rac1, RhoA, and Pak2 activation in THP-1 cells, affecting their F-actin cytoskeleton formation and migration.](Image)

![Figure 11. Schematic diagram that shows the potential signaling mechanism by which thrombin induces monocyte/macrophage migration.](Image)
shown in Fig. 11, the present observations reveal that thrombin stimulates monocyte/macrophage migration, and this response requires PAR1-Gαq-dependent Pyk2-mediated Gab1 and p115 RhoGEF interactions, leading to Rac1-RhoA-Pak2 activation and lamellipodia/filopodia formation.

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