Cell motility during wound healing and inflammation is often dependent on the ability of the cell to sense a gradient of agonist. The first step in this process is the extension of a pseudopod in the direction of the agonist, and a diverse set of signals mediate pseudopod extension by different receptors. We have reported previously that protease-activated receptor-2 (PAR-2), a proinflammatory receptor that is highly expressed in motile cells such as neutrophils, macrophages, and tumor cells, is one of a growing family of receptors that utilizes a β-arrestin-dependent mechanism for activation of the 42–44-kDa members of the MAPK family (extracellular signal-regulated kinases 1 and 2; ERK1/2). β-Arrestin-bound PAR-2 serves as a scaffold to sequester a pool of activated ERK1/2 in the cytosol; however, a specific role for the sequestered scaffold to sequester a pool of activated ERK1/2 (PAR-2), a proinflammatory receptor that is highly expressed in motile cells such as neutrophils, macrophages, and tumor cells, is one of a growing family of receptors that utilizes a β-arrestin-dependent mechanism for activation of the 42–44-kDa members of the MAPK family (extracellular signal-regulated kinases 1 and 2; ERK1/2). β-Arrestin-bound PAR-2 serves as a scaffold to sequester a pool of activated ERK1/2 in the cytosol; however, a specific role for the sequestered kinase activity has not been established. We now show that PAR-2 activation promotes ERK1/2- and β-arrestin-dependent reorganization of the actin cytoskeleton, polarized pseudopodia extension, and chemotaxis. Using subcellular fractionation, confocal microscopy, and physical isolation of pseudopodial proteins, we demonstrate that the previously identified PAR-2/β-arrestin/ERK1/2 scaffolding complex is enriched in the pseudopodia, where it appears to prolong ERK1/2 activation. These studies suggest that the formation of a β-arrestin/ERK1/2 signaling complex at the leading edge may be involved in localized actin assembly and chemotaxis and provide the first example of a distinct cellular consequence of β-arrestin-sequestered ERK1/2 activity.

A number of stimuli are able to promote cytoskeletal reorganization and random migration, but directed migration (or chemotaxis) is dependent upon the ability of the cell to sense and respond to a gradient of agonist (1). The first step in chemotaxis is the formation of a leading edge, where a pseudopod is extended in the direction of the receptor. Receptors that promote chemotaxis must then be able to communicate with actin machinery to direct the formation of a leading edge. A number of common signaling proteins are implicated in pseudopod extension and chemotaxis, including members of the MAPK family: extracellular signal-regulated kinases 1 and 2 (ERK1/2), and p38 MAPK. ERK1/2 phosphorylation of one member of the Wiscott-Aldrich syndrome family of proteins (Wave-1/Scar-1) and myosin light chain kinase have been implicated in platelet-derived growth factor, integrin, and lysophosphatidic acid-induced cell motility (2–4), whereas p38 MAPK activity was found to be important for CXCR-4 receptor-induced chemotaxis (5). More recently, localization of ERK1/2 to the leading edge of migrating fibroblasts was shown to be involved in lysophosphatidic acid-induced pseudopod extension, although the mechanism by which it is localized is unclear (4). Although the nuclear effects of ERK1/2 on gene regulation are more widely studied, an understanding of how ERK1/2 activity is controlled in the cytosol by association with different scaffolding complexes is emerging. Recent studies suggest that the cytosolic effects of ERK1/2 may not occur in response to any signal that converges on this pathway and are not a default result of ERK1/2 that is left over in the cytosol (6). This model would require that an additional level of control over MAPK activity be exerted. Studies on a number of cell surface receptors have demonstrated a role for components of the endocytotic machinery, such as the clathrin adapter protein, β-arrestin, in both the activation and subcellular localization of MAPKs (7). There is a growing body of evidence to suggest that these “endosomal scaffolds” can both link activation of MAPKs to specific receptors and determine the ultimate subcellular localization of the active kinases (7). Furthermore, recent genetic studies (5, 8) have suggested that β-arrestins are required for immune cell chemotaxis, and an enticing hypothesis is that endosomal scaffolds can restrict kinase activity to the leading edge to promote localized actin assembly and filament organization.

One of the first examples of β-arrestin-dependent localization of MAPKs came from work on protease-activated receptor-2 (PAR-2), a member of the recently identified family of G protein-coupled receptors that are self-activated by tethered ligands exposed upon proteolytic cleavage of their extracellular N termini. PAR-2 is cleaved and activated by pancreatic trypsin.

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sin, mast cell tryptase, coagulation factors (factor VII/Xa) and a membrane-bound serine protease (MBSPII) to mediate proinflammatory responses (9–11). A synthetic peptide (PAR-2 activating peptide or P2AP), corresponding to the tethered ligand sequence, will specifically activate PAR-2 in the absence of enzymatic cleavage (12). Our early studies showed that ERK1/2 activation by PAR-2 is facilitated by β-arrestin and involves the formation of a large scaffolding complex (Stokes radius ~6.7 nm) that sequesters the activated kinases in the cytosol, thus preventing their nuclear translocation and presumably leading to specific phosphorylation of cytosolic or membrane-associated proteins. PAR-2 also uses a ras-independent pathway to activate ERK1/2, in contrast to the classic pathway used by receptor tyrosine kinases and many G protein-coupled receptors (13). Similar results have been observed for angiotensin AT1a receptor-stimulated ERK1/2 and c-Jun N-terminal kinase activation (14–16) and for vasopressin V2 receptor activation of ERK1/2 (17). Surprisingly, other β-arrestin-dependent pathways of ERK1/2 activation, such as demonstrated for β2-adrenergic receptor, neurokin-1 receptor, and insulin-like growth factor-I receptor (IGF-1R), do the opposite, facilitating nuclear transport (18–20). Still other receptors, such as the thrombin receptor, PAR-1 (structurally similar to PAR-2), do not require β-arrestin for activation of ERK1/2 (21). Thus, one cannot make assumptions about the specific effects of MAPK activation by a given receptor as they vary depending on cell type, mechanism of activation, and scaffolding complexes with which the kinases associate.

PAR-2 is highly expressed in neutrophils, mast cells, and tumor cells, where it has been suggested to promote cytoskeletal reorganization, but the mechanism by which it does so remains unclear (22). We hypothesized that PAR-2 induces chemotaxis through the formation of a scaffolding complex, retaining ERK1/2 and possibly other kinases near the membrane where they can activate actin machinery and lead to polarized pseudopod formation. Here, we focus on the regulation of ERK1/2 pathway and show the following: 1) that PAR-2 activation results in actin reorganization, pseudopod formation, and chemotaxis whereas two other receptors that activate ERK1/2 by different mechanisms (IGF-1R and PAR-1) do not; 2) that MEK1/2 inhibitors and dominant negative mutants of ERK1/2 prevent PAR-2-mediated chemotaxis but not IGF-1R-induced random migration; and 3) that phosphorylated ERK1/2 is enriched along with β-arrestin-bound PAR-2 in the pseudopodia, where its activity is prolonged.

**EXPERIMENTAL PROCEDURES**

**Materials**—All chemicals were from Sigma or Fisher Biotech unless stated otherwise. Human and mouse P2AP, SLIGKV-NH2, and SLIGRL-NH2 were synthesized and purified by Sigma Genosys. Human PAR-1 activating peptide (P2AP) was synthesized and purified by the Peptide Synthesizing Facility (University of Calgary, Calgary, Alberta, Canada). Both peptides were of high purity, as determined by liquid chromatography-mass spectrometry and amino acid analysis. ALLN (20 μmol/L) and ALLN-2 (82 μmol/L) were gifts from Dr. Robert Lefkowitz (Duke University Medical Center); human PAR-2 cDNA, GFP-tagged β-arrestin-1, and GFP-tagged β-arrestin-1319-418 were gifts from Dr. Nigel Bunnett (University of California, San Francisco, CA). Dominant negative ERK2 (ERK-KR) was a gift from Dr. Melanie Cobb (University of Texas Southwestern Medical Center). GFP-tagged ERK-KR was made by PCR amplification of ERK-KR coding sequence and insertion into BamHI sites of EGFPN1 and confirmed by DNA sequencing. The following antibodies were used: anti-β-arrestin (44MAPK) was from Transduction Laboratories; PAR-2 (SAM11), histone H1, and actin primary antibodies and protein A were from Santa Cruz Biotechnology; fluorescence-conjugated secondary antibodies were from Pierce; and Texas Red-conjugated phalloidin was from Molecular Probes Inc.

Cell Culture and Construction of Stable Cell Lines—NIH3T3 cells were a gift from Dr. Nigel Bunnett (University of California, San Francisco, CA), and MDA MB-468 cells were from American Type Tissue Collection. NIH3T3 cells were grown in CellGro medium (Mediatech) supplemented with 10% Cosmic calf serum (Hyclone Laboratory) and were grown in Leibovitz’s L-15 medium (Invitrogen), supplemented with 10% Cosmic calf serum and 14 μM NaHCO3. All cell lines were maintained at 37 °C with 5% CO2. NIH3T3 cells were stably transfected with FLAG/HA.11-tagged PAR-2 alone or with ARR-GFP by calcium phosphate precipitation followed by drug selection. Clonal cell lines were screened for expression by flow cytometry and immunofluorescence as described previously (13), and immunoblotting with antibodies to PAR-2 (SAM11) and β-arrestin-1. Three clones of each cell line were tested for trypsin and P2AP-stimulated colocalization with β-arrestin and internalization, as described previously (13), and for membrane localization of phosphorylated ERK1/2. For transient transfections, Lipofectin (Invitrogen) was used according to the manufacturer’s instructions.

**Subcellular Fractionation and Immunoprecipitations**—5 × 106 cells per 60-mm dish were grown for 24 h, serum-starved for 16 h, and treated with 50 μM P2AP, 50 μM P2AP, or 100 ng/ml insulin for 0–60 min at 37 °C. Cells were washed twice and lysed by dounce homogeni- zation (10 strokes) in 0.25 ml of Hypotonic lysis buffer (HLB; 10 mm Tris–HCl pH 7.6, 1 mM EGTA, 0.25% sucrose + 10 μg/ml aprotinin, leupeptin, and benzamidine, 1 mM phenylmethylsulfonyl fluoride, 10 mM NaF, 2 mM sodium vanadate, and 0.5 mM dithio- tetrathitol). Lysates were centrifuged at 10,000 × g for 10 min; crude nuclear pellets were washed one time in HLB and recentrifuged for 10 min at 10,000 × g. Nuclear fractions were resuspended in Laemmli sample buffer. Supernatants were combined and centrifuged at 100,000 × g for 60 min to yield membrane and cytosolic fractions. Membrane fractions were resuspended in HLB + 1% Triton X-100. Equal amounts of protein from each fraction were analyzed by SDS-PAGE, followed by Western blotting with antibodies to phospho-ERK (p-ERK, actin (cytoskeletal control), and histone H1 (nuclear control). A small amount of actin immunoreactivity was observed in membrane and nuclear fractions, and some histone immunoreactivity was ob- served in membrane fractions, suggesting that minor cross-contamina- tion of fractions occurred. For ERK/β-arrestin co-immunoprecipita- tions, cytosolic and membrane fractions were adjusted to contain 150 μg NaCl, 1% Triton X-100, and 0.1% bovine serum albumin in HLB and incubated with 1 μg of ERK2 antibody bound to 25 μl of protein A-agarose overnight at 4 °C.

**Immunofluorescence and Confocal Microscopy**—106 cells were seeded onto coverslips coated with 1 mg/ml extracellular matrix or poly-L-lysine, allowed to attach for 6 h, and serum-starved overnight. To observe p-ERK localization, NIH3T3-PAR-2 cells, treated with or without P2AP (100 μM for 5 min or insulin (100 ng/ml) for 5 min, were fixed in 4% paraformaldehyde, blocked in 5% normal goat serum, and stained with anti-p-ERK (1:100) for 1 h at 25 °C followed by fluorescein isothio- cyanate-conjugated anti-rabbit IgG (1:200) for 1 h at 25 °C. During the second wash, 1 mg/ml DAPI was added to visualize nuclei. To observe actin-cytoskeletal reorganization, NIH3T3-PAR-2 + ARR-GFP cells were treated with 50 μM P2AP for 0–30 min, washed twice in cytoske- letal stabilization buffer (10 mM MES, pH 6.0, 138 mM NaCl, 3 mM MgCl2, 2 mM EGTA), fixed in 4% paraformaldehyde in cytoskeletal stabilization buffer for 5 min at 25 °C, permeabilized in phosphate-buffered saline + 0.1% Triton X-100, and stained with Texas Red-conjugated phalloidin for 1 h at 25 °C. All images were observed by using a Leica TCS SP microscope on a Zeiss Axiovert 35 microscope. NIH3T3 cells preincubated with anti-p-ERK stained cells were also taken using a Nikon upright fluores- cence microscope (×40 objective).

**Pseudopodia and Cell Migration Assays**—Migration and pseudopod extension were monitored using 24-well Costar transwell polycarbonate membranes with 8.0-μm (migration) or 3.0-μm (pseudopod) pores, coated on one side with fibronectin (10 μg/ml). After overnight serum starvation, cells were allowed to attach for 6 h, and serum-starved overnight. To observe p-ERK localization, NIH3T3-PAR-2 cells, treated with or without P2AP (100 μM for 5 min or insulin (100 ng/ml) for 5 min, were fixed in 4% paraformaldehyde, blocked in 5% normal goat serum, and stained with anti-p-ERK (1:100) for 1 h at 25 °C followed by fluorescein isothio- cyanate-conjugated anti-rabbit IgG (1:200) for 1 h at 25 °C. During the second wash, 1 mg/ml DAPI was added to visualize nuclei. To observe actin-cytoskeletal reorganization, NIH3T3-PAR-2 + ARR-GFP cells were treated with 50 μM P2AP for 0–30 min, washed twice in cytoskel- etal stabilization buffer (10 mM MES, pH 6.0, 138 mM NaCl, 3 mM MgCl2, 2 mM EGTA), fixed in 4% paraformaldehyde in cytoskeletal stabilization buffer for 5 min at 25 °C, permeabilized in phosphate-buffered saline + 0.1% Triton X-100, and stained with Texas Red-conjugated phalloidin for 1 h at 25 °C. All images were observed by using a Leica TCS SP microscope on a Zeiss Axiovert 35 microscope. NIH3T3 cells preincubated with anti-p-ERK stained cells were also taken using a Nikon upright fluores- cence microscope (×40 objective).

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β-Arrestin-dependent ERK Sequestration and Actin Reorganization

Our previous studies have suggested that PAR-2 activates ERK1/2, resulting in the incorporation of the kinases into a scaffolding complex that sequesters their activity in the cytosol, by a mechanism distinct from that utilized by receptor tyrosine kinases such as the IGF-1R. To investigate the possibility that PAR-2 induces actin reorganization by an MEK-dependent pathway, two PAR-2 expressing cell lines were used. Values for GFP migration indices and GF/CT ratios were nearly identical; a value closer to one indicates little effect of transfection on cell migration.

Pseudopodia Purification—Pseudopodia purification was performed as described (23). 15 × 10⁶ NIH3T3-PAR-2, transiently transfected with ARR-GFP, were seeded into the upper chamber of transwell filters (24-mm diameter, 3-μm pore size) as described above and treated with 50 μM AP for 90–90 min to induce pseudopodia extension. Cells were rinsed in excess cold phosphate-buffered saline and rapidly fixed in 100% ice-cold methanol. To isolate pseudopodial proteins, cell bodies on the upper membrane surface were manually removed with cotton swab, and pseudopodia on the undersurface were scraped into lysis buffer in excess cold phosphate-buffered saline and analyzed by flow cytometry using Cellquest software. Regions were set based on background fluorescence of untransfected NIH3T3 cells, with and without addition of Celltracker Red (CT). Region 1 contained cells that were both CT and GFP positive, and Region 2 contained cells that were only CT positive. Non-viable cells that did not take up CT were eliminated from the analysis. Total cell numbers from top and bottom sides of each filter were counted, density plots of each region were generated, and migration was determined using a transwell filter assay, and random migration was distinguished from chemotaxis by observing the migratory response of cells to a uniform concentration of agonist (added to top and bottom sides of filters) compared with a gradient of agonist (added only to the bottom chamber). This technique is commonly used to identify a chemotactic response, because cells that are able to sense a gradient of agonist will become confused by a uniform concentration and try to migrate in all directions, resulting in a reduced number of migratory cells. In contrast, cells that exhibit random migration will migrate equally well in response to a gradient and a uniform concentration of agonist (23, 25). A gradient of P2AP induced a 5.2 ± 0.35-fold increase in NIH3T3 cell migration over basal levels (Fig. 2A) and 16 ± 3-fold increase in MDA MB-468 cell migration (Fig. 2B). In response to a uniform concentration of P2AP, migration was reduced by ~60%. In contrast, P2AP did not induce migration in either cell line. Insulin (probably acting through IGF-1R) appeared to be an important factor in inducing migration in these cell lines, as denoted by Western blotting (data not shown).

To address the possibility that PAR-2 stimulates chemotaxis whereas other receptors that activate ERK1/2 by alternative mechanisms do not, we compared the effects of PAR-2 activation on cell migration with that of IGF-1R (as a model of the receptor tyrosine kinase pathway) and PAR-1 (a receptor for thrombin that activates ERK1/2 by a β-arrestin-independent pathway). Two PAR-2 expressing cell lines were used in these studies: NIH-3T3 cells and a breast cancer cell line, MDA MB-468. Insulin-like growth factor-1 receptor is highly expressed in NIH3T3 cells, and PAR-1 is expressed in both cell lines, as denoted by Western blotting (data not shown). Migration was determined using a transwell filter assay, and random migration was distinguished from chemotaxis by observing the migratory response of cells to a uniform concentration of agonist (added to top and bottom sides of filters) compared with a gradient of agonist (added only to the bottom chamber). This technique is commonly used to identify a chemotactic response, because cells that are able to sense a gradient of agonist will become confused by a uniform concentration and try to migrate in all directions, resulting in a reduced number of migratory cells. In contrast, cells that exhibit random migration will migrate equally well in response to a gradient and a uniform concentration of agonist (23, 25). A gradient of P2AP induced a 5.2 ± 0.35-fold increase in NIH3T3 cell migration over basal levels (Fig. 2A) and 16 ± 3-fold increase in MDA MB-468 cell migration (Fig. 2B). In response to a uniform concentration of P2AP, migration was reduced by ~60%. In contrast, P2AP did not induce migration in either cell line. Insulin (probably acting through IGF-1R) appeared to be an important factor in inducing migration in these cell lines, as denoted by Western blotting (data not shown).

In summary, PAR-2 stimulates chemotaxis, IGF-1R stimulates random migration, and PAR-1 does not stimulate motility in the cell lines examined, despite the fact that all three are known to activate ERK1/2.
To examine the role of MAPK activity in PAR-2-stimulated chemotaxis, we repeated migration assays in the presence of two MEK1/2 inhibitors, U0126 and PD98059, and a p38 MAPK-specific inhibitor, SB203580. Both U0126 and PD98059 inhibited PAR-2-induced chemotaxis by 70 and 89%, respectively, in NIH3T3 cells, whereas SB203580 had no significant effect (Fig. 2C). In MDA MB-468 cells, U0126 and PD98059 inhibited PAR-2-stimulated chemotaxis by 56 and 60%, respectively, whereas SB203580 actually stimulated PAR-2-induced migration (Fig. 2D), suggesting that ERK1/2 but not p38 MAPK activation is involved in PAR-2-stimulated chemotaxis (p38 MAPK and ERK1/2 can antagonize each other, so the stimulatory effect of p38 MAPK may be because of enhanced ERK1/2 activity). In contrast, insulin-stimulated random migration was only mildly inhibited by U0126 (<40%) and was not affected by SB203580 or PD98059 (Fig. 2C), suggesting random and directed migration might have differential requirements for MAPK activity.

To confirm that the sensitivity to U0126 and PD98059 reflected a requirement for ERK1/2 rather than nonspecific effects of these inhibitors, we repeated motility experiments in the presence of ERK-KR-GFP. NIH3T3 cells were transiently transfected with ERK-KR-GFP or GFP alone and grown on 8-μm transwell filters. After 16 h of P2AP or insulin stimulation, cells from both sides of the filters were collected, labeled with Cell Tracker Red, and analyzed by flow cytometry. Migration indices (MI) and GFP MI were determined as described under “Experimental Procedures.” A representative density plot of PAR-2-stimulated migration in ERK-KR-GFP-transfected cells is shown in Fig. 3A. P2AP-stimulated chemotaxis was inhibited by ~80% (80 ± 3% MI inhibition and 89 ± 4% GFP MI inhibition) by ERK-KR-GFP expression (Fig. 3B). Insulin-induced random migration, however, was reduced less than 50% (28 ± 6% MI inhibition and 42 ± 4% GFP MI inhibition) by expression of ERK-KR-GFP (Fig. 3B). These results confirmed observations obtained with MEK1/2 inhibitors, demonstrating that ERK1/2 is required for PAR-2-stimulated chemotaxis. Although ERK1/2 may be a contributing factor to IGF-1R-mediated random migration, it is not an absolute requirement, and these data strongly suggest the two receptors utilize different mechanisms for inducing directed and random migration.

**PAR-2 Activation Results in a Membrane-localized Pool of Activated ERK1/2**—We demonstrated previously that PAR-2 activates ERK1/2 by a β-arrestin-dependent mechanism, resulting in retention of a pool of activated kinase in the cytosol. In contrast, IGF-1R and PAR-1 have been shown to stimulate nuclear translocation of ERK1/2 (13, 24, 26). We proposed that upon PAR-2 activation, the endosomal scaffold-associated ERK1/2 is retained in a membrane compartment, where it might regulate actin assembly at the leading edge of a cell. To investigate whether PAR-2 could specifically sequester a pool of activated ERK1/2 to a membrane-associated compartment, we performed subcellular fractionation on NIH3T3-PAR-2 cells treated with P2AP, insulin, or P1AP for 0–90 min and compared levels of activated ERK1/2 in crude cytosolic, nuclear, and membrane fractions. In both NIH3T3 and MDA MB-468 cells, PAR-2 activation increased cytosolic and membrane p-ERK immunoreactivity, but little nuclear ERK1/2 was observed (Fig. 4A). In contrast, insulin stimulated a strong increase in cytosolic and nuclear p-ERK in NIH3T3 cells, and P1AP stimulated a significant increase in both cytosolic and nuclear p-ERK in MDA MB-468 cells and a mild increase in cytosolic p-ERK in NIH3T3 cells (Fig. 4A). No significant increase in p-ERK was observed in membrane fractions in response to either insulin or P1AP (Fig. 4A). Stripping and reprobing blots with anti-histone (nuclear marker) and anti-actin demonstrated the fidelity of fractionation (data not shown).
We confirmed the localization of activated ERK1/2 to a membrane compartment in response to PAR-2 by immunofluorescence using anti-p-ERK (Fig. 4, B and C), followed by fluorescein isothiocyanate-conjugated secondary antibody and DAPI co-staining to identify nuclei. Although insulin induced a stronger nuclear translocation of ERK1/2, very little activated ERK1/2 was observed in the cytosol (Fig. 4 B). In contrast, PAR-2 activation resulted in retention of activated ERK1/2 in the cytosol, where it may be associated with the plasma membrane through interactions with other membrane proteins (Fig. 4 C), and very little ERK1/2 co-localized with DAPI in the nucleus (Fig. 4 B).

**Prolonged ERK1/2 Activation in the Pseudopodia Is Associated with a PAR-2/β-Arrestin Scaffolding Complex**—Recently, a method for analyzing protein redistribution during pseudopodia formation was established (23). NIH3T3 cells lend themselves well to this assay, because they have large pseudopodia; some cell lines such as MDA MB-468 cells extend smaller pseudopodia and thus the pseudopodial protein acquired is insufficient for analysis. This method is similar to the migration assays described above, except that membranes with 3-μm pores (which are too small to allow cell body translocation) are used, allowing physical separation of cell body from pseudopodia. To demonstrate P2AP-stimulated pseudopodia formation, filters stained with crystal violet are shown in Fig. 5A. Similar to what was observed in migration assays, P2AP induced a 6 ± 0.33-fold increase in pseudopodia density. To purify pseudopodial proteins, both the cell bodies from the top or the pseudopodia from the underside are scraped into lysis buffer, and equal amounts of proteins are then analyzed by SDS-PAGE. Equivalent amounts of protein from control cells not been induced to extend pseudopodia are included as a control. By immunoblotting with antibodies to proteins shown previously (13) to be in PAR-2 endosomal scaffold, we were able to determine that PAR-2, β-arrestin, Raf-1, and phosphorylated ERK1/2 are all found in the pseudopodia. Both Raf-1 and p-ERK are highly

![Fig. 3. Dominant negative ERK2 blocks PAR-2-stimulated chemotaxis.](image-url)
FIG. 4. PAR-2 induces sequestration of ERK1/2 with β-arrestin in a membrane-associated fraction. A, NIH3T3 cells or MDA MB-468 cells were treated with P2AP (50 μM in NIH3T3 and 5 μM in MDA MB-468), P1AP (50 μM), or insulin (100 ng/ml) for 0–90 min and separated by subcellular fractionation. Crude membrane and cytosolic and nuclear fractions were analyzed by SDS-PAGE followed by Western blotting for presence of activated ERK (anti-p-ERK). The images are representative of three experiments. B, NIH3T3-PAR2 cells were treated with nothing, 50 μM P2AP, or 100 ng/ml insulin for 5 min, stained with anti-p-ERK followed by fluorescein isothiocyanate-conjugated secondary (upper panels) and DAPI (middle panels), and observed on an inverted microscope with a ×40 objective. Bottom panels are overlays of p-ERK and DAPI. Arrowheads indicate cytosolic, and arrows indicate nuclear ERK1/2. C, p-ERK stained slides from B were observed by confocal microscopy. Arrowheads indicate membrane staining; scale bar = 10 μ.

FIG. 5. Prolonged ERK activation in pseudopodia is associated with PAR-2/β-arrestin scaffolding complex. A, NIH3T3 cells, transiently transfected with ARR-GFP, were induced with P2AP to extend pseudopodia for 90 min on transwell filters (3-μm pore size); cell bodies were removed and stained with crystal violet. Quantification of pseudopod density was determined by histogram analysis of crystal violet staining in four different fields and quantification of eluted dye by absorbance at 600 nm. Fold increase in average density was determined (n = 5; p = 0.005). B, pseudopodia formation was induced as described in A, and proteins (10 μg) isolated from total cell lysate, cell bodies on the upper surface of the membrane, or pseudopodia from the underside were analyzed by SDS-PAGE followed by Western blotting with antibodies to p-ERK, total ERK1/2, Raf-1, β-arrestin-1, PAR-2, and histone H1 (cell body control). Enrichment of proteins in either pseudopodia or cell body was determined by histogram analysis of band density and presented as a percentage of the total (cell body + pseudopodia) band densities. C, crude cytosol/membrane preparations from NIH3T3 + ARR-GFP cells, treated with and without P2AP for 90 min, were subjected to immunoprecipitation with anti-ERK1/2 followed by Western blotting with anti-β-arrestin (upper panel) or total ERK1/2 (lower panel).
enriched, and β-arrestin is slightly enriched in the pseudopodia compared with the cell body (Fig. 5B), suggesting that PAR-2-stimulated ERK1/2 activity is sequestered within the growing pseudopodia of polarized cells where it might be modifying actin machinery or other proteins associated with cell motility. Histone H1 immunoreactivity was found only in the cell body, indicating that there was very little cell body contamination of pseudopodial preparations. In addition, total ERK1/2 levels were not enriched in the plasma membrane. Interestingly, whereas in either whole cell (not shown) or fractionated lysates (see Fig. 4), ERK1/2 activation appears to return to baseline by 60 min of PAR-2 stimulation, strong ERK1/2 activation is observed in the pseudopodia after 90 min (Fig. 5B), suggesting that a pool of the total ERK1/2 is sequestered by β-arrestin-bound PAR-2 at the leading edge, where its activity is prolonged. To confirm that this pool of activated ERK1/2 was still interacting with β-arrestin (the complex was originally purified after 5 min of PAR-2 stimulation), we performed co-immunoprecipitations of ERK1/2 and β-arrestin. Even after 90 min of PAR-2 stimulation, β-arrestin and ERK1/2 can be co-precipitated (Fig. 5C).

PAR-2-induced Cytoskeletal Reorganization and Pseudopod Formation Requires β-Arrestin—Our previous studies demonstrated that expression of a GFP-tagged dominant negative fragment of β-arrestin-1, corresponding to the clathrin binding domain (ARR319–418-GFP), inhibits PAR-2-stimulated ERK1/2 activation and cytosolic sequestration (13). Having established that inhibiting ERK1/2 activity with U0126, PD98059, or a dominant negative ERK2 mutant reduces PAR-2-stimulated chemotaxis and that components of the previously identified β-arrestin-dependent scaffolding complex are localized to the pseudopodia, we investigated the effect of expressing ARR319–418-GFP on PAR-2-mediated pseudopodia formation and chemotaxis. NIH3T3 cells were transiently transfected with ARR319–418-GFP or GFP alone, treated with P2AP or insulin. Cell migration as described in Fig. 3 and pseudopodia formation as described in Fig. 5A were assayed. ARR319–418-GFP inhibited pseudopodia formation by 60 ± 6% (Fig. 6A). In contrast, insulin-stimulated pseudopodia extension was slightly enhanced by expression of ARR319–418-GFP, suggesting that prevention of desensitization and internalization of IGF-1R might enhance random migration. That β-arrestin was required for chemotaxis was confirmed using the migration assay described in Fig. 3. In cells transfected with ARR319–418 P2AP-stimulated migration was inhibited by ~60% (68 ± 5% inhibition of MI, 62 ± 2% inhibition of GFP MI), whereas insulin-stimulated migration was unaffected (Fig. 6B). Phalloidin staining revealed that ARR319–418-GFP expression prevented P2AP-induced formation of broad pseudopodia, as compared with a nearby untransfected cell (Fig. 6C).

**DISCUSSION**

PAR-2 is one of a growing family of receptors that requires the scaffolding function of β-arrestin for activation and localization of MAPKs (7). There is evidence that these endosomal scaffolds play a major role in determining the subcellular location of MAPKs and thereby their substrate specificity and physiological responses (13, 15, 17, 19, 27). Although one study clearly shows that overexpression of β-arrestin can prevent angiotensin AT1a receptor-induced gene expression through cytosolic retention of ERK2 (16), a role for β-arrestin-dependent endosomal scaffolds in a normal cellular response has never been shown. Our previous work demonstrated that PAR-2/β-arrestin complexes retain activated ERK1/2 in the cytosol (13), but the precise localization and role of the sequestered kinase activity in normal PAR-2 responses were unknown. Here we demonstrate that PAR-2-stimulated chemotaxis is dependent on both ERK1/2 and β-arrestin. Using a combination of biochemical, physical, and microscopic methods, we also show that phosphorylated ERK1/2 is retained at the plasma membrane in response to activation of PAR-2, but not in response to either IGF-1R or PAR-1 stimulation, and that it is highly enriched in the extending pseudopodia of actively migrating cells. Furthermore, the active ERK1/2 is found in purified pseudopodia, along with other components (β-arrestin, PAR-2, and Ral) of a previously identified endosomal scaffold, suggesting that the complex might serve to retain ERK1/2 and possibly other kinases at the leading edge of motile cells.

**Endosomal Scaffolds and Prolonged ERK1/2 Signaling**—A requirement for β-arrestin in cell motility is a newly emerging idea that, to date, has only been demonstrated in immune cells. Genetic studies have demonstrated that β-arrestins are essential for CXCR-1, CXCR-4, and FMLP-directed cytoskeletal reorganization and cell motility (8, 28), but the studies described here provide the first evidence for β-arrestin-dependent endosomal scaffolds directing cell polarization and pseudopodia formation.

Interestingly, PAR-1, a receptor for thrombin that does not require β-arrestin for activation of ERK1/2, did not direct chemotaxis in the cell lines described here, and insulin stimulated random, but not directed, migration. PAR-1 has been shown to
direct chemotaxis in hematopoietic, immune cells, and some tumor cells (29–31), while inhibiting migration in others (22); the inability to promote chemotaxis in these systems, although it can still activate ERK1/2, suggests a unique role for \( \beta \)-arrestin-scaffolded ERK1/2. Similarly, the fact that insulin-stimulated random migration was not dependent on ERK1/2 or \( \beta \)-arrestin further supports this hypothesis. Importantly, the ability of PAR-2 to promote localization of ERK1/2 at the membrane, where it is associated with \( \beta \)-arrestin for up to 90 min, while neither IGF-1R nor PAR-1 activation results in membrane localization of the kinases, is consistent with a model in which the PAR-2 endosomal scaffold sequesters a pool of active ERK1/2 at the leading edge to modify actin machinery. This result does not imply that all receptors that induce membrane localization of ERK1/2 will promote chemotaxis, nor does it imply that all chemotactic receptors activate membrane ERK1/2. It is widely accepted that there are multiple mechanisms for promoting pseudopod extension; the mechanism described here for PAR-2 is novel and may prove to be utilized by other receptors.

We were surprised to find that whereas ERK1/2 activation appeared to return to baseline after 60 min of PAR-2 activation either in whole cell lysates (not shown) or in total microsomal preparations (Fig. 4A), activated ERK1/2 was enriched in pseudopodial preparations even after 90 min of PAR-2 activation (Fig. 5). The pseudopodia comprise only a small fraction of total membrane protein, and equivalent amounts of protein from each cell fraction were analyzed in Fig. 5; therefore, if a small pool of active ERK1/2 is concentrated there, we would be able to detect it although we might not be able to see it in total membrane samples. Because total ERK1/2 was not enriched in the pseudopodia, we propose that its activity is somehow prolonged at the leading edge of the cell. Pseudopodal ERK1/2 may be protected from dephosphorylation by its association with the scaffolding complex. Alternatively, dephosphorylated ERK1/2 might rapidly exchange for phosphorylated ERK1/2 during pseudopodial extension, or it might be rephosphorylated by MEK1/2 retained in the complex.

**A Model for PAR-2-mediated Chemotaxis**—Based on these and our previous studies (13), we propose a model for PAR-2-induced cell migration that is depicted in Fig. 7. First, cells sense a gradient of PAR2 agonist, either enzymatic (e.g. trypsin, mast cell tryptase, or coagulation factors VII and Xa) or peptide, resulting in receptor activation mobilization of \( \mathrm{Ca}^{2+} \) and activation of protein kinase C and RhoA GTPases. Second, activation of PAR-2 results in \( \mathrm{Ca}^{2+} \) mobilization and activation of protein kinase C and RhoA GTPases. Third, protein kinase C-dependent phosphorylation of the PAR-2 C terminus promotes \( \beta \)-arrestin binding and association of the receptor with clathrin-coated pits. An endosomal scaffold that contains \( \beta \)-arrestin-bound PAR-2, Raf-1, MEK1, and ERK1/2 forms, promoting prolonged activation of ERK1/2 (shown as ERK-P). Fourth, membrane localization ERK1/2 activity, along with other undetermined factors, promotes localized activation of actin machinery, resulting in actin assembly and cell migration. Fifth, alternatively, pseudopodia formation occurs at a region of the membrane near the clathrin-coated pit, where activated ERK1/2 and potentially Raf-1 and other proteins, are released from the PAR2/\( \beta \)-arrestin complexes. The complex is then disassembled, PAR-2 is internalized, and a new cycle of activation occurs.

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2 Y. Ly and K. DeFea, unpublished observations.
RhoA-GTPase effectors. These events promote localized actin assembly, ultimately resulting in phosphodius extension and cell migration. Alternatively (Fig. 7, 4b), the association of ERK1/2 with a β-arrestin scaffold on the clathrin-coated pit may serve to cluster ERK1/2 and potentially other kinases such as Raf-1 near the leading edge, but the complex may actually disassemble as the pseudopodia extends leaving ERK1/2 free to phosphorylate proteins involved in cell migration. Prolonged ERK1/2 activity may result from exchange of dephosphorylated ERK1/2 for phosho-ERK, or other signaling molecules at the leading edge may reactivate it. Cell migration requires a coordinated cycle of signal activation and inactivation, and initiation of this pathway is tightly coupled to its termination by virtue of the requirement for β-arrestin in ERK1/2 activation. Thus, in the final step, the complex is probably disassembled, and PAR-2 is internalized and degraded, resulting in transport of new PAR-2 to the plasma membrane for another round of signaling (33).

Understanding the mechanism of PAR-2 mediated chemotaxis is important from a biomedical, as well as a basic cell biological perspective. PAR-2 is highly expressed in a number of both chemotactic and static cells, the former including leukocytes and metastatic tumor cells and the latter including vascular endothelial and both respiratory and intestinal epithelial cells. From a biomedical perspective, there is substantial evidence linking PAR-2 to wound healing and tumor metastasis (22, 34–36) suggesting that PAR-2-induced chemotaxis has both protective and pathophysiological roles. From a more basic cell biological perspective, these studies provide us with new insights into the importance of β-arrestin-mediated sequestration of ERK1/2. Further investigation may reveal that the PAR-2 endosomal scaffold is associated with a number of cytoskeletal regulating proteins at the leading edge of the cell.

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