The G protein-coupled thrombin receptor, protease-activated receptor 1 (PAR1), mediates many of the actions of thrombin on cells including chemotaxis. In contrast to the reversible agonist binding that regulates signaling by most G protein-coupled receptors (GPCRs), PAR1 is activated by an irreversible proteolytic mechanism. Although activated PAR1 is phosphorylated, uncoupled, and internalized like typical GPCRs, signal termination is additionally dependent on lysosomal degradation of cleaved and activated receptors. In the present study we exploit two PAR1 mutants to examine the link between chemotaxis and receptor shutoff. One, a carboxyl tail deletion mutant (Y397Z), is defective in phosphorylation and internalization. The other, a carboxyl tail chimeric receptor (P/S), is phosphorylated and internalized upon activation but recycles to the plasma membrane like reversibly activated GPCRs. Expression of these receptors in a hematopoietic cell line disrupted cell migration along thrombin gradients. Thrombin activation of cells expressing P/S or Y397Z resulted in persistent signaling independent of the continued presence of thrombin. Signaling in response to the soluble agonist peptide SFLLRN was reversible for P/S but persisted for Y397Z. Strikingly, cells expressing P/S responded chemokinetically to thrombin but chemotactically to SFLLRN. In contrast, Y397Z-mediated migration was largely chemokinetic to both agonists. These studies suggest that termination of PAR1 signaling at the level of the receptor is necessary for gradient detection and directional migration.

Cell migration induced by specific environmental stimuli is central to a host of biological processes including embryonic development, inflammation, and wound healing. Cells can respond to chemoattractants by directional movement along attractive gradients (chemotaxis) or by random locomotion (chemokinesis) (1). In both cases, chemoattractants elicit migration of cells by activating surface receptors that communicate with the cytoskeletal machinery that is ultimately used to achieve movement (2, 3). Leukocytes in particular have evolved to respond to a spectrum of chemoattractants via G protein-coupled receptors (GPCRs) (3). The chemotactic response in leukocytes is mediated by receptors that couple to members of the G12 subfamily of G proteins and requires signaling via G protein βγ subunits (4, 5). However, the precise path(s) from receptor to cytoskeletal movement and cell migration is not yet known. Even more remains to be learned about how cells use receptors to perceive both static and changing chemoattractant gradients to direct or arrest movement (6–8). Recent studies have begun to address the effects of mutations that alter receptor signaling on chemotaxis. Uncoupling of GPCRs from signaling by phosphorylation and internalization of activated receptors might be anticipated to be important for cells to repeatedly sense their surroundings or to reset their sensitivity to chemoattractants as they move along a gradient. However, independent studies using the formyl peptide receptor, CCR2B, or cAMP receptor found that defects in agonist-dependent receptor phosphorylation and/or internalization did not affect chemotaxis (9–11).

The G protein-coupled thrombin receptor, protease-activated receptor 1 (PAR1), can mediate many of the actions of thrombin on cells including chemotaxis (12–14). Unlike most GPCRs, PAR1 is activated by an unusual proteolytic mechanism in which thrombin cleaves the amino-terminal exodomain of PAR1 to expose a new amino terminus that then serves as a tethered ligand (15, 16). The synthetic peptide SFLLRN, which mimics the first six amino acids of this newly unmasked amino terminus, can activate PAR1 independent of receptor cleavage and thrombin. PAR1 can thus be activated by both irreversible proteolytic and reversible ligand-binding mechanisms, the former being physiological and the latter a pharmacological device. The irreversibility of the proteolytic activation mechanism of PAR1 contrasts with the reversible agonist binding that activates classical GPCRs and suggests that PAR1 may require a distinct signal termination mechanism. Signal termination for classical GPCRs is accomplished in part by receptor phosphorylation and consequent arrestin binding, which disrupts further interaction with G proteins (17). Receptors are then internalized and enter endosomal compartments where they dissociate from their ligands and become dephosphorylated. They subsequently return to the cell surface ready to signal again. Activated PAR1 is similarly phosphorylated and internalized, but rather than recycling to the surface it is preferentially sorted to lysosomes and degraded (18). The importance of this process in terminating thrombin-induced signaling was demonstrated by using PAR1 receptor mutants that lack normal internalization or lysosomal sorting (19, 20). Cells expressing a PAR1 carboxyl tail truncation mutant defective in internalization and phosphorylation showed persistent signaling after only transient exposure to thrombin (20). Similarly, a mutant defective in lysosomal sorting also mediated persistent signaling because of recycling of thrombin-activated receptors to the cell surface (19).

Exploiting the unique activation mechanism of PAR1 and the properties of these mutants, we set out to determine the relationship between the mechanisms of receptor shutoff and
the ability of a cell to sense gradients and undergo chemotaxis. Our results show that directional movement correlated with an ability to terminate signaling and that a lack of PAR1 shut off did not ablate motility but rather produced a chemokinetic response.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Monoclonal anti-FLAG antibody, M1, was purchased from Eastman Kodak Co.). α-Thrombin was obtained from Enzyme Research Labs, and SDF-1β was obtained from R&D Systems. The PAR1 agonist peptide SFLLRN was synthesized as a carboxy amide and purified by high pressure liquid chromatography. Pertussis toxin was obtained from List Biological Laboratories.

**DNA and Cell Culture**—Human PAR1 cDNA containing a prolactin leader sequence and amino-terminal FLAG epitope in the mammalian expression vector pBJ1 (provided by Mark Davis, Stanford University) was used for generating mutants as described previously (21). BaF3 cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 2-mercaptoethanol, and 10% WEHI supernatant as a source of interleukin-3. Cells were transfected by electroporation. Apoptosis was induced by treating cells with 10 mg/ml of G418 (Geneticin, Invitrogen). Surviving colonies were picked and expanded for propagation of cells, a heterogeneous population of stably transfectants for each case.

**Reagents**—Thrombin was obtained from Enzyme Research Laboratories and was used at 1 mg/ml.

**Signal Termination in PAR1 Chemotaxis**

**RESULTS AND DISCUSSION**

In the present study we employed the murine hematopoietic progenitor cell line, BaF3, which demonstrates a potent chemotactic response to agonists such as SDF-1β and thrombin. This cell line provided an excellent chemotaxis model because of robust agonist-induced migration and suitability for the Transwell assay system (5). To examine PAR1-dependent migration, BaF3 cells were stably transfected with FLAG-tagged forms of the wild type human PAR1, a PAR1 cytoplasmic tail deletion mutant designated Y397Z, or a chimeric receptor designated P/S in which the carboxyl tail of PAR1 was replaced with that of the substance P (NK-1) receptor (Fig. 1). To avoid clonal differences that might arise during selection and propagation of cells, a heterogeneous population of stably transfected BaF3 cells was isolated after each transfection and propagated for a limited amount of time. Transfection with the P/S chimera consistently resulted in higher expression levels than transfection with the Y397Z mutant. Transfectants expressing comparable levels of the wild type receptor were used as controls for each case.

**P/S and Y397Z** were previously characterized in a fibroblast cell line derived from PAR1-deficient mouse lung fibroblasts and in Rat1 fibroblasts (18–20). Like wild type PAR1, P/S was phosphorylated and internalized upon activation, but unlike wild type PAR1, P/S recycled to the plasma membrane instead of sorting to lysosomes. Thrombin activation of P/S resulted in persistent signaling, even after removal of thrombin, because of recycling of permanently activated (cleaved) receptors. In contrast, P/S activation by the tethered ligand peptide SFLLRN was reversible, presumably because SFLLRN and P/S dissociated after receptor internalization. Y397Z was impaired in both agonist-triggered phosphorylation and internalization and like P/S showed persistent signaling after thrombin activation, presumably because of defective uncoupling, internalization, and degradation of activated receptors.

The signaling and trafficking behavior of wild type and mutant receptors was re-examined in BaF3 cells. Agonist-induced internalization was measured as the loss of surface receptor tagged with monoclonal M1 antibody (Fig. 2). Surface levels of wild type PAR1 decreased by approximately 65% within 30 min of stimulation with SFLLRN, consistent with agonist-triggered receptor internalization. Surface levels of Y397Z decreased by only 35% over the same period, consistent with the relative

![Signal Termination in PAR1 Chemotaxis](image.png)
internalization defect described previously for this mutant (20). Surface levels of the P/S receptor decreased by an intermediate amount, consistent with this receptor undergoing agonist-triggered internalization and recycling (18, 19). Recovery of the internalized receptor on the cell surface following agonist stimulation was also measured and confirmed that P/S recycled in these cells (data not shown).

Signaling properties of the transfectants were examined by measuring calcium mobilization and phosphoinositide hydrolysis after agonist stimulation. Treatment with pertussis toxin abolished both thrombin-triggered increases in cytoplasmic calcium and migration in cells expressing wild type PAR1, P/S, and Y397Z (not shown), demonstrating that the wild type PAR1 and mutant receptors all signaled via Gi, in BaF3 cells. Thrombin and SFLLRN-stimulated increases in cytosolic calcium showed distinct tempos in cells expressing wild type PAR1 versus the mutant receptors (Fig. 3). Cells expressing wild type PAR1 showed an immediate response that rapidly returned to near baseline levels. In contrast, P/S or Y397Z-expressing cells showed a response that was both prolonged and increased in magnitude, a profile that is consistent with defective signal termination by the mutant receptors. It is noteworthy that tracings for both thrombin and SFLLRN are similar despite the peptide having a reversible mode of activation. Thus, in the continuous presence of saturating agonist, P/S and Y397Z showed a prolonged signaling response that was independent of the activation mechanism.

Studies of signaling shutoff after removal of agonist revealed an interesting difference between the P/S and Y397Z mutants (Fig. 4). To assess shutoff, [3H]inositol-labeled cells were first exposed to thrombin or SFLLRN in the absence of LiCl. Under these conditions, phosphoinositide hydrolysis occurs but [3H]inositol phosphates do not accumulate. After 10 min, agonists were removed; LiCl was added, and accumulation of [3H]inositol phosphates over the next 60 min was measured as an index of persistent receptor signaling. Cells expressing the P/S chimera showed no shutoff of signaling after removal of thrombin. [3H] Inositol phosphate accumulation was indistinguishable whether thrombin and LiCl were present simultaneously or thrombin was added and removed before the LiCl was introduced. In contrast, activation of P/S-expressing cells by SFLLRN was reversible; little ongoing inositol phosphate ac-
Y397Z signaling persists after SFLLRN removal is unknown. For classical GPCRs, agonist-receptor dissociation occurs at least in part in an endosomal compartment (17). Hence, the slow shutoff of Y397Z after removal of SFLLRN might result from prolonged residence of activated receptors on the cell surface and slow SFLLRN dissociation from the receptor in the absence of internalization. Regardless, these cell lines provided useful tools to study the relationship of shutoff to chemotaxis.

Next we examined the ability of wild type PAR1, P/S, and Y397Z to mediate cell migration in BaF3 cells using the Transwell assay system. In this Boyden chamber-like system, a chemoattractant gradient is presented across a polycarbonate membrane that separates the upper cell-containing chamber from the lower chemoattractant-containing chamber. Migration was measured as the total number of cells that enter the lower chamber during a 4 h incubation. Untransfected BaF3 cells, which express a low level of endogenous PAR1 and BaF3 cells transfected with wild type PAR1, showed a clear chemotactic response to thrombin. Maximum migration in a dose-response experiment occurred between 100 pM and 1 nM thrombin and progressively decreased at higher concentrations generating a bell-shaped curve typical of chemotactic responses (Fig. 5) (1, 2). In a “checkerboard” analysis, movement occurred only when thrombin was present at higher concentrations in the lower chamber than in the upper chamber and not when thrombin was evenly distributed in both chambers or present in excess in the upper chamber (Table I). Cells expressing wild type PAR1 also showed a chemotactic response to SFLLRN, with optimal migration observed at 1 μM (Fig. 5 and Table II). Both agonists induced maximum migration at the same concentration in cells expressing high or moderate levels of transfected wild type PAR1 as well as in cells expressing low levels of endogenous receptor, suggesting that the surface receptor number does not significantly influence sensitivity to chemoattractant in this system (data not shown).

BaF3 cells expressing the P/S chimeric receptor showed migratory responses to thrombin that were exaggerated compared with those seen with wild type PAR1-expressing cells (Fig. 5 and Table I), consistent with enhanced signaling by P/S (Fig. 3). More interestingly, this response was primarily chemokinetic. The presence of thrombin in the upper chamber alone was sufficient to stimulate migration. Additionally, migration at high thrombin concentrations was not decreased, producing a sigmoidal response curve indicative of chemokinetic movement. Like their response to thrombin, migration of P/S-expressing BaF3 cells to SFLLRN was exaggerated compared with wild type receptor-expressing cells, but strikingly, it was predominantly chemotactic. The addition of SFLLRN to the lower chamber elicited cell migration with a bell-shaped concentration response (Fig. 5). Furthermore, SFLLRN in the upper chamber elicited little cell movement by itself and caused diminished movement in response to low concentrations of SFLLRN in the lower chamber (Table II).

BaF3 cells expressing Y397Z also showed an exaggerated migration response but in this case migration to both thrombin and SFLLRN was predominantly chemokinetic in character. The concentration response curves to thrombin and SFLLRN were sigmoidal rather than bell-shaped, and checkerboard analysis demonstrated migration along gradients and in evenly distributed agonist.

Cells expressing wild type PAR1 or the P/S or Y397Z mutant receptors responded chemokinetically to SDF-1α, a chemokine that acts on the endogenous receptor CXCR4 in BaF3 cells (data not shown) (23). Thus the chemokinetic response to thrombin in the P/S and Y397Z cells was not because of a global change in the chemoattractant responses of these cells but...
rather was likely because of the properties of the P/S and Y397Z receptors themselves. The unusual properties of the P/S receptor-expressing cells, which showed chemokinesis to thrombin and chemotaxis to SFLLRN, also strongly suggest that the altered migratory properties of the transfectants were because of the signaling characteristics of the transfected receptors.

These data show that mutation of the carboxyl tail of PAR1 yielded receptors with an altered ability to mediate chemotactic migration. The wild type receptor, which showed rapid shutoff of signaling to thrombin or SFLLRN, mediated chemotaxis to both agonists. The P/S chimeric receptor showed reversible signaling to SFLLRN but persistent signaling after activation by thrombin. Activation by SFLLRN resulted in a chemotactic response in cells expressing this receptor, whereas irreversible activation by thrombin resulted in chemokinesis. The Y397Z mutant showed persistent signaling after activation by thrombin and, to a lesser extent, SFLLRN. This mutant yielded chemokinetic responses to both agonists. These results suggest that efficient shutoff of signaling is necessary for chemotaxis but not for chemokinesis. In particular, the ability of the P/S mutant to mediate chemotaxis when activated reversibly by peptide but chemokinesis when activated irreversibly by thrombin suggests that it was the lack of shutoff rather than another effect of the mutation that was responsible for loss of directional movement.

Chemotaxis is thought to necessitate adaptation of cells to their local environment such that they ignore agonist concentrations that have been previously encountered and respond only to increased agonist concentrations (24–26). Directional migration must also require repeated sampling of the local environment for cells to detect changes in agonist concentration and gradient direction. Thus, activation of naive receptors and/or reactivation of recycled receptors must occur during

TABLE I
Checkerboard analysis of thrombin-stimulated cells

Migration of transfected BaF3 cells in response to thrombin added to the upper and/or lower chambers at the indicated concentrations was assessed. Data are the mean ± S.E. of duplicate values from one of three representative experiments and are expressed as the total number of cells (×1000) that migrated to the lower chamber.

| Thrombin | Wt | P/S | Y397Z |
| --- | --- | --- | --- |
| Lower well | Upper well | Lower well | Upper well | Lower well | Upper well | Lower well | Upper well |
| 0 | 0.1 ± 0.07 | 0.1 ± 0.07 | 0.3 ± 0.14 | 0.7 ± 0.17 | 0.13 ± 0.09 | 0.13 ± 0.09 | 0.33 ± 0.13 | 0.7 ± 0.17 |
| 1 nM | 6.0 ± 0.41 | 6.0 ± 0.41 | 6.0 ± 0.41 | 6.0 ± 0.41 | 4.5 ± 0.23 | 4.5 ± 0.23 | 4.5 ± 0.23 | 4.5 ± 0.23 |
| 10 nM | 2.4 ± 0.07 | 2.4 ± 0.07 | 2.4 ± 0.07 | 2.4 ± 0.07 | 1.5 ± 0.03 | 1.5 ± 0.03 | 1.5 ± 0.03 | 1.5 ± 0.03 |
| 100 pM | 1.3 ± 0.09 | 1.3 ± 0.09 | 1.3 ± 0.09 | 1.3 ± 0.09 | 0.9 ± 0.07 | 0.9 ± 0.07 | 0.9 ± 0.07 | 0.9 ± 0.07 |
| 1 nM | 15.4 ± 0.90 | 15.4 ± 0.90 | 15.4 ± 0.90 | 15.4 ± 0.90 | 13.0 ± 0.69 | 13.0 ± 0.69 | 13.0 ± 0.69 | 13.0 ± 0.69 |
| 10 nM | 41.8 ± 0.90 | 41.8 ± 0.90 | 41.8 ± 0.90 | 41.8 ± 0.90 | 39.9 ± 0.36 | 39.9 ± 0.36 | 39.9 ± 0.36 | 39.9 ± 0.36 |
| 100 pM | 42.8 ± 3.61 | 42.8 ± 3.61 | 42.8 ± 3.61 | 42.8 ± 3.61 | 39.0 ± 0.15 | 39.0 ± 0.15 | 39.0 ± 0.15 | 39.0 ± 0.15 |
| 1 nM | 17.1 ± 2.11 | 17.1 ± 2.11 | 17.1 ± 2.11 | 17.1 ± 2.11 | 16.2 ± 1.05 | 16.2 ± 1.05 | 16.2 ± 1.05 | 16.2 ± 1.05 |
| 10 nM | 74.8 ± 8.89 | 74.8 ± 8.89 | 74.8 ± 8.89 | 74.8 ± 8.89 | 63.0 ± 2.41 | 63.0 ± 2.41 | 63.0 ± 2.41 | 63.0 ± 2.41 |

FIG. 5. Migration of BaF3 cells expressing wild type PAR1 and shutoff-defective mutant receptors. Migration of BaF3 cells expressing wild type, P/S, or Y397Z constructs in response to the indicated concentrations of thrombin or SFLLRN was determined using the Transwell assay system. Data represent total number of cells (mean ± S.E.; n = 2) that migrated to the lower chamber following a 4-h incubation at 37 °C. This experiment was replicated at least five times with similar results. Wt, wild type.
Migration of transfected BaF3 cells in response to SFLLRN added to the upper and/or lower chambers at the indicated concentrations was assessed. Data are the mean ± S.E. of duplicate values from one of three representative experiments and are expressed as the total number of cells (>1000) that migrated to the lower chamber.

| Receptor | Lower well 0 | 100 nM | 1 µM | 10 µM | 100 nM | 1 µM | 10 µM | 100 nM | 1 µM | 10 µM |
|----------|--------------|--------|------|-------|--------|------|-------|--------|------|-------|
| Wt       | 0 ± 0        | 0.1 ± 0.03 | 0.4 ± 0.23 | 0.7 ± 0.20 |
| P/S      | 5.7 ± 3.31   | 1.2 ± 0.44 | 0.4 ± 0.02 | 0.4 ± 0.10 |
| 1 µM     | 17.8 ± 6.62  | 10.4 ± 1.05 | 4.1 ± 0.50 | 1.3 ± 0.67 |
| 10 µM    | 3.8 ± 0.15   | 4.1 ± 0.67 | 3.5 ± 0.45 | 1.5 ± 0.40 |
| Y397Z    | 3.6 ± 0.12   | 4.0 ± 0.66 | 7.7 ± 1.78 | 6.5 ± 0.57 |
| 1 µM     | 56.8 ± 3.46  | 21.7 ± 1.81 | 18.4 ± 1.51 | 7.8 ± 0.60 |
| 10 µM    | 102.7 ± 9.94 | 84.9 ± 7.83 | 48.5 ± 0.30 | 37.4 ± 4.80 |
| 0.90     | 78.6 ± 2.71  | 25.3 ± 1.81 | 69.5 ± 10.54 | 63.9 ± 1.81 |
| 1.60     | 10.0 ± 0.30  | 5.1 ± 0.90 | 11.4 ± 1.81 | 30.1 ± 0.60 |
| 3.30     | 30.4 ± 3.31  | 5.4 ± 0.60 | 13.0 ± 0.30 | 24.7 ± 0.60 |
| 1 µM     | 38.9 ± 0.30  | 33.4 ± 1.51 | 28.0 ± 4.52 | 31.0 ± 6.33 |
| 10 µM    | 46.7 ± 5.12  | 43.4 ± 4.82 | 43.7 ± 3.31 | 36.4 ± 5.12 |

**FIG. 6. Persistent chemokinetic response of P/S-expressing BaF3 cells after thrombin stimulation.** P/S-expressing BaF3 cells were incubated in the presence or absence of agonist (100 nM thrombin or 1 µM SFLLRN) for 10 min at 37 °C. Cells were then washed and transferred to Transwell chambers and further incubated in the presence (4 hr) or absence (10 min) of agonist. Migration was assessed after 4 hr. Data are expressed as the number of cells that migrated to the lower chamber (mean ± S.E.; n = 2). This experiment was replicated three times.

**Acknowledgments**—We thank Israel Charo and Henry Bourse of the University of California, San Francisco for their critique of this manuscript.

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Chemokinetic migration, however, might simply require cell activation without a need for such repeated sensing of the extracellular environment. The P/S receptor provided an opportunity to test these predictions (Fig. 6). BaF3 cells expressing P/S were exposed to thrombin or SFLLRN for 10 min, washed, and tested for a subsequent chemokinetic response. A brief exposure to thrombin resulted in a migratory response during the subsequent 4 h incubation; the magnitude of this response was comparable to that seen when thrombin was continuously present in the upper chamber. Such responses are obviously chemokinetic. On the other hand, transient exposure to SFLLRN did not induce significant migration consistent with its reversible activation of P/S and chemotactic rather than chemokinetic action.

Taken together, these studies strongly suggest that signal termination at the receptor level is important for PAR1-mediated chemotaxis. Curiously, phosphorylation and/or internalization mutants of the CCR2B, cAMP, and formyl peptide receptors were previously shown to mediate chemotaxis like their respective wild type receptors (9–11, 27). Clearly, the P/S chimera is a special case in which the receptor was “engineered” to recycle and resignal indefinitely. P/S-expressing cells exposed to thrombin in essence sample their environment for thrombin only once. The Y397Z mutant is more analogous to the mutant CCR2B, cAMP, and formyl peptide receptors previously studied (9–11, 27). Why Y397Z mediated chemokinesis to SFLLRN, whereas similar mutations in other GPCRs allowed chemotaxis to their respective ligands, is not clear. Perhaps the shutoff defect of Y397Z is more profound than that of other mutant receptors studied, or perhaps dissociation of SFLLRN from Y397Z is slower and/or more dependent on internalization than is the case for the other ligands and receptors. Alternatively, mechanisms unaffected by mutation of receptor carboxyl tails might terminate signaling and permit repeated sampling of gradients in these other systems. Such mechanisms might function at the receptor or post-receptor levels.

The process by which cells detect the direction of a chemotactrant gradient and orchestrate their movement accordingly is not well understood. Our data show a close correlation between the ability of a cell to move directionally in response to an agonist with an ability to terminate signaling upon removal of that agonist. Defective signal termination resulted in a loss of chemotactic migration but persistence of agonist-triggered movement in the form of chemokinesis. The P/S chimera makes a particularly strong case for a relationship between shut off and chemotaxis versus chemokinesis. P/S demonstrates that a single receptor can trigger chemokinesis or chemotaxis depend-
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