The Chemotactic Response to PDGF-BB: Evidence of a Role for Ras

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Abstract. The PDGF receptor-β mediates both mitogenic and chemotactic responses to PDGF-BB. Although the role of Ras in tyrosine kinase-mediated mitogenesis has been characterized extensively, its role in PDGF-stimulated chemotaxis has not been defined. Using cells expressing a dominant–negative ras, we find that Ras inhibition suppresses migration toward PDGF-BB. Overexpression of either Ras-GTPase activating protein (Ras-GAP) or a Ras guanine releasing factor (GRF) also inhibited PDGF-stimulated chemotaxis. In addition, cells producing excess constitutively active Ras failed to migrate toward PDGF-BB, consistent with the observation that either excess ligand or excess signaling intermediate can suppress the chemotactic response. These results suggest that Ras can function in normal cells to support chemotaxis toward PDGF-BB and that either too little or too much Ras activity can abrogate the chemotactic response. In contrast to Ras overexpression, cells producing excess constitutively active Raf, a downstream effector of Ras, did migrate toward PDGF-BB. Cells expressing dominant–negative Ras were able to migrate toward soluble fibronectin demonstrating that these cells retained the ability to migrate. These results suggest that Ras is an intermediate in PDGF-stimulated chemotaxis but may not be required for fibronectin-stimulated cell motility.

The platelet-derived growth factor (PDGF) is both a mitogen and a chemoattractant (Grotendorst et al., 1981; Seppa et al., 1982; Grotendorst, 1984). PDGF exists as a homodimer (AA or BB), or as a heterodimer (AB), (Heldin et al., 1986; Hammacher et al., 1988; Stroobant and Waterfield, 1984). There are two PDGF receptor subunits, alpha and beta. β-receptor dimers bind PDGF-BB, β-receptor heterodimers bind both PDGF-AB or BB and α-receptor dimers bind all three forms of PDGF (AA, BB, and AB) (Hammacher et al., 1989; Matsui et al., 1989; Seifert et al., 1989). The PDGF receptors are tyrosine kinases which are activated by dimerization and autophosphorylation after ligand stimulation. Upon activation, the receptor associates with a number of secondary signal transduction molecules including phosphatidylinositol-specific phospholipase C-γ (PLC-γ; Kumjian et al., 1989), Ras-GTPase activating protein (GAP; Kaplan et al., 1990; Kazlauskas et al., 1992), phosphatidylinositol-3 kinase (PI-3) kinase (Coughlin et al., 1989; Kazlauskas and Coo-

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regulators of guanine nucleotide exchange that have been reported include Sos, the mammalian son of sevenless protein which binds directly to Ras and is linked to the EGF receptor by a complex that includes GRB-2 (Gale et al., 1993; Li et al., 1993; Rozakis-Adcock, 1993) or to the PDGF receptor via GRB-2 and the SH-PTP2 tyrosine phosphatase (Syp; Li et al., 1994), and Vav, which may link the T cell receptor–CD3 complex to Ras activation in hematopoietic cells (Gulbins et al., 1993).

Ras regulates both differentiation and mitogenesis. For example, tyrosine kinases that induce neurite outgrowth of PC-12 cells (Hagag et al., 1986), R7 photoreceptor development in Drosophila (Simon et al., 1991), or vulval development in Caenorhabditis elegans (Han and Sternberg, 1990) all use a Ras-dependent signal transduction pathway. In addition, Ras has been shown to regulate growth factor-induced fibroblast mitogenesis (Cai et al., 1990; Mulchay et al., 1985; Stacey et al., 1991). Fibroblasts expressing v-ras, for example, can grow in defined media which does not contain FGF or PDGF (Zhan and Goldfarb, 1986). A few downstream effectors of Ras have also been characterized. Ras associates with the serine/threonine kinase Raf (Vojtek et al., 1993) which has been reported to be downstream of Ras for mitogenesis (Kolch et al., 1990) all use a Ras-dependent signal transduction pathway. In addition, Ras has been shown to regulate growth factor-induced fibroblast mitogenesis (Cai et al., 1990; Mulchay et al., 1985; Stacey et al., 1991). Fibroblasts expressing v-ras, for example, can grow in defined media which does not contain FGF or PDGF (Zhan and Goldfarb, 1986). A few downstream effectors of Ras have also been characterized. Ras associates with the serine/threonine kinase Raf (Vojtek et al., 1993) which has been reported to be downstream of Ras for mitogenesis (Kolch et al., 1990), yet a role for Raf in chemotactic signaling pathways has not been directly demonstrated.

In order to migrate along a chemotactic gradient, a cell must distinguish a greater concentration of ligand at one end versus its opposite end. Classically, chemotaxis follows a bell-shaped curve in which excess ligand inhibits chemotaxis (Devreotes and Zigmond, 1988). We have shown that overexpression of constitutively active chemotactic signaling molecules inhibits migration toward PDGF (Kundra et al., 1994b). In the current study, we have investigated whether overexpression of either constitutively active Ras or Raf could lead to inhibition of PDGF-stimulated chemotaxis. To further study the role of Ras in chemotaxis, we tested the ability of cells transfected with a dominant negative Ras mutant to migrate to PDGF as well as to other attractants such as lysophosphatidic acid (LPA) (van Corver et al., 1989) and fibronectin (Andelmann et al., 1989) that stimulate motility through non-tyrosine kinase receptor pathways. Our results suggest that Ras functions as an intermediate in chemotactic signaling by the PDGF β-receptor.

**Materials and Methods**

**Cell Lines**

ras-transfected BALB/c 3T3 cells were provided by Drs. Charles Stiles and brf transfectant BALB/c 3T3 cells were provided by Dr. Thomas Roberts. ras,raf, and dominant−negative ras-expressing NIH(M17) cells were donated by Dr. Geoffrey Cooper. GRF expressing NIH 3T3 cells were produced as previously described (Shou et al., 1992). NIH 3T3 V8 and GAP4 cell lines were provided by Drs. Jackson Gibbs and Michael Weber (Merck Research Laboratories, West Point, PA). All cell lines were grown in DME (GIBCO BRL, Gaithersburg, MD), supplemented with glutamine, penicillin, streptomycin, and 10% calf serum at 37°C in a humidified 10% CO₂ incubator. NIH(M17), ras and raf transfectant cell lines were grown in 500 µg/ml G418 (Genetecin, GIBCO, BRL). The media for V8 and GAP4 cells was α-MEM containing dialyzed fetal calf serum (HyClone, Logan, UT) and 1 µM methotrexate (Sigma Chemical Co., St. Louis, MO). Cells grown in our laboratory were tested periodically by Western blotting for their continued expression of the transfected genes as well for expression of the PDGF receptor-β.

**Chemotaxis Assay**

Migration was assayed using a multiwell chamber assay (after Boyden, 1962). 25 × 80-mm 8 µm polycarbonate free filters (Nucleopore, Corp., Pleasanton, CA) were coated for one or two days with 100 µg/ml collagen type I (Collaborative Biomedical Products, Bedford, MA) in 0.2 N acetic acid. For some experiments, filters were coated with 1.33 µg/ml fibronectin for 15 min. A dry, coated filter was placed on a 48-blind well chamber (Neuroprobe, Cabin John, MD) over wells containing attractant diluted in DME (JRH Biosciences, Lenexa, KS) or DME alone. The gasket and upper part of the chamber were then assembled. After trypanosmization and dilution, 15,000 cells in 50 µl of DME were added to the top wells. The chamber was then placed in a 37°C, 10% CO₂ incubator for 4 h. Next, the chamber was disassembled and the side of the filter to which the cells were added was scraped. The migrating cells were then fixed in formalin, washed in PBS, and stained overnight in Gill’s triple strength hematoxylin (Polysciences, Warfington, PA). After three washes in water, the filter was mounted in glycerol. All cells within an area representing a well were counted visually. Error bars represent the standard error of three or four replicates.

**Results**

**Dominant Negative Ras Inhibits Migration toward PDGF-BB**

To examine whether Ras is along the signal transduction pathway for PDGF-BB-mediated chemotaxis, we tested the effect of suppressing Ras activity using a dominant-negative Ras mutant. Ras⁴[Asn-17] with an asparagine for serine substitution at position 17 (Asn-17) has a 20-40-fold decreased affinity for GTP without a significant change in its affinity for GDP (Feig and Cooper, 1988). By competing with an upstream Ras regulator (Farnsworth and Feig, 1991; Medema et al., 1992), the Asn-17 Ras mutant inhibits endogenous Ras activity, thereby acting as a dominant-negative mutant. To study PDGF-BB-mediated chemotaxis, we used NIH3T3 cells transfected with the Asn-17 ras mutant under the control of the dexamethasone inducible mouse mammary tumor virus long terminal repeat promoter, NIH(M17) cells (Cai et al., 1990). After a 2-d incubation with 5 × 10⁻⁷ M dexamethasone, the number of NIH(M17) cells crossing the filter in response to PDGF-BB decreased compared to non-induced cells (Fig. 1 A). Induction of Ras expression by dexamethasone had no significant effect on the amount of PDGF receptor-β on these cells (Fig. 1 A, inset). In contrast, nontransfected 3T3 cells showed similar migration toward PDGF-BB in the presence or absence of dexamethasone induction (data not shown).

To determine whether the requirement for activated Ras was specific to tyrosine kinase receptor-mediated cell motility, we tested two additional attractants that bind to
differing receptor types. LPA is a serum component that mediates mitogenesis and chemotaxis, operating via a pertussis toxin-sensitive Gi protein (van Corven et al., 1989; Jalink et al., 1993). As shown in Fig. 2 A, we find that LPA-stimulates M17 cell motility in the Boyden chamber assay and that this is inhibited by induction of dominant-negative Ras. In contrast, soluble fibronectin, a known attractant (Fukai et al., 1992; Aznavoorian et al., 1990) that operates via an integrin-mediated pathway, stimulates motility that is insensitive to the effects of dominant-negative ras expression (Fig. 2 B). This result indicates that cell migration toward gradients of PDGF and LPA but not of fibronectin, are mediated by a pathway that contains activated Ras as an essential intermediate. The result with fibronectin also demonstrates that the dominant-negative ras expression does not result in a general inhibition of cell motility or in a disruption of all directional cell motility.

**Constitutive v-ras Activity Inhibits Migration toward PDGF-BB**

Chemotaxis is a gradient-dependent process that requires a cell to distinguish a higher ligand concentration on one side relative to the opposite side. We have shown previously that the chemotactic response to PDGF-BB can be reduced by either excess ligand or by excess receptor tyrosine kinase activity (Kundra et al., 1994b). To analyze whether excess constitutive Ras activity could also affect PDGF-BB mediated chemotaxis, we employed cells producing constitutively active v-Ras. As shown in Fig. 3 A, NIH 3T3 cells expressing v-ras failed to migrate toward PDGF-BB, although they did display normal random motility in the absence of PDGF. In contrast, control NIH 3T3 cells responded to PDGF in a dose-dependent manner. Similarly, BALB/c 3T3 cells producing constitutively active EJ-Ras did not migrate toward PDGF-BB whereas the control BALB/c 3T3 displayed directional migration toward PDGF-BB (Fig. 3 B).

**Modulators of Ras Activity Affect PDGF-stimulated Chemotaxis**

The experiments described above suggest that the chemotactic response to PDGF-BB is inhibited when Ras activity is altered. To confirm this finding, we used intracellular modulators of Ras that regulate the phosphorylation state of the bound guanine nucleotide. GAP, for example, stimulates the GTPase activity of normal Ras, thus, inhibiting Ras activity (Trahey et al., 1988; Vogel et al., 1988). In GAP4 cells, which overexpress GAP ~110-fold, Ras-GTP levels are reduced in both quiescent and PDGF-stimulated cells relative to cells transfected with vector alone (Gibbs et al., 1990). As shown in Fig. 4, these cells show reduced migration toward PDGF relative to control cells transfected with vector alone. Both cell types displayed a low level of unstimulated random motility in the absence of stimulant, implying that the cells have the ability to migrate across the filter.

As noted above, excess constitutive Ras activity supplied by transfection with mutant v-ras resulted in decreased chemotactic responsiveness to PDGF-BB. Ras can also be activated by expression of a guanine nucleotide-releasing factor such as the brain-derived GRF (Shou et al., 1992). GRF accelerates the rate of GDP release from Ras, increasing the formation of Ras-GTP. Cells expressing constitutive GTPase activity showed reduced migration toward PDGF-BB (Fig. 5) in the Boyden chamber assay confirming that constitutive Ras activity inhibits PDGF-BB mediated chemotaxis. No significant difference in PDGF receptor-β number was observed in the GRF or GAP overexpressing

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**Figure 1.** Migration of M17 cells which have dexamethasone inducible expression of dominant-inhibitory ras toward PDGF-BB. (A) Cells were treated with vehicle (○) or vehicle containing 5 × 10^{-7} M dexamethasone (●) for 2 d before plating onto 8 μm porous filters in a Boyden multiwell chemotactic chamber containing the indicated concentrations of PDGF in the bottom well. Expression of the PDGF-receptor-β was not altered after dexamethasone induction of dominant negative Ras (inset).

**Figure 2.** Migration of M17 cells in response to lysophosphatidic acid (LPA) or fibronectin. LPA or fibronectin were added in the indicated concentrations to the lower chambers of Boyden multiwell chemotactic chambers. M17 cells transfected with dexamethasone-inducible dominant negative Ras were tested for their ability to migrate toward LPA (A) or fibronectin (B) in the presence or absence of 48-h pretreatment with dexamethasone.
cell lines (data not shown) and several groups have previously demonstrated that Ras overexpression does not markedly alter PDGF receptor-β number or affinity (Benjamin et al., 1987; Zullo and Failer, 1988; Kaplan et al., 1990). Thus, our results are unlikely to be due to decreased levels of PDGF receptors in these cell lines. Our current results do suggest that a window of appropriate Ras activity is necessary for optimal chemotactic movement toward PDGF-BB. Substantially increased or decreased levels disable the chemotactic response.

**Excess Raf Activity Does Not Affect Migration toward PDGF-BB**

To identify potential downstream Ras effectors involved in mediating chemotaxis toward PDGF-BB, we tested the effect of constitutive Raf activity on PDGF induced chemotaxis. Raf binds Ras directly (Vojtek et al., 1993) and appears to be downstream of Ras in a number of signal transduction pathways (Adelmann et al., 1989; Cantley et al., 1991; Carthew and Rubin, 1990) including those leading to mitogenesis (Kolch et al., 1991). If Raf operates downstream of Ras in PDGF-induced chemotaxis, one would expect constitutively active Raf to inhibit PDGF-BB mediated chemotaxis as does constitutively active Ras. Our results, shown in Fig. 6, demonstrate that either NIH 3T3 or BALB/c 3T3 cells producing constitutively active Raf migrate effectively toward PDGF-BB. The data imply that unlike constitutive Ras activity, constitutive Raf activity does not inhibit PDGF-BB induced chemotaxis.

**Discussion**

Using cells in which Ras activity has been modulated, we find that either stimulation or suppression of Ras activity results in reduced cellular chemotaxis toward PDGF-BB. Ras has previously been shown to be an essential intermediate in PDGF-stimulated mitogenesis (Zhan and Goldfarb, 1986). Our current results imply that Ras is also an important signaling intermediate in PDGF-stimulated chemotaxis. Previous results from our laboratory (Kundra et al., 1994a) indicated that the signaling pathways for mitogenesis and chemotaxis are not identical. Consequently, the finding that Ras is involved in the chemotactic pathway highlights this molecule as an important intermediate in two distinct responses to a single ligand.

Evidence for an involvement of Ras in chemotactic signaling is provided by experiments using a dominant negative Ras construct in which asparagine is substituted for serine normally present at amino acid 17 (NIH-M17 cells). Expression of dominant-negative ras resulted in severely limited chemotaxis toward PDGF-BB. Expression of dominant-negative ras also blocked cell motility induced by lysophosphatidic acid but not that stimulated by fibroectin. These results indicate that Ras is not essential for all forms of stimulated cell motility in 3T3 cells.

Previously, it was shown that the level of dominant-negative Ras activity can influence Ras-dependent functions such as fos induction in fibroblasts (Amrosio et al., 1989) and neurite outgrowth in PC-12 cells (Szederenyi et al., 1990). Ras activation has recently been shown to have a
role in wound-stimulated cell motility in corneal and vascular endothelial cells (Sosnowski et al., 1993; Fox et al., 1994) whereas conflicting results have been reported for the role of Ras in epithelial cell scattering in response to scatter factor (Takaishi et al., 1994; Hartmann et al., 1994). The level of downstream Ras signaling activity appears to influence chemotaxis as well since PDGF stimulates chemotaxis but not random motility in 3T3 cells (Kundra, 1994b) and since cells expressing dominant negative Ras remain able to move toward fibronectin.

Ras p21 can be regulated at the level of GTPase activity and at the level of nucleotide exchange. Ras is active when bound to GTP (Grand and Owen, 1991). GAP accelerates the intrinsic GTPase activity of normal Ras by up to 100-fold (Trahey and McCormick, 1987). Cells producing PDGF receptor mutants unable to bind GAP display wild-type levels of mitogenesis (Fantl et al., 1992; Kashishian et al., 1992), but increased migration in response to PDGF-BB (Kundra et al., 1994a). Thus, although GAP binding to the PDGF receptor does not effect mitogenesis, it can negatively regulate chemotaxis toward PDGF-BB.

If Ras activity were involved in PDGF-BB mediated chemotaxis, one would expect that GAP overexpression could potentially influence migration toward PDGF by reducing the percentage of Ras activated by PDGF. GAP4 cells overexpress GAP 110-fold and have a reduced percentage of Ras complexed to GTP when unstimulated and when stimulated with PDGF (Gibbs et al., 1990). These cells were inhibited in their ability to migrate toward PDGF-BB, adding support to the hypothesis that GAP can regulate the chemotactic response to PDGF-BB, possibly by suppressing Ras activity.

Classically, the chemotactic response is characterized by a bell-shaped curve with either too little or too much ligand limiting chemotaxis (Devreotes and Zigmond, 1988). Inhibition of the chemotactic response can be accomplished either by autocrine production of excess ligand or by expression of constitutive tyrosine kinase activity (Kundra et al., 1994b). If the reduced chemotactic response to excess ligand were mimicked at the level of the downstream effector, excess Ras activity should also suppress chemotaxis in response to PDGF-BB. In support of this hypothesis, we found decreased chemotaxis toward PDGF in cells in which Ras activity was upregulated by two different methods. In the first case, we employed cells overexpressing brain-derived GRF, a guanine-releasing factor that facilitates exchange of Ras-GDP to Ras-GTP, resulting in increased levels of activated Ras (Shou et al., 1992). In the second case, we employed cells producing constitutively activated EJ-Ras or v-HA-Ras. In all cases tested, cells with increased ras expression showed diminished chemotaxis toward PDGF.

We did not observe a significant increase in the unstimulated random motility of our ras expressing cells, although such an increase has been reported previously (Groten-dorst, 1984). These differences may be due to the type of Ras used. We have shown previously, however, that signal transduction pathways that modulate PDGF-stimulated chemotaxis are independent of those mediating unstimulated random motility (Kundra et al., 1994b). The loss of the chemotactic response in Ras transfected cells cannot be ascribed to cellular transformation since cells transformed by the serine/threonine kinase Mos do migrate toward PDGF (Kundra et al., 1994b) and data presented here show that cells producing constitutively active Raf are also capable of migrating toward PDGF-BB. Thus, transforming oncogene expression does not necessarily diminish the chemotactic response to PDGF-BB.

Raf has been reported to be downstream of Ras in a number of signal transduction systems, including mitogenesis of fibroblasts (Amrosio et al., 1989; Cantley et al., 1991; Kolch et al., 1991). Raf is recruited by Ras to the plasma membrane where it is subsequently activated (Dent et al., 1992; Stokoe et al., 1994; Leevers et al., 1994). Raf kinase activity directly leads to activation of MAP kinase (Mek-1) and subsequent activation of MAP kinase (Dent et al., 1992; Huang et al., 1993; Kyriakis et al., 1992; Williams et al., 1993). Complexes containing Ras-GTP, Raf and MAP kinase have been isolated (Moody et al., 1993). If Raf were downstream of Ras for migration toward PDGF, constitutive Raf activity, like constitutive Ras activity, might be expected to suppress chemotaxis toward PDGF. However, both BALB/c 3T3 cells and NIH 3T3 cells producing constitutive Raf activity migrated toward PDGF-BB.

Although Raf appears to be along the pathway for PDGF mediated mitogenesis, it may lie outside the pathway for PDGF-BB mediated chemotaxis. This would imply that the signal transduction pathways leading from a single tyrosine kinase receptor to either mitogenesis or chemotaxis diverge at a point distal to Ras activation in 3T3 cells. If Raf is not involved in signaling for PDGF-induced chemotaxis, it would imply either that MAP kinase is not involved or that an alternate route of MAP kinase activation was being employed such as has been proposed for MAP kinase activation in rat fibroblasts (Kizakondoh and Okayama, 1993). Bornfeldt et al. (1994) have also reported that IGF-1 can induce chemotaxis independent of MAP kinase activation. Ras can interact directly with the catalytic subunit of PI3 kinase and inhibition of Ras activity suppresses growth factor-induced PI3 kinase activity (Rodriguez-Viciana et al., 1994). Vojtec et al. (1994b) have found additional Ras-binding proteins other than Raf and such proteins may play a role in chemotaxis. Together, these results imply that novel signaling intermediates that are downstream of Ras may function in the intracellular regulation of PDGF-BB mediated chemotaxis. Future work will be necessary to determine whether activation of MAP kinase is necessary for PDGF-induced chemotaxis since MAP kinase is necessary for mitogenesis and can be activated by both Raf-dependent and Raf-independent mechanisms (Dent et al., 1992).

Using studies employing both inhibited and excess Ras activity, we identify Ras as a member of the signal transduction pathway for PDGF-BB mediated chemotaxis. We also support the idea that one may be able to identify downstream effectors or mediators of downstream effectors involved in chemotaxis toward a particular ligand by either inhibiting or over-producing signaling molecule activity (Kundra et al., 1994b). Our data suggests that Ras is a downstream modulator of PDGF-BB mediated chemotaxis. Further such studies should help distinguish signaling pathways involved in regulating chemotaxis and in finding post-Ras pathways that transduce signals involved in chemotaxis.
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