RAPID COMMUNICATION

Receptor Tyrosine Kinase Stimulates Cell-Matrix Adhesion by Phosphatidylinositol 3 Kinase and Phospholipase C-γ1 Pathways

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Receptor tyrosine kinases are known to be important in growth and differentiation. We have recently found that c-kit, the tyrosine kinase receptor for steel factor, also regulates cell-matrix adhesion. Because Steel factor helps regulate cell migration and localization, this may be an important biologic function. Integrin adhesiveness is regulated within minutes by c-kit. The signaling pathways for tyrosine kinase stimulation of integrin adhesiveness and their relation to pathways that regulate growth and differentiation over much longer time periods remain uncharacterized. We have studied the effector pathways by which receptor tyrosine kinases regulate cell-matrix adhesion using wild-type and mutant forms of the platelet-derived growth factor (PDGF) receptor, which is closely related to c-kit. The PDGF receptor expressed in mast cells is as potent as c-kit in stimulating adhesion to fibronectin. We show that induction of adhesion is regulated through two independent pathways of phosphatidylinositol 3 kinase (PI3K) and phospholipase C-γ1 (PLCγ)-protein kinase C by elimination of autophosphorylation sites required for activation of PI3K and PLCγ or in combination with downregulation of protein kinase C or wortmannin. By contrast, a receptor mutated in both the PI3K and PLCγ association sites can still stimulate mast cell growth, indicating a crucial role of these effector molecules in regulating adhesion rather than cell growth.

STEEL FACTOR stimulation of bone marrow-derived mast cell adhesion to fibronectin (FN)[1,2] requires the tyrosine kinase activity of c-*kit* and is mediated at least in part by the integrin VLA-5 ($\alpha5\beta1$). Activation of adhesion is transient and occurs at concentrations of Steel factor 100-fold lower than those required for growth stimulation. Steel factor does not induce any change of cell surface density of VLA-5, suggesting that the affinity or some other qualitative change is stimulated.[1] This is reminiscent of the increased adhesiveness in lymphocyte function-associated antigen-1 (LFA-1) stimulated by T-cell receptor cross-linking.[3] T-cell receptor cross-linking was shown to result in transient adhesiveness of LFA-1. It is believed that cytoplasmic signals initiated by T-cell receptor cross-linking modulate avidity of integrins (inside-out signaling).[4] However, it is unclear which signaling pathways initiated by tyrosine kinases are required to achieve avidity modulation of integrins.[5] We use here the platelet-derived growth factor (PDGF) receptor to understand these molecular mechanisms. Upon binding to PDGF, the PDGF receptor dimerizes and autophosphorylates tyrosine residues in its cytoplasmic region, creating binding sites for Src-homology-2 (SH2) domains from signaling molecules such as ras GTPase-activating protein (GAP), phosphatidylinositol 3 kinase (PI3K), and phospholipase C-γ1 (PLCγ).[6] Point mutations at these sites selectively block binding of these molecules to the receptor.[7,8] Studies using these mutant PDGF receptors indicated that PI3K and PLCγ may play a role in initiation of DNA synthesis, although the exact effect of mutations varies in the context of different cells.[7,9-11] Recent study also showed that mutation of PI3K binding sites in c-*kit* reduced Steel factor-induced adhesion partially.[12] However, it is unclear from this study whether PI3K could bind to other sites or whether other effector molecules could be involved. We show here that the PDGF receptor is as potent as c-*kit* in stimulating adhesion to FN and that adhesiveness of integrin to FN is regulated in two pathways independently by PI3K and PLCγ-protein kinase C, respectively, which are activated by the PDGF receptor. These pathways are dispensable for growth of mast cells, indicating crucial roles of these effector molecules in regulating the adhesiveness of integrin.

MATERIALS AND METHODS

Cell lines. Mast cells were established from mouse bone marrow, as described,[1] and retrovirus-mediated transfection was used to introduce the above-mentioned genes. Retrovirus-producing packaging cell lines were established by transfecting GP + E86[13] with a retrovirus vector, pBabe,[14] alone (neoR) or containing cDNA encoding the wild-type mouse PDGFβ receptor or PDGF mutant receptors for kinase activity (K602A),[15] the GAP binding site (Y739F), the PI3K binding site (Y708 and 719F),[7] or the PLCγ1 association site (Y977 and 989F).[16] Mast cells (2×10^6 cells in 30 mL of RPMI 1640 containing 10% fetal calf serum, 50 μmol/L β-mercaptoethanol, 10% WEHI-3 conditioned medium as a source of interleukin-3) 4 weeks after establishment were cocultured with irradiated (3,000 rad) subconfluent GP + E86 cells. Infected mast cells were selected with G418 (0.8 mg/mL) for 2 weeks and expanded.

Adhesion assays. The adhesion assay to FN was performed as described.[1] Briefly, mast cells labeled with 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF) in 96-well plates precoated with FN (2 μg/well) were incubated in triplicate at 37°C for 30 minutes in the presence of phorbol 12-myristate 13-acetate (PMA; 10 ng/mL; Sigma, St Louis, MO), Steel factor (1 U/mL; Genzyme, Cambridge, MA), human PDGF-BB (10 ng/mL; Oncogene Science, La Jolla, CA), or medium alone as indicated. The amounts of these factors were chosen to give the maximum response. After washing

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the plate four times, levels of bound fluorescence were measured with a Pandex fluorescence concentration analyzer (IDEXX Laboratories, Inc, Westbrook, ME). The level of adhesion was calculated by dividing bound fluorescence by input fluorescence. Mast cells did not adhere to wells precoated with bovine serum albumin with or without stimulation (data not shown). For the pretreatment with PMA, mast cells were incubated with 100 ng/mL of PMA for 40 hours. The pretreatment had no effect on the viability of mast cells. PMA was washed away before the adhesion assay. For the experiment with wortmannin (Wako Pure Chemical Ltd, Tokyo, Japan), cells were treated with wortmannin at room temperature for 10 minutes, as described.[17]

Western blotting analysis. Infected mast cells were prepared for cell lysates as described.[7] Equal amounts of protein (50 μg corresponding to about 8×10^5 cells) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), Western blotting, and detection with rabbit anti-PDGF receptor antibodies[7] or isotype-specific rabbit anti-PKCα, β, or ζ antibodies (GIBCO-BRL, Gaithersburg, MD), followed with ^{125}I-protein A (ICN, Irvine, CA). In some experiments, mast cells were stimulated with PDGF (100 ng/mL) for 1 minute at 37°C, and cell lysates were immunoprecipitated with anti-PDGF receptor antibodies. Western blots were probed with rabbit polyclonal antibodies specific for phosphotyrosine,[18] GAP, PI3K, and PLCγ (UBI, Lake Placid, NY) and for ^{125}I-protein A (ICN).[7]

Measurement of DNA synthesis. Mast cells were cultured in 96-well plates (5×10^4 cells in 0.2 mL/well) for 24 hours with or without growth factors. Methyl-^{3}H-thymidine (6.7 Ci/mmol; NEN, Boston, MA) was added into wells (0.5 μCi/well) 4 hours before cells were harvested. Incorporated ^{3}H-thymidine was collected onto glass filters and counted by liquid scintillation.

RESULTS AND DISCUSSION

To test whether the PDGF receptor can substitute for c-*kit,* wild-type and mutant forms of the PDGF receptor were introduced into mast cells and examined for ability to induce binding to FN (Fig 1A). When treated with PDGF, the transfectants expressing the wild-type PDGF receptor bound FN to the same extent as when treated with Steel factor or phorbol ester. Mast cells transfected with vector alone did not respond to PDGF. PDGF-stimulated adhesion was at least in part mediated by VLA-5 because the anti–VLA-5 antibody (MFR-5) reduced adhesion by 60% to 70% and 1.5 mmol/L RGDS but not RGES peptide reduced adhesion by more than 90% (data not shown), as was the case for PMA- or Steel factor-stimulated adhesion.[1] Mast cells expressing the PDGF receptor kinase defective mutant, K602A,[15] were unable to respond to PDGF but responded well to PMA and Steel factor, indicating that the tyrosine kinase activity of the PDGF receptor is prerequisite in stimulating the binding to FN, as is the case with c-*kit.*[1] The PDGF receptors mutated in the tyrosines required for association with the SH2 domains of either GAP, PI3K, or PLCγ bound FN in response to PDGF as well as PMA and Steel factor. However, mutation of both PI3K and PLCγ binding sites almost eliminated PDGF-induced adhesion to FN, whereas the response to PMA or Steel factor was comparable to that of wild-type. The level of expression of the PDGF receptors was similar, and Western blots verified that mutations had the expected effects on association of GAP, PI3K, and PLC-γ (Fig 2).

PLCγ produces diacylglycerol and inositol phosphates that release Ca^{2+}. In turn, these activate protein kinase C

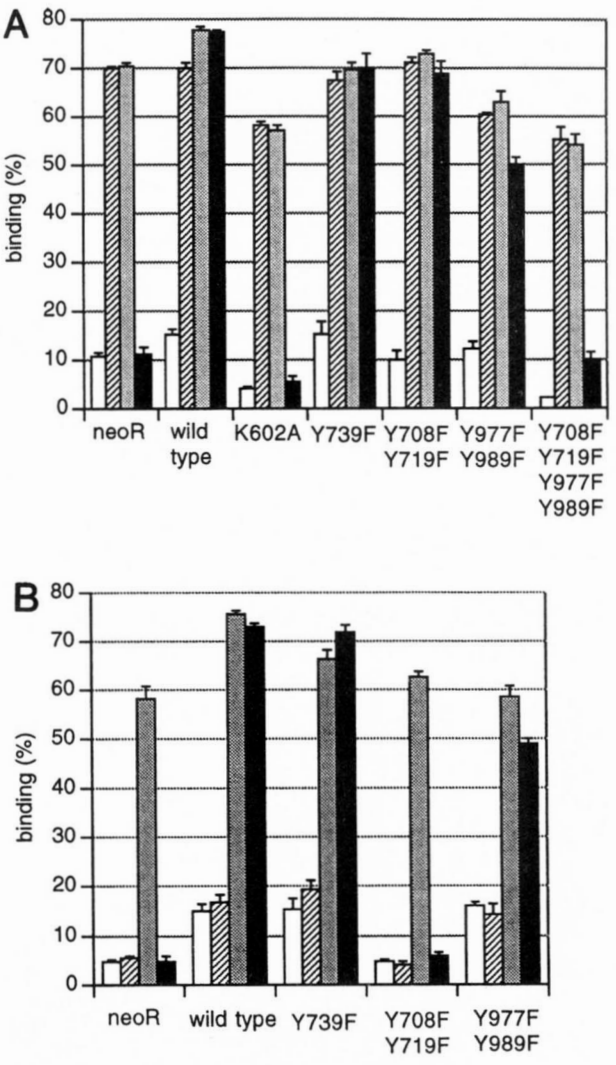

Fig 1. (A) Adhesion to FN of mast cells unstimulated (□) or stimulated with PMA (▨), Steel factor (▤), or PDGF (■). Mast cells were transfected with genes encoding neomycin resistance (neoR), with the wild-type mouse PDGFβ receptor (wild), or with PDGF mutant receptors for kinase activity (K602A), the GAP binding site (Y739F), the PI3K binding site (Y708 and 719F), the PLCγ1 association site (Y977 and 989F), or both PI3K and PLCγ binding sites (Y708, 719, 977, and 989F). (B) Adhesion to FN of mast cells pretreated with PMA. The PMA pretreatment was performed as described in the Materials and Methods and assayed for adhesion to FN as in (A). Bars show the standard error.

(PKC).[19] To study how PKC contributes to stimulation of binding to FN, mast cells were pretreated with PMA to down-regulate PKC (Fig 1B). Mast cells pretreated with PMA failed to bind to FN in response to further PMA, but did respond to Steel factor to a degree similar to that of untreated mast cells. PMA pretreatment did not affect PDGF-induced FN binding by mast cells with the PDGF receptors of wild-type or mutated at the GAP and PLCγ sites. However, PMA-pretreated mast cells expressing the PDGF receptor mutated in the PI3K site became unresponsive to PDGF. By contrast, these cells adhered to FN in response to Steel factor. Mast

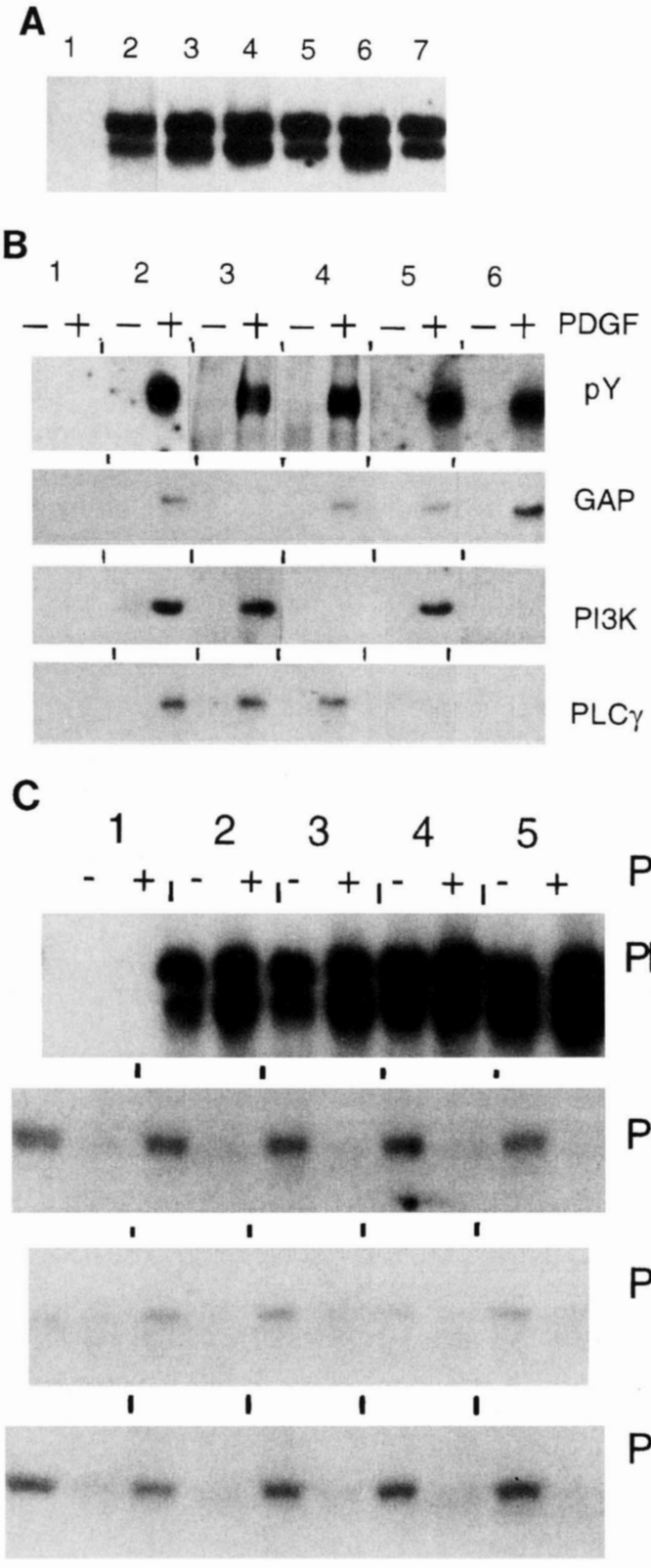

Fig 2. Expression and the specificity of mutations of the PDGF receptor. (A) Western blot analysis with the polyclonal anti-PDGF receptor antibody of whole lysates from mast cells infected with retroviruses carrying the neomycin resistance gene only (lane 1), the wild-type PDGF receptor (lane 2), K602A (kinase defective, lane 3), Y739F (GAP site mutant, lane 4), Y708, 719F (PI3K site mutant, lane 5), Y977, 989F (PLCγ site mutant, lane 6), or Y708, 719, 977, 989F (PI3K and PLCγ site mutant, lane 7). Ligand binding assay using ^{125}I-human PDGF BB showed that the PDGF receptor was expressed at approximately 0.9, 0.8, 0.9, 1.5, 0.9, and 1.4 × 10^4 receptors per cell for wild-type and above mutants, respectively. (B) The association of the PDGF receptor and its mutants with specific molecules. Cell lysates of mast cells stimulated with (+) or without (−) PDGF immunoprecipitated with anti-PDGF receptor antibody were subjected to Western blotting with antibodies against phosphotyrosine (pY), GAP, PI3K, or PLCγ. Lane 1, K602A; lane 2, wild-type; lane 3, Y739F; lane 4, Y708, 719F; lane 5, Y977, 989F; lane 6, Y708, 719, 977, 989F. (C) Effect of PMA treatment on the expression of the PDGF receptor and PKCα, β, and ζ. Lane 1, neoR; lane 2, wild-type; lane 3, Y739F; lane 4, Y708, 719F; lane 5, Y977, 989F. Mast cells pretreated with (+) or without (−) PMA as in Fig 1 were analyzed with Western blotting using antibodies against proteins as indicated.

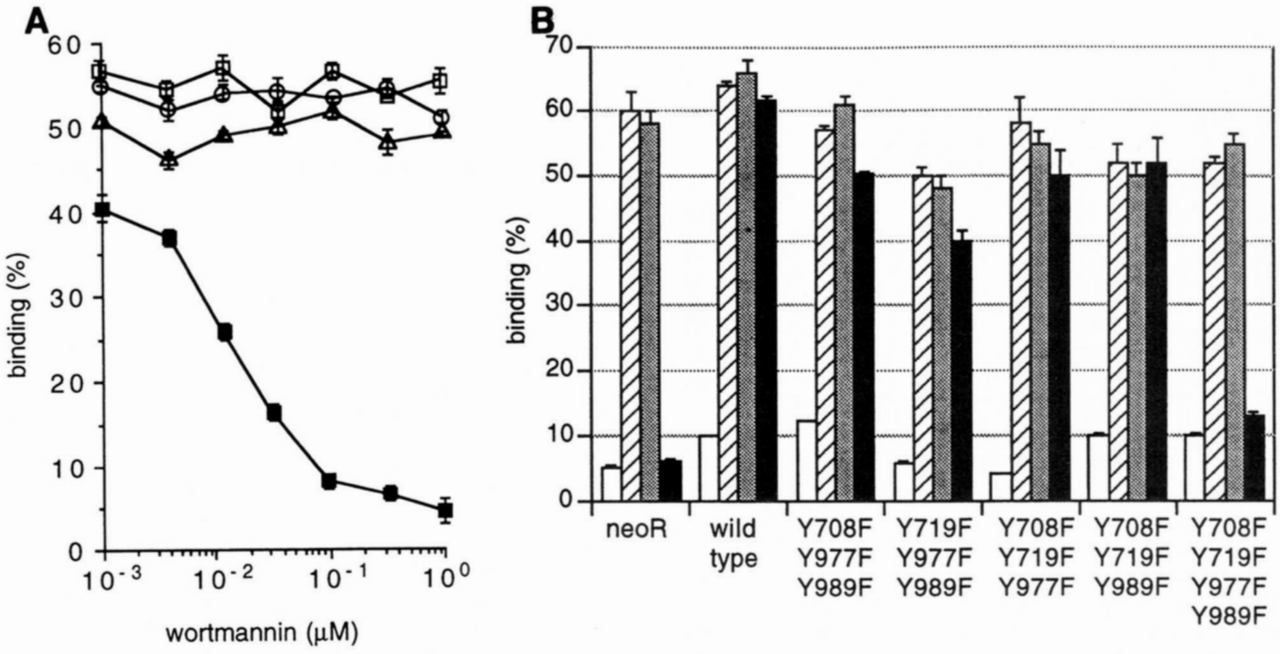

Fig 3. (A) Inhibition of PDGF-stimulated adhesion with wortmannin. Mast cells expressing the wild-type PDGF receptor (○), Y739F (□), Y708, 719F (△), or Y977, 989F (■) were treated with indicated amounts of wortmannin at room temperature for 10 minutes. FN binding stimulated with PDGF was assayed as in the legend to Fig 1. (B) The effect of single tyrosine mutation in PI3K binding sites or PLCγ binding sites. Adhesion to FN of mast cells expressing wild-type or the series of mutations of tyrosine to phenylalanine at the indicated position without stimulation (□) or with PMA (▨), Steel factor (▤), or PDGF (■).

cells expressed PKCα, β, and ζ, but not γ, δ, and ϵ. The pretreatment with PMA caused downregulation of all isotypes expressed, including PKCζ, which was reported to be rather resistant to the PMA pretreatment in other cell types,[20] but no reduction in the expression of the PDGF receptors (Fig 2C), c-*kit,* VLA-5, or effector molecules, including PI3K, PLCγ, and vav (data not shown). This result indicates that the mutant lacking PI3K binding sites is dependent on PKC, perhaps through PLCγ, to induce adhesion to FN.

Moreover, wortmannin, a PI3K inhibitor, inhibited PDGF-induced adhesion of mast cells expressing the PDGF receptor lacking PLCγ sites in the dose-dependent manner, but did not affect wild-type or the one lacking PI3K or GAP binding sites at all (Fig 3A). This result indicates that the effect of wortmannin on adhesion is specific and that the PDGF receptor lacking PLCγ sites requires PI3K to induce adhesion to FN.

Taken together, the above-mentioned results indicate that there are two pathways from the PDGF receptor in which PI3K and PLCγ independently transmit signals that activate integrin adhesiveness. The pathway initiated by PLCγ is dependent on PKC, as shown by downregulation by pretreatment with PMA of the response through the receptor with the PI3K association site mutation. The receptor with the mutation in the PLCγ site depends on the PI3K pathway, which was shown by inhibition with wortmannin. Mutation of the binding site of PI3K in c-*kit* inhibited adhesion to FN partially.[12] Steel factor-induced adhesion was shown to be inhibited completely by treatment with wortmannin only when mast cells were pretreated with PMA.[21] These results suggest that c-*kit* also stimulates adhesion in a fashion similar to the PDGF receptor. Downstream effectors of these pathways are uncertain at present. The product of PI3K, inositol-3,4,5 trisphosphate was reported to be a potent stimulator for PKCζ in vitro.[22] However, the downregulation of PKCζ by PMA pretreatment (see Fig 2C) suggests that effector molecules other than PKCζ are important in PI3K-stimulated signaling in mast cells.

Furthermore, to rule out the possibility that other effectors such as Nck and Syp, which could bind to these sites (Y719 for Nck[23]; Y977 is more important for Syp than Y989[24]) play a role in stimulating adhesion, we examined the effect of the single tyrosine mutation in the PI3K binding sites and PLCγ binding sites. Because the PI3K binding sites and PLCγ binding sites compensate each other, the single mutation was studied with the other binding sites being mutated (Fig 3B). Single tyrosine mutations in PI3K binding sites or PLCγ binding sites did not show any significant difference in adhesion, indicating potential bindings of other effectors such as Nck and Syp were not likely involved in PDGF-stimulated adhesion.

The tyrosine kinase activity of the PDGF receptor was crucial for stimulation of growth[15] (Fig 4) as for adhesion. However, the mutant PDGF receptor that was unable to associate with GAP, PI3K, PLCγ, or both PI3K and PLCγ still stimulated DNA synthesis to an extent similar to wild-type (Fig 4). Although PI3K and PLCγ were reported to be important for growth control and chemotaxis in fibroblasts lineages,[7,9,25] exact effects vary in different lineages[10,11] and apparently these sites are not indispensable for growth in

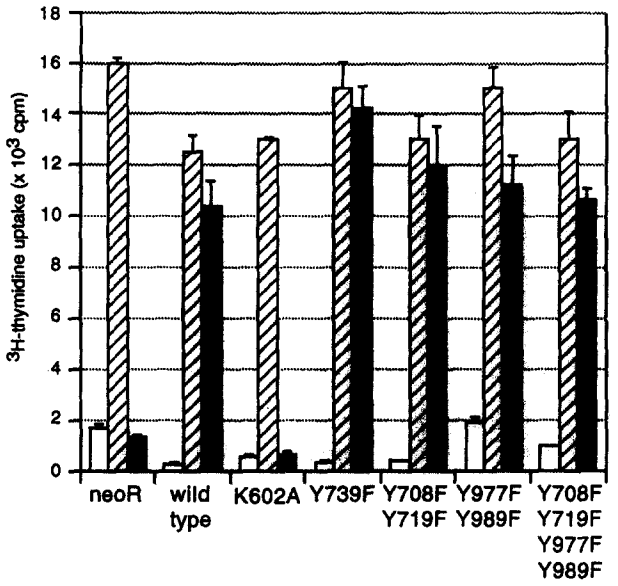

Fig 4. Growth response of mast cells transfected with the PDGF receptors. Mast cells transfected with vector alone, the wild-type PDGF receptor, or its mutants, as described in the legend to Fig 1, were cultured with Steel factor (■; 100 U/mL), PDGF-BB (■; 100 ng/mL), or medium alone (□). Uptake of methyl-^{3}H-thymidine was measured as described in the Materials and Methods.

mast cells. Consistent with this result, activation of p21ras was not affected with mutation at either or both of PI3K and PLCγ binding sites (data not shown).

Our results show that PI3K and PLCγ play a pivotal role in regulation of cell-matrix adhesion by a tyrosine kinase receptor and that regulation of adhesion to the extracellular matrix can be dissociated from regulation of cell growth.

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