Phosphatidylinositol-3'-Kinase Is Not Required for Mitogenesis or Internalization of the Flt3/Flk2 Receptor Tyrosine Kinase*

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Flt3/Flk2 is a receptor tyrosine kinase that is expressed on early hematopoietic progenitor cells. Flt3/Flk2 belongs to a family of receptors, including Kit and colony-stimulating factor-1R, which support growth and differentiation within the hematopoietic system. The Flt3/Flk2 ligand, in combination with other growth factors, stimulates the proliferation of hematopoietic progenitors of both lymphoid and myeloid lineages in vitro. We report that phosphatidylinositol 3'-kinase (PI3K) binds to a unique site in the carboxy tail of murine Flt3/Flk2. In distinction to Kit and colony-stimulating factor-1R, mutant receptors unable to couple to PI3K and expressed in rodent fibroblasts or in the interleukin 3-dependent cell line Ba/F3 provide a mitogenic signal comparable to wild-type receptors. Flt3/Flk2 receptors that do not bind to PI3K also normally down-regulate, a function ascribed to PI3K in the context of other receptor systems. These data point to the existence of other unidentified pathways that, alone or in combination with PI3K, transduce these cellular responses following the activation of Flt3/Flk2.

Vertebrate hematopoiesis is maintained throughout the life of the organism by a small number of stem cells that have the capacity to give rise to all mature myeloid and lymphoid lineages and to repopulate their own numbers through a process known as self-renewal (1). Murine hematopoietic stem cells (HSCs) express characteristic cell surface proteins (AA4.1, Sca1, Thy1.1, Lin−) (2, 3) and are functionally heterogeneous, based upon their state within the cell cycle (4). The majority of HSCs are in a dormant G0/G1 state and are capable of radio-protection and long term reconstitution, while approximately 20% of the cells are in the S-G2-M phases of the cell cycle and have reduced reconstituting ability. The cycling fraction of HSCs may represent an early step toward the commitment of these cells to differentiate into lineage-restricted progenitor cells (4). Little is currently known about the signal transduction molecules that influence the cell cycle of hematopoietic stem cells and the activation of lineage-specific developmental programs. Kit (5-10) and Flt3/Flk2, (11, 12) receptor tyrosine kinases, are expressed on HSCs and may participate in these processes. Kit is expressed on AA4.1 Sca1 fetal liver cells capable of long-term reconstitution of irradiated recipient mice at high frequency (12). Flt3/Flk2 is expressed on cells of similar phenotype, which in distinction to the Kit-positive cells, are actively cycling and have reduced reconstitution capacity (12). W mice, which have loss-of-function mutations within the Kit locus, demonstrate diminished stem cell activity and have developmental defects in the erythroid and mast cell lineages (13-15). Mice in which the Flt3/Flk2 locus has been disrupted by homologous recombination have no defect in stem cell self-renewal but are deficient in the steady-state numbers of CD43+ pro-B cells and in repopulating both the lymphoid lineages during competition restitutive assays (16). The Flt3/Flk2 ligand, FL, by itself is a weak proliferative stimulus for Lin−Sca11 progenitors; however, in combination with other growth factors such as granulocyte-CSF, granulocyte/macrophage-CSF, IL-3, IL-6, IL-7, IL-11, and IL-12, it acts as a potent mitogen (17-20). AA4.1 Sca1 B220 progenitor cells from day 12.5 fetal liver when cultured in combination with IL-11 and FL give rise to IL-7-responsive pre-B cells at high frequency (105). Although Flt3/Flk2 does not appear to play a role in maintaining the process of HSC self-renewal, Flt3/Flk2 in collaboration with other cytokine factors may function to expand the cycling pool of HSCs destined to commit to the lymphoid lineage.

To elucidate the biochemical basis for Flt3/Flk2 mitogenic function in early B-cell ontogeny, we set out to determine the proximal signaling molecules that interact with Flt3/Flk2. Flt3/Flk2 belongs to the class III family of RTKs, based on structural similarities that include the PDGF α and β receptors, CSF-1R, Kit, and Flt3/Flk2. These molecules are characterized by a ligand-binding extracellular domain composed of five immunoglobulin-like domains and a kinase domain biased by a non-catalytic region, known as the kinase insert. The kinase insert contains tyrosine autophosphorylation sites and, in the case of Kit, serine residues that are phosphorylated by protein kinase C (21). Phosphorylation of tyrosine residues in the PDGF β receptor kinase insert leads to the creation of
high affinity binding sites for SH2-containing signaling proteins such as Grb2 (22), phosphatidylinositol 3'-kinase (PI3K) (23, 24), Nck (25), Gap (26, 27), and Shc (28). In this report, we demonstrate that PI3K, a lipid kinase that has been implicated in mediating mitogenesis and receptor internalization, binds to Flt3/Flik2 in a region outside of the kinase insert domain. In distinction to PDGFRβ (26, 27, 37), CSF-1R, and Kit (38), Flt3/Flik2 receptors which are uncoupled from PI3K down-modulate normally and are still capable of providing a full mitogenic signal. These data suggest that Flt3/Flik2 may interact with unique mitogenic signaling pathways that are operative in the absence of PI3K activation.

**MATERIALS AND METHODS**

**Growth Factors, Antibodies, GST Fusion Proteins, and Cell Lines**

Recombinant human CSF-1 was kindly provided by Dale Ando (Chiron Corp.) Supematant from transfection X63Ag8-653 myeloma cells was used as a source of IL-3 (39). The anti-Flt3/Flik2 antibodies were provided in rabbits against a TrpE interkinase fusion protein as described previously (40). The Shc and Gap antisera were generously provided by J. Jane McClade (Amgen Institute, Toronto, Ontario, Canada) and Mike Moran (Banting and Best Institute, Toronto, Ontario, Canada), respectively. p85(NΔa) and p85(NΔada) and Mike Moran (Banting and Best Institute, Toronto, Ontario, Canada), respectively. p85 antibodies were produced in rabbits against p85(NΔada) and Mike Moran (Banting and Best Institute, Toronto, Ontario, Canada), respectively. p85 antibodies were produced in rabbits against p85(NΔada) and Mike Moran (Banting and Best Institute, Toronto, Ontario, Canada), respectively. The Shc and Gap antisera were generously provided by J. Jane McClade (Amgen Institute, Toronto, Ontario, Canada), respectively. The Shc and Gap antisera were generously provided by J. Jane McClade (Amgen Institute, Toronto, Ontario, Canada), respectively.

**Cell Transfection and Infection**

Cos7 cells were transfected using 1 μg of vector DNA and Lipofectin (Life Technologies, Inc.) according to manufacturer’s protocol. GP-1+E cell lines producing helper-free retrovirus expressing FF3 or mutant receptors were prepared as described previously (42), and used to infect Rat2 cells and BA/F3 cells.

**In Vitro Kinase Assays, PI3K Assays, and Western Blotting**

**RESULTS**

Expression of Wild-type and Phenyalanine-mutant FF3 Receptors in Rat2 Fibroblasts—We and others have shown previously that Flt3/Flik2 binds to PI3K (46, 47). To study the function of PI3K in the context of Flt3/Flik2 signaling, we first needed to identify its binding site on Flt3/Flik2. PI3K binds to unique sites defined by the consensus motif YXXM, within the kinase insert domain of PDGFRα (48), PDGFRβ (23, 24), CSF-1R (49), and Kit (50–52). Flt3/Flik2 also has a kinase insert domain, but it contains no PI3K binding sites, conforming to the consensus motif or the variant sequences YVXY found in the hepatocyte growth factor/scatter factor receptor (53) or YVLV in the erythropoietin receptor (54). However, two putative PI3K binding sites exist within the carboxy tail of Flt3/Flik2 surrounding codons 922 and 958, respectively. We have previously shown, by peptide competition experiments, that a phosphopeptide containing 958 but not 922 was able to block PI3K association with Flt3/Flik2 in vitro (47).

To determine the location of the PI3K binding site in vivo and to assay the function of PI3K-uncoupled Flt3/Flik2 receptors in biological assays, we substituted tyrosine at codons 922 and 958 with phenylalanine. These studies used a chimeric receptor FF3, composed of the CSF-1R ligand-binding ectodomain fused to the transmembrane and cytoplasmic domains of murine Flt3/Flik2, which we have previously shown transduces both biochemical and biological signals in response to CSF-1 (40). Wild-type and mutant FF3 receptors (F922 and F958) were expressed in Rat2 cells, and the IL-3-dependent hematopoietic cell line, BA/F3, using retroviral infection. Neomycin-resistant pooled populations and individual clones were selected by FACs analysis for comparable receptor expression.
levels (data not shown). In vitro kinase and in vivo ligand-induced autophosphorylation assays were performed to investigate whether mutations at codons 922 and 958 interfered with the intrinsic catalytic activity of the receptors. FF3 immune complexes derived from 10^6 cells Rat2 expressing FF3, -F922, and -F958, and lysed in RIPA buffer, were used in in vitro kinase assays. Fig. 1A shows that mutant and wild-type receptor kinase activities were comparable. Rat2-infected cells (5 x 10^6) expressing similar levels of wild-type and mutant receptors were stimulated with CSF-1 and analyzed by FF3 with wild-type receptors sine phosphorylated at levels slightly below those observed in vivo ligand stimulation, F922 and F958 receptors become tyrosine phosphorylated at 4G10. FF3 is indicated as the 150-kDa migrating protein. Phosphotyrosine-containing proteins were identified by Western blotting with 4G10. FF3 is indicated as the 150-kDa migrating protein.

PI3K binds Flt3/Flik2 at Position 958 within the Carboxy Tail—PI3K is a heterodimer composed of a M, 110,000 catalytic polyepitope and a p85 modular protein containing two SH2 domains, a p110-binding insert, two proline-rich SH3-binding sequences, a sequence suggestive of a rho-Gap function, and an N-terminal SH3 domain. The SH2 domains of p85 bind to phosphorylated tyrosines on receptors and thereby link PI3K to activated receptors. We have shown previously that the C-terminal SH2 has higher affinity to Flt3/Flik2 than the N-terminal SH2 and that both SH2 domains in tandem bind with greater affinity than either one alone (47). We assayed FF3 receptors mutated at each of the two potential PI3K binding sites for their capacity to bind to glutathione S-transferase-p85 (N+C) SH2 domains in vitro and to associate with p85 and p85 Western blotting, and PI3K assays in vitro. Fig. 1B shows that mutant and wild-type FF3 receptors coprecipitated with the SH2 domains of both Grb2 and p85; F958 FF3 associated normally with the Grb2 SH2, whereas it lost the capacity to bind to the p85-derived SH2 domain (data not shown). These associations were dependent on prior activation of the receptors with the CSF-1 ligand.

The association of endogenous p85 with the wild-type and mutant FF3 receptors was examined next. Fig. 2A represents an anti-Flt3/Flik2 immunoprecipitation, followed by an anti-p85 Western blot on lysates derived from cells containing the wild-type, F922, and F958 FF3 receptors. Both FF3 and F922 bind p85 at comparable levels, whereas no coprecipitating p85 was detectable with F958. The association of p85 with FF3 and F922 was induced following stimulation of the cells with CSF-1. The capacity of the F958 mutant receptor to associate with PI3K activity was assayed in comparison with wild-type and F922 receptors. Cellular lysates from FF3, F922, and F958 were immunoprecipitated with an anti-Flt3/Flik2 antibody and subjected to a PI3K assay. Consistent with our previous findings, wild-type FF3 and mutant FF3 receptors associated with PI3K. Mutation at tyrosine 958 but not tyrosine 922 resulted in a loss of this association (Fig. 2B, lanes 1 and 2) and mutant receptors (F922, lanes 3 and 4; F958, lanes 5 and 6) were subjected to immunoprecipitation with an anti-Flt3 antibody and blotted with anti-p85 anti-sera. B, FF3 wild-type and mutant receptors were immunoprecipitated from cellular lysates as in A with anti-Flt3 antibody and subjected to a PI3K assay.
determine whether mutation of tyrosine 958 would disrupt either of these two pathways, we performed antiphosphotyrosine Western blot analysis on F958 in comparison to wild-type and F922.

Shc is expressed as three polypeptides ranging in size from 46–66 kDa (58). Phosphorylated Shc complexes with Grb-2 (59) and in some cell types with the inositol polyphosphate phosphatase, p150SHIP (60, 61, 106, 107). Shc contains a second phosphotyrosine binding (PTB/PID) domain in its amino terminus in addition to its carboxyl terminus SH2 domain, through which it binds to phosphorylated proteins (62, 63). Although its biological function is unknown, Shc can transform cells when overexpressed, suggesting that it participates in mitogenic pathways (58). Cellular lysates from FF3, F922, and F958 cells stimulated with CSF-1 were immunoprecipitated with anti-Shc antibodies, and the precipitated proteins were blotted with anti-phosphotyrosine antibodies. Shc proteins isolated from unstimulated cells were poorly phosphorylated, whereas Shc tyrosine phosphorylation was markedly increased in stimulated cells containing FF3, F922, or F958 receptors (Fig. 3A, lanes 2, 4, and 6). Shc immunoprecipitates from cells treated with epidermal growth factor and subsequently blotted with anti-phosphotyrosine antibodies revealed the presence of activated epidermal growth factor receptors in the Shc immune complexes, suggesting a high stoichiometric interaction between Shc and epidermal growth factor receptor (64). We detected a high molecular weight phosphoprotein in Shc immunoprecipitates that is compatible with the electrophoretic mobility of the activated form of FF3 (Fig. 3A, lanes 2, 4, and 6). The ras-GAP associated protein, p62, is a major tyrosine phosphoprotein in transformed and growth-factor-treated cells (65, 66). Although its exact function is not known, it can bind directly to Src family tyrosine kinases and to Grb2 and phospholipase Cγ. It has been implicated as a linker protein bridging activated Src family tyrosine kinases with downstream effectors (67–69). P62 binds RNA in a tyrosine phosphorylation-regulated manner through its KH domain and thus may link transcription control to tyrosine kinase signaling pathways (70). P62, or a molecule highly related to p62, p68, has been identified as a target of Src during mitosis (71, 72). P62 becomes tyrosine phosphorylated in response to multiple receptor tyrosine kinases including Flt3/Flk2 (47, 73–75). Gap was immunoprecipitated from the lysates of CSF-1 stimulated FF3, F922, and F958 Rat2 cells, and the Gap-associated proteins were resolved by gel electrophoresis and probed with an antiphosphotyrosine antibody. Fig. 3B shows that p62 was normally phosphorylated by the wild-type and mutant FF3 receptors. Therefore, mutagenesis of tyrosine 958 uncouples FF3 from PI3K but does not affect the biosynthesis, cell surface expression, or kinase activity of FF3 or the phosphorylation of its targets, Shc and p62.

The F958 Mutant FF3 Receptor Provides a Normal Mitogenic Signal in Rat2 Fibroblasts and Ba/F3—PI3K activity increases in response to numerous ligands, including those that signal through RTKs (76), cytokine receptors (77–80), and G protein-coupled receptors (81–83). The association of PI3K with activated receptors and the accumulation of its lipid products have been associated with mitogenic responses (84). We were, therefore, interested in testing the requirement of PI3K in Flt3/Flk2-mediated mitogenic responses, both in rodent fibroblasts and in an IL-3-dependent hematopoietic cell line, Ba/F3.

We have shown previously that Flt3/Flk2 could sustain anchorage-independent cell growth of Rat2 fibroblasts (40). We used growth in soft agar as an assay to analyze the transforming activity of the mutant F958 Flt3/Flk2 receptor in comparison with the wild-type. Neomycin-resistant pools (Fig. 4, upper panel) or individual colonies (Fig. 4, lower panel) of Rat2 fibroblasts expressing either wild-type or F958 mutant FF3 recep-
tors were seeded into soft agar in equal numbers and cultured for 14 days in the presence or absence of CSF-1. Both FF3 and F958 transfected cells were capable of forming colonies in soft agar. The kinetics of colony formation and number of colonies per seeded input cells were equivalent for both cell types; however, the colony size of the F958 transfected cells was consistently smaller than the wild-type-expressing fibroblasts.

To study the CSF-1-induced changes in growth rate, Rat2 fibroblast clones or the IL-3-dependent cell line Ba/F3 expressing wild-type or F958 FF3 receptors were grown in medium containing 1.5% FCS in the absence or presence of CSF-1. The CSF-1-dependent growth rate of both cell types expressing wild-type or F958 were comparable (data not shown). The mitogenesis assays in rodent fibroblasts and in the Ba/F3 hematopoietic cells suggest that PI3K is not an essential component of the mitogenic machinery activated by Flt3/Flk2.

PI3K Is Not Required for Efficient Internalization of Flt3/Flk2—Stimulation of RTKs results in rapid receptor internalization and the activation of cellular pathways that lead either to receptor recycling and re-expression at the cell surface or to degradation through endocytic fusion with lysosomal vesicles (85). Down-modulation limits the duration of receptor kinase activity at the cell surface and has also been linked to an essential step in the transduction of mitogenic signals. The cellular machinery and the gene products that govern receptor internalization are not yet fully characterized. PI3K has been shown to be both necessary and sufficient for PDGFRβ internalization and targeting to post-endosomal perinuclear vesicles (86–88). In contrast, Kit mutants unable to bind PI3K behaved similarly to wild-type receptors in their rate of internalization and degradation (89). We studied the kinetics of F958 internalization in comparison to wild-type FF3. Ba/F3 cells transfected with both FF3 and F958 were stimulated with biotinylated CSF-1 at 37°C for variable times. The cells were then stained with phycoerythrin-labeled streptavidin at 4°C and were analyzed by FACS to determine the rate of receptor internalization. Fig. 5 shows that Flt3/Flk2 uncoupled from PI3K binding internalized with the same kinetics as did the wild-type Flt3/Flk2 receptor. These data show that Flt3/Flk2, similar to Kit but distinct from PDGFRβ, does not require PI3K to internalize following ligand binding.

DISCUSSION

In this study, we showed that PI3K binds to a unique site in the carboxy terminus of the cytoplasmic tail. The location of this binding site distinguishes Flt3/Flk2 from the other four members of the class III family of RTKs, Kit, CSF-1R, PDGFRα, and PDGFRβ, which contain PI3K binding sites within their kinase insert domains (26, 27, 37, 50, 90). Flt3/Flk2 binds to the p85 subunit of PI3K in an indubitable fashion to a sequence surrounding tyrosine 958, YQNM. This sequence conforms to the optimal p85 binding consensus sequence, YYXM, defined by Songyang et al. (91) and is present in Kit, CSF-1, and PDGFRs. We were unable to detect a secondary p85 binding site, as seen in the case of PDGFR (24, 48, 92, 93), although another potential binding site, YFFM at codon 922, is present in the carboxy tail. The amino acids neighboring tyrosine 958 contain an asparagine residue at the –2 position and thus conform to a Grb2 SH2 binding sequence (91). Preliminary data show that Grb2 binds directly to Flt3/Flk2 at Y958 and to at least one other site present in the carboxy tail. Tyrosine 958 may, therefore, function as a dual-specific site for binding p85 and Grb2. A similar site, which specifies both p85 and Grb2 binding, is present in the Met RTK (57).

PI3K has been linked to a variety of RTK-mediated biological responses including mitogenesis (84), chemotaxis (94), cell survival (95), receptor down-modulation (86, 88), and cell polarization (96). The diversity of these biological responses may be related to the complex biochemical interactions with which PI3K is involved. PI3K physically interacts with or up-stream of several signaling molecules that participate in mitogenic responses, including the Src family kinases (97, 98), Ras (30–32), the Ras-related GTPase Cdc42HS (33), the serine/threonine kinase Akt (34, 35), and pp70/85 S6 kinase (36). The requirement for PI3K to mediate receptor tyrosine kinase mitogenesis is variable and depends on both the receptor and the cell type. PI3K is both necessary and sufficient for PDGF-dependent mitogenesis through the β receptor (26, 27) but not the α receptor (48). The CSF-1R mutant at the PI3K binding site and ectopically expressed in rat fibroblasts has a diminished mitogenic response (99), whereas Kit mutant receptors that no longer bind PI3K have only a slight mitogenic defect in mast cells (38). We studied the necessity of PI3K on Flt3/Flk2-dependent mitogenesis in both rat fibroblasts and Ba/F3 cells by analyzing the capacity of the Flt3/Flk2-F958 mutant to induce anchorage-independent cell growth, cell proliferation, and DNA synthesis. Rat2 cells expressing F958 were able to form colonies in soft agar and to proliferate with a doubling time comparable to wild-type receptors in response to ligand. Flt3/Flk2 receptors uncoupled from PI3K expressed in Ba/F3 cells also stimulated a mitogenic response at levels similar to wild-type receptors. We showed that two downstream targets of Flt3/Flk2, Shc and p62, are normally phosphorylated by the F958 Flt3/Flk2 mutant.

Receptor coupling to Shc may be sufficient to maintain the mitogenic response in some receptor systems. For example, epidermal growth factor receptor mutants with a truncation in the carboxy terminus that removes the major autophosphorylation sites and the binding sites for phospholipase Cγ, Grb-2, PI3K, and Shc are still able to promote the phosphorylation of Shc, induce formation of Grb2-Shc complexes, and provide a

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mitogenic response (100, 101). Therefore, in distinction to PDGFR and to a lesser degree CSF-1R, the capacity of Flt3/Flk2 to maintain anchorage-independent growth or stimulate proliferative responses does not require PI3K and may be mediated through a Grb2-Shc pathway.

RTKs rapidly internalize following ligand binding. Internalized receptors are then sorted to distinct subcellular pathways that lead either to degradation or recycling to the cell surface. PDGFR mutants that do not bind to PI3K have attenuated rates of internalization and degradation (86–88). These studies have suggested that mammalian PI3K may share with its yeast homologue Vps34, a highly conserved cellular function that directs membrane-associated proteins to sort to postendocytic degradative vesicles (102). The detailed biochemical basis for this function is not known; however, PI3K has been shown to interact with several GTPases involved in endocytosis and reorganization of the actin cytoskeleton. PI3K, possibly acting through SH3 domain of dynamin (103), a large GTPase required for the reorganization of the actin cytoskeleton. PI3K binds, via its

interact with several GTPases involved in endocytosis and degradative vesicles (102). The detailed biochemical basis for
distinction to PDGFR

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