Fiz1, a Novel Zinc Finger Protein Interacting with the Receptor Tyrosine Kinase Flt3∗

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Ingrid Wolff‡ and Larry R. Rohrschneider

From the Fred Hutchinson Cancer Research Center, Division of Basic Sciences, Seattle, Washington 98109-1024

The receptor tyrosine kinase Flt3 has been shown to play a role in proliferation and survival of hematopoietic progenitor cells as well as differentiation of early B lymphoid progenitors. However, the signaling events that control growth or differentiation are not completely understood. In order to identify new signaling molecules interacting with the cytoplasmic domain of Flt3, we performed a yeast two-hybrid screen. In addition to several SH2 domain-containing proteins, we have isolated a novel Flt3 interacting zinc finger protein (Fiz1) with 11 C2H2-type zinc fingers. Fiz1 binds to the catalytic domain of Flt3 but not to the structurally related receptor tyrosine kinases Kit, Fms, and platelet-derived growth factor receptor. This association is independent of kinase activity. The interaction between Flt3 and Fiz1 detected in yeast was confirmed by in vitro and in vivo coprecipitation assays. Fiz1 mRNA is expressed in all murine cell lines and tissues tested. Anti-Fiz1 antibodies recognize a 60-kDa protein, which is localized in the nucleus as well as in the cytoplasm. Together, these results identified a novel class of interaction between a receptor tyrosine kinase and a signaling molecule which is independent of the well established SH2 domain/phosphotyrosine binding.

Flt3 receptor tyrosine kinase (RTK) is a member of the class III RTKs (1–5). Common structural features include the extracellular region composed of five immunoglobulin-like domains and an intracellular tyrosine kinase made up of an ATP-binding loop and a catalytic domain separated by a kinase insert domain. Additional members of this receptor family are the receptors for macrophage-colony-stimulating factor, and steel factor, encoded by the FMS (6–8) and KIT (9, 10) protooncogenes, respectively, and the receptors for α- and β-platelet-derived growth factors (PDGFRα and -β). The RTKs Flt3, Fms, and Kit play a key role in hematopoiesis by stimulating proliferation and/or differentiation of various hematopoietic cell types (11, 12). Mice lacking a functional Flt3 receptor have normal mature hematopoietic populations; however, they exhibit reduced numbers of early B cell precursors and multipotent stem cells (13). The recently cloned Flt3 ligand (FL) (14–16), in combination with other cytokines, has been shown to stimulate proliferation of human and murine hematopoietic progenitor/stem cells in vitro as well as in vivo (14–20). FL also promotes growth of early B cell progenitor cells in combination with IL-7 (21, 22) and was shown to induce adhesion of the progenitor B cell line BaF3/Flt3 to fibronectin by activating the fibronectin receptors VLA-4 and VLA-5 integrins (23).

Little is known about the signaling pathways of Flt3. By using a chimeric Fms/Flt3 receptor, several groups have shown phosphorylation of and/or association with phospholipase C-γ1, Ras GTPase-activating protein, Vav, Shc, the p85 subunit of phosphatidylinositol 3′-kinase, and Grb2 in fibroblasts and pro-B cells (24, 25) upon receptor activation. The site of p85 interaction with murine Flt3 was mapped to tyrosine 958 (YQNM) in the C-terminal tail of Flt3 (24, 26, 27) which fits the consensus sequence favored by the SH2 domains of p85 (28). In contrast, p85 does not interact with human Flt3 (29) which is lacking a potential p85-binding site in the C terminus. In addition, ligand stimulation of the human Flt3 receptor results in phosphorylation of SHP-2, SHIP, and a 100-kDa protein in monocytic THP-1 cells (29), phosphorylation of SHC and CBL in myeloid cells, and SHC and CBL in pro-B cells (30, 31).

In order to understand the specific function of Flt3, it is necessary to understand its signaling pathways in more detail. Therefore, we used the cytoplasmic domain of Flt3 as bait in a two-hybrid screen to identify new signaling molecules that interact with this RTK. This technology has been used successfully to isolate novel substrates of various other RTKs, such as the association of SH2 domains of phospholipase C-γ2 and Mono with Fms (32, 33), the SH2 domain of the adaptor molecule APS with Kit (34), the PTB domain of Dok-R with Tek/Tie2 (35), and the zinc finger protein ZPR1 with the epidermal growth factor receptor (EGFR) (36). In this report we describe the isolation of a novel C2H2 zinc finger protein and its specific interaction with the catalytic domain of Flt3 in a kinase-independent manner.

EXPERIMENTAL PROCEDURES

All recombinant DNA work was done using standard techniques (37). Additional details of plasmid constructions and oligonucleotide sequences are available upon request.

Cells and Culture—The murine hematopoietic cell lines BaF3, BaF3/Flt3, and BaF3/MT-Fiz1/Flt3 were grown in RPMI 1640 supplemented with 5% fetal calf serum (HyClone), 5% iron-supplemented calf serum (HyClone), and 0.2% conditioned medium of X63-IL-3 cells expressing recombinant IL-3 (38). The BaF3/Flt3 cell line was generated by infection of BaF3 cells with the pJZen2-Flt3 retroviral vector. Infected cells
were sorted for receptor expression by flow cytometry using a polyclonal antibody (antibody 8580) against the extracellular domain of Flt3. The BaF3/M-TF-Fz1/Flt3 cell line was generated by electroporating the expression plasmid Rc-CMV-MT-Fz1 into the parental cell line BaF3 and selecting for G418-resistant clones. After screening for MT-Fz1 expression, three clones were identified above. HEK293 cells expressing the murine Fms receptor and codons 917 and 918 of the murine Fms receptor were mutated to introduce an R917V and Q918P mutation, respectively, by using the NcoI and KpnI restriction sites, the swap mutants Lexa-F3T-K2I111Fz1 and Lexa-Fms-TKI111-Fz1, respectively, were cloned by standard restriction digestion. All constructs were sequenced to confirm correct mutagenesis. To test for interaction, Lexa plasmids were transformed into yeast strain AMR70. VP16 plasmids were transformed into yeast strain L40. Mating assays were performed as described previously (40), and interaction of various mutants (Fig. 3) was visualized by spotting cells onto selective plates containing 50 μg/ml 3-amino-1,2,4-triazole and X-gal. Color of colonies was recorded after 3 days.

Color in Vitro Binding Assays Using GST Fusion Proteins—GST-Fz1 wild type fusion protein was prepared by cloning the BamHI-ECOR fragment of the originally isolated clone VP16-KM13 in frame into pGEX-3X (Amersham Pharmacia Biotech). After transformation into Escherichia coli BL21(DE3) (Novagen), GST or GST-Fz1 wild type fusion proteins were induced and were affinity purified on glutathione-agarose resin (Amersham Pharmacia Biotech) in PBS, 1% Triton X-100, 1 μM PMSF.

Metionine-labeled Flt3 was cloned by obtaining the cytoplasmic domain into the vector pET1b (Novagen), and in vitro transcription/translation was done using the T7 in vitro transcription/translation system (Novagen). Lysates were cleared by centrifugation and then used for binding reactions with 1 μg of GST or 1 μg of GST-Flt3 protein in IVT-150 buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 μM ZnCl2, 1% Nonidet P-40). Interacting complexes were washed 3 times with IVT-750 buffer (IVT buffer with 750 mM NaCl) and eluted by boiling in 2% Laemmli buffer. Proteins were separated on a 10% SDS-polyacrylamide gel, and bound proteins were visualized by autoradiography.

1.3 × 107 cells of the BaF3 or BaF3/Ft3 cell line were lysed by sonication in IVT-150 buffer (supplemented with 1 μM PMSF, 20 μg/ml aprotinin). Lysates were cleared by centrifugation and preclared by incubation with GST protein bound to beads. Supernatants were incubated with 5 μg of GST or GST-Fz1 protein in IVT-150 buffer, washed 3 times with IVT-150 buffer, and eluted by boiling in 2% Laemmli buffer. Proteins were separated on a 7.5% SDS-polyacrylamide gel, transferred to nitrocellulose membrane, and blotted with anti-Fz1 antisemur 270J.

Immunoprecipitation and Immunoblotting—BaF3/M-TF-Fz1/Ft3 cells (2.5 × 107/sample) were starved in RPMI, 1% FBS for 3 h, washed in PBS, stimulated with 1 μM Flt3 ligand (FL, a kind gift of Immunex, Seattle, WA), incubated by rotation at 37°C, and lysed by sonication in Nonidet P-40 lysis buffer. Membranes were separated by SDS-PAGE, transferred to nitrocellulose membrane, and blotted with anti-Fz1 antibody and 20 μl of protein A-Sepharose. IPs were washed 3 times with lysis buffer and eluted by boiling in 2% Laemmli buffer. Proteins were separated on a 6.5% SDS-polyacrylamide gel, transferred to nitrocellulose membrane, and visualized using enhanced chemiluminescence (NEN Life Science Products).

Cell Fractionation—Fractionation was done as described previously (44). In brief, cells (2.5 × 107/fractionation) were washed once in ice-cold PBS and once in hypotonic buffer (10 mM HEPES, pH 7.6, 1.5 mM MgCl2, 1 mM PMSF, 20 μg/ml aprotinin). Cells were lysed in hypotonic buffer containing 20 μl of glutathione-agarose. Nuclei were lysed by sonication in Nonidet P-40 lysis buffer (0.5% Nonidet P-40). Membranes were separated from the cytoplasmic fraction by high speed centrifugation (450,000 g) of the pellet containing the membranes was resuspended in Nonidet P-40 lysis buffer. Fractions were analyzed by IP/Western blot.

Immunofluorescence—NIH3T3 cells were plated sparsely on fibronectin (Sigma)-coated coverslips. Cells were grown for 1–2 days, washed with cold PBS, fixed with 4% paraformaldehyde (Sigma) in PBS, and permeabilized with 0.2% Triton X-100 in PBS. Cells were blocked for nonspecific antibody-binding sites by incubation with PBS, 2 G. M. Myles, unpublished results.
Isolation of Fiz1, a Novel Zinc Finger Protein—To identify novel signaling intermediates of the receptor tyrosine kinase Flt3, we employed the yeast two-hybrid system. The hybrid baits consisted of the DNA binding/dimerization domain of LexA and the cytoplasmic region of murine Flt3 (4, 5). A screen of 9 × 10^6 primary transformants of an EML-C1 cDNA expression library (41) yielded five potential positive clones detected by anti-Fiz1 antibody 9704 (affinity purified, diluted 1:50) followed by fluorescein isothiocyanate-conjugated donkey anti-rabbit antibody (H+L) (Jackson ImmunoResearch). Nuclei were stained using 4,6-diamidino-2-phenylindole reagent (Sigma). Indirect fluorescence was detected with a Deltavision SA3.1 Wide-field Deconvolution Microscope (Applied Precision Inc.).

Northern Blot Hybridization—Total RNA of various cell lines or mouse tissues was extracted with guanidinium isothiocyanate and purified by centrifugation through a 5.7 M CsCl gradient (45). By using oligo(dT)-cellulose (New England Biolabs) affinity purification, poly(A)^+ RNA was isolated from mouse tissue total RNA. 20 μg of total RNA or 3 μg of poly(A)^+ RNA were separated on 1.2% agarose, 0.6 M formaldehyde gels, transferred to GeneScreen membrane (NEN Life Science Products), hybridized, and washed as recommended by the manufacturer.

RESULTS

Expression of Fiz1 in Cells and Tissues—The pattern of Fiz1 expression was examined by Northern blot analysis of RNA from various mouse cell lines and tissues (Fig. 2). A single RNA species of 2.6 kilobase pairs was detected in all hematopoietic and non-hematopoietic cell lines tested but to a lower degree in brain, heart, kidney, liver, lung, skeletal muscle, pancreas, spleen, thymus, and uterus.

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Fig. 1. Amino acid sequence of Fiz1. A, deduced amino acid sequence of Flt3 cDNA. Zinc finger motifs are underlined, and conserved cysteine and histidine residues are highlighted by a black box. GenBank™ accession numbers are AF126746 and AF126747. B, alignment of the amino acid sequence of the 11 zinc finger domains, conforming to the C_2H_2 consensus (C_X_C_X_F_X_L_X_H). Sequences linking adjacent fingers are also shown; they frequently conform to the H/C link consensus (C_G_k_R_P_F_Y). C, schematic diagram of the structure of Fiz1. Zinc finger domains ZF1–11 are symbolized by ovals.

Fiz1 Interacts Specifically to the Catalytic Kinase Domain of Flt3—To analyze the interaction of Flt3 with Fiz1 in more detail, different Flt3 mutants were tested for interaction in the yeast two-hybrid system (Fig. 3). Replacing the lysine 645 within the ATP-binding site of the kinase domain with an alanine (K645A) results in a kinase-defective Flt3 mutant that interferes with Fiz1 binding. In fact, deletion of JM results in a kinase-independent manner.

To determine what region of the Flt3 receptor is bound by Fiz1, several Flt3 deletion mutants were examined (Fig. 3B). Deletion of the kinase insert domain or the C terminus (CT) had no effect on binding of Fiz1 to Flt3. However, deletion of the juxtamembrane domain (JM) completely eliminated the interaction. Expression of JM alone or JM plus tyrosine kinase domain I (TKI, ATP-binding loop) failed to restore Fiz1 interaction. These results suggest that Fiz1 does not bind to the cytoplasmic domain of the structurally related receptor tyrosine kinases Fms, Kit, and PDGFR (Fig. 3B). Therefore, Fiz1 binds specifically to Flt3 in a kinase-independent manner.

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Flt3-interacting Protein Fiz1

Fiz1 Interacts with Flt3 in Vitro—To confirm the specific interaction between Fiz1 and Flt3 observed in yeast cells, we examined the potential direct interaction of Fiz1 with Flt3 in vitro. Full-length Fiz1 was expressed in E. coli as a GST fusion protein. Fusion proteins were immobilized on glutathione-agarose beads and incubated with in vitro translated, [35S]methionine-labeled cytoplasmic domains of Flt3, wild-type Fms, or PDGFR cytoplasmic domains. Interactions were monitored by blue color after spotting yeast on plates lacking WHULK but containing 50 mg/ml 3-amino-1,2,4-triazole and X-gal. Kinase activity of each bait was monitored by Western blotting yeast total cell lysates and probing with a α-phosphotyrosine antibody (4G10). C, yeast mating assays between S. cerevisiae L40 containing VP16-Fiz1 or VP16 fused to the C-terminal SH2 domain of p85, as a positive control, and S. cerevisiae L40 containing Flt3-TER or Fms/TKI-Fiz1 expressing cytoplasmic domains of wild-type Flt3, wild-type Fms, or the catalytic swap mutants Flt3/TKI-Fms and Fms/TKI-Flt3.

In Vivo Association of Fiz1 and Flt3—To examine the interaction between Fiz1 and Flt3 in mammalian cells, we utilized a rabbit polyclonal antiserum reactive against the C-terminal half of Fiz1 (ZF7-ZF11, Fig. 1C). The antiserum was affinity purified using the antigen. This antiserum detected a band of the ~160-kDa size was detected suggesting that the product encoded by the isolated cDNA corresponds to the endogenous protein (Fig. 5A).

To confirm this result, GST-Fiz1 fusion protein was incubated with total cell lysates prepared from the pre-B cell line BaF3 or a subline BaF3/Flt3 expressing the wild-type Flt3 receptor. As shown in Fig. 4B, GST-Fiz1 interacted with the immature as well as the mature glycosylated Flt3 receptor molecule. GST alone did not interact with any of the receptor isoforms. These results verify that Fiz1 is able to associate in vitro with Flt3 without receptor activation.
at 37 °C). Cell extracts were incubated with affinity purified α-Fiz1 antibody, and immunoprecipitated proteins were analyzed by Western blotting with an α-Flt3 antibody. As shown in Fig. 5B, the glycosylated as well as the unmodified form of Flt3 specifically coprecipitated with Fiz1 in extracts from stimulated, but not unstimulated, cells. The blot was reprobed with α-Fiz1 antibody and the blot was probed with α-Flt3 antibody. The blot was stripped and reprobed with α-Fiz1 antibody. IB, immunoblot.

**DISCUSSION**

In this study we describe the identification of a novel C_{2}H_{2} zinc finger protein Fiz1 via its interaction with the receptor tyrosine kinase Flt3. The association found in yeast was verified with in vitro and in vivo coprecipitation assays.

Fiz1 protein is composed of 11 C_{2}H_{2} zinc finger domains spread over the entire protein in clusters of two, three, or four fingers. The classical C_{2}H_{2} zinc finger domains were first identified in the transcription factors TFIIIA and Krüppel and were shown to be DNA binding domains (54). However, recent stud-
and early hematopoietic progenitor cells, thymus, spleen, lymph nodes, placenta, brain and gonads (4, 5, 46). In contrast, Fiz1 is expressed ubiquitously, suggesting that Fiz1 may interact with other proteins besides Flt3 and proteins different from class III RTKs. To address this question, a yeast two-hybrid screen using Fiz1 as bait has been performed. The additional Fiz1-interacting proteins will be reported elsewhere.

In conclusion, we have identified a novel C4H2 zinc finger protein Fiz1 by its interaction with the catalytic domain of the receptor tyrosine kinase Flt3. Although the physiological function remains to be clarified, Fiz1 represents a new class of receptor-interacting molecules.

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Be relevant in the Linan cells, eventually by receptor oligomerization or even receptor enhances binding affinity of Fiz1 and Flt3 in mammalian cells, eventually by receptor oligomerization or even receptor phosphorylation. Receptor dimerization is known to occur in the yeast system due to the dimerization of the LexA-DNA binding domain. However, receptor oligomerization might not be relevant in the in vitro system. Thus, Flt3/Fiz1 binding in the yeast and in vitro systems might be facilitated by higher protein concentrations and therefore independent of receptor stimulation. Further investigation needs to be done to clarify the exact mechanism of this interaction. As shown in Fig. 3B, Fiz1 interacts specifically with Flt3. This specificity is surprising with respect to the expression pattern of Fiz1. Flt3 expression is restricted to bone marrow...
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