Activation of FIP1L1-PDGFRα requires disruption of the juxtamembrane domain of PDGFRα and is FIP1L1-independent

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Genetic abnormalities that result in expression of chimeric tyrosine kinase proteins such as BCR-ABL1 and ETV6-PDGFRβ are common causes of hematopoietic malignancies. The paradigm for constitutive activation of these fusion tyrosine kinases is enforced homodimerization by self-association domains present in the fusion partner proteins. The unique interstitial deletion on chromosome 4q12 that leads to expression of the FIP1L1-PDGFRα fusion tyrosine kinase was recently identified as a cause of chronic eosinophilic leukemia. In this report, we demonstrate that FIP1L1 is completely dispensable for PDGFRα activation *in vitro* and *in vivo*. Instead, truncation of PDGFRα between two conserved tryptophan residues in the juxtamembrane (JM) domain is required for kinase activation and transforming potential of FIP1L1-PDGFRα. The presence of a complete JM domain in FIP1L1-PDGFRα is inhibitory, but this autoinhibition can be overcome by enforced homodimerization. Similar effects of the JM domain in the context of PDGFRβ were observed. These results suggest that disruption of the autoinhibitory JM domain is an alternative, dimerization-independent mechanism by which chimeric tyrosine kinases are constitutively activated and induce leukemogenesis.

fusion gene | leukemia | oncogene | tyrosine kinase

Protein tyrosine kinase genes are frequent targets of chromosomal rearrangements in hematopoietic malignancies and, to a minor extent, in some solid tumors (1, 2). As a consequence, such chromosomal abnormalities result in expression of fusion tyrosine kinases, in which the tyrosine kinase domains are fused in-frame to distinct N-terminal partner proteins that contain homotypic oligomerization domains. Homodimerization of the fusion tyrosine kinases by the N-terminal oligomerization domains leads to autophosphorylation at tyrosine residues and constitutive kinase activation, as well as activation of critical downstream signaling effectors that induce cellular proliferation (1). Well studied examples include *BCR-ABL*, *ETV6-PDGFRB*, *ZNF198-FGFR1*, and *NPM-ALK*, in which the presence of the oligomerization domains of the fusion partners have been shown to be indispensable for kinase activation and for transforming properties of the fusion proteins (2–6).

FIP1L1-PDGFRA is a fusion gene that has been identified in patients with hypereosinophilic syndrome/chronic eosinophilic leukemia (CEL) and systemic mast cell disease (7, 8). *FIP1L1* is fused to *PDGFRA* due to an interstitial deletion on chromosome 4q12, in contrast to other fusion tyrosine kinases that are typically generated by chromosomal translocations (7–13). We and others have shown that the FIP1L1-PDGFRα fusion protein is a constitutively activated tyrosine kinase that confers growth factor-independent growth to hematopoietic cells and is sensitive to inhibition by imatinib (7, 12, 14). FIP1L1 is a protein involved in polyadenylation, but it is unknown whether FIP1L1 confers dimerization properties, because it does not harbor any known protein–protein interaction domains (15).

Interestingly, among all of the different variants of the FIP1L1-PDGFRα fusion identified in clinical studies of hypereosinophilic syndrome/CEL and systemic mast cell disease patients, the autoinhibitory juxtamembrane (JM) domain of PDGFRα is consistently disrupted. Although the breakpoints within FIP1L1 of the FIP1L1-PDGFRα fusion have been found to be variably distributed among introns 7–13, all breakpoints in PDGFRA identified to date are located within PDGFRA exon 12, which encompasses the JM domain (7–13). In addition, a fusion of *BCR* to *PDGFRA* in patients with atypical chronic myelogenous leukemia has been described, with the breakpoints in *PDGFRA* again localized within exon 12 (16, 17). It is plausible that the predominance of JM domain disruption in FIP1L1-PDGFRα reflects a functional significance of the JM domain in PDGFRα activation. The JM domain is notable for its autoregulatory role of receptor tyrosine kinases (RTKs) (18). Recent structural and biochemical studies of type III RTKs PDGFRβ, FLT3, and KIT suggest a model in which the JM domain inhibits RTK catalytic activity, most significantly by displacing the critical αC helix in the N-lobe of the kinase domain and disrupting the conformation of the ATP-binding site. Furthermore, this inhibition can be overcome by receptor phosphorylation or mutation of the JM region (18–21).

Results and Discussion

FIP1L1 Is Not Required for Transforming Properties of FIP1L1-PDGFRA *in Vitro*. Constitutive kinase activity of chimeric oncogenic tyrosine kinases was shown to depend on the presence of oligomerization domains in the partner proteins (3–6). To identify the regions of *FIP1L1* that are required for activation of the FIP1L1-PDGFRα tyrosine kinase, we generated deletion constructs *FIP1L1 (1–29)-W-PDGFRA* (containing only the first 29 aa of FIP1L1) and *Myc-W-PDGFRA* or *HA-W-PDGFRA*, in which FIP1L1 sequences were completely replaced by the Myc or hemagglutinin epitopes (Fig. 1). In these constructs, the *PDGFRA* segment starts within the JM domain, at the same fusion point as the first cloned *FIP1L1-PDGFRA* fusion (7), and incorporates only the second of the two conserved tryptophan residues (W_{586}) within the JM domain (Fig. 1). To emphasize this point, the *FIP1L1-PDGFRA* construct is hereafter referred to as *FIP1L1-W-PDGFRA*.

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Abbreviations: CEL, chronic eosinophilic leukemia; JM, juxtamembrane; RTK, receptor tyrosine kinase.

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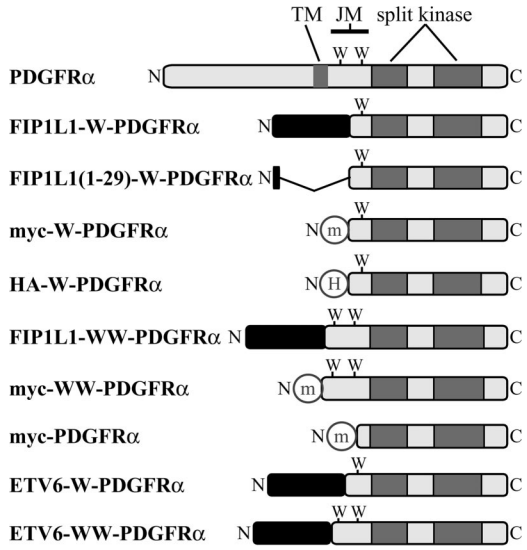

Fig. 1. Schematic representation of the proteins studied in this work. JM, JM domain; TM, transmembrane domain. ''m'' indicates the myc epitope tag; ''H'' indicates the hemagglutinin epitope tag.

Ba/F3 is a murine hematopoietic cell line that requires IL3 for proliferation and cell survival. We have shown previously that FIP1L1-W-PDGFRα transforms Ba/F3 cells to IL3-independence (7). Expression of FIP1L1 (1–29)-W-PDGFRα, Myc-W-PDGFRα, and HA-W-PDGFRα also conferred IL3-independence to Ba/F3 cells (Fig. 2*A*), and Western blot analysis demonstrated that these proteins were autophosphorylated (Fig. 2*B*). In contrast, expression of wild-type PDGFRα did not result in transformation or autophosphorylation (Fig. 2 *A* and *B*). To further confirm these findings, these proteins were transiently expressed in 293T cells. FIP1L1-W-PDGFRα, FIP1L1 (1–29)-W-PDGFRα, Myc-W-PDGFRα, and HA-W-PDGFRα proteins were autophosphorylated, indicating that all of these proteins behaved as constitutively activated tyrosine kinases. Consistent with this observation, expression of these proteins resulted in tyrosine phosphorylation of a variety of proteins in 293T cells, whereas this was not observed in 293T cells transfected with empty vector or wild-type PDGFRA (Fig. 2*C*). These results illustrate that FIP1L1 is completely dispensable for constitutive kinase activity of FIP1L1-W-PDGFRα and its transforming properties *in vitro*.

FIP1L1 Is Dispensable for Transforming Properties of FIP1L1-W-PDGFRA *in Vivo*. To test whether FIP1L1 was also dispensable for FIP1L1-W-PDGFRα-mediated transformation of hematopoietic cells *in vivo*, we performed a murine bone marrow transplant experiment by using bone marrow donor cells transduced with FIP1L1-W-PDGFRA or Myc-W-PDGFRA retroviral vectors. We have previously shown that expression of FIP1L1-W-PDGFRA in bone marrow cells of mice causes a fatal myeloproliferative disease (14). In this study, both FIP1L1-W-PDGFRA and Myc-W-PDGFRA expression induced a similarly fatal myeloproliferative disease, with a median survival of 35 and 27 days, respectively (Fig. 3*A*). Diseased animals in both groups exhibited significantly elevated white blood cell counts and a considerable degree of hepatosplenomegaly (data not shown). Peripheral blood smears in both groups displayed marked leukocytosis predominantly comprised of maturing myeloid forms (Fig. 3*B*). Histopathologic examination of both groups revealed complete effacement of normal splenic and bone marrow architecture by a massive infiltrate of maturing myeloid elements, with an extensive amount of extramedullary hematopoiesis observed in the liver, consistent with our previous studies (Fig. 3*B* and data not shown) (14). In corroboration with these histopathologic findings, FACS analysis of spleen cells showed significant myeloid expansion and a concomitant nearly complete loss of normal B and T lymphoid cells in both groups of animals (data not shown). Taken together, these observations demonstrate that expression of *Myc-W-PDGFRA* induces a myeloproliferative disease that is indistinguishable from the disease produced by *FIP1L1-W-PDGFRA*, indicating that FIP1L1 is also

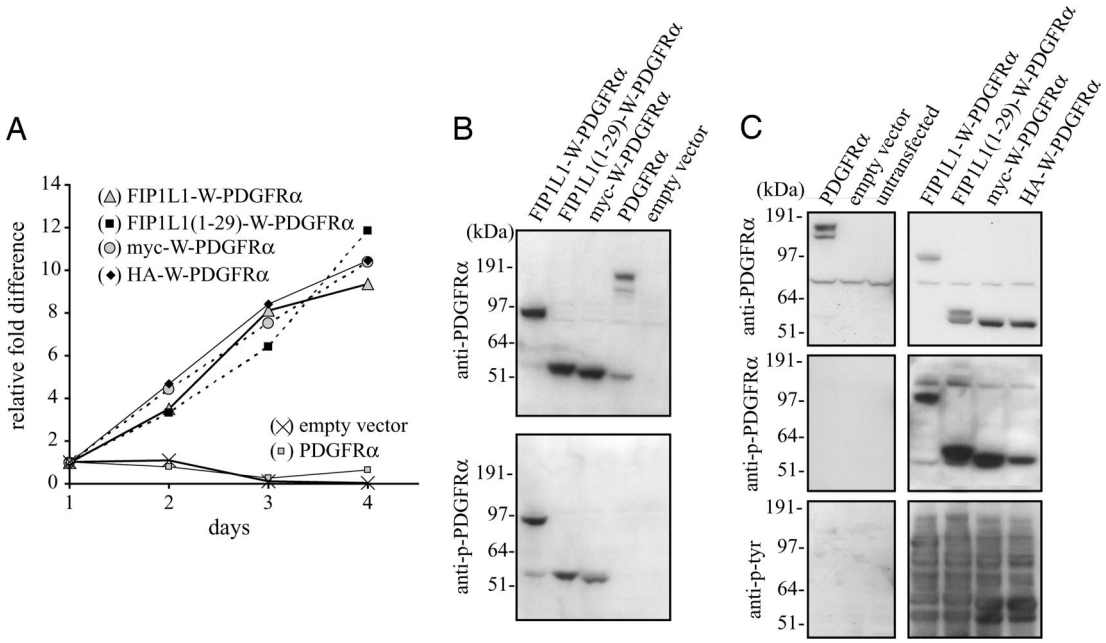

Fig. 2. FIP1L1 is not required for the activation of FIP1L1-PDGFRα *in vitro*. (*A*) Transformation assay of Ba/F3 cells in the absence of IL3. (*B*) Phosphorylation of the chimeric PDGFRα proteins when expressed in Ba/F3 cells. (*C*) Phosphorylation of the chimeric PDGFRα proteins when expressed in 293T cells and general phosphorylation of proteins in 293T cells expressing the different constructs.

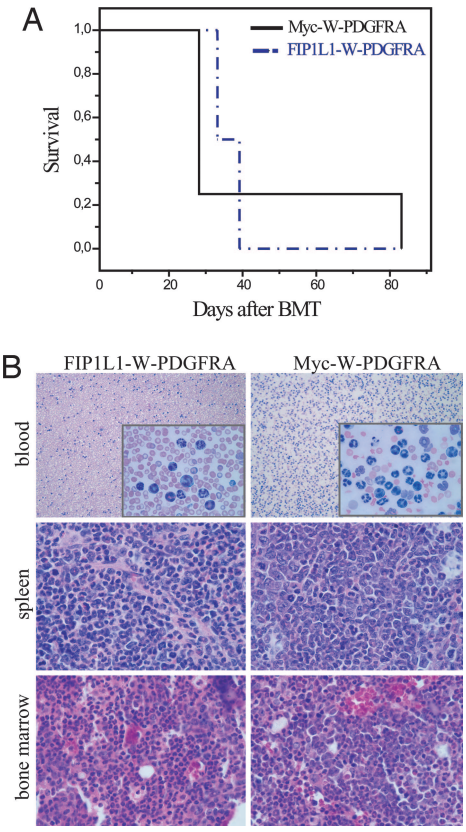

Fig. 3. FIP1L1 is not required for the transforming potential of FIP1L1-PDGFRα *in vivo*. (*A*) Kaplan–Meier plot showing the short latency of disease onset in FIP1L1-W-PDGFRA and Myc-W-PDGFRA bone marrow transplant assays. (*B*) Histology of FIP1L1-W-PDGFRA and Myc-W-PDGFRA bone marrow transplant assays showing smears of peripheral blood (magnification: *Top*, ×100; *Inset*, ×600; Wright–Giemsa staining) and histopathology in representative sections of spleen and bone marrow (magnification: ×600; hematoxylin/eosin staining). Complete effacement of normal splenic architecture and replacement of normal maturing trilineage hematopoietic elements by a prominent population of maturing myeloid forms, many with folded or ring-like nuclei, is observed.

completely dispensable for the transforming properties of FIP1L1-W-PDGFRα *in vivo*.

Interruption of the JM Domain of PDGFRα Is Required for Activation of FIP1L1-PDGFRα. Our *in vitro* and *in vivo* results demonstrate that the FIP1L1 moiety is not required for either the constitutive kinase activity of FIP1L1-W-PDGFRα or transformation of hematopoietic cells by FIP1L1-W-PDGFRα. This finding implies that other features of the fusion protein are responsible for its oncogenic properties. A consistent feature of the FIP1L1-W-PDGFRα fusions in CEL and systemic mast cell disease patients is the interruption of the JM domain of PDGFRα (Fig. 1) (7–13). Because the JM domain has an autoinhibitory function in type III RTKs (18), we next tested whether disruption of the JM domain was required for activation of the kinase activity of FIP1L1-W-PDGFRα.

We examined the effect of reintroducing the full-length JM domain on the transforming potential and kinase activity of FIP1L1-W-PDGFRα and Myc-W-PDGFRα. We generated Myc-FIP1L1-WW-PDGFRA and Myc-WW-PDGFRA constructs in which the complete JM domain of PDGFRA was present. In addition, we tested the effects of deleting the JM domain entirely by using a Myc-PDGFRA construct (Fig. 1). These constructs were transiently expressed in 293T cells. All proteins with an interrupted or absent JM domain clearly demonstrated autophosphorylation, whereas FIP1L1-WW-PDGFRα and Myc-WW-PDGFRα were not phosphorylated (Fig. 4*A*). Furthermore, Western blot analysis of total cell lysates with an anti-phospho-tyrosine antibody demonstrated extensive phosphorylation in cells expressing constructs with a truncated or deleted JM domain but complete absence of tyrosine phosphorylation in cells expressing FIP1L1-WW-PDGFRα or Myc-WW-PDGFRα (Fig. 4*A*). These results support the conclusion that the FIP1L1-WW-PDGFRα and Myc-WW-PDGFRα proteins, each of which contain intact JM domains, are inactive kinases.

To confirm these findings, the constructs were also expressed in Ba/F3 cells. Ba/F3 cells expressing FIP1L1-W-PDGFRα, Myc-W-PDGFRα, and Myc-PDGFRα were transformed to IL3-independent growth (Fig. 4*B*), and these proteins were phosphorylated (Fig. 4*C*). In contrast, Ba/F3 cells expressing FIP1L1-WW-PDGFRα or Myc-WW-PDGFRα showed a marked reduction in proliferation and survival in the absence of IL3 (Fig. 4*B*). However, after selection of these cells for >5 days in the absence of IL3, the Myc-WW-PDGFRA-expressing cells started to proliferate (data not shown). In agreement with this finding, the Myc-WW-PDGFRα protein did show evidence for autophosphorylation, but the phosphorylated protein migrated at a smaller-than-predicted size (Fig. 4*C*). Upon reinvestigation of the sequence of the Myc-WW-PDGFRA construct, we noted that a possible alternative start codon was present within the JM domain (Fig. 5*A*). As a consequence, a smaller protein derived from initiation of translation from this ATG could potentially be synthesized from the Myc-WW-PDGFRA transcript and would lack the first half of the JM domain. On the basis of data presented above, the resultant Myc-W-PDGFRα protein would be predicted to be a fully activated kinase. To verify this hypothesis, variants of Myc-WW-PDGFRA were created in which the methionine within the JM domain was mutated to valine or alanine (Myc-WW-M578V-PDGFRA and Myc-WW-M578A-PDGFRA). Ba/F3 cells stably expressing these constructs were unable to grow in the absence of IL3, and the corresponding proteins were not phosphorylated (Fig. 5 *B* and *C*). These results confirm that the Myc-WW-PDGFRα kinase itself is not activated but that a minority of cells that expressed a variant protein with a truncated JM domain escaped inhibition and were able to proliferate. Taken together, these data provide convincing support for the hypothesis that the presence of an intact JM domain inhibits the kinase activity of the FIP1L1-PDGFRα fusions and that truncation between the conserved WW residues is sufficient for kinase activation.

Disruption of the JM Domain Between Conserved Tryptophan Residues also Activates PDGFRB. Our results indicate that the presence of FIP1L1 is not sufficient to cause kinase activation when the JM domain is intact. In contrast, ETV6-PDGFRβ (which will be written as ETV6-WW-PDGFRβ for similarity with FIP1L1-WW-PDGFRα), which contains the complete JM domain of PDGFRβ, was described as a constitutively active kinase that transforms hematopoietic cells *in vitro* and *in vivo* (4, 22). Previous studies have shown that homodimerization of ETV6-WW-PDGFRβ through the pointed domain of ETV6 is strictly required for kinase activation and transformation of hematopoietic cells (4).

To determine whether the observations in the context of the FIP1L1-W-PDGFRα fusion could be extrapolated to other RTKs, we tested whether disruption of the JM domain in PDGFRβ would be sufficient for kinase activation in the absence of homodimerization. We generated two truncated PDGFRβ constructs with an interrupted JM domain (Myc-W-PDGFRβ clone 1 and 2) that harbor breakpoints within the JM domain that correspond to those found in the two major

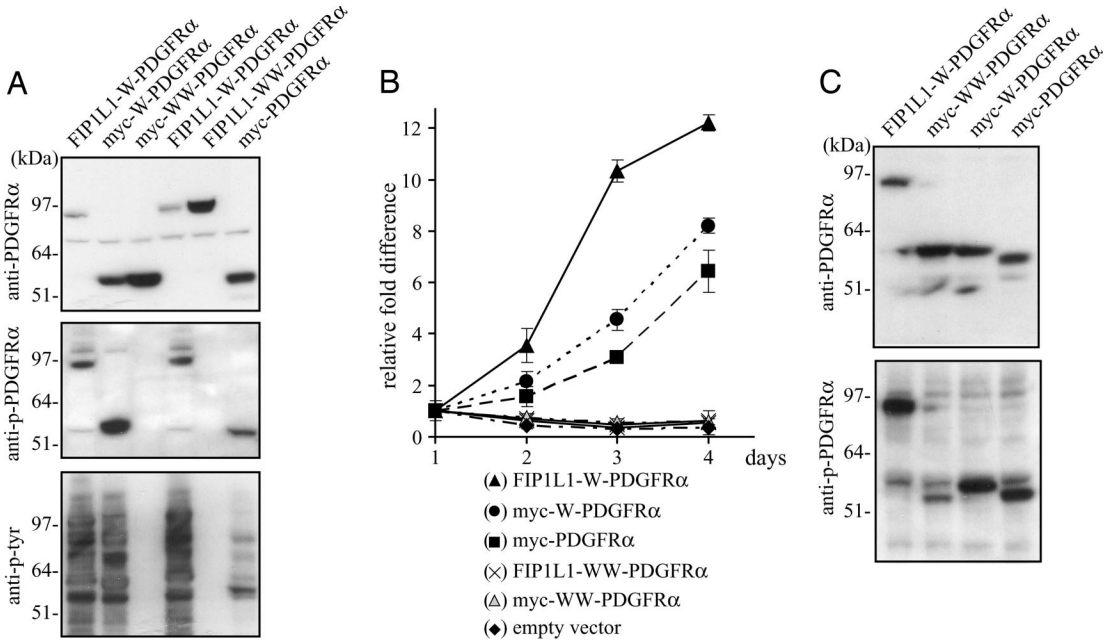

Fig. 4. Interruption of the JM domain of PDGFRα is required for activation of FIP1L1-PDGFRα. (*A*) Phosphorylation of the chimeric PDGFRα proteins when expressed in 293T cells and general phosphorylation of proteins in 293T cells expressing the different constructs. (*B*) Transformation assay of Ba/F3 cells in the absence of IL3 over a period of 4 days. Note that after longer selection cells expressing Myc-WW-PDGFRA also started to proliferate. (*C*) Phosphorylation of the chimeric PDGFRα proteins when expressed in Ba/F3. Cells were harvested after selection for IL3-independent growth.

isoforms of FIP1L1-PDGFRA identified in CEL patients (Fig. 7, which is published as supporting information on the PNAS web site). As shown previously, the ETV6-WW-PDGFRβ fusion protein conferred IL3-independent growth to Ba/F3 cells and was autophosphorylated (Fig. 7). Notably, expression of two truncated Myc-W-PDGFRβ constructs also resulted in kinase activation (autophosphorylation) and IL3-independent growth, even in the absence of the dimerization partner ETV6 (Fig. 7). These results are in agreement with our observations with PDGFRα and also illustrate that, in the context of PDGFRβ, the inhibitory effect of the JM domain can be overcome by interruption of this domain, even in the absence

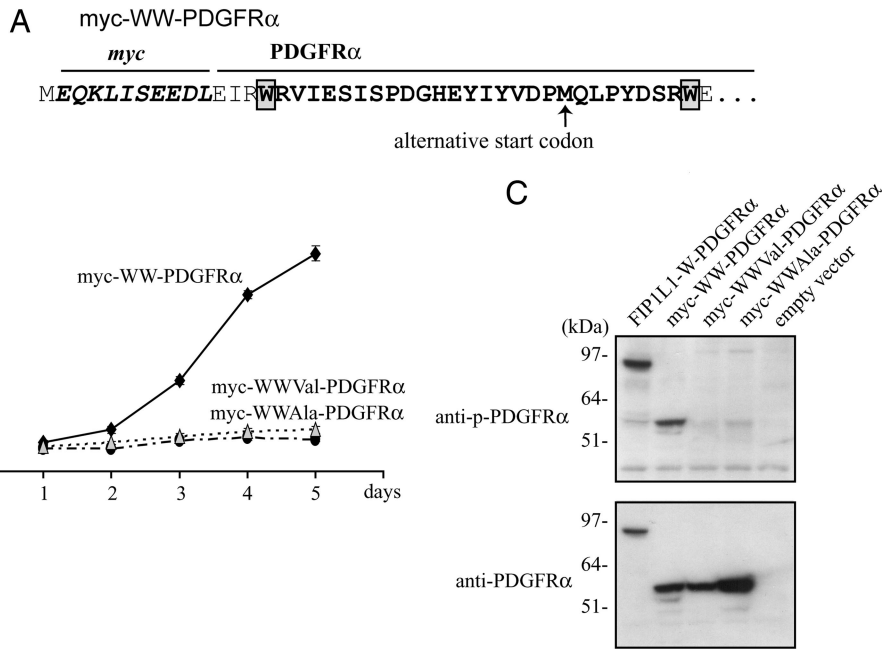

Fig. 5. Myc-WW-PDGFRα is an inactive kinase. (*A*) Structure of the N-terminal part of the Myc-WW-PDGFRα protein, with indications of the positions of the Myc tag, JM domain, and alternative start codon. (*B*) Transformation assay of Ba/F3 cells in the absence of IL3, showing that Ba/F3 cells expressing the Myc-WW-PDGFRA construct become IL3-independent, whereas the constructs with a mutated internal ATG do not. (*C*) Phosphorylation of the chimeric PDGFRα proteins when expressed in Ba/F3 cells, showing the absence of phosphorylation of the proteins when the internal ATG is mutated.

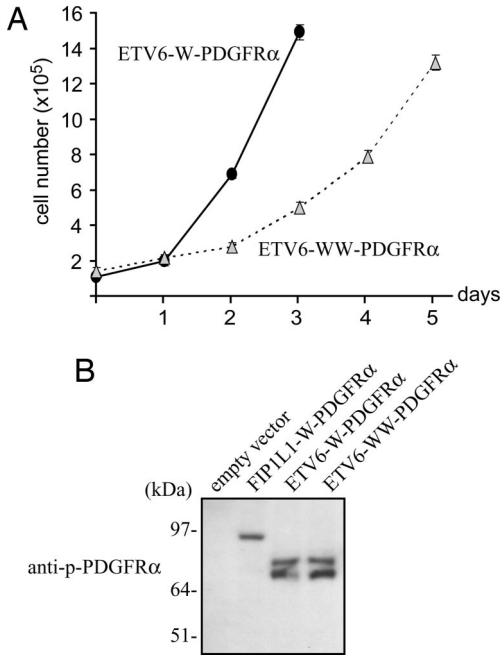

Fig. 6. Enforced dimerization can overcome the inhibition by the JM domain. (*A*) Transformation assay of Ba/F3 cells in the absence of IL3. (*B*) Phosphorylation of the chimeric PDGFRα proteins.

of dimerization. This observation may also be applicable to KIT, as demonstrated by the viral gag-kit oncoprotein, in which the JM domain of KIT is also interrupted (23).

Fusion of the Homodimerization Domain of ETV6 to PDGFR Overcomes the Inhibitory Function of the JM Domain. To test the effects of ETV6 compared with FIP1L1 in the context of PDGFRα, we created constructs in which the oligomerization domain of ETV6 was fused to PDGFRα with an intact JM domain (ETV6-WW-PDGFRA) or an interrupted JM domain (ETV6-W-PDGFRA) (Fig. 1). Both the ETV6-W-PDGFRα and ETV6-WW-PDGFRα proteins transformed Ba/F3 cells to IL3 independence, although proliferative potential in this assay was slightly lower for the ETV6-WW-PDGFRα-expressing compared with the ETV6-W-PDGFRA-expressing cells (Fig. 6*A*). In agreement with this result, both ETV6-W-PDGFRα and ETV6-WW-PDGFRα were found to be strongly phosphorylated (Fig. 6*B*).

These data indicate that enforced dimerization of PDGFRα or PDGFRβ with an intact JM domain can overcome the inhibitory effect of an intact JM domain. In contrast, our results, shown in Fig. 4*A*, indicate that FIP1L1 is not capable of doing so. These observations may explain the differences between the structures of the FIP1L1-W-PDGFRA and the ETV6-WW-PDGFRB fusions observed in patients. Whereas in all ETV6-PDGFRB and most variant PDGFRB fusions the entire transmembrane and JM domains of PDGFRB are present, the JM domain of PDGFRA is always truncated in the different FIP1L1-PDGFRA fusions. Our results may explain the reason for this difference, because enforced dimerization of PDGFRβ is able to overcome the inhibition of the JM domain, whereas FIP1L1 is clearly not able to do so.

Oligomerization by a fusion partner is a well-established mechanism for activation of fusion tyrosine kinases, but we found that, in the context of FIP1L1-PDGFRα, the FIP1L1 moiety is dispensable for kinase activation. In contrast, this study indicates that the interruption of the JM domain of PDGFRα is absolutely required to constitutively activate the FIP1L1-PDGFRα fusion kinase. This observation is consistent with the structure of the fusion gene identified in CEL and systemic mast cell disease (7–13). These results are notable in the context of previous studies of type III RTKs that have demonstrated an autoinhibitory role for the JM domain (18). Point mutations and insertions/deletions in the JM domain in type III RTKs have been identified in several malignancies, lending credence to the concept that interfering with the structure or binding interactions of the JM domain may be a critical mechanism of activation of tyrosine kinases in human cancers (19, 24–27). This finding is also supported by the crystal structure of FLT3, in which it has been shown that the JM domain maintains the kinase domain in the closed inactive state (20). Our results extend these findings and show that interruption of the JM domain of PDGFRα by fusing part of PDGFRα to a partner protein (FIP1L1) is a previously unrecognized mechanism of interfering with JM inhibitory function. As a consequence, our data predict that breakpoints in FIP1L1 can be highly variable or that any other protein could hypothetically be fused to PDGFRα and that all these fusions would result in the generation of an activated kinase, as long as the JM domain is interrupted or deleted. From a genetic perspective, however, it could be that FIP1L1 has a different significant function, especially in the context of eosinophils or mast cells. Expression of the fusion kinase is driven under control of the FIP1L1 promoter, which may be important in regulating the level, timing, or distribution of kinase expression. In addition, the genomic region of the FIP1L1 gene could be sensitive to DNA breakage, explaining why FIP1L1 is the preferred partner of PDGFRA.

In conclusion, our results indicate that the FIP1L1-PDGFRα fusion protein encodes an activated tyrosine kinase that does not depend on any functional contribution from FIP1L1 for constitutive tyrosine kinase activation. These observations are in agreement with the structure of FIP1L1-PDGFRA fusions identified in patients. Our data extend previous observations that mutations, deletions, and insertions of JM domains of type III RTKs can result in the activation of kinase activity. We show that interruption of the JM domain is crucial for the activity of chimeric RTKs in which the partner protein alone cannot overcome the inhibitory function of the JM domain. Our results demonstrate that enforced dimerization is not an absolute requirement for the transforming properties of a fusion kinase and indicate that the activity of these chimeric kinases can also depend on the removal of important inhibitory domains present in the native tyrosine kinases.

Materials and Methods

Constructs. *FIP1L1-PDGFRA* cDNA from a hypereosinophilic syndrome patient cloned into modified murine stem cell virus (MSCV)-internal ribosome entry site (IRES)-GFP and *ETV6-PDGFRB* cloned into MSCV-neomycin are described in refs. 4 and 7. All other constructs were generated by PCR and were cloned in MSCV-puromycin/neomycin (Clontech) or in MSCV-IRES-GFP.

Cell Culture. Cells (293T) were cultured in DMEM supplemented with 10% FBS. Ba/F3 cells were cultured in RPMI medium 1640 supplemented with 10% FBS and 1 ng/ml mouse IL3. Transfection of 293T cells was performed with FuGENE 6 (Roche, Gipf-Oberfrick, Switzerland). Production of retroviral vectors, transduction of Ba/F3 cells, and transduction of bone marrow cells are described in ref. 14. To test IL3-independent growth, stable Ba/F3 cell lines were washed three times in PBS and cultured in RPMI medium 1640 plus FBS without IL3 for 3–4 days. Cell growth was quantitated at 24-h intervals by using the CellTiter 96 AQueous One assay (Promega) or trypan blue exclusion and normalized to values at the start of the experiment.

Western Blotting. Total cell lysates were obtained by lysing cells in cold lysis buffer (PBS with 1 mM Na_2EDTA/1M NaF/0.1% Triton X-100/5 mM Na_3VO_4/200 mM phenylarsine oxide, pH 7.2) (Calbiochem) and complete protease inhibitor tablets (Roche). Thirty to 50 μg of protein lysate was combined with SDS loading buffer plus DTT (Cell Signaling Technology, Beverly, MA) before electrophoresis on SDS/10–12% PAGE gels or 4–12% Bistris gradient gels (Invitrogen) and transferred to nitrocellulose membranes. Antibodies used were as follows: anti-phospho-PDGFRα (Tyr 720), anti-PDGFRα 951 (Santa Cruz Biotechnology), anti-phospo-tyr (4g10; Upstate Biotechnology, Lake Placid, NY), and anti-mouse peroxidase and anti-rabbit peroxidase (Amersham Pharmacia Biotech).

Bone Marrow Transplant Assay and Analysis of Animals. Murine bone marrow transplant experiments were performed as described in ref. 14. Animals were killed when they had palpable splenomegaly or were moribund. Peripheral blood was collected from the retroorbital cavity by using heparinized glass capillary tubes and analyzed by automated complete and differential blood cell counts (Advia 120; Bayer, Wuppertal, Germany) and smears (stained with Wright–Giemsa). For histopathology, tissues were fixed in 10% neutral buffered formalin, dehydrated in alcohol, cleared in xylene, and infiltrated with paraffin on an automated processor (Leica, Vienna). Tissue sections (4-μm) from paraffin-embedded tissue blocks were stained with hematoxylin and eosin. Flow cytometric analysis was performed with a FACS-Calibur flow cytometer (Becton Dickinson); at least 10,000 events were acquired and analyzed by using CELLQUEST software.

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