The fusion proteins TEL-PDGFRβ and FIP1L1-PDGFRα escape ubiquitination and degradation

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The online version of this article contains a supplementary appendix.

ABSTRACT

Background

Chimeric oncogenes encoding constitutively active protein tyrosine kinases are associated with chronic myeloid neoplasms. TEL-PDGFRβ (TPβ, also called ETV6-PDGFRB) is a hybrid protein produced by the t(5;12) translocation, FIP1L1-PDGFRα (FPα) results from a deletion on chromosome 4q12 and ZNF198-FGFR1 is created by the t(8;13) translocation. These fusion proteins are found in patients with myeloid neoplasms associated with eosinophilia. Wild-type receptor tyrosine kinases are efficiently targeted for degradation upon activation, in a process that requires Cbl-mediated monoubiquitination of receptor lysines. Since protein degradation pathways have been identified as useful targets for cancer therapy, the aim of this study was to compare the degradation of hybrid and wild-type receptor tyrosine kinases.

Design and Methods

We used Ba/F3 as a model cell line, as well as leukocytes from two patients, to analyze hybrid protein degradation.

Results

In contrast to the corresponding wild-type receptors, which are quickly degraded upon activation, we observed that TPβ, FPα and the ZNF198-FGFR1 hybrids escaped down-regulation in Ba/F3 cells. The high stability of TPβ and FPα hybrid proteins was confirmed in leukocytes from leukemia patients. Ubiquitination of TPβ and FPα was much reduced compared to that of wild-type receptors, despite marked Cbl phosphorylation in cells expressing hybrid receptors. The fusion of a destabilizing domain to TPβ induced protein degradation. Instability was reverted by adding the destabilizing domain ligand, Shield1. The destabilization of this modified TPβ reduced cell transformation and STAT5 activation.

Conclusions

We have shown that chimeric receptor tyrosine kinases escape ubiquitination and down-regulation and that their stabilization is critical to efficient stimulation of cell proliferation.

Key words: PDGF receptor, oncogenes, protein degradation, ubiquitin.

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Introduction

Platelet-derived growth factor (PDGF) receptors (PDGFRα and PDGFRβ) belong to the family of type III receptor tyrosine kinases (RTK), which also includes c-KIT, FLT3 and the M-CSF receptor.[1] They are composed of an extracellular ligand binding domain, a transmembrane segment and an intracellular part containing a split tyrosine kinase domain. Ligand-induced dimerization of these receptors leads to activation of intrinsic kinase activity and subsequent autophosphorylation of tyrosine residues in the intracellular part. These phosphotyrosines act as docking sites for SH_2-domain-containing signaling proteins.[2] Signal transduction by PDGFR leads to the activation of transcription factors, which ultimately control the proliferation of cells such as fibroblasts and neurons.[3-5] However, PDGF-BB, which binds to both PDGFRα and PDGFRβ, is a poor mitogen for hematopoietic cells, although PDGFRα is expressed in platelets and megakaryocytes and PDGFRβ is expressed in megakaryocytes, macrophages and other hematopoietic cells.[6-8]

Activation of PDGFR also promotes ubiquitination of lysine residues leading to receptor down-regulation. The proto-oncogene Cbl is recruited by PDGFR in a ligand-dependent manner and mediates receptor ubiquitination.[9] Attachment of ubiquitin monomers to multiple lysines (multi-monoubiquitination) targets the receptor for endocytosis and degradation by lysosomes.[10] In contrast, ligation of polymers of at least four ubiquitin moieties to the receptor (polyubiquitination) targets for degradation by proteasomes. The importance of the latter pathway in RTK down-regulation is not clear.[10,11]

Alteration of RTK genes contributes to cancer development. Mutations in the activation loop of the kinase domain or in the juxtamembrane domain can lead to constitutive receptor activation, as shown by PDGFRα D842V in gastrointestinal stromal tumors.[12] Chromosomal translocations of RTK genes create hybrid oncogenes, which are the primary cause of a number of hematopoietic malignancies. In the resulting fusion proteins, the RTK ligand binding domain is replaced by the N-terminus of an intracellular protein, which generally includes a motif mediating oligomerization. This domain induces ligand-independent clustering of the hybrid receptor, leading to constitutive activation of the kinase portion, constitutive signaling and uncontrolled cell proliferation. PDGFR and fibroblast growth factor receptors (FGFR) are activated by this mechanism in myeloid neoplasms associated with eosinophilia. About 20 different PDGFRβ translocation products have been described, the most common being TEL-PDGFRβ (TPβ), which results from the t(5;12) (q33;p13) chromosomal translocation between the genes *ETV6* (*TEL*) and *PDGFRB* in a subset of myeloid neoplasms previously classified as chronic myelomonocytic leukemia (CMML).[13,14] The TPβ fusion protein consists of the first 154 amino acids of the TEL transcription factor fused to the transmembrane and intracellular domains of PDGFRβ. The pointed domain of TEL (PNT, also called HLH or SAM domain) mediates TPβ oligomerization,[13,15,16] which is essential for its activation and its oncogenic properties.

The fusion protein FIP1L1-PDGFRα (FPα) results from an interstitial deletion on chromosome 4q12, which is found in patients with myeloid neoplasms that were previously classified as chronic eosinophilic leukemia (CEL).[17] This fusion protein contains a variable number of amino acids from the FIP1L1 protein depending on the position of the breakpoint, which spreads from exon 7 to exon 10 in the *FIP1L1* gene. The breakpoint in PDGFRα is located within the juxtamembrane region and causes the disruption of an inhibitory WW-like domain resulting in constitutive activation of the FPα kinase domain.[18] By contrast to most other chimeric oncogenes, FPα does not harbor any known motif mediating protein-protein interactions. The FIP1L1 part of FPα is dispensable for constitutive receptor activation in Ba/F3 cells,[19] but plays a role in proliferation and differentiation of transduced human $CD34^+$ hematopoietic progenitors.[20]

Down-regulation of RTK is essential in limiting the duration and intensity of signaling initiated by ligand stimulation. Evasion from such negative mechanisms is emerging as one of the processes implicated in cell transformation.[21-23] However, little information is available on hybrid PDGFR degradation. We, therefore, studied the stability and ubiquitination profile of TPβ and FPα proteins and wild-type receptors, examining the consequences for efficient cell proliferation.

Design and Methods

Patients

Patient #1, a 65-year old man, was diagnosed as having FIP1L1-PDGFRα-positive CEL in blast crisis.[24] At the time of sampling, his white blood cell count was 25×10^9 cells/L, of which 75% were blast cells. Peripheral blood mononuclear cells were isolated from fresh blood on a Ficoll gradient.

Patient #2, a 47-year old man, was diagnosed as having ETV6-PDGFRB-positive CMML associated with eosinophilia and myelofibrosis. His white blood cell count was elevated at 49.8×10^9 cells/L, including 2.5×10^9 eosinophils/L. At that time, a sample was frozen after white blood cell purification on a density gradient. The patient responded well to imatinib therapy and remains in complete cytogenetic remission 2 years after initial diagnosis.

This study was approved by the local biomedical ethics committee of the medical faculty and university hospital (ref #F/2005/02).

In both cases, cells were maintained in Iscove's modified Dulbecco's medium supplemented with 20% fetal bovine serum (FBS), glutamine, asparagine, arginine, penicillin and streptomycin (Invitrogen).

Antibodies and reagents

Anti-PDGFRα (951), anti-PDGFRβ (958), anti-phosphotyrosine (pY99), anti-FLAG (D8), anti-STAT5 and anti-FGFR1 (Flg) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-phospho-STAT5 (tyrosine 694) antibody was purchased from Cell Signaling. Mouse monoclonal anti-HA tag antibody (clone12CA5) was obtained from Roche. Mouse mono-

clonal anti-β-catenin and anti-Cbl antibodies were purchased from BD Transduction Laboratories. Mouse monoclonal antibodies against β-actin (clone AC-15) and FLAG (M5) were purchased from Sigma. The antibody against stearoyl-coenzyme A desaturase was described previously.[4] G418, MG132, puromycin and aprotinin were purchased from Sigma. Human PDGF-BB and FGF-2 were purchased from Peprotech.

Constructs and mutagenesis

The human PDGFRα D842V in pMSCV-puro vector, and TPβ and FPα in pMSCV-eGFP vector have been described previously.[13,18,25] Human HA-PDGFRβ was cloned in pEF-BOS-puro as reported elsewhere[26] and subcloned in pcDNA3 (Invitrogen) for transient transfection experiments. Murine PDGFRβ D849V in pcDNA3 has also been described earlier.[27] Human PDGFRα was cloned in pMX-ires-GFP and subcloned in pcDNA3 (Invitrogen) for transient transfection experiments.

ZNF198-FGFR1 was cloned in pcDNA3 and was a kind gift from Dr. Nicholas Cross (Salisbury, UK).[28] Flag-ubiquitin in pcDNA3 and c-Cbl in pRK5 was obtained from Dr. Philippe Soubeyran (INSERM, Marseille, France). HA-ubiquitin and flag-4KR mutant in pcDNA3 were a kind gift of Dr. Yosef Yarden (Rehovot, Israel). Human FGFR1 cDNA was a kind gift of Dr. Lena Claesson-Welsh (Uppsala, Sweden) and was subcloned into pcDNA3. The cDNA encoding TPβ was subcloned by polymerase chain reaction in pRetroX-Tuner (Clontech) in a Not1 site in-frame with the destabilizing domain (DD). Mutagenesis was performed using QuickChange XL-II kit (Stratagene) according to the manufacturer's instructions. All constructs were checked by sequencing.

Cell culture, transfection, infection and thymidine incorporation assay

Ba/F3, BOSC and HEK-293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma) with 10% FBS. Ba/F3 cell culture was supplemented with interleukin 3 (IL-3).

Transient transfections of BOSC and HEK-293T cells were performed using the calcium phosphate method as follows. The day before transfection, cells were seeded in 6-well plates. Plasmid DNA (3 μg) was diluted in 56.25 μL of water and mixed with 62.5 μL of BBS buffer (50 mM N,N-bis-(2-hydroxyethyl)-2-aminoethane-sulfonic acid, pH 7, 280 mM NaCl, 1.5 mM Na_2HPO_4). Following addition of 6.25 μL of $CaCl_2$ 2.5M, the solution was incubated for 20 min at room temperature to allow the formation of DNA precipitates. Precipitates (125 μL) were added to each well in the presence of medium (1 mL) with 10% calf serum (Invitrogen) for 4 h. Cells were washed once with PBS and incubated in medium with 10% FBS. For immunoprecipitation experiments, cells were lysed 24 h after transfection.

Ba/F3 cell lines stably expressing receptors were created as previously reported.[29] Briefly, retroviral supernatants were generated by co-transfection of the BOSC packaging cell line with plasmid DNA encoding the receptor and the ecotropic envelope protein using the calcium phosphate method, as described above. After 48 h, the supernatants were harvested and used in a spin infection protocol. One million Ba/F3 cells were centrifuged for 2 h at 37°C and 300 x g in the presence of viral supernatant (1 mL) and Polybrene (20 μg, Sigma). Cells were then resuspended in medium with 10% FBS and IL-3. After 24 h, GFP-positive cells were isolated by fluorescence-activated cell sorting (FACS) and maintained in DMEM with FBS and IL-3.

Alternatively, 10^7 Ba/F3 cells were electroporated with 60 μg of DNA and diluted in 30 mL DMEM with 10% FBS and IL-3 as described earlier.[26] After 48 h, cells were selected with 3 μg/mL puromycin or 3 mg/mL G418 for 14 days.

Homogenous Ba/F3 cell lines expressing PDGFRα or PDGFRβ were sorted by flow cytometry after staining with specific anti-receptor antibodies.

Ba/F3 cell lines expressing DD-TPβ were created by retroviral infection as described above. Cells were selected with 3 μg/mL puromycin for 1 week.

In [^{3}H]-thymidine incorporation assays, Ba/F3 cells stably expressing receptors were washed extensively and seeded in triplicate in a 96-well plate in DMEM with 10% FBS in the presence of the indicated growth factor. After 20 h, [^{3}H]-thymidine (0.5 μCi/well, GE Healthcare) was added for 4 h. Cells were then harvested and the incorporation of [^{3}H]-thymidine was quantified using a TopCount instrument (Perkin Elmer).

Cycloheximide treatment, western blot and immunoprecipitation

Stability assays were performed in the presence of cycloheximide (50 μg/mL, Sigma) for the indicated periods of time. At each time point, 10^6 cells were washed once with ice-cold PBS and then lysed in 200 μL Laemmli buffer. Samples were subjected to 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to PVDF membranes. Blots were blocked in the presence of 5% milk and incubated with 1 μg/mL of primary antibody overnight at +4°C. After incubation with secondary antibodies conjugated to horseradish peroxidase (Cell Signaling Technology), bands were visualized by enhanced chemiluminescence (Western blot Luminol Reagent, Santa Cruz, CA, USA). Bands were quantified after incubation with secondary fluorescent antibodies (IRDye) using the Odyssey system (Li-Cor).

In immunoprecipitation experiments, cells were lysed in 25 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 10% glycerol, 1% Triton X-100, 1 mM Pefabloc (Roche), 1 μg/mL aprotinin and 1 mM Na_3VO_4. After clearing by centrifugation, cell lysates were incubated overnight with 1 μg antibody at +4°C. Antibody complexes were collected by adding protein A/G Ultralink (Pierce) for 1 h at +4°C, washed extensively, and then analyzed by western blotting.

Results

PDGFR hybrids are less degraded than wild-type receptors

Upon activation by their ligands, PDGFR are quickly internalized and degraded with a half-life of about 30

min.[9,30,31] To compare the degradation of constitutively activated chimeric PDGFR with wild-type receptors, we created different Ba/F3 cell lines stably expressing PDGFRα, PDGFRβ, or the hybrid receptors TPβ or FPα. In agreement with published data, TPβ and FPα stimulated Ba/F3 cell growth in the absence of IL-3, whereas PDGFR did not, even in the presence of PDGF-BB (Figure 1A).[16] We next analyzed the degradation kinetics of these receptors in cells that were treated with cycloheximide to block new protein synthesis. As shown in Figure 1B, wild-type receptors stimulated with PDGF-BB were efficiently degraded and blot quantification revealed that less than 20% of the protein was left after 4 h, in accordance with published results.[30,31] Similar data were obtained for PDGFRα D842V, a constitutively active receptor that harbors a mutation in the activation loop. In contrast, we found that hybrid receptors were not effectively degraded in the presence of cycloheximide. More than 50% of the TPβ protein still remained in the cells after 12 h, while FPα degradation was negligible. Western blot against β-actin, which is a stable protein with a long half-life, is shown as a control of cell viability. Western blot against β-catenin, which is instead quickly degraded by proteasomes in normal cells, was used as a control for the efficiency of the cycloheximide treatment. To confirm our observations in human leukemia cells, we evaluated the degradation of hybrid receptors in cells isolated from patients with CEL or CMML (Figure 1C). After 8 h of cycloheximide treatment, FPα and TPβ protein were poorly degraded, in line with our results on Ba/F3 cells. We were not able to detect β-catenin in the patients' samples. As an alternative control, we performed western blots to probe stearoyl-coenzyme A desaturase, which was detectable only in the FPα-positive sample (*Online Supplementary Figure S1*). The degradation kinetics of stearoyl-coenzyme A desaturase was compatible with published data[32] suggesting that the degradation machinery was still active in these cells. Altogether, these experiments indicated that hybrid PDGFR are much more stable than full-length membrane receptors.

PDGFR hybrids are scarcely ubiquitinated

We next analyzed the ubiquitination of fusion receptors. HA-tagged ubiquitin was transfected with PDGFRα or PDGFRβ, FPα or TPβ in HEK-293T cells. To make a comparison between constitutively active receptors, we also examined the ubiquitination of PDGFRα D842V and PDGFRβ D849V. We adjusted the amounts of transfected DNA to obtain similar levels of expression of wild-type and hybrid PDGFR, as judged by western blots. Following immunoprecipitation with anti-PDGFR antibodies, ubiquitination was evaluated by anti-HA immunoblotting (Figure 2). PDGFRα and PDGFRβ were heavily ubiquitinated. This was observed even in the absence of stimulation with PDGF-BB as a result of ligand-independent activation of over-expressed receptors in HEK-293T cells, which we had noticed previously.[33] In contrast to membrane PDGFR, ubiquitination of hybrid receptors was very weak and not always detectable by immunoblotting. In conclusion, the level of ubiquitination of TPβ and FPα was much lower than that of membrane receptors, which could explain their increased stability.

Cbl is constitutively phosphorylated in the presence of hybrid PDGFR

The E3 ubiquitin ligase Cbl is phosphorylated upon PDGF stimulation and acts as negative regulator of PDGFR.[9,31] Oncogenic mutants of Cbl enhance PDGFR signaling by preventing PDGFR ubiquitination and degrada-

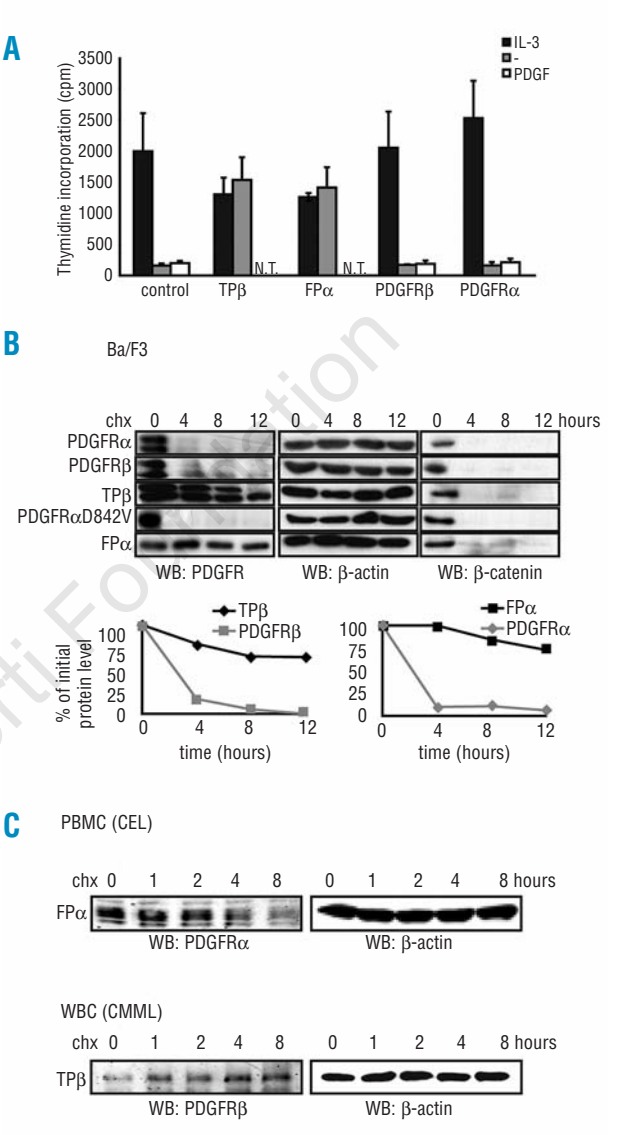

Figure 1. Hybrid PDGFR are stable proteins. (A) Wild-type PDGFR or hybrid PDGFR were stably expressed in Ba/F3 cells. The cell lines were incubated for 24 h with the indicated growth factor and proliferation was measured by a [³H]-thymidine incorporation assay as described in the Design and Methods section. Untransfected Ba/F3 cells were used as a control. One representative experiment is shown with S.D. N.T., not tested. (B) Wild-type PDGFRα or PDGFRβ, constitutively active PDGFRα D842V or hybrid PDGFR were stably expressed in Ba/F3 cells. These cell lines were treated with cycloheximide (chx, 50 μg/mL) for the indicated periods of time. Wild-type PDGFRα or PDGFRβ were simultaneously stimulated with PDGF-BB (20 ng/mL). Samples were collected at each time point and cell lysates were analyzed by western blot with anti-PDGFR antibody. Western blot against β-actin is shown as a control. Western blot anti-β-catenin was used as a control of the efficiency of the cycloheximide treatment. Quantifications shown are representative of the results of at least three independent experiments. (C) Peripheral blood mononuclear cells from a patient with FPα-positive leukemia (upper panel) or leukocytes from a patient with TPβ-positive myeloid neoplasm (lower panel) were treated as in (B).

tion in a dominant negative manner.[30,34] We first examined the phosphorylation of Cbl in Ba/F3 cells expressing wild-type or hybrid PDGFR. As shown in Figure 3A, PDGF stimulation increased the level of Cbl phosphorylation in cells expressing PDGFRα or PDGFRβ. A strong and constitutive Cbl phosphorylation was found in cells expressing TPβ or FPα, suggesting that Cbl was recruited to chimeric PDGFR complexes, at least transiently. We next investigated whether hybrid receptor ubiquitination could be induced by Cbl. We transfected HEK-293T cells with TPβ and flag-tagged ubiquitin in the presence or absence of Cbl (Figure 3B). Cbl over-expression increased TPβ ubiquitination, suggesting that Cbl is capable of mediating the ubiquitination of hybrid receptors. While polyubiquitination of cytosolic proteins induces proteasomal degradation, monoubiquitination in the cytosol controls different processes that do not necessarily lead to protein degradation.[35] We, therefore, examined which kind of ubiquitination of TPβ occurred in the presence of Cbl. We used a branching-defective mutant of ubiquitin, in which the four main lysine residues used to form polymers (in position 11,29, 48 and 63) are mutated to arginine (4KR mutant). This mutant has been previously used to demonstrate protein monoubiquitination.[36] Wild-type or 4KR mutant ubiquitin was expressed together with TPβ or PDGFRβ and Cbl. *Online Supplementary Figure S2* shows that ubiquitinated TPβ can be detected in the presence of both forms of ubiquitin indicating that Cbl mediates monoubiquitination of the hybrid receptor. We also observed that in the presence of 4KR mutant the amount of ubiquitinated TPβ was increased. Since monoubiquitinated forms of TPβ reside in the cytosol and not at the plasma membrane, this type of ubiquitination may not effectively induce hybrid receptor degradation. Altogether these experiments showed that TPβ can be ubiquitinated by Cbl only if Cbl is over-expressed.

TPβ and FPα stability does not require signaling

The degradation of RTK is accelerated by ligand binding and receptor activation. Inactivation of the kinase activity

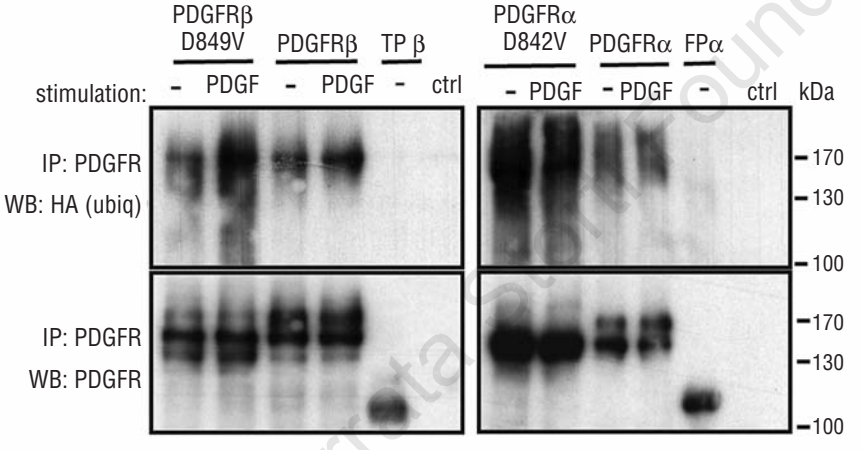

Figure 2. Hybrid PDGFR are poorly ubiquitinated. The indicated PDGFR were transfected in HEK-293T cells together with HA-tagged ubiquitin. Cells were starved for 2 h and stimulated with PDGF-BB (20 ng/mL) for 15 min where indicated. Receptors were immunoprecipitated with specific anti-PDGFR antibodies and ubiquitination was analyzed by anti-HA immunoblotting.

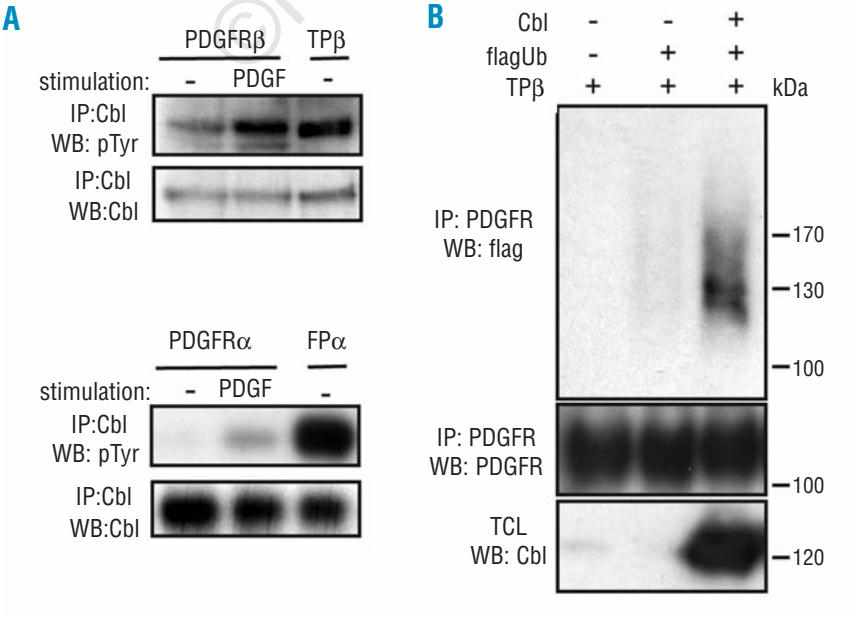

Figure 3. Cbl is constitutively phosphorylated in the presence of PDGFR hybrids. (A) Ba/F3 cells expressing wild-type PDGFR were starved for 4 h and then stimulated with PDGF-BB (20 ng/mL) or control medium for 5 min. Cbl was immunoprecipitated from cell lysates and phosphorylation was detected by immunoblotting with anti-phosphotyrosine antibody. (B) HEK-293T cells were transfected with TPβ , flag-ubiquitin and Cbl as indicated. Immunoprecipitation was performed with an anti-PDGFRβ antibody and ubiquitination was analyzed by anti-flag immunoblotting.

in PDGFRβ has been shown to reduce the level of turnover of the receptor.[37] However, in some cases, constitutively active signaling can prevent RTK degradation. For example, constitutively active Cdc42 protects EGF receptors from degradation by inhibition of Cbl activity.[38] We, therefore, created kinase-dead versions of TPβ and FPα by introducing a lysine to arginine mutation in the ATP binding pocket at a position corresponding to 627 and 634 in human PDGFRα and PDGFRβ, respectively.[27,39] Ba/F3 cell lines stably expressing these mutants were tested in a stability assay in the presence of cycloheximide (Figure 4). Kinase-dead TPβ and FPα were as stable as the active oncogenes, arguing against the possibility that degradation of hybrid receptors is prevented by constitutive signaling.

The prolonged half-life of TPβ promotes cell transformation

To determine whether the high stability of PDGFR hybrids has an impact on their ability to transform hematopoietic cells, we used a reversible method for controlling protein stability which is based on the fusion of a destabilizing domain (DD) to the protein of interest.[40] This domain targets proteins for degradation unless it is bound to the small membrane-permeable molecule Shield1, which stabilizes the target protein. We fused the DD to the N-terminus of TPβ (DD-TPβ). As shown in Figure 5A, the level of DD-TPβ expression in Ba/F3 cells was increased after treatment with Shield1 for 8 h. To confirm that the higher level of expression was the result of a longer half-life of DD-TPβ, we evaluated degradation kinetics in the presence of cycloheximide (Figure 5B). Cells were initially pre-treated with Shield1 for 24 h to stabilize DD-TPβ and to start the experiment with comparable DD-TPβ levels. Shield1 was then removed in the control condition and maintained in the other one. We observed that in the presence of Shield1, DD-TPβ was more stable than TPβ. The effect of DD-TPβ stabilization on cell proliferation was assessed by performing a Ba/F3 proliferation test in the absence of IL3 (Figure 5C). Vector-transduced cells or DD-TPβ-transduced cells were incubated with or without Shield1 for 72 h. When DD-TPβ was stabilized with Shield1, cell proliferation was much greater than in the absence of Shield1. The critical role of the transcription factor STAT5 in TPβ-mediated cell transformation has been previously demonstrated.[41,42] In agreement with these reports, the stabilization of DD-TPβ with Shield1 increased the amount of phosphorylated STAT5 in Ba/F3 cells (Figure 5D). Altogether these experiments indicated that stabilization of hybrid receptors favors the transformation of hematopoietic cells.

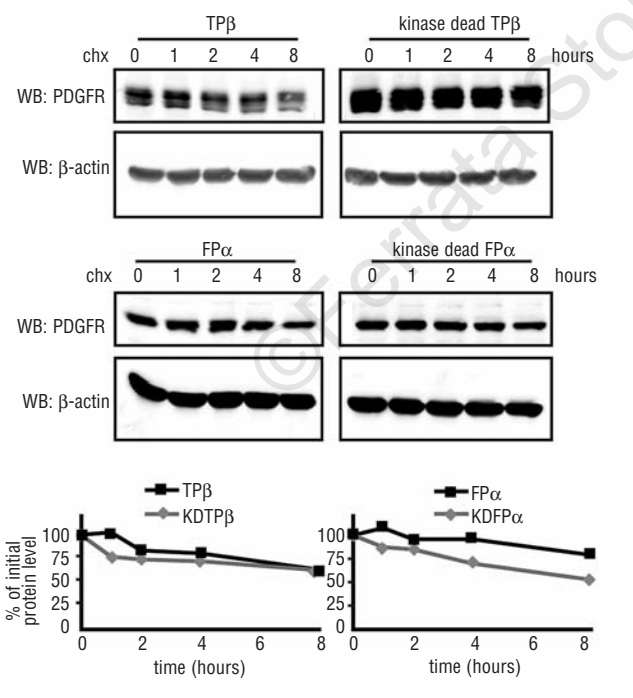

Figure 4. Hybrid PDGFR kinase activity is not required for stabilization. Ba/F3 cells stably expressing TPβ or FPα or the corresponding kinase-dead mutants (mutations corresponding to K627R in human PDGFRα and K634R in human PDGFRβ) were treated with cycloheximide (50 μg/mL) for the indicated periods of time. At each time-point, samples were collected and analyzed by immunoblotting with anti-PDGFR antibody as in Figure 1B. Western blot against β-actin is shown as a control.

FGFR1 hybrid receptors are stable proteins

To test whether our findings could be applied to other translocation products, we studied the stability of the FGFR1 hybrid receptor ZNF198-FGFR1. This fusion protein has been associated with the 8p11 myeloproliferative syndrome (now re-classified as myeloid neoplasm associated with FGFR1 rearrangement).[28,43] We performed stability assays in the presence of cycloheximide with Ba/F3 cells stably expressing either wild-type FGFR1 stimulated with FGF2 and heparin, or ZNF198-FGFR1. As for PDGFR, more than 80% of the mature form of FGFR1 was degraded after 4 h of treatment (Figure 6) while the precursor form, differently from the precursor form of PDGFR, was not degraded. ZNF198-FGFR1 was highly stable, similarly to the PDGFR hybrid proteins.

Discussion

In this study, we show that three RTK fusion proteins associated with chronic myeloid neoplasms, namely FPα, TPβ and ZNF198-FGFR1, are highly stable compared to the corresponding activated wild-type receptors. Using a destabilizing domain-tagged version of TPβ, we demonstrated that the stabilization of TPβ strongly enhanced its ability to stimulate cell proliferation. Thus, the fusion of TEL/ETV6 with PDGFRB does not only induce constitutive activation of the kinase domain, but also prevents normal and efficient degradation of the activated receptor.

Our results indicate that FPα and TPβ are poorly ubiquitinated, a process that is required to enter the degradation pathway.[10,44] This prompted us to analyze the activation of Cbl, which is described as the key E3 ubiquitin ligase for RTK. Indeed, most reports of impaired RTK down-regulation in cancer cells have pointed to a defect in Cbl recruitment or activation. For instance, several oncogenic mutations in RTK, such as FLT3, PDGFR and TRP-MET, disrupt the recruitment of Cbl, preventing Cbl-mediated ubiquiti-

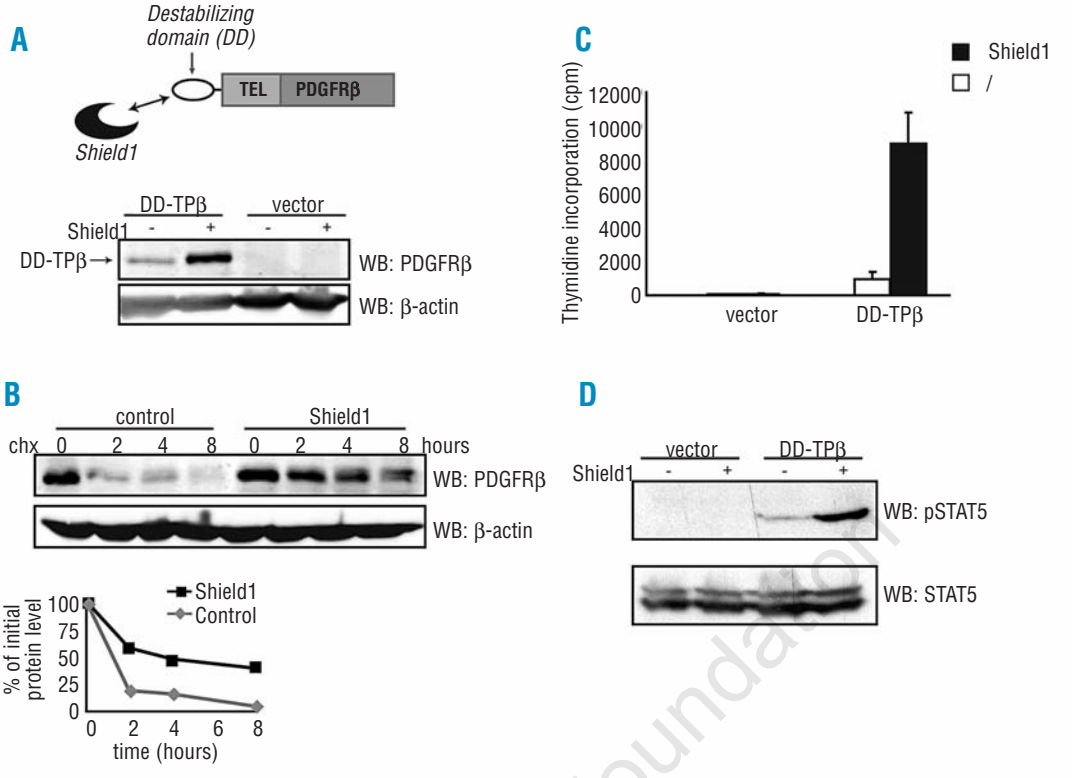

Figure 5. TPβ stabilization induces Ba/F3 transformation. (A) Ba/F3 cells stably expressing DD-TPβ or empty vector were treated with 1 μM Shield1 (Clontech) for 8 h and the cell lysates were then analyzed by western blot with anti-PDGFRβ antibodies. Western-blot against β-actin is shown as a control. (B) Ba/F3 cells expressing DD-TPβ were pre-treated for 24 h with Shield1 (100 nM). Shield1 was removed or maintained in the culture medium where indicated. Cells were incubated with cycloheximide (50 μg/mL) for the indicated periods of time. Cell lysates were analyzed by western blot against PDGFRβ. Western blot against β-actin is shown as a control. (C) Ba/F3 cells expressing empty vector or DD-TPβ were washed extensively with medium without IL3. Cells were incubated for 72 h with or without 1 μM Shield1. Proliferation was measured by a [^{3}H]-thymidine incorporation assay. (D) Ba/F3 cells expressing empty vector or DD-TPβ were treated for 24 h with Shield1 (100 nM) or left untreated and then washed extensively with medium without IL3. Shield1 was added again in the medium as indicated. After 4 h in the absence of IL3, cells were harvested and STAT5 phosphorylation (tyrosine 694) analyzed by immunoblotting.

nation, endocytosis and degradation of the receptors.[21,22,34,45] Mutations inactivating Cbl itself have also been described.[23] A different mechanism accounts for the lack of down-regulation of hybrid receptors observed in the present study. We found that Cbl was heavily phosphorylated, which was shown to correlate with its activation.[46] Although Cbl was capable of mediating TPβ ubiquitination when over-expressed, endogenous Cbl was clearly not able to induce hybrid receptor ubiquitination and degradation.

It has been shown in the case of CSF-1 receptor, which belongs to the family of PDGFR, that ligand stimulation triggers Cbl activation and translocation to the plasma membrane where it mediates ubiquitination of the receptor.[47,48] The lack of ubiquitination of chimeric receptors could, therefore, be associated with their altered localization, as they are not inserted in the plasma membrane, like the wild-type receptors, but reside in the cytosol[49] (*and our unpublished results*). In line with this hypothesis, membrane targeting of TRP-MET, a hybrid kinase generated by *in vitro* carcinogenesis, was recently shown to promote the down-regulation of TRP-MET by over-expressed Cbl.[21]

The transcription factor STAT5 plays a key role in sig-

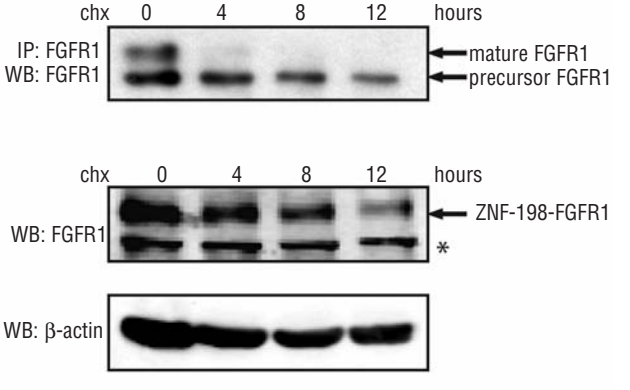

Figure 6. The hybrid ZNF198-FGFR1 is also a stable protein. Ba/F3 cells were electroporated with wild-type human FGFR1 or ZNF198-FGFR1 and selected in the presence of G418. Cells stably expressing the receptors were treated with cycloheximide (50 μg/mL) for the indicated periods of time. At each time point, samples were collected. ZNF198-FGFR1 expression was directly assessed by immunoblotting with anti-FGFR1 antibody. Wild-type FGFR1 was immunoprecipitated with specific antibodies and revealed by western blot. The symbol (*) indicates a background band. Western-blot anti-β-actin is shown as a control.

nal transduction by chimeric tyrosine kinases *in vitro* and *in vivo*.[20,41,42,50] Genetic studies have shown that the loss of both STAT5a alleles does not allow the development of a TPβ–induced myeloproliferative disease in mice.[42] Interestingly, the loss of a single STAT5 allele greatly impairs the development of the disease, indicating that the level of expression of STAT5 is crucial for TPβ-mediated cell transformation. This may be due to a weak affinity of STAT5 for PDGFR, or to competition between STAT5 and other factors for binding to PDGFR. Our results demonstrated that the level of TPβ expression is also important for cell proliferation. The accumulation of DD-TPβ increased the level of phosphorylation of STAT5 and resulted in a higher rate of IL3-independent proliferation of Ba/F3 cells. This provided a direct link between oncogene stabilization and malignant transformation of cells.

The idea of improving cancer treatment by using drugs that destabilize oncogenes has emerged in recent years and has found a promising example in the use of inhibitors of HSP90.[51] However, we did not observe degradation of TPβ following treatment of cells with the HSP90 inhibitor 17-allylamino-17-demethoxygeldanamycin (17AAG) (*data not shown*). Uncovering the mechanism behind fusion receptor stabilization could provide new and useful targets for drug-mediated degradation of oncogenes that are not HSP90 clients.

In conclusion, our data indicate that chimeric receptor tyrosine kinases are resistant to degradation, as a result of decreased ubiquitination. We suggest that, in conjunction with constitutive tyrosine kinase activity, high stability promotes hematopoietic cell transformation by hybrid receptors.

Authorship and Disclosures

FT performed all experiments and wrote the paper; AK contributed by establishing several cell lines and provided support to the project; LM, PV and PP took care of the patients and provided samples; JC provided key reagents; J-BD designed the study, supervised the work and wrote the paper.

The authors reported no potential conflicts of interests.

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