Src Family Kinases Negatively Regulate Platelet-derived Growth Factor α Receptor-dependent Signaling and Disease Progression*

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We tested the hypothesis that Src family kinases (SFK) contribute to c-Cbl-mediated degradation of the platelet-derived growth factor (PDGF) α receptor (αPDGFR). Using either a receptor mutant that does not engage SFKs (F72/74), or cells that lack SFKs, we found that SFKs contributed to degradation of the αPDGFR. Overexpression of c-Cbl also reduced the receptor half-life, but only if the receptor was able to engage SFKs. In cultured cells, prolonging the half-life of the receptor correlated with enhanced signaling and more efficient S phase entry, whereas accelerating receptor degradation had the opposite effect. Consistent with these tissue culture findings, there was a statistically significant increase in the onset of a proliferative retinal disease when animals were injected with cells expressing the F72/74 receptor, as compared with cells expressing the WT receptor. Our findings suggest that SFKs cooperate with c-Cbl to negatively regulate the αPDGFR, and that the SFK/c-Cbl suppression of αPDGFR output is relevant to the onset and progression of a proliferative disease.

Platelet-derived growth factor (PDGF)1 receptors (PDGFRs) mediate a number of cellular responses including proliferation, migration, and survival. Signaling via PDGFRs plays a critical role in development, as well as in physiological repair mechanisms and in the pathogenesis of proliferative diseases (1–3). Two related PDGFR subtypes, termed α and β, have been identified, and the two PDGFRs differ from one another in both their signaling mechanisms and biological functions (3).

Exposure of cells to PDGF induces dimerization of PDGFRs, and the various PDGF isoforms (PDGF-AA, -AB, and -BB) differ in their ability to associate with the two PDGFRs. PDGFR-BB is the universal ligand, and it assembles αβ-homodimers, as well as αβ-heterodimers, whereas PDGF-AA will assemble only αα-homodimers (4). Receptor dimerization leads to activation and phosphorylation of the αPDGFR, and the phosphorylated receptor is able to associate with a number of SH2-containing proteins. One class of such proteins is the Src family tyrosine kinases (SFK), and they associate with the αPDGFR once tyrosine 572 and/or 574 are phosphorylated (5, 6). This leads to an increase in SFP kinase activity, however, PDGFR-dependent cell cycle progression and growth in soft agar are not compromised in cells expressing the αPDGFR mutant which does not bind or activate SFKs (5, 6). These observations suggest that αPDGFR-dependent DNA synthesis does not require an increase in SFP activity at the start of the cell cycle.

So why are SFKs engaged when a cell is exposed to PDGF? One possibility is that SFKs contribute to phosphorylation of cellular proteins, which regulate PDGFR-dependent responses. For instance, we have found that a receptor mutant that fails to bind or activate SFKs is also unable to mediate efficient tyrosine phosphorylation of a subset of the proteins that are phosphorylated in a PDGF-stimulated cell (5, 7). Thus the early activation of SFKs, or their association with the αPDGFR may be required to engage responses that are dependent on the phosphorylation of cellular proteins.

c-Cbl is tyrosine phosphorylated in response to a variety of agonists, and hence appears to contribute to many different types of signaling systems (8, 9). At least in certain settings c-Cbl negatively regulates receptor tyrosine kinases. Genetic studies in C. elegans show that the function of Let 23 (an EGF receptor homologue) is suppressed by Sli-1 (to which c-Cbl is homologous) (10, 11). In mammalian cells, c-Cbl promotes the ubiquitination, endocytic sorting, and/or degradation of the α and βPDGFR, colony stimulating factor-1 receptor and epidermal growth factor (EGF) receptor (EFGFR) (12–16). Identification of the ring finger domain of c-Cbl as an E3 ubiquitin-protein ligase (17, 18) supports the idea that c-Cbl is directly involved in degradation of receptor tyrosine kinases. The ability of c-Cbl to promote clearing of receptors from the cell surface is at least one mechanism by which c-Cbl negatively regulates growth factor-dependent responses.

A growing body of literature indicates that PDGFRs play a non-trivial role in the pathogenesis of a variety of proliferative diseases, such as tumorigenesis, atherosclerosis, and fibrosis. While important advances have been made in elucidating the signaling pathways downstream of each of the PDGFRs, this information is not readily translated to the in vivo disease state. This is in part because these diseases are complex, often involve both of the PDGFRs, and hence it is difficult to evaluate the relative importance of the α and βPDGFR subtypes during disease progression. We have recently identified the αPDGFR as a key contributor to a proliferative retinal disease, prolifer-
ative vitreoretinopathy (PVR) (19). This model provides a valuable approach to study the importance of signal relay mechanisms for disease progression in vivo.

In this study we have tested the hypothesis that SFKs act through c-Cbl to decrease the half-life of the αPDGFR. We also tested the consequences of altered receptor half-life on PDGFR-dependent signaling and DNA synthesis. Finally, the relevance of these in vitro findings was tested in an in vivo model of a proliferative disease.

MATERIALS AND METHODS

Cell Lines—The mouse embryo 3T3 Patch B (Ph) cell line was derived from F0/P mouse embryos and was kindly provided by Dr. Dan Bowen-Pope (48). These are 3T3-like cells which express the βPDGFR at approximately 1×10⁶ receptors per cell, but have no endogenous αPDGFRs. Ph cells were maintained in Dulbecco’s modified Eagle’s (DME) medium supplemented with 5% calf serum. The human WT and F72/74 αPDGFRs were stably expressed in Ph cells to 1×10⁶ receptors per cell, as described previously (5). The WT c-Cbl construct (kindly provided by Dr. Hamid Band) was subcloned into the pH licking’ retroviral vector, whose polymer linker contains the following unique restriction sites: Hind III, Sal I, Bgl II, and Not I. The c-Cbl construct in the retroviral vector was transfected into 293GPG cells (49), the virus supernatant collected for 7 days, and concentrated by centrifugation (25,000 × g, 90 min, 4 °C). Equal amounts of colony forming units from the concentrated virus were used to infect Ph cells expressing either WT or F72/74 mutant receptors.

The SYF cells are SV-40 large T antigen-immortalized fibroblasts that were derived from mouse embryos harboring functional null mutations for Src family members, Src, Yes, and Fyn. Murine wild type src-S were stably expressed in SYF cells to generate the SYF + Src cells (20). The SYF panel of cells was maintained in DME medium supplemented with 10% fetal bovine serum. Since these cells expressed very low and dissimilar levels of αPDGFRs, the WT human αPDGFR was stably expressed to similar levels in the SYF panel of cells, using the 293GPG system as described above.

The F7 cells are SV-40 large T antigen-immortalized mouse embryo fibroblasts established from E9 PDGFR-double-knockout embryos, which were kindly provided by M. Tallquist and P. Soriano (19). F cells were maintained in DME medium supplemented with 10% fetal bovine serum. The human WT and F72/74 αPDGFRs were stably expressed in F cells using the 293GPG system as described above.

Antibodies—The rabbit polyclonal of αPDGFR antibodies recognize either the carboxy terminus (27P) or a portion of the first immunoglobulin domain (80.8) of the human αPDGFR (5). The c-Cbl antibody used for immunoprecipitation and Western blot analysis of c-Cbl was purchased from Santa Cruz (C-15; sc-170). For anti-phospho-Erk Western blot analysis, a phospho-specific p44/42 MAP kinase (Thr202/Tyr204) antibody purchased from New England BioLabs Inc. (number 9101L) was used at a 1:500 dilution. Anti-phospho-Erk-specific antibody (M-20; sc-93) and 30 μg of protein were resolved on a 10% SDS-polyacrylamide electrophoresis gel. The resolved proteins were transferred to Immobilon, and probed with a phospho-Erk-specific antibody. [3H]Thymidine Uptake—PDGFR-stimulated [3H]thymidine uptake was assayed as follows. Cells were plated at 8×10⁵ cells/ml in DME containing 10% isopropl alcohol and 10% acetic acid for 10 min, washed twice in low-grade acetic acid for 10 min, incubated in 30% 2,5-diphenyloxazole/ acetic acid for 1 h, and washed for 1 h in H2O. The gel was dried and subjected to autoradiography. The amount of mature and immature receptor was quantified by densitometry, the amount of mature receptor was normalized by the amount of immature receptor in each sample, which does not appreciably change over the course of the experiment (data not shown). The resulting values were plotted as a function of time.

Erl Activation—Cells were grown to subconfluence, serum starved in DME containing 0.1% calf serum overnight, and left resting or stimulated with 50 ng/ml PDGF-AA at 37 °C for times indicated. The cells were washed twice with H/S, lysed in 1 ml of EB (without BSA), and centrifuged at 13,000 rpm for 15 min to remove insoluble debris. The amount of protein in the lysates was determined by the BCA protein assay (Pierce), and 30 μg of protein were resolved on a 10% SDS-polyacrylamide electrophoresis gel. The resolved proteins were transferred to Immobilon, and probed with a phospho-Erl-specific antibody. [3H]Thymidine and harvested as described previously (7). Duplicate samples were performed at each data point, and four independent experiments gave similar results. The data are expressed as a fold increase over the buffer control.

PVR—F cells expressing either the WT or the F72/74 αPDGFR were used in the rabbit PVR model exactly as described previously (19). Briefly, rabbits were anesthetized and gas compression vitrectomy performed, and after 3 days the gas was replaced with balanced saline solution. One hundred thousand F cells expressing either WT or F72/74 αPDGFR were then injected into the vitreous cavity through a 30-gauge needle 4 mm posterior to the limbus. The fundus was checked after the injection to exclude iatrogenic retinal damage, and out of a total of 37 animals, six were excluded from the study due to iatrogenic complications including retinal tear or lens damage.

The experiments were examined by the same examiner on days 1, 4, 7, 14, and 28 by slit-lamp and indirect ophthalmoscope with a +20 diopters fundus lens through dilated pupils (1% cyclopentolate HCl eye drops and 2.5% phenylephrine HCl eye drops, 0.05 ml of each). Each animal was examined at the outset of the experiment to rule out the presence of any pre-existing anterior and posterior segment ocular abnormalities. All procedures were performed under aseptic conditions and pursuant to the regulations of the ARVO Statement for the use of...
RESULTS

SFKs Are Required for the Ligand-induced Degradation of the αPDGFR—Our first goal was to test the possibility that SFKs contributed degradation of the αPDGFR in PDGF-stimulated cells. To this end, we compared the half-life of the WT receptor to that of a mutant αPDGFR in which the tyrosines required for binding and activation of SFKs (tyrosines 572 and 574) were replaced with phenylalanine (F72/74). The WT and F72/74 receptors were stably expressed in Patch (Ph) cells, which express normal levels of βPDGFRs, but no endogenous αPDGFRs. In this system, the F72/74 receptor is unable to bind or activate SFKs in response to PDGF-AA (5). Pulse-chase analysis indicated that the half-life of the two receptors was indistinguishable in resting cells (Fig. 1, A and B). Exposure to PDGF dramatically reduced the half-life of the WT receptor. PDGF also promoted degradation of the F72/74 receptor, yet its half-life was much longer than the WT receptor (Fig. 1B). Thus, mutation of tyrosines 572 and 574 affects the half-life of the receptor in activated, but not resting cells.

Since the F72/74 receptor is unable to engage SFKs, it is possible that the PDGF-dependent acceleration of receptor degradation requires the assistance of SFKs. Other interpretations of the data in Fig. 1, A and B, include that tyrosines 572 and 574 are required for the binding of other proteins that are required for receptor degradation, or that substitution of these tyrosines to phenylalanines resulted in structural changes.

One way to evaluate the likelihood of these interpretations is to compare PDGF-dependent receptor degradation is cells that have a WT αPDGFR, and do or do not express SFKs. In this experimental approach we turned to SYF cells, which lack the three ubiquitously expressed SFKs: Src, Yes, and Fyn; and SYF + Src cells, which are SYF cells in which c-Src was re-expressed (20). The half-life of the αPDGFR was similar in both cell lines, provided that they were not exposed to ligand (Fig. 1C). PDGF enhanced receptor degradation in each cell line, however, it was faster in the cells expressing Src (Fig. 1C). Thus expression of c-Src in SYF cells accelerated PDGF-dependent receptor degradation. Furthermore, these data support the idea that the prolonged half-life of the F72/74 αPDGFR relates to its inability to engage SFKs. Finally, two distinct experimental approaches indicate that SFKs promote degradation of the αPDGFR.

The Effect of c-Cbl on Receptor Half-life—To begin to investigate the mechanism by which SFKs contribute to the ligand-enhanced degradation of the αPDGFR, we focused on c-Cbl, which appears to be involved with SFKs in a number of signal transduction systems, and promotes degradation of receptor tyrosine kinases. We compared the effect of overexpression of c-Cbl on the half-lives of resting and PDGF-stimulated WT and F72/74 receptors. The c-Cbl cDNA was stably overexpressed (Fig. 2A), and this did not alter the morphology or growth characteristics of cells cultured in serum-containing medium (data not shown). To assess the effect of c-Cbl overexpression on the degradation rate of the WT and F72/74 receptor, pulse-chase experiments were performed as described in the legend to Fig. 1. Despite high levels of c-Cbl expression, there was no effect on the half-lives of the WT or of the F72/74 receptor in the absence of PDGF (Fig. 2, B and C). However, when cells were exposed to PDGF, the half-life of the WT receptor was markedly reduced in cells overexpressing c-Cbl (Fig. 2B). This effect was most pronounced at the early time points. In contrast, increasing the cellular levels of c-Cbl had no effect on the degradation rate of the F72/74 αPDGFR (Fig. 2C). Thus c-Cbl overexpression shortens the receptor half-life, but only in PDGF-stimulated cells, and only if the αPDGFR is able to engage SFKs.

Enhanced Tyrosine Phosphorylation of c-Cbl Correlates with Receptor Degradation—To begin to investigate how SFKs are involved with promoting the c-Cbl-dependent degradation of the αPDGFR, we tested whether tyrosine phosphorylation of c-Cbl correlated with the ability of c-Cbl to shorten the half-life of the receptor. Quiescent cultures of cells expressing the WT or F72/74 receptor (expressing either endogenous levels of Cbl or overexpressing c-Cbl) were left resting or stimulated with 50 ng/ml PDGF-AA for 5 min, the cells were lysed, and c-Cbl was immunoprecipitated and subjected to autoradiography (A). The “~60” lane represents cells that were harvested after 60 min in the chase medium without PDGF. The signals were quantified with a PhosphorImager, normalized, and plotted as a function of time (B and C). Each point is the average of three independent experiments, ± S.D. The solid lines and dashed lines are for the PDGF stimulated or unstimulated cells, respectively.

Animals in Ophthalmic and Vision Research. Clinical observations were graded according to the Fastenberg classification (stage 0–5), sketches made, and representative eyes were photographed (23). The animals were sacrificed on day 28.

FIG. 1. Receptors that do not engage Src have a prolonged half-life. Ph cells expressing either the WT or F72/74 αPDGFR, or SYF and SYF + Src cells expressing the WT αPDGFR, were radiolabeled overnight in methionine-free DME containing 0.1% calf serum and 50 μCi/ml [35S]methionine. The medium was changed to DME containing 2 mg/ml BSA, 300 mg/liter L-methionine, and 300 mg/liter L-cysteine, and cells were exposed to buffer or 50 ng/ml PDGF-AA for times indicated. The cells were lysed, and the αPDGFR was immunoprecipitated using the 27P antibody. Immunoprecipitates representing approximately 1 × 10^6 cells were resolved by SDS-polyacrylamide gel electrophoresis. The proteins were fixed, the gel enhanced, and then subjected to autoradiography (A). The “~60” lane represents cells that were harvested after 60 min in the chase medium without PDGF. The signals were quantified with a PhosphorImager, normalized, and plotted as a function of time (B and C). Each point is the average of three independent experiments, ± S.D. The solid lines and dashed lines are for the PDGF stimulated or unstimulated cells, respectively.
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B
D

Fig. 2. Overexpression of c-Cbl enhances the degradation rate of the WT, but not the F72/74 αPDGFR. A, expression levels of c-Cbl. Triton X-100 soluble cell lysates from Ph-αWT or Ph-αF72/74 cells, expressing either endogenous levels of c-Cbl or overexpressing c-Cbl (+c-Cbl), were subjected to Western blot analysis using c-Cbl antibodies (top panel) or αPDGFR antibodies (bottom panel). Degradation of the WT and F72/74 receptor: αPDGFR degradation rate was determined as described in the legend of Fig. 1 for WT (B) or F72/74 (C) Ph cells expressing either endogenous levels of c-Cbl or overexpressing c-Cbl. Each point is the average of three independent experiments, ± S.D. The solid lines and dashed lines are for the PDGF stimulated or unstimulated cells, respectively.

Fig. 3. Tyrosine phosphorylation of c-Cbl promotes receptor degradation. Ph-WT or Ph-F72/74 cells, that do or do not overexpress c-Cbl, were grown to 80% confluence, starved for 12 h in DMEM containing 0.1% calf serum, and then left resting (−) or stimulated with 50 ng/ml PDGF-AA (+) for 5 min at 37 °C. The cells were lysed, and the lysates were immunoprecipitated with an antibody that recognizes c-Cbl. Immunoprecipitates representing 3 × 10⁶ cells were resolved by SDS-polyacrylamide gel electrophoresis, transferred to Immobilon, and anti-tyrosine phosphorylation (P-Y) Western blot analysis was performed (top panel). The Western blots were then stripped and re-probed with an anti-c-Cbl antibody (bottom panel). The samples in panel A are from cells expressing endogenous levels of c-Cbl, and the overexpressors are in panel B.

solved on a 10% SDS-polyacrylamide electrophoresis gel, and subjected to Western blot analysis using a phospho-Erk-specific antibody (Fig. 4A). Addition of PDGF-AA to empty vector expressing cells did not activate Erk, as these cells have no αPDGFRs. In WT receptor expressing cells, Erk was maximally phosphorylated 5 min after PDGF stimulation, and the signal declined to near baseline levels by 15 min. In contrast, the F72/74 receptor triggered a much stronger and prolonged response, which also was maximal at 5 min and only declined slightly during the first 60 min of PDGF stimulation (Fig. 4A). PDGF-induced Ras activation was also enhanced in the F72/74 cells (data not shown). An increase in PDGF-dependent Erk activation was also seen in SYF cells, whereas re-expression of c-Src reduced the PDGF-induced activation of Erk (Fig. 4B). These findings suggested that prolonging the half-life of the αPDGFR resulted in enhanced signal relay.

If the half-life of the receptor is indeed responsible for the differences in Erk activation between WT and F72/74 receptor, then overexpression of c-Cbl in WT receptor expressing cells should lead to diminished Erk phosphorylation upon PDGF stimulation, since it enhances the degradation rate of the receptor. As shown in Fig. 4C, the PDGF-stimulated activation of Erk is weaker in cells overexpressing c-Cbl. The data in panels A-C of Fig. 4 indicate that the magnitude and duration of Erk activation reflects the half-life of the αPDGFR in PDGF-stimulated cells.

To determine the relevance of these changes in signaling to biological responses, we compared PDGF-AA-induced DNA synthesis in WT and F72/74 receptor expressing Ph cells, as well as in WT receptor expressing cells that overexpressed c-Cbl. To measure entry into S-phase, quiescent cells were stimulated with increasing doses of PDGF-AA, pulsed with [³H]thymidine, harvested, and the incorporated radioactivity was counted. As shown in Fig. 4D, stimulation of cells with PDGF-AA resulted in a dose-dependent increase in [³H]thymidine uptake in all three cell types. At every concentration of PDGF the response of the F72/74 cells was better than that of the WT receptor expressing cells. In contrast, overexpression of c-Cbl reduced the ability of the WT receptor to promote cell

Changes in the Receptor Half-life Correlate with Altered Downstream Signaling Events and Biological Responses—The prolonged half-life of the F72/74 receptor may lead to an enhancement of PDGF-induced downstream signaling events as compared with the WT receptor. To test this hypothesis, we compared the kinetics of PDGF-dependent activation of extracellular-regulated kinase 1 and 2 (Erk-1/2) in WT and F72/74 expressing cells, as well as the WT receptor-triggered response in cells that did or did not express Src. The cells were grown to subconfluence, arrested by serum starvation, and were left resting or stimulated with 50 ng/ml PDGF-AA for up to 1 h. The cells were washed, lysed, equal amounts of protein were re-

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cycle progression. These data indicate that the degradation rate of the αPDGFR tightly correlates with the magnitude and duration of PDGF-induced signaling events. In addition, changes in the half-life of the receptor appear to have an impact on biological responses such as DNA synthesis.

The FT72/74 Receptor Facilitates Progression of Proliferative Vitreoretinopathy in a Rabbit Model of the Disease—The finding that the FT72/74 receptor was more efficient than the WT receptor in mediating PDGF-dependent responses in tissue culture cells (Fig. 4) raised the possibility that this mutant receptor was more efficient than the WT receptor in vivo. The same was true for the FT72/74 receptor. Using a receptor mutant, the disease is strongly dependent on the αPDGFR (19), and using this model we compared the PVR potential of cells expressing the WT or FT72/74 receptor. The two receptors were expressed in F cells, which are derived from embryos nullizygous for both of the α- and βPDGFRs and thus express no endogenous PDGFRs. This cell type was previously used in the PVR model (19). Fig. 5A shows that the WT or FT72/74 receptor was expressed at comparable levels. This level of expression was similar to that of the αPDGFR in Ph WT cells, which express approximately $1 \times 10^5$ receptors/cell (5). Rabbits were first subjected to gas vitrectomy, and then injected with either Fx or FaFT72/74 cells. The rabbits were examined up to 28 days after cell injection, and clinical findings were graded according to the Fastenberg classification system (0 = normal retina; 1 = vitreal strand; 2 = retinal focal traction; 3 = focal retinal detachment (RD); 4 = extensive RD; 5 = total RD) (23). Rabbits that had been injected with cells expressing the F72/74 receptor began to develop the disease earlier than most of the rabbits that received cells expressing the WT receptor. For instance, formation of membranes and vitreal strands was first seen in rabbits that were injected with the FT72/74 expressing cells (Fig. 5, B and C). This resulted in a statistically significant difference ($p < 0.05$) between the two groups at early time points (Table I). As the experiment was extended, both groups of rabbits developed disease. While the trend of the FT72/74 group leading the WT group persisted throughout the experiment, there was not a statistically significant difference between the groups by the end of the first week. At the end of the experiment, rabbits in both groups had developed the severe stages of the disease (Fig. 5, B and C). Thus, the onset and early progression of PVR was faster with cells expressing αPDGFRs that fail to activate SFKs and had a prolonged receptor half-life. Although the pathological mechanisms mediating proliferative diseases are complex and likely to involve a number of different growth factors, our findings suggest that the enhanced signaling of the FT72/74 receptor in vitro plays a role in vivo.

**DISCUSSION**

The present study reveals that the degradation rate of the αPDGFR is a key element in controlling the magnitude and duration of PDGF-induced responses. Whereas c-Cbl has been previously implicated in αPDGFR degradation, the studies presented herein suggest that SFKs are also involved. Importantly, our findings in tissue culture cells appear to readily translate to at least one in vivo setting.

**SFKs Plays a Negative Role Downstream of the αPDGFR**—A large body of evidence implicates SFKs as positive regulators of signaling, although under certain circumstances, they are either neutral or even have a negative impact. Src positively regulates signaling downstream of the EGFR, promoting cell proliferation, and transformation (24). In contrast, PDGF-dependent activation of SFKs is dispensable for PDGF-dependent cell proliferation (25), although basal SFK activity may (26), or may not (20), be required. Recent studies provide evidence for a negative regulatory role for SFKs in other systems. B lymphocytes from Lyn-deficient mice exhibited enhanced Erk activation and increased proliferation following B cell receptor engagement (27). Furthermore, Erk activation by mitogenic stimuli was repressed in v-Src-transformed cells (28). Thus the capacity of SFKs to contribute to signaling systems may not be restricted to enhancing cellular responses.

Here we used multiple approaches to study the role of SFKs in αPDGFR signaling. Our results show that SFKs negatively regulate signal relay by the αPDGFR. Using a receptor mutant, which does not bind or activate SFKs, we cannot exclude the possibility that mutating tyrosines 572 and 574 does more than...
eliminate the binding and activation of SFKs. Recently the juxtamembrane domain of the PDGFR class of receptor tyrosine kinases was shown to behave as WW domain, although replacing the tyrosines corresponding to 572 and 574 had no effect on association of the juxtamembrane domain with proline-containing peptides in vitro (29). Even so, these conservative tyrosine to phenylalanine substitutions may alter the structure, or some unknown function of the receptor. The F72/74 receptor is indistinguishable from the WT receptor with respect to receptor half-life in resting cells, kinase activity, and extent of receptor phosphorylation. Additional approaches to pharmacologically inhibit SFKs in the same cell type were unsuccessful, since a concentration of the tyrosine kinase inhibitor PP1 that inhibited SFKs also attenuated the kinase activity of the αPDGFR (30). A useful approach was to use the SYF panel of cell lines, which harbor the WT αPDGFR, but are altered with respect to the expression of SFKs (20). Our results using the SYF panel were similar to those obtained with the mutant receptor. Both of these approaches indicated that SFKs promote degradation of the αPDGFR. The data in Figs. 4 and 5 support the idea that, as with many other receptor tyrosine kinases, reducing the half-life of the αPDGFR attenuates PDGF-dependent signaling and responsiveness. Taken together, these findings give rise to the novel idea that SFKs negatively regulate PDGF-dependent responses emanating from the αPDGFR.

Our findings that increased tyrosine phosphorylation of c-Cbl correlates with receptor degradation suggest that these events somehow contribute to receptor degradation. One possibility is that SFKs promote phosphorylation of c-Cbl by either directly phosphorylating it, or by acting as an adapter to bring c-Cbl to the receptor. Whether phosphorylation of c-Cbl enables

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**Table 1**

Stages of PVR in rabbits injected with F cells expressing WT or F72/74 receptors

| Time point | PVR (Fastenberg stage) | p value |
|------------|------------------------|---------|
| day        |                        |         |
| 1          | 0.40 ± 0.21            | 1.06 ± 0.21 | 0.046 |
| 4          | 0.73 ± 0.21            | 1.44 ± 0.22 | 0.039 |
| 7          | 1.00 ± 0.24            | 1.63 ± 0.27 |         |
| 14         | 2.40 ± 0.47            | 2.81 ± 0.39 |         |
| 21         | 2.93 ± 0.41            | 3.19 ± 0.29 |         |
| 28         | 3.13 ± 0.43            | 3.81 ± 0.34 |         |

Data are expressed as mean ± S.E. Statistical analysis was assessed using the Mann-Whitney U test. A p value of ≤0.05 was considered statistically significant.
it to promote receptor degradation is also speculative, and is currently under investigation. Work from several other groups also suggest a relationship between SFKs and c-Cbl. In osteoclasts, c-Cbl is poorly phosphorylated in cells that lack Src, and Src and c-Cbl are both necessary for bone resorption (31). Fyn and Syk are required for tyrosine phosphorylation of c-Cbl in T cells (32). In some types of integrin signaling, SFKs and c-Cbl are involved with activation of phosphatidylinositol 3-kinase, which is required for adhesion and migration of macrophages (33).

While our data suggest that SFKs cooperate with c-Cbl in promoting receptor degradation, this may not be the whole story. SFKs contribute to receptor trafficking by pathways that apparently do not involve c-Cbl. A role for SFKs in receptor trafficking has been demonstrated for the EGF receptor. Overexpression of c-Src in fibroblasts enhances the endocytic internalization of the EGF receptor, but did not affect receptor half-life, thus leading to a larger steady-state pool of internalized EGF receptors (34). In this case, Src is required for EGF-mediated phosphorylation of clathrin which may promote EGF receptor endocytosis (35). The overall effect of c-Src overexpression, however, is increased mitogenesis and tumorigenesis induced by EGF (36–38), which may be explained by evidence suggesting that the complex of EGF and its receptor continues to signal in the endosomal compartment until it is degraded (39). Alternatively, the enhanced responsiveness of c-Src overexpressing cells may arise from c-Src contributing to events other than EGF trafficking.

The Half-life of the Receptor Affects Its Output—Our results suggest that the degradation rate is a critical determinant of receptor-mediated signals, as the prolonged half-life of the F72/74 receptor correlated with enhanced PDGF-dependent Erk activation and initiation of DNA synthesis. These findings contrast a study by Hooshmand-Rad et al. (6) who found no difference in mitogenicity between WT and F72/74 αPDGFRs when expressed in porcine aortic endothelial cells. However, in these cells failure to activate SFKs also did not affect PDGF-induced chemotaxis and actin rearrangement, whereas in Ph cells the F72/74 receptor was impaired in its ability to mediate phosphorylation of signaling molecules (5) and PDGF-stimulated chemotaxis (40). This difference may be explained by cell-type specific effects of SFKs, possibly due to the expression levels of signaling molecules such as c-Cbl. Consistent with our findings, a recent study demonstrated a correlation between activation of ERK and ligand-induced down-regulation of the EGF receptor (41). Moreover, a decrease in the degradation rate of the βPDGFR also resulted in increased mitogenic signaling (42). Together, these findings indicate that receptor down-regulation indeed determines the strength and duration of ligand-induced signals. However, the signal output of a receptor appears to be more complex than the half-life of the receptor. The βPDGFR half-life is apparently 6 times longer than that of the αPDGFR (43, 44), yet when the two receptors are expressed in the same cell type they induce comparable levels of Erk activation and cell cycle progression (19).

Receptor Mutants Can Identify the Role of Signaling Enzymes in Disease—Receptor tyrosine kinases play a critical role in the pathogenesis of proliferative diseases (45, 46). The complexity of pathogenic events in vivo makes it difficult to dissect the role of a single receptor tyrosine kinase and its signal relay pathways in disease progression. Thus, the translation of in vitro studies to in vivo settings is important, yet challenging. PDGF was recently shown to be of particular importance in a proliferative retinal disease, PVR. This was done by comparing the PVR potential of cells that did or did not express receptors for PDGF. Cells that expressed no PDGFRs were unable to induce the later stages of the disease, whereas expression of the αPDGFR greatly increased the PVR potential of the cell line. Note that with the exception of the PDGFRs, these cells are otherwise normal, and are expected to express receptors for numerous other growth factors. Hence their low PVR potential suggests that these other receptors are not able to initiate the processes leading to disease. This idea is further supported by the observation that expression of the βPDGFR in these cells did not increase the PVR potential of the cells, even though the βPDGFR was at least as good as the αPDGFR in triggering signaling events and progression to S phase (19).

In this PVR animal model we were able to relate our in vitro findings to an in vivo pathological setting. We were surprised to find that the failure to engage the SFK pathway would promote αPDGFR-dependent disease progression. This is because proliferative diseases like PVR are thought to result from the contribution of many growth factors, not only PDGF. Furthermore, SFKs were previously shown to contribute to cell proliferation, and activated versions of c-Src cause transformation of cells (47). Thus, it appears that SFKs can be used in many different ways. For the αPDGFR, our in vitro studies show that SFKs negatively regulate signaling and mitogenicity. Importantly, these findings are not only restricted to tissue culture cells, but appear to relate to prevention of disease progression in vivo. Future studies using additional αPDGFR mutants may reveal which other signaling enzymes are involved with proliferative disease formation.

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