PI3K and PLCγ Play a Central Role in Experimental PVR

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Purpose. It has been reported that the platelet-derived growth factor (PDGF)-α receptor (αPDGFR) is required for experimental proliferative vitreoretinopathy (PVR) in rabbits. This study investigated which of the signaling enzymes downstream of the αPDGFR participate in PVR.

Methods. A panel of cell lines that expressed αPDGFR signaling mutants were made and characterized. These cell lines were used in a rabbit model of PVR and in an in vitro collagen type I contraction assay.

Results. Phosphoinositide 3-kinase (PI3K) and, to a lesser extent, phospholipase C (PLC)-γ were the signaling enzymes required for the αPDGFR to mediate PVR. Furthermore, the cells lines that were the most effective at inducing PVR displayed the most potent activity in the in vitro contraction assay.

Conclusions. PI3K and PLCγ are necessary downstream effectors of the αPDGFR in experimental PVR. Consequently, these two signaling enzymes are required for one or more of the cellular responses (chemotaxis, proliferation, extracellular matrix production, contraction) that contribute to PVR. (*Invest Ophthalmol Vis Sci.* 2002;43:483–489)

Proliferative vitreoretinopathy (PVR) occurs in up to 10% of patients undergoing surgery to reattach the retina.[1] Approximately 60% to 80% of the patients that undergo repeated surgery achieve anatomic success.[2,3]

There are numerous studies indicating that growth factors contribute to PVR.[4,5–14] Using a simple rabbit model of the disease, we have found that platelet-derived growth factor (PDGF)-α receptor (αPDGFR) plays a major role in PVR.[4] Unlike the PDGF β receptor, which is activated by a small subset of the PDGF family, the αPDGFR is activated by many PDGF isoforms, including PDGF-AA, -AB, -BB, and -CC.[15–17] Ligand binding activates the receptor, whereupon it becomes tyrosine phosphorylated and associates with a variety of SH2 domain–containing signaling enzymes.[18] These include Src family kinases, the phosphotyrosine phosphatase SHP-2, phosphoinositide 3-kinase (PI3K), and phospholipase C (PLC)-γ. These signaling enzymes are required to mediate PDGF-dependent cellular responses, and different pathways seem to be involved in the distinct cellular responses. For instance, PI3K is required to drive cells into the S phase, whereas the combination of Src family kinases, PI3K, and PLCγ are necessary for PDGF-dependent chemotaxis.[19] The Src family kinases are cytosolic tyrosine kinases, whereas PLCγ is a phospholipase that cleaves phosphatidylinositol-4,5-bis phosphate to generate diacylglycerol and inositol trisphosphate, which are potent second messengers.[20] PI3K is a family of enzymes that are best known for their ability to phosphorylate phospholipids, which engage numerous other enzymes and signaling cascades.[21]

The PDGFR is particularly well suited to the investigation of signaling cascades that contribute to PDGF-dependent responses. This is because at least some of the signaling enzymes that are used by the PDGFR are activated after phosphorylation of the PDGFR at specific tyrosine residues. Mutating individual tyrosine phosphorylation sites selectively prevents activation of a given branch of the signaling cascade. Hence, a panel of PDGFR phosphorylation site mutants has been used to determine the relative contribution of signaling enzymes to cellular responses such as proliferation, migration, and protection from apoptosis.[19,22–24] Mice expressing various PDGFR phosphorylation site mutants have recently been generated and used to evaluate the role of signaling enzymes in development and disease.[25,26]

One of the pathologic events in PVR is the contraction of the epiretinal membrane, which results in tractional retinal detachment (TRD).[2] Contraction of cells within the epiretinal membrane is commonly believed to cause TRD. A large fraction of this epiretinal membrane is composed of extracellular matrix (ECM) proteins, such as type I collagen.[27] The contraction event appears to require binding of cellular integrins, such as α2β1, to the ECM, in that administration of peptides that interfere with this interaction prevent PVR.[28–31]

In this study we investigated which of the known αPDGFR effectors is required for PVR and for cellular contraction. Using a panel of cell lines that express αPDGFR-signaling mutants, we found that PI3K and, to a lesser extent, PLCγ are required for both contraction and PVR. Furthermore, in vitro contraction correlates well with in vivo PVR, suggesting that cellular contraction is an important component of PVR.

Materials and Methods

Cells

F cells are a simian virus (SV)-40 immortalized line of mouse embryo fibroblasts derived from mice nullizygous for both the α and β PDGFRs. They were generously provided by Michelle Tallquist and Philippe Soriano (Fred Hutchinson Cancer Research Center, Seattle, WA). The cells were routinely maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS); the serum concentration was reduced to 1% when the cells were serum starved.

The αPDGFRs were expressed in F cells, by using a retroviral system.[19,32,33] Briefly, wild-type (WT) or mutated PDGFR cDNA was subcloned into the retroviral vector pLHDCX^2. The cDNA constructs were transfected into the virus-producing 293 GPG cell line, using a reagent for transformation of eukaryotic cells (Lipofectamine; Life Technologies, Gaithersburg, MD). The virus-containing supernatant was collected for 5 days and then concentrated by centrifugation at 25,000*g* at 4°C for 90 minutes. The virus was resuspended in a small volume overnight and frozen at −70°C until use. F cells were infected with the appropriate retrovirus in the presence of 4 μg/mL Polybrene (Sigma Chemical Co., St. Louis, MO) overnight and then selected by growth in medium containing 5 mM histidinol. In all cases, mass populations of drug-resistant cells were used. In Figure 3 the CX^2 cells were generated by infecting F cells with an empty-expression vector and selecting by growth in medium containing 5 mM histidinol.

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TABLE 1. Properties of αPDGFR Mutants

Name	Mutation	Properties
F72/74	Tyr to Phe substitution at 572 and 574	Fails to recruit or activate Src family kinases
F720	Tyr to Phe substitution at 720	Fails to recruit SHP-2
F31/42	Tyr to Phe substitution at 731 and 742	Fails to recruit PI3K
F1018	Tyr to Phe substitution at 1018	Fails to recruit or activate PLCγ
F7	Tyr to Phe substitution at 572, 574, 720, 731, 742, 988, and 1018	Fails to recruit Src family kinases, SHP-2, PI3K, and PLCγ
R627	Lys to Arg substitution at 627	Kinase inactive

Information compiled from the current study and from Rosenkranz et al.,[19] Gelderloos et al.,[32] and Bazenet et al.[34]

Tyr, tyrosine residue; Phe, phenylalanine residue; SHP-2, tyrosine phosphatase SHP-2; PI3K, phosphoinositide 3-kinase; PLCγ, phospholipase C-[gamma]; Lys, Lysine residue; Arg, Arginine residue.

αPDGFR Mutagenesis and Expression

The αPDGFR mutants were generated using site-directed mutagenesis, as previously described.[19] The nature of each of the mutants and their capacity to recruit signaling enzymes are described in Table 1.

Immunoprecipitation and Immunoblotting

Cells were grown to 80% confluence and then starved with DMEM containing 1% FBS for 20 hours. To activate the receptors, the cells were exposed to 50 ng/mL PDGF-BB for 5 minutes. After treatment, the cells were washed twice with 20 mM HEPES (pH 7.4), 150 mM NaCl (H/S) and then lysed in EB (10 mM Tris-HCl [pH 7.4], 5 mM EDTA, 50 mM NaCl, 50 mM NaF, 1% Triton X-100, 0.1% BSA, 20 μg/mL aprotinin, 2 mM Na_3VO_4, and 1 mM phenylmethylsulfonyl fluoride [PMSF]). Lysates were centrifuged for 15 minutes at 13,000*g*, the pellet was discarded, and the soluble fraction was used as the total cell lysate. The protein concentration was measured using a protein assay kit (Pierce Chemical Co., Rockford, IL), according to the manufacturer's instructions. Receptors were immunoprecipitated from the soluble fraction with the 27P antibody (described below). Immune complexes were bound to formalin-fixed membranes of *Staphylococcus aureus*, spun through an EB sucrose gradient, and washed twice with EB, then with PAN (10 mM piperazine-*N*-*N*′-bis(2-ethanesulfonic acid [PIPES; pH 7.0], 100 mM NaCl, and 1% aprotinin) with 0.5% Nonidet P [NP]-40), and the final two washes were with PAN. Total cell lysates containing 20 μg protein or receptor immunoprecipitates from 1.0×10^6 cells were resolved on a 7.5% SDS-PAGE gel under reducing conditions. Proteins were transferred onto nitrocellulose membranes (Immobilon; Millipore, Bedford, MA), and the membranes were blocked using blocking reagents: either Block (10 mM Tris-HCl, [pH 7.5], 1.5 M Tris base, 150 mM NaCl, 10 mg/mL BSA, 10 mg/mL ovalbumin, and 0.05% Tween 20; Pierce Chemical Co.) for anti-phosphotyrosine antibodies, or Blotto (10 mM Tris-HCl [pH 7.5], 1.5 M Tris base, 150 mM NaCl, 10 mg/mL nonfat dry milk, and 0.05% Tween 20; Santa Cruz Biotechnology, Santa Cruz, CA) for other antibodies. The membranes were incubated with primary antibodies for 1 hour at room temperature, and washed five times with Western rinse solution (150 mM NaCl, 10 mM Tris-HCl [pH 7.5], and 1.5 mM Tris base). Afterward, they were incubated with secondary antibody for 1 hour at room temperature and washed five times with Western rinse, and the signal was developed using the enhanced chemiluminescence (ECL) reagent (Amersham Pharmacia Biotech, Piscataway, NJ). Multiple exposures of each were obtained, and the data presented were within the linear range of the film.

Reagents and Antibodies

Recombinant human PDGF-BB was purchased from R&D Systems (Minneapolis, MN). The 27P (anti-αPDGFR), 80.8 (anti-αPDGFR), and 69.3 (anti-Ras guanosine triphosphate [GTP]–activating protein [RasGAP]) rabbit crude antisera have been described and characterized previously.[35,36] Crude rabbit anti-p85 antibody was kindly provided by Alex Toker (Beth Israel Hospital, Harvard Medical School, Boston, MA). 4G10 and PY20 mouse monoclonal anti-phosphotyrosine antibodies were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY) and Transduction Laboratories (San Diego, CA), respectively. The monoclonal antibodies against PLCγ and SHP-2 were purchased from Upstate Biotechnology Inc. For Western blot analysis the following dilutions were used for each primary antibodies: anti-αPDGFR, a 1:1 mixture of the 27P and 80.8 antibodies, 1:1000; anti-phosphotyrosine, 4G10:PY20 (1:1), 1:5000; 69.3, 1:4000; anti-PLCγ, 1:1000; and anti-SHP-2, 1:1000. Secondary antibodies were horseradish peroxidase–conjugated anti-rabbit (catalog no. NA934; Amersham Pharmacia Biotech) or anti-mouse (catalog no. NA931; Amersham Pharmacia Biotech) antibodies diluted 1:5000.

PI3K Assay

The PI3K assay was performed as previously described.[37] Briefly, immunoprecipitated αPDGFR from approximately 5×10^5 cells were incubated with phosphatidylinositol in the presence of [^{32}P]-γ-adenosine triphosphate (ATP). The reactions were terminated, and the phospholipids were extracted and resolved by thin-layer chromatography. The radioactive product of the reaction (phosphoinositide-3-phosphate) was detected by autoradiography.

Collagen I Contraction Assay

The contraction assay was performed as previously described,[38] with slight modifications. Briefly, cells were suspended in 1.5 mg/mL neutralized collagen I (Cohesion Vitrogen 100, Palo Alto, CA) at a density of 10^6 cells/mL and placed in a 24-well plate (Falcon, Franklin Lakes, NJ) that had been preincubated for 12 to 16 hours with 5 mg/mL BSA in phosphate-buffered saline (PBS). The gel was solidified by incubating at 37°C for 90 minutes, and the wells were flooded with DMEM and 5 mg/mL BSA supplemented with the agent to be tested. The gels were incubated at 37°C with 5% CO_2. The media were replaced every 24 hours, and the gel diameter was measured after 24, 48, and 72 hours. The extent of contraction was calculated by subtracting the diameter of the well at a given time point from the initial diameter (15 mm). Each experimental condition was assayed in triplicate, and at least three independent experiments were performed.

Rabbit Experimental PVR Model

PVR was induced in rabbit eyes according to a method previously described.[39] Briefly, rabbits were anesthetized and subjected to gas vitrectomy by injection of 0.4 mL of expanding perfluoropropane gas (C_3F_8) into the vitreous cavity. Three days later, 1×10^5 cells were suspended in 0.1 mL DMEM and coinjected into the vitreous with 0.1 mL platelet-rich plasma. A single investigator evaluated the retinal status in an unmasked fashion using an indirect ophthalmoscope at 1, 4, 7, 14, 21, and 28 days after cell injection. PVR grading was according to the method of Fastenberg et al.,[40,41] as follows: 0, no abnormality; 1,

vitreous strand; 2, traction of the retina; 3, retinal detachment involving less than two quadrants; 4, extended retinal detachment including more than two quadrants; and 5, total retinal detachment. All procedures involving the animals were performed under aseptic conditions, pursuant to the regulations of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Statistic Analysis

An unpaired *t*-test was performed to detect statistical differences in the contraction assay; a nonparametric Mann-Whitney test was used for animal PVR experiments. In all cases, $P < 0.05$ was considered significant.

Results

Characterization of αPDGFR Mutants

The first step of this project was to create and characterize a panel of cell lines that express αPDGFR mutants, which selectively fail to associate with signaling enzymes (Table 1). We used F cells, which is a mouse embryo fibroblast cell line devoid of any PDGFR proteins, because they are derived from embryos that have neither of the PDGFR genes. Their having no endogenous PDGFRs makes these cells uniquely suited for experiments using PDGFR mutants, because endogenous PDGFRs activate the complete set of signaling enzymes and hence negate the selective engagement of a subset of signaling pathways made possible by the use of receptor mutants. An additional feature of these cells is that the parental line induces PVR poorly when injected into rabbits, and re-expression of the αPDGFR greatly increases their PVR potential.[4] The mutant αPDGFRs were stably expressed in F cells using a retroviral expression system. Western blot analysis of the resultant cell lines indicated that each of the mutants were expressed to within twofold of the level of the WT receptor (Fig. 1A). The mature form of the receptor is glycosylated (Fig. 1A, arrow) and runs as a smear, whereas the immature, unglycosylated receptor is the better-resolved lower molecular weight species.

To characterize the αPDGFR mutants, we examined their ability to undergo ligand-dependent tyrosine phosphorylation and to associate with signaling enzymes. The top panels of Figure 1B show that all the receptors, with the exception of the kinase-inactive mutant (R627) were tyrosine phosphorylated after exposure of the cells to PDGF. Seven tyrosine phosphorylation sites are missing in the F7 receptor, and this is probably why this receptor is less phosphorylated. The WT αPDGFR coprecipitated with PLCγ, p85 and SHP-2, whereas the F7 and R627 receptors failed to recruit any of these signaling enzymes (Fig. 1B, bottom panels). The F720, F31/42, and F1018 receptors displayed a more selective defect, so that they failed to efficiently associate with SHP-2, p85, and PLCγ, respectively. To measure the PI3K activity that coprecipitated with the PDGFRs, the receptor immunoprecipitates were subjected to an in vitro PI3K assay. Consistent with the findings of the p85 Western blot (Fig. 1B), PI3K activity coprecipitated with the WT receptor, and the amount of activity was greatly enhanced by PDGF stimulation (Fig. 1C). In contrast, PI3K activity did not detectably coprecipitate with the F31/42 receptor isolated from either resting or PDGF-stimulated cells (Fig. 1C). We have previously characterized the F72/74 receptor expressed in these cells, and it selectively failed to associate with Src family kinases.[42] Finally, the behavior of the αPDGFR mutants in F cells is very similar to our previous observation when the receptor was expressed in a different fibroblast cell line (Ph cells).[19] In summary, this panel of αPDGFR mutants is suitable for assessing the importance of several parameters of receptor function. These include kinase activity, global ability to recruit signaling enzymes, and the importance of specific signaling pathways that are initiated by PI3K, PLCγ, SHP-2, or the Src family kinases.

Involvement of the PI3K and PLCγ Pathways in Experimental PVR

To identify the signaling enzymes that participate in PVR, we assayed cells expressing the various αPDGFR mutants in a rabbit PVR model. In this model, rabbits first undergo gas vitrectomy, and then cells are coinjected with platelet-rich plasma. The formation of an epiretinal membrane, retinal traction, and retinal detachment is observed in living animals over 28 days. By day 14, clear differences were noted between the groups of rabbits, and these differences persisted and intensified over the following 2 weeks (Fig. 2, Table 2). By day 28, extensive retinal detachment (score of 3 or greater) was observed in most of the animals in the WT and F720 groups, whereas the disease was markedly less severe in the R627, F7, and F31/42 groups (Fig. 2, Table 2). Most of the animals in these latter groups had no retinal detachment. Instead, they remained disease free (score of 0), showed development of a membrane (score of 1), or showed a membrane with tractional force (score of 2). This very modest PVR response was comparable to that seen when rabbits were injected with the parental cells, which have no PDGFRs.[4] Although the response of the F1018 group was quite heterogeneous, there was a statistically significant difference between the mean score of the WT and F1018 groups (Fig. 2, Table 2). We have found that cells expressing the F72/74 αPDGFR induces PVR with slightly accelerated kinetics.[42] Because this mutant fails to activate Src family kinases, it appears that this class of signaling enzymes modestly attenuates PVR. Taken together, the data in Figure 2 and Table 2 indicate that the kinase activity of the αPDGFR and its ability to engage signaling pathways such as PI3K and PLCγ are critical components of the signaling cascades that drive PVR.

Involvement of the PI3K and PLCγ Pathways in PDGF-Dependent Contraction

An important component of PVR is the tractional force generated when cells of the epiretinal membrane contract. To investigate the signaling pathways that participate in contraction, we subjected each of the cells in the panel to an in vitro collagen gel contraction assay. The cells expressing the WT receptor contracted when PDGF was added to the medium, whereas no contraction was seen in cells expressing the kinase-inactive (R627) receptor or the receptor unable to recruit signaling enzymes (F7; Fig. 3C). The response of all three cell types was comparable under the negative (buffer) and positive (10% FBS) control conditions (Figs. 3A, 3B, respectively). Thus, contraction required that the receptor have kinase activity and be able to recruit signaling enzymes.

Analysis of the contraction response of the other members of the panel revealed which of the signaling enzymes was most important. A complete loss of the contraction response was observed in cells that expressed receptors unable to recruit PI3K (F31/42) (Fig. 3C). This observation is consistent with previous reports showing that PDGF-dependent contraction is blocked when cells are treated with inhibitors of PI3K, or express PDGFR mutants unable to associate with PI3K.[25,43] Failure to engage PLCγ (in the case of the F1018 receptor) also reduced the ability of the receptor to promote contraction, although not as severely as the loss of PI3K (Fig. 3C). In contrast, eliminating the contribution of PDGF-driven activation of Src family kinases (F72/74 receptor) had no effect on contraction (Fig. 3C). The cells expressing the receptor that did not recruit SHP-2 (F720) displayed a somewhat reduced

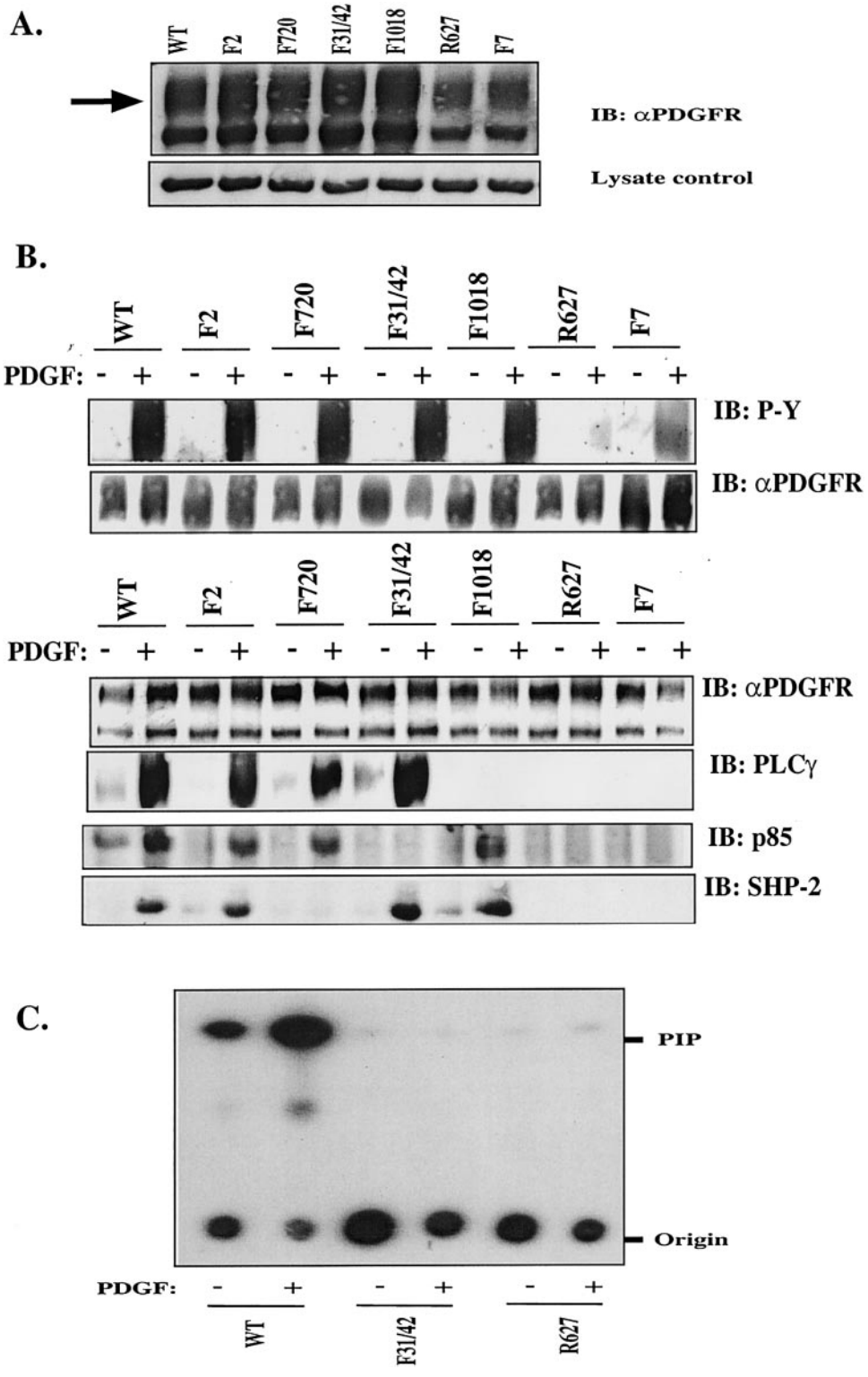

FIGURE 1. Characterization of F cells expressing αPDGFR mutants. (**A**) Western blot analysis of total cell lysates. Cells were grown to 80% confluence, serum starved overnight, and lysed. Twenty micrograms of protein was subjected to Western blot analysis, using an antibody specific for either αPDGFR (*top*) or RasGAP, which was included as a control for the amount of protein loaded (*bottom*). *Arrow*: glycosylated mature form of the receptor (180 kDa), whereas the 160-kDa species is the immature form of the receptor. RasGAP is 120 kDa. Multiple exposures of each of the blots in (**A**) and (**B**) were obtained, and the results presented were within the linear range of the film. (**B**) PDGF-dependent tyrosine phosphorylation and recruitment of signaling enzymes. F cells expressing various αPDGFR mutants were grown to 80% confluence and serum starved overnight. Cells were either left resting (−) or stimulated with 50 ng/mL PDGF-BB (+) for 5 minutes and then lysed. The αPDGFR was immunoprecipitated with an αPDGFR antibody, and the resultant immunoprecipitates were resolved by 7.5% SDS-PAGE and transferred onto nitrocellulose membrane. The segment of the blot containing the αPDGFR was first blotted with anti-phosphotyrosine antibodies and then stripped and reprobed with an anti-αPDGFR antibody (*top*). Portions of the membrane that contained the proteins of the appropriate molecular mass were immunoblotted for PLCγ (148 kDa), the regulatory subunit of PI3K (p85, 85 kDa), or SHP-2 (64 kDa). (**C**) Receptor-associated PI3K activity. Aliquots of the immunoprecipitates used in (**B**) were subjected to a PI3K activity assay. This assay detects PI3K that has coprecipitated with the αPDGFR. Immunoprecipitates were incubated with phosphatidylinositol and [^{32}P]-γATP, the reaction was stopped, and the phospholipids were extracted and separated by chromatography. The resultant chromatogram was exposed to x-ray film to detect radioactive phosphatidylinositol phosphate (PIP). IB, immunoblot; the antibodies used for the immunoblot assays are indicated to the right; P-Y, anti-phosphotyrosine.

response (Fig. 3C), but this was not routinely observed. We conclude that PDGF-dependent contraction requires that the αPDGFR be kinase active and be able to engage PI3K and, to a lesser extent, PLCγ.

DISCUSSION

We used a novel system to identify signaling enzymes that participate in PVR. Our results indicate that PI3K and, to a lesser extent, PLCγ are essential for PVR in a rabbit model of the disease.

Because all the retinal evaluations were performed by a single investigator in an unmasked fashion, it is possible that the results contain some degree of bias. The major conclusion of the study is that only a subset of the experimental groups (those injected with cells expressing the WT or F720 αPDGFR) developed extensive retinal detachments. The retinal status of these animals was easily distinguished from the other groups

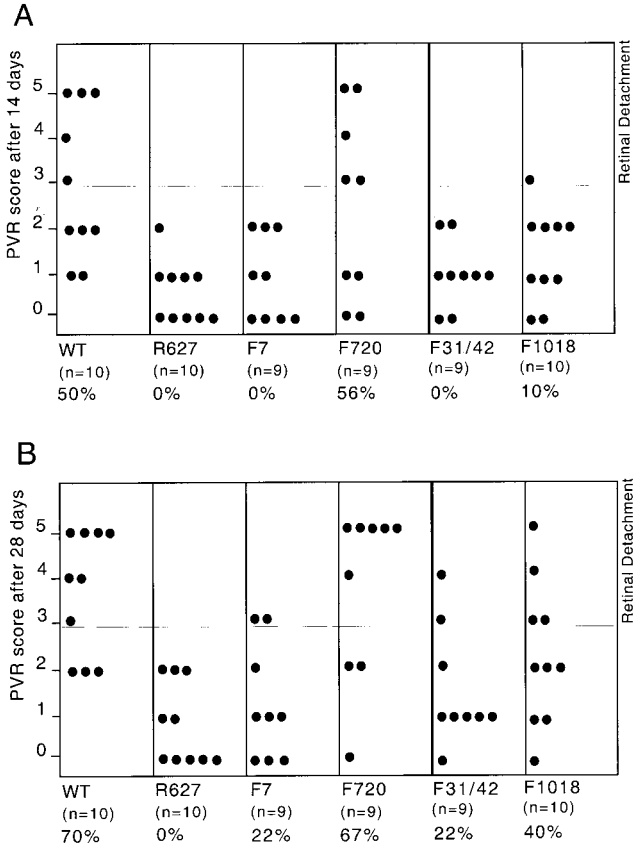

FIGURE 2. Identification of signaling enzymes that are required for PVR. PVR was induced by injecting F cells expressing the indicated αPDGFR into the vitreous of the rabbit eyes. The PVR score was evaluated on days 1, 4, 7, 14, 21, and 28 after surgery. The data for days 14 and 28 are presented in (**A**) and (**B**), respectively. (●) Score for individual rabbits; at the bottom of each column is the percentage of animals that had retinal detachments (graded 3 or higher). The response to parental F cells was previously reported[4] and was the same as the R627, F7, or F31/42 groups. There was a statistically significant difference in the median value between the WT and all other groups, with the exception of the F720 group. Details of the statistical analysis are presented in Table 2. A description of each of the receptors is in Table 1; the numbers in parentheses indicate the number of rabbits in each group.

that formed only membranes or membranes and retinal traction. The large differences between the two types of outcomes reduce the possibility of bias in these experiments.

Is it possible to relate the findings obtained with this animal model of PVR to the clinical setting? As with all models, this is not a perfect recreation of the disease that afflicts humans, and perhaps the most obvious difference is the cell type used to induce PVR. Whereas retinal pigment epithelial cells are commonly believed to be the predominant cell type of the epiretinal membrane isolated from humans,[2,3,44,45] we used a mouse embryo fibroblast cell line. Although the use of RPE cells would have better approximated the disease, it is not possible to use receptor mutants in cells that naturally express the PDGFR, because the endogenous receptor activates all signaling pathways and hence would compensate for the signaling defect of mutant receptors. These signaling mutants are useful only in cells that have no endogenous PDGFRs. For this reason we chose the F cells, which are isolated from embryos that have both of the PDGFR genes eliminated by gene targeting. Unfortunately, the αPDGFR is required for mouse development,[46,47] and the double-knockout animals die at midgestation[4]; therefore, it does not seem to be possible to isolate RPE cells with the appropriate genetic background at the present time. Although the F cells enabled us to use the panel of PDGFR mutants, additional studies are needed to evaluate the clinical relevance of the novel and exciting information that has emerged using this particular PVR model.

How do PI3K and PLCγ participate in the events leading to PVR in this model? One possibility is that these signaling enzymes mediate cellular responses that are essential for the development of PVR. Indeed, we have found that PI3K is one of the effectors of αPDGFR-dependent cell cycle progression, chemotaxis,[19] and contraction (Fig. 3). Similarly, PLCγ is required for chemotaxis[19] and contraction (Fig. 3). These experimental results support the commonly held belief that cell proliferation, migration, and contraction are essential cellular responses that contribute to PVR. It is also possible that PI3K and PLCγ are required for some essential process in disease progression beyond these cellular events. For instance, the synthesis and organization of an extracellular matrix is likely to be central to PVR, because the bulk of an epiretinal membrane is ECM.[27]

An additional benefit of knowing which signaling enzymes are essential for PVR is that inhibitors of these signaling enzymes become potential therapeutic agents to prevent and/or treat PVR. This is particularly exciting in the case of PI3K, the most important signaling enzyme in PVR, because several well-characterized inhibitors already exist, and there is a sizable effort to develop additional agents that selectively and effectively block the activity of PI3K.

The development of a panel of cell lines that display a range of responses in a contraction assay has enabled us to assess the importance of this cellular parameter in PVR. Comparison of the data in Figures 2 and 3 indicates that those receptors that fail to promote contraction of collagen gels in the in vitro assay were the ones with low PVR potential. Similarly, the receptors that have high PVR potential were the ones that stimulated the best response in the in vitro contraction assay. This suggests that the ability of cells to contract is an important component of the disease. Contraction is commonly believed to involve the interaction between integrins and ECM.[48] Consequently, it is possible that signaling enzymes such as PI3K and/or PLCγ are involved with upregulation of integrins or the secretion of ECM. Alternatively, these signaling enzymes may play a role in regulating enzymes such as myosin light chain kinase that mediate the intracellular events responsible for the mechanics of the contraction event.[38,49–52] Additional experiments are needed to investigate these possible scenarios, and to further evaluate the relative importance of cellular contraction in the development of PVR.

There is a pressing need for agents that effectively prevent PVR. To this end, in vitro–based assays are much more ame-

TABLE 2. The PVR Potential of Cells Expressing the αPDGFR Mutants

Days	WT	R627	F7	F720	F31/42	F1018
1	0/0	0/0	0/0	0/0	0/0	0/0
4	1.0/10	0/0	0/0	1.0/0	0/0	0.5/0
7	1.0/10	0/0*	0/0*	1.0/11	0.5/0	0.5/10
14	2.5/50	0.5/0†	1.0/0†	3.0/56	1.0/0†	1.5/10*
21	3.0/70	0.5/0†	1.0/11†	4.0/67	1.0/11†	2.0/20†
28	4.0/70	0.5/0†	1.0/22†	5.0/67	1.0/22†	2.0/40*

The first number is the median PVR grade in either 9 or 10 rabbits, and the second number is the percentage of rabbits with retinal detachment (stage 3, 4 or 5). Each of the groups was compared with the WT group, using the Mann-Whitney test. All the data points for the 14- and 28-day times are presented in Figure 2.

* $P < 0.05$.

† $P < 0.01$.

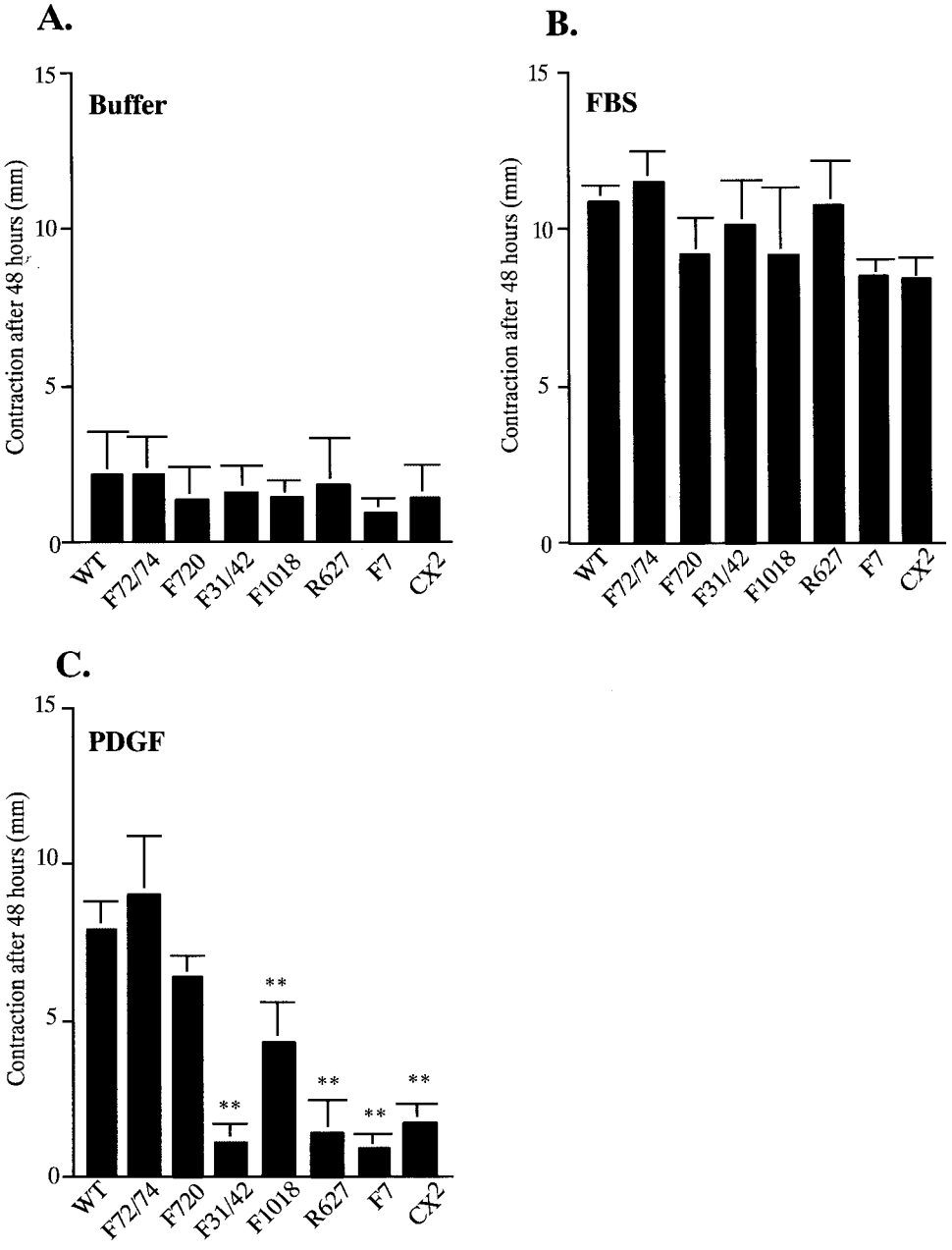

FIGURE 3. Identification of signaling enzymes that are required for αPDGFR-mediated contraction. Cells expressing the indicated αPDGFRs were subjected to the in vitro collagen gel assay in the presence of buffer (**A**), 10% FBS (**B**), or 50 ng/mL PDGF-BB (**C**). The data presented are the mean ± SD measured at the 48-hour time point. Each experimental condition was assayed in triplicate, and at least three independent experiments were performed. Cells that have no PDGFRs (CX^2) or express a kinase-inactive receptor (R627) failed to respond to PDGF. Similarly, receptors that fail to recruit multiple signaling enzymes (F7) responded poorly. PDGF-dependent contraction was either eliminated or reduced when receptors were unable to engage PI3K (F31/42) or PLCγ (F1018), respectively. In contrast, the inability to recruit Src family kinases (F72/74) or SHP-2 did not markedly compromise PDGF-dependent contraction. **Significant difference ($P < 0.01$ by unpaired *t*-test) compared with the response of the WT cells.

nable to large-scale screening of libraries than in vivo assays. In vitro assays for cell proliferation, migration, and contractions are all well established and can be used individually or in combination to identify candidates that can be subsequently evaluated for their potential to block PVR in the in vivo setting.

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