Dominant-negative mutants of a platelet-derived growth factor gene

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Using site-directed mutagenesis of a PDGF-A cDNA clone, we identify two domains that are required to generate stable, mitogenically active PDGF-AA homodimers. Alteration of the tetra-basic amino acid sequence (Arg⁸⁴-Arg-Lys-Arg to Arg-Ser-Asn-Gly) results in the formation of stable pro-PDGF-A homodimers that lack mitogenic activity. Substitution of serine for Cys¹²⁹ destabilizes PDGF-A subunits within the cell. Genes incorporating either the processing lesion or the cysteine substitution suppress wild-type PDGF-A gene expression in a *trans*-dominant fashion. Suppression occurs because the mutant PDGF subunits dimerize with wild-type subunits to form inactive or unstable heterodimers. Suppression is exerted across phylogenetic boundaries; thus, the mouse PDGF-A chain mutants inhibit the activity of the wild-type *Xenopus* PDGF-A. The cysteine mutant gene suppresses expression of PDGF-B (*c-sis*), as well as PDGF-A. The processing mutant gene, however, suppresses only PDGF-A. Dominant-negative mutations of PDGF and other growth factors which, like PDGF, function as dimers may prove useful for creating animal models of growth factor deficiency disease states and for revealing the function of growth factors during early embryonic development.

[**Key Words:** Mouse PDGF-A cDNA clone; PDGF-AA homodimers; *Xenopus*; PDGF-A]

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Platelet-derived growth factor (PDGF) consists of two subunits, A and B, encoded by separate genes that have been conserved from mammals through amphibians and probably further (Singh et al. 1982; Doolittle et al. 1983; Waterfield et al. 1983; Betsholtz et al. 1986; Mercola et al. 1988; Bowen-Pope et al. 1989). PDGF-A and PDGF-B dimerize to form three isoforms (AA, AB, and BB) that are mitogenic for cultured cells (Heldin et al. 1986; Bywater et al. 1988; Kazlauskas et al. 1988; Nister et al. 1988). Likewise, two receptor proteins, α and β , are encoded by separate genes and dimerize to form functional receptor molecules that differ in their specificity for the PDGF isoforms (Yarden et al. 1986; Hart et al. 1988; Heldin et al. 1988; Claesson-Welsh et al. 1989; Gronwald et al. 1989; Matsui et al. 1989; Seifert et al. 1989). The phylogenetic conservation of two distinct receptor and ligand genes, as well as the differential affinities of the receptors for the different ligands, implies that the PDGF isoforms have different functions in different contexts.

We and others have shown recently that PDGF-A and α -receptor genes are selectively expressed in early mouse and *Xenopus* development, as well as in embryonal carcinoma cell lines (Mercola et al. 1988, 1990; Rappolee et al. 1988). The dominant mouse mutation patch (*Ph*) encompasses a deletion of most of the α -receptor gene (Stephenson et al. 1990). *Ph* homozygotes usually die in

utero between 8.5 and 12.5 days gestation and are characterized by gross anatomical abnormalities (Grüneberg and Truslove 1960), suggesting that defects in PDGF-A function result in anatomical defects and lethality. The function of PDGF-A during the earliest stages of embryogenesis is certainly different from that postulated previously for PDGF in the adult. In the adult, normal PDGF function has been implicated in wound healing and connective tissue remodeling, and aberrant expression has been linked to proliferative diseases such as atherosclerosis (for review of PDGF function, see Ross et al. 1986). Collectively, these findings suggest that PDGF may be required transiently during embryogenesis for functions unrelated to its role in the adult. However, no experiments have yet assessed the function of PDGF during development. Moreover, the role of PDGF in adult tissues has been inferred largely from cell culture studies and its pattern of expression in relevant tissues.

The *in vivo* functions of a hormone are displayed most vividly by the physiological defects of animals incapable of its synthesis. In this paper we describe the creation of dominant-negative mutants of a PDGF cDNA that could be introduced into animals transgenically. PDGF lends itself to the genesis of dominant-negative mutations because a dimeric structure is required for activity (Antonjades et al. 1979; Heldin et al. 1979; Hannink et al. 1986). We reasoned that an inactive subunit could be de-

signed that would dimerize with wild-type PDGF to yield an inactive dimer, thereby functioning as a dominant-negative mutant. Two mutations of murine PDGF-A, one substituting a serine for a crucial cysteine and one altering the proteolytic processing site, were made by site-directed mutagenesis. Each mutant appears capable of inhibiting wild-type PDGF-A activity. The cysteine mutant, but not the processing mutant, is also capable of suppressing PDGF-B activity. These mutants may prove invaluable to the study of PDGF-A action in animal and cell culture models. The function of numerous mammalian genes encoding growth factors, transcription factors, and other proteins requiring an oligomeric state for function may be studied using this form of genetic analysis.

Results

In vitro mutagenesis of murine PDGF-A

PDGF dimerization occurs through disulfide linkage between cysteine residues that are conserved between the A and B chains (Giese et al. 1987; Sauer and Donoghue 1988), as well as between species at least as divergent as human and *Xenopus* (Mercola et al. 1988). The mutation of any one of four distinct cysteines has been shown to abolish the transforming potential of *v-sis* (Giese et al. 1987; Sauer and Donoghue 1988) and is presumed crucial to the formation of an active dimer. Because of the similarity between the PDGF isoforms, mutations at these crucial cysteines might perturb function in PDGF-A as well as *v-sis*. Although proteolytic processing of the *v-sis* gene product has been observed not to be required for biological activity (Hannink and Donoghue 1986), processing is required for the activation of other growth factors, e.g., transforming growth factor- β (TGF- β), insulin, and insulin-like growth factors (Blundell and Humbel 1980; Wakefield et al. 1988). Moreover, the pro-PDGF-A protein, expressed recombinantly or from endogenous genes, appears to be proteolytically processed efficiently. Therefore, mutations that prevent processing may disrupt PDGF-A activity. We constructed two mutants of a murine PDGF-A cDNA, F9A5 (Mercola et al. 1990), by site-directed mutagenesis. In mutant 1317, three of the four basic amino acids at the proteolytic cleavage site for the processing of pro-PDGF to the mature form were altered (Fig. 1). A second mutant, 1308, contains a serine in place of one of the four cysteines shown to be essential for the transforming activity of the retroviral homolog of PDGF-B, *v-sis* (Fig. 1, schematic). We reasoned that these mutations would result in biologically inactive PDGF-A proteins that would nonetheless dimerize with wild-type PDGF.

Expression of the mutant proteins in transfected COS cells

The mutant constructs were expressed in COS cells to confirm that the predicted proteins could be expressed from the mutant genes. Conditioned media, labeled with

Figure 1. COS cell expression of mutant proteins analyzed under reducing conditions. The schematic diagram (top) shows the site-directed mutagenesis scheme. Pre-pro-PDGF is shown schematically with the solid bar representing the leader portion, the hatched bar representing the pro portion, and the open bar representing the mature PDGF-A chain. Heavy vertical lines represent cysteines of the mature protein, with asterisks (*) on those essential for transformation by *v-sis* (Giese et al. 1987; Sauer and Donoghue 1988). The two mutant PDGF cDNA clones are indicated below, with the wild-type sequence indicated above the changes. (A) Radiolabeled proteins were analyzed by immunoprecipitation with PDGF-A-specific antibodies. Where indicated, 1 μ g of recombinant PDGF-AA was added as a competitor to conditioned medium from a transfection including 1317 and wild-type PDGF-A to demonstrate the specificity of the immunoprecipitation. (B) Synthetic mRNAs of the wild-type, 1308, and 1317 constructs were translated *in vitro* using rabbit reticulocyte lysates in the presence of [35 S]cysteine, and the products were analyzed directly by polyacrylamide gel electrophoresis and autoradiography.

[35 S]cysteine, were immunoprecipitated with an anti-PDGF-A antiserum. The wild-type PDGF-A cDNA construct expresses two protein species, as analyzed under reducing conditions (Fig. 1A). The lower molecular weight species is fully processed PDGF-A (~16 kD). The larger species (~21 kD) may be a processing intermediate or the result of aberrant processing. As predicted, the 1317 mutant construct encodes a ~24-kD peptide exclusively, a size consistent with unprocessed pro-PDGF-A. The 1308 mutant product was not detectable in the conditioned medium when analyzed by immunoprecipitation (Fig. 1A). Reasoning that the antibody may not recognize the 1308 protein, conditioned media were analyzed directly on gels. Mutant 1308 was not detected under these conditions, whereas wild-type and 1317 proteins were readily visible (data not shown). In addition, 1308 is not retained within the COS cells (data not shown). Northern blot analysis confirms the expression of an abundant level of 1308 mRNA within transfected COS cells (identical to that of 1317 or wild-type PDGF-

A; data not shown but see Fig. 5, below). Furthermore, each of the three cDNAs encodes mRNAs that are translated to peptides of the same molecular weight (consistent in size with pre-pro-PDGF-A) with equal efficiency in vitro (Fig. 1B). Thus, we conclude that our inability to detect the 1308 product in transfected COS cells reflects its instability in vivo.

When analyzed under nonreducing conditions (Fig. 2A, top), the wild-type construct alone produces three dimers of ~36, ~32.5, and ~31.5 kD, corresponding to the three possible combinations of the two wild-type monomers (Fig. 1A). The 1317 construct produces a protein of ~40 kD consistent in size with the dimer of the monomer observed in Figure 2A (top).

The PDGF-A processing mutant (1317) forms a heterodimer with wild-type PDGF-A

The strategy for creation of a dominant negative mutant requires that the mutant and wild-type subunits dimerize. To visualize heterodimers, ³⁵S-labeled conditioned media from COS cells transfected with a fixed amount of wild-type and increasing amounts of 1317 constructs were analyzed under nonreducing conditions (Fig. 2A, top). Increasing amounts of the 1317 construct results in a clear reduction in the amount of wild-type PDGF-A homodimers of ~31.5 and ~32.5 kD. This decrease is concomitant with the appearance of a new protein species at ~38 kD and the persistence of a band at ~36 kD. The protein of ~38 kD is consistent in size with a heterodimer of the larger (~21 kD) wild-type PDGF-A subunit and the 1317 subunit. A second heterodimer would be expected to form between the smaller wild-type species and the 1317 chain. This protein would be expected to comigrate with the heaviest wild-

type homodimeric protein (at ~36 kD). Thus, the persistence of the ~36-kD band is most likely due to the appearance of this heterodimer replacing the ~36-kD wild-type homodimer.

To further clarify the constituents of the dimeric proteins, samples identical to those in Figure 2 (top) were electrophoresed under reducing conditions (Fig. 2A, bottom). The higher molecular weight species (the ~38- and ~36-kD dimeric proteins) are resolved into their expected monomeric components of a 1317 subunit and a wild-type subunit. Thus, the production of increasing amounts of 1317 protein does not alter the structure of the wild-type PDGF-A but results in the wild-type protein forming a heterodimer with the mutant protein. Furthermore, quantitation of the radioactivity in each of the bands using the PhosphorImager indicates that the overall production of PDGF-A is not affected significantly by overexpression of the 1317 protein (data not shown). However, the slight decrease of processed wild-type subunits (the ~16- and ~21-kD proteins on the reducing gel; Fig. 2A, bottom) seen upon overexpression of 1317 protein may suggest that processing of the wild-type subunit is inhibited slightly by 1317 overexpression.

Expression of the 1308 mutant construct decreases the amount of wild-type PDGF-A produced in transfected COS cells

The cysteine mutant [1308] did not produce detectable protein in conditioned media (Fig. 1A); nonetheless, we tested its effect on wild-type PDGF (Fig. 2B). Consistent with the results shown in Figure 1, the 1308 protein product is not detectable under reducing conditions. However, cotransfection with increasing amounts of

Figure 2. The effect of the mutant constructs on PDGF-A production. (A) The effect of 1317. (Top) Conditioned media from transfections containing 2.5 μ g (= 1 unit) of the wild-type PDGF-A construct and increasing amounts of the 1317 construct (including pMT2 to bring the total DNA transfected to 20 μ g) were analyzed under nonreducing conditions. The ratio of the amounts of wild-type to 1317 constructs are indicated above each lane. (Insert) A high-resolution image of a portion of the gel. (Bottom) The same samples were analyzed under reducing conditions. (B) The effect of 1308 on PDGF-A production. Conditioned media from transfections containing 2.5 μ g (= 1 unit) of the wild-type PDGF-A construct and increasing amounts of the 1308 construct were analyzed under reducing conditions as in A.

1308 reduced the abundance of the wild-type PDGF-A in a dose-dependent manner. The same decrease is observed in radiolabeled cell lysates as in conditioned media (data not shown).

1317 forms a heterodimer with PDGF-B

Figure 3A (left) shows the products of cotransfections with PDGF-B and 1317 DNA analyzed under nonreducing conditions. Binding of the PDGF products by SP-Sephadex C-50 resin was found superior to commercially available antibodies in quantitatively enriching PDGF-B. SP-Sephadex C-50 binds basically charged proteins and has been used previously to enrich for PDGF (Deuel et al. 1981; Heldin et al. 1986). COS cells transfected with the PDGF-B construct express a protein of ~50 kD, consistent in size with homodimers of pro-PDGF-B. As expected, the same sample analyzed under reducing conditions shows a protein of ~30 kD consistent in size with monomeric pro-PDGF-B (data not shown). These results are identical to those of Hannink and Donoghue (1986), who showed that transfected COS cells produce predominantly a pro-PDGF-B molecule that is biologically active.

Cotransfection of 2.5 μ g of the PDGF-B construct with a sevenfold excess (17.5 μ g) of the 1317 construct results in a reduction in the amount of homodimeric PDGF-B and the appearance of a protein at ~44-kD, intermediate in size between PDGF-B and 1317 homodimers (Fig. 3A, left). This result suggests that a heterodimer of PDGF-B and 1317 is produced. To determine whether the new protein (~44 kD) in Figure 3A (left) contains PDGF-A, a fraction of each sample was immunoprecipitated with the anti-mouse PDGF-A antiserum and analyzed under nonreducing conditions (Fig. 3A, right). As expected, the PDGF-B protein is not precipitated by the anti-PDGF-A antibody. The ~44 kD protein that appears in cotransfections of the PDGF-B and 1317 constructs is precipitated with the anti-PDGF-A antiserum, indicating that it contains a 1317 subunit. Furthermore, analysis of these samples under reducing conditions indicates that the ~44-kD protein contains a PDGF-B subunit (data not shown). These data indicate that an excess of 1317

expression will drive the wild-type PDGF-B into heterodimeric complexes with 1317.

Expression of the 1308 mutant construct decreases the amount of wild-type PDGF-B produced in transfected COS cells

Similar to its effect on PDGF-A, a sevenfold excess of 1308 DNA resulted in a decrease in the amount of PDGF-B detected in the conditioned media (Fig. 3B). Quantitation by the PhosphorImager (Materials and methods) showed that a sevenfold excess of the 1308 construct resulted in an average decrease in the amount of PDGF-B of 57.1% (with a range of 43–71%, SD = 15, and $n = 4$).

Biological assay shows that both mutants can suppress expression of PDGF-A genes

Conditioned media from transfected COS cells were tested for PDGF mitogenic activity. PDGF activity was assayed by a standard PDGF bioassay that measures the ability of the conditioned media to stimulate the incorporation of [³H]thymidine by quiescent BALB/c-3T3 cells (Antoniades et al. 1979). Figure 4 shows the biological activity of each of the mutants, various wild-type PDGF constructs, and a cotransfection including a 1 : 7 ratio of wild-type to mutant DNA. In each transfection, total DNA was adjusted to a constant 20 μ g per transfection by addition of pMT2 vector to minimize variations in transfection efficiency. Figure 4, A and B, shows that 17.5 μ g of either mutant construct results in little or no detectable PDGF production, shown compared to the amount produced by cells transfected with 2.5 μ g of the wild-type mouse PDGF-A construct (generally ~250 ng/ml COS-conditioned media). Thus, both mutants show greatly reduced biological activity. Cotransfection of 17.5 μ g of either mutant with 2.5 μ g of wild-type PDGF-A (a sevenfold excess) resulted in a significant displacement of the curve, indicating a substantial reduction in the biological activity recovered (Fig. 4A,B). Cumulative results (Table 1) show that a 7-fold excess of mutant DNA reduces PDGF-A activity by 11.3-fold for

Figure 3. The effect of the mutant constructs on PDGF-B production. (A) The effect of 1317. (Left) Conditioned media from transfections containing 2.5 μ g of the wild-type PDGF-B construct and 17.5 μ g of the 1317 construct were analyzed as in Fig. 2 under nonreducing conditions. However, instead of immunoprecipitation, PDGF was enriched by retention on an SP-Sephadex C-50 resin, as described in Materials and methods. (Right) Aliquots of the same samples were analyzed following immunoprecipitation with the anti-mouse PDGF-A antiserum. (B) The effect of 1308. Conditioned media from transfections containing 2.5 μ g of the wild-type PDGF-B construct and 17.5 μ g of the 1308 construct were analyzed as in A under nonreducing conditions.

Figure 4. Trans-dominant inhibition of wild-type PDGF from several sources. As positive controls, COS cells were transfected with 2.5 μ g of a construct encoding wild-type PDGF-A of mouse (A and B) or *Xenopus* (C), or 2.5 μ g of a construct encoding wild-type human PDGF-B (D). To demonstrate suppressor activity, the wild-type constructs were cotransfected with 17.5 μ g of either 1317 or 1308 constructs as indicated. As negative controls, vector or mutant constructs alone were transfected. In all transfections, pMT2 vector DNA was added as required to bring the total amount of transfected DNA to 20 μ g. Conditioned media from these transfected cells were assayed for their ability to stimulate cell growth in quiescent BALB/c-3T3 cells in a standard bioassay (Antoniadou et al. 1979).

1317 ($n = 11$) and 5.4-fold for 1308 ($n = 6$; summarized in Table 1).

We tested whether our murine mutants could suppress *Xenopus* PDGF-A (Fig. 4C). The open reading frame of pO1, a cDNA clone encoding *Xenopus* PDGF-A (Mercola et al. 1988), was expressed from the pMT2 vector. Typically, this construct produces less mitogenic activity when assayed on 3T3 cells than murine or human PDGF, possibly because of sequence divergence at its carboxyl terminus (Mercola et al. 1988). Coexpression of the *Xenopus* PDGF construct with a sevenfold excess of either of the murine mutant PDGF constructs 1308 or 1317 also inhibited biological activity (Fig. 4C; Table 1). Thus, the murine mutant constructs can suppress PDGF-A from species at least as divergent as *Xenopus*.

The cysteine mutant 1308, but not the processing mutant 1317, suppresses PDGF-B activity

The predominant form of PDGF from human platelets is thought to be heterodimers of PDGF-A and PDGF-B, termed PDGF-AB (Hammacher et al. 1989). Because heterodimer formation occurs readily upon coexpression (Ostman et al. 1988), we examined whether the PDGF-A mutants would suppress PDGF-B activity. The open reading frame of a cDNA clone encoding human PDGF-B (*c-sis*, as described in Rao et al. 1986) was inserted into pMT2. The results of a cotransfection experiment of the PDGF-B construct and the PDGF-A mutant constructs are shown in Figure 4D. A sevenfold excess of 1308 resulted in a significant decrease in mitogenic activity, similar to its effect on coexpressed PDGF-A activity.

Table 1. Summary of cotransfection data: fold suppression of PDGF mitogenic activity by a sevenfold excess of various constructs

Test construct	Suppressing constructs		
	1317	1308	pMT2-JE
PDGF-A	11.3 ($n = 11$, SD = 4.3)	5.4 ($n = 6$, SD = 2.3)	1.3 ($n = 2$, SD = 0.5)
PDGF-B	1.0 ($n = 5$, SD = 0.6)	4.2 ($n = 6$, SD = 2.6)	ND

The activity of COS cells transfected with a wild-type PDGF construct (2.5 μ g) and a mutant construct (17.5 μ g) is compared to that of cells transfected with a wild-type PDGF construct (2.5 μ g) and vector DNA (17.5 μ g). The dilutions giving half-maximal PDGF activity are compared to determine fold suppression. The fold suppression is shown in the figure (a fold suppression of 1.0 equals no suppression). Because the bioassay is unreliable when the initial concentration of PDGF in the COS-conditioned media falls below ~ 20 ng/ml, fold suppression > 15 is simply assessed as 15; thus, the reported values represent a minimum estimate. Abbreviations: (n) Number of experiments done in duplicate; (ND) not determined; (SD) standard deviation.

Cumulative results show a 4.2-fold reduction in activity ($n = 6$; Table 1). The processing mutant 1317 did not inhibit mitogenic activity of PDGF-B (Fig. 4D; Table 1), although the 1317 subunit dimerized with the PDGF-B subunit (Fig. 3A).

Cotransfection of the mutant PDGF constructs does not suppress the expression of wild-type PDGF mRNA

Mixing experiments with the conditioned media indicate that the 1317 homodimeric product is not a competitive inhibitor of PDGF-AA (data not shown). Therefore, inhibition does not result from an interaction between the secreted products of the mutant and wild-type genes but, rather, relies on coexpression of the mutant and wild-type genes. Most likely, inhibition requires specific dimer formation between the mutant and wild-type PDGFs. However, in other experiments, we noticed a COS cell artifact that could have altered the interpretation of our data with the 1308 mutant. In brief, we found that TGF- β cDNA constructs inhibit the expression of mRNA from cotransfected plasmids derived from pMT2 as well as pSV2CAT. We were concerned that our mutations could have inadvertently created toxic sequences capable of interacting with factors required for expression of the wild-type genes. Thus, we examined whether the inhibition of PDGF activity reflects an artifactual block at either a transcriptional or a post-transcriptional level.

To rule out the possibility of a transcriptional block, we examined whether the mRNA from the wild-type constructs was affected by cotransfection with mutant DNA. It should be noted that each transfection contained an identical amount of DNA, with the differences being made up by the addition of pMT2, the expression vector. Because pMT2 expresses dihydrofolate reductase from the same promoter, competition for cellular transcription and translation factors by additional exogenous genes is otherwise well controlled in these experiments. RNase protection analysis was used to examine the levels of mRNA. The results in Figure 5 show that neither mutant had a significant effect on the levels of PDGF-A or PDGF-B mRNA. Because 1308 and wild-type PDGF-A differ by only a single point mutation, it was necessary to increase the amount of RNase A to discriminate between the two mRNAs. The increased RNase A levels account for the numerous bands seen in each lane.

Mutant constructs inhibit PDGF specifically

Another potential artifact could have altered our interpretation of the data from experiments using the processing mutant 1317. We were concerned that the overexpression of this secreted protein was acting nonspecifically to affect the synthesis or secretion of other proteins. For this reason, we tested whether an unrelated secretory protein, JE, would block the synthesis or secretion of wild-type PDGF in the COS cell transfection assay. In addition, we tested whether the mutants would block the expression of JE. JE is a heavily glycosylated,

Figure 5. RNA analysis of transfected COS cells. RNA from COS cells transfected with wild-type and mutant constructs as in Figs. 2–4 was analyzed by RNase protection analysis. The DNA constructs used in the transfection are listed above each lane. (A) Hybridization to a probe from wild-type PDGF-A spanning the region mutated in the 1308 construct [bases 345–518 of the wild-type PDGF-A cDNA (Mercola et al. 1990)]. (B) Hybridization to a probe from wild-type PDGF-A spanning the region mutated in the 1317 construct [bases 258–345 of the wild-type PDGF-A cDNA]. (C) Hybridization to the PDGF-B probe (a 180-bp probe extending from base 1601 of the sequence of Rao et al. (1986) into the 3'-untranslated region of our PDGF-B expression construct).

cytokine-like protein that migrates at ~25 kD (Rollins et al. 1988). Increasing amounts of pMT2-JE were cotransfected with the wild-type PDGF-A construct. Figure 6A shows that unlike the mutant PDGF constructs, pMT2-JE has no effect on PDGF-A production. Similarly, no effect on PDGF biological activity was observed (Table 1). A sevenfold excess (17.5 μ g) of the 1308 or 1317 constructs was cotransfected with a constant amount of pMT2-JE (Fig. 6B). The processing mutant 1317 had no effect on JE synthesis or secretion. Cotransfection of the 1308 construct resulted in a slight decrease in the amount of JE produced (21.6% reduction, $SD = 6.9\%$, $n = 2$, done in duplicate). This level of nonspecific suppression is too low to account for the suppression of PDGF-A or PDGF-B, either at the level of biological activity (76.2% reduction in activity or 4.2-fold, Table 1) or protein synthesis (57.1% reduction, Figs. 2B and 3B). However, it is possible that the suppression observed with the 1308 construct may include this nonspecific component under the conditions of the COS cell transfections. Total secretory protein production, as determined by SDS-PAGE analysis of total [35 S]cysteine-labeled conditioned media, is unaffected by transfection with either mutant PDGF construct (data

Figure 6. Analysis of the specificity of the mutants for PDGF. (A) Analysis of the effect of JE on wild-type PDGF-A production. COS cells were transfected with a constant amount of the wild-type PDGF-A construct (2.5 μ g) and increasing amounts of pMT2-JE in an experimental design identical to that in Fig. 2. Radiolabeled conditioned media were immunoprecipitated with anti-JE (top) or anti-PDGF-A (bottom) antisera and analyzed under reducing conditions. (B) Analysis of the effect of mutant PDGF production on JE. COS cells were transfected with 2.5 μ g of pMT2-JE and 17.5 μ g of the mutant PDGF constructs or pMT2 alone. Radiolabeled conditioned media were immunoprecipitated with an anti-JE antiserum and analyzed under reducing conditions.

not shown). These results, taken together with the observation that mRNA levels are unchanged by the coexpression of 1308 and 1317 (Fig. 5), indicate that a specific interaction between the mutants and PDGF is the primary mechanism responsible for the suppression of biological activity.

Discussion

We have described two mutant PDGF genes that encode biologically inactive molecules. Both mutants are effective in inhibiting coexpressed wild-type PDGF-A activity. Only the 1308, or cysteine mutant, inhibited PDGF-B activity. Collectively, our results indicate that inhibition occurs because hybrid dimers form between the wild-type and mutant PDGF molecules. This conclusion is drawn from several lines of evidence. First, the mutants do not block the accumulation of wild-type PDGF mRNA (Fig. 5). Second, suppression of wild-type activity appears to be specific for PDGF and not other secretory proteins (Fig. 6; Table 1). Third, dimers of 1317 protein (which is the only one of the two mutants secreted in detectable amounts) do not compete with PDGF-AA for biological activity and, hence, most likely do not bind to receptors (data not shown). In the case of

1308, we speculate that mutant/wild-type dimers form and are unstable in vivo. This is supported by the decrease in wild-type PDGF-A and PDGF-B protein produced with an excess of 1308 construct DNA (Figs. 2B and 3B). In the case of 1317, our conclusion that dimerization is a requirement for suppression is supported by the dose-dependent formation of 1317/wild-type dimers (Figs. 2A and 3A) concomitant with a loss of biological activity.

The 1317 mutant dimerizes with PDGF-B (Fig. 3A) yet does not suppress biological activity (Fig. 4; Table 1). A sevenfold overexpression of 1317 protein is sufficient to drive most of the PDGF-B into a heterodimer with the mutant PDGF-A (Fig. 3A). We conclude, therefore, that the 1317/PDGF-B heterodimer is biologically active, although its specific activity relative to PDGF-AA, PDGF-AB, and PDGF-BB remains to be determined. Hannink and Donoghue (1986) have observed that PDGF-B does not need to be fully processed to function and that pro-PDGF-B is the biologically active recombinant PDGF-B expressed following transfection in COS cells. In light of this observation, it is interesting that the 1317 protein is biologically inactive when dimerized with itself (or with wild-type PDGF-A) but exhibits activity when in the context of a dimer with PDGF-B. This result suggests that an AA homodimer must contain two processed subunits to be mitogenically active, but an AB heterodimer could contain unprocessed subunits. We are currently attempting to determine which of the two PDGF receptor subunits recognizes the 1317/PDGF-B dimer.

We and others have recently reported that mRNA for PDGF-A and the α -receptor is expressed at early times in vertebrate development (Mercola et al. 1988, 1990; Rapoport et al. 1988). Moreover, the potential allelism between the α receptor and *Ph* (Stephenson et al. 1990) suggests that PDGF-A function is crucial for normal development. However, the function of PDGF during early embryogenesis remains unknown. Also, roles for PDGF have been postulated in the processes of wound healing, eye and kidney function, and vascular proliferative disease (Ross et al. 1986; Brewitt and Clark 1988; Kartha et al. 1988; Campochiaro et al. 1989). These mutants may prove invaluable in defining the action of PDGF in these processes by permitting the facile creation of transgenic animals incapable of expressing the growth factor. The use of dominant negative mutants is simpler than homologous recombination techniques and may function in instances where antisense technology has proved difficult, such as in early *Xenopus* development (Bass and Weintraub 1987; Rebagliati and Melton 1987; Shuttleworth et al. 1988).

Materials and methods

Site-directed mutagenesis

A fragment spanning the open reading frame of a murine PDGF-A cDNA, F9A5 (Mercola et al. 1990), was cloned into pBSKS+ (Stratagene). Site-directed mutagenesis was carried out by the method of Kunkel (Kunkel et al. 1987) and confirmed by sequencing.

COS cell transfections

Wild-type [F9A5 (Mercola et al. 1990)] and 1317 and 1308 mutants were inserted into the expression vector pMT2 [pMT2 is a derivative of pXM1 (Yang et al. 1986)]. Transfections and [³⁵S]cysteine labeling of the secreted proteins was as described (Rollins et al. 1988). Transfections included 2.5 µg of each DNA as indicated (and included pMT2 DNA to bring the total amount transfected in each case to 20 µg). The conditioned media were collected and 100 µl was immunoprecipitated (Harlow and Lane 1988), using antibodies to human PDGF-A (Fig. 1) or a polyclonal antiserum to recombinant mouse PDGF-A homodimers (Figs. 2, 3, and 6) or to recombinant mouse JE (a generous gift of B. Rollins, Dana-Farber Cancer Institute; Fig. 6). In some cases (Fig. 2), PDGF was enriched using SP-Sephadex C-50 as described (Heldin et al. 1986). One milliliter of radiolabeled conditioned medium was incubated with 125 µl of preswollen SP-Sephadex C-50 (Pharmacia) for 4 hr. The resin was washed with wash buffer (10 mM sodium phosphate at pH 7.5, 0.1 M NaCl, 5 mM EDTA) and eluted with two volumes of elution buffer (10 mM sodium phosphate at pH 7.5, 0.5 M NaCl, 5 mM EDTA). Samples were analyzed on 15% polyacrylamide gels, which were fixed and impregnated with autoradiography enhancer (for X-ray film autoradiography, Enlightening, NEN) prior to exposure. With the exception of Figures 1 and 6, all radioactive gel images were displayed on a PhosphorImager machine (Molecular Dynamics, Sunnyvale, CA) to produce the autoradiographic images. Quantitation of radioactivity in the gels was also done using the PhosphorImager. The PhosphorImager is more sensitive to radioactivity than X-ray film and responds linearly over a 10⁷-fold range. Therefore, analysis is more accurate and is possible over a broader range of values than previously possible by densitometric analysis of X-ray film.

In vitro translation

RNA transcripts were synthesized in vitro as described previously (Melton et al. 1984). Rabbit reticulocyte lysates (Promega) were used to translate 0.3 µg of each of the synthetic transcripts in the presence of [³⁵S]cysteine according to the manufacturer's instructions.

PDGF bioassay

Following transfection, cells were placed in Dulbecco's modified Eagle medium + 0.5% platelet-poor plasma (Antoniades et al. 1979) for 48 hr. Conditioned media were then collected and assayed by a standard PDGF bioassay (Antoniades et al. 1979). In multiple experiments, 17.5 µg of the mutant constructs produced <1% of the activity of transfections with 2.5 µg of wild-type construct.

RNA analysis

RNA was purified by the guanidium isothiocyanate procedure (Chirgwin et al. 1979) and assayed for specific gene expression by an RNase protection analysis (Melton et al. 1984). Antisense RNA hybridization probes were transcribed in vitro in the presence of [³²P]CTP and hybridized to 5 µg of total RNA from the transfected cells. Following hybridization for 16 hr (45°C) and digestion (37°C), the protected fragments were analyzed on a 6% polyacrylamide–8 M urea sequencing gel. RNase conditions were as described, except for the experiment shown in Figure 5A, in which the RNase A levels were increased fivefold to detect the single-base mismatch between the probe and the 1308 mRNA.

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