Detection of High Molecular Weight Forms of Platelet-Derived Growth Factor by Sequence-Specific Antisera

Abstract. *Antisera to synthetic peptides representing sequences of both chains of platelet-derived growth factor (PDGF) were used to structurally analyze PDGF isolated from outdated human platelets and PDGF-like proteins in normal and transformed cells. Most PDGF isolated from platelets did not contain the carboxyl portion of PDGF-2 in contrast to p20^{sis}, the major form of p28^{sis} detected in simian sarcoma virus–transformed cells. In addition, higher molecular weight forms of molecules containing PDGF-1 and PDGF-2 sequences were detected in all cell lines tested. These lines were heterogeneous with respect to species, cell type, and transforming agent.*

The structural (*1*, *2*) and immunological (*3*–*5*) relatedness of the transforming *sis* gene product (p28^{sis}) of simian sarcoma virus (*6*) to platelet-derived growth factor (PDGF) (*7*, *8*) provides the most solid link between the transforming properties of oncogenes and the mitogenic action of growth factors. The *sis* gene is one of many oncogenes that have been transduced by retroviruses (*9*). These captured genes have been highly conserved through evolution, suggesting they have important physiological functions. Platelet-derived growth factor is a very potent mitogen for many connective tissue cell types in culture (*10*). It is stored in the alpha granules of platelets and is released at sites of vascular damage. PDGF binding to specific cell surface receptors triggers a tyrosine-specific protein kinase activity (*11*). This event identifies a common mechanism used by a wide variety of growth factors and oncogenes. Insulin, gastrin, epidermal growth factor, and transforming growth factors all bind to receptors that are associated with tyrosine protein kinase activity (*12*). The proteins of oncogenes *src*, *yes*, *fes*, *fps*, *ros*, *abl*, and *fgr* have tyrosine kinase activity while the oncogenes *mos*, *raf*, *mht*, and *erb*B have sequence homology to the region encoding the kinase activity (*13*). Furthermore, fragments of the epidermal growth factor receptor show a very close homology with the predicted sequence of *erb*B (*14*). Thus the binding of a growth factor to a receptor with tyrosine kinase activity appears to be a common event in mitogenesis and transformation.

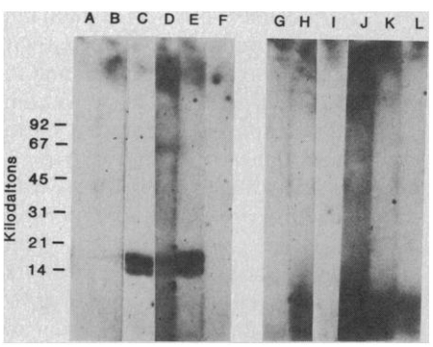

Fig. 1. Immunological detection of PDGF. Approximately 100 ng of PDGF-2 per lane (*16*) was boiled in the presence (lanes A to F) or absence (lanes G to L) of 10 percent 2-mercaptoethanol before electrophoresis (*5*). Used were two antisera against PDGF-1-(1–12) (lanes A and G or B and H) and PDGF-2-(73–89) (lanes D and J or E and K) or antiserum against PDGF-2-(1–17) (lanes C and I), and PDGF-2-(126–145) (lanes F and L). Antibody was visualized with rabbit antibody to mouse immunoglobulin and ^{125}I-labeled protein A (*5*).

The precise molecular mechanisms of this interaction are not known. PDGF isolated from platelets contains two polypeptide chains that form disulfide-bonded complexes that migrate on denaturing polyacrylamide gels between 28 and 35 kD (*15*, *16*). Reduction destroys the biological activity of these complexes and produces proteins that migrate between 14 and 18 kD (*15*, *16*). Sequence analysis of the material migrating at 18 kD identifies two homologous but distinct sequences (*7*). One of these, PDGF-2, is highly homologous to the protein (p28^{sis}) predicted by the nucleotide sequence of the simian sarcoma virus (SSV) oncogene (*sis*) (*6*). The homology begins at residue 67 and extends at least to residue 171 (*1*, *2*). Recently, the isolation and sequencing of a human c-*sis* clone extended this homology to the predicted carboxyl terminus (*8*). The open reading frame encoding the sequenced PDGF-2 region continues upstream, indicating that PDGF isolated from platelets is derived from a larger precursor, consistent with the 4.2-kb *sis*-related messenger RNA detected in various cell lines (*17*).

Synthetic peptides representing various regions of both PDGF sequences were made. The amino termini of PDGF-1 and PDGF-2 and the central and carboxyl portions of PDGF-2 were synthesized, conjugated to the immunogenic carrier keyhole limpet hemocyanin (KLH), and injected into mice. The peptide representing the unique region of PDGF-2 contains the first 17 amino acids of this sequence and will be called PDGF-2-(1–17). The unique region of PDGF-1 is represented by a peptide, PDGF-1-(1–12), which contains the first 12 amino acids of this sequence. Six of the 12 amino acids are shared with PDGF-2 but only three are consecutive. The third peptide, PDGF-2-(73–89), has been described elsewhere (*5*). It represents the predicted residues 139 to 155 of p28^{sis} and contains an additional cysteine at its carboxyl terminus for coupling. It produces antibodies that recognize the reduced subunits of purified PDGF, proteins of 31, 30, 21, and 18 to 14 kD in a platelet extract, and a 56-kD protein in SSV-infected marmoset cells (*5*). The fourth peptide, PDGF-2-(126–145), was also predicted by the v-*sis* sequence (residues 192 to 211 of p28^{sis}).

To analyze the specificity of the antisera generated against these synthetic peptide conjugates, PDGF was probed with these antisera. Purified PDGF was reduced and electrophoresed into a polyacrylamide gel and onto nitrocellulose (lanes A to F in Fig. 1). Two antisera directed against PDGF-1-(1–12) reacted with a protein of approximately 18 kD (lanes A and B). Sequence analysis of purified PDGF indicates that most of the PDGF-1 chain migrates at this position (*7*). The weakness of the reactivity ob-

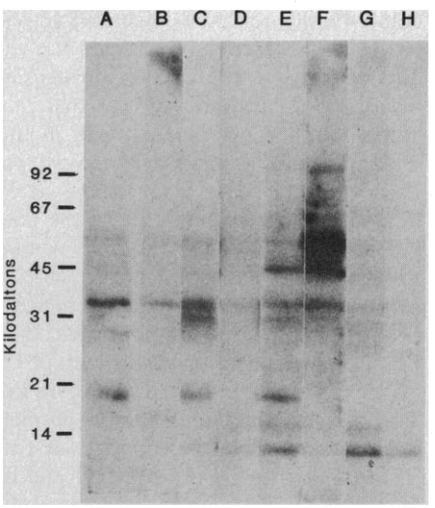

Fig. 2. Detection of p20^{sis} and other PDGF-like molecules in SSV-NRK cells. An extract of approximately 10^6 SSV-NRK cells per lane was transferred to nitrocellulose (*5*). Antisera used to probe the extracts were against PDGF-2-(1–17) (lanes A and B), PDGF-2-(73–89) (lanes C and D), PDGF-2-(126–145) (lanes E and F), and PDGF-1-(1–12) (lanes G and H). These antisera were incubated for 60 minutes at 37°C with 100 μg of PDGF-2-(1–17) (lanes B and G), PDGF-2-(73–89) (lanes D and E), PDGF-2-(126–145) (lanes C and F), or PDGF-1-(1–12) (lanes A and H) before being used to probe extracts. Longer exposure revealed a 70-kD protein, which was the only band specifically detected in lane G.

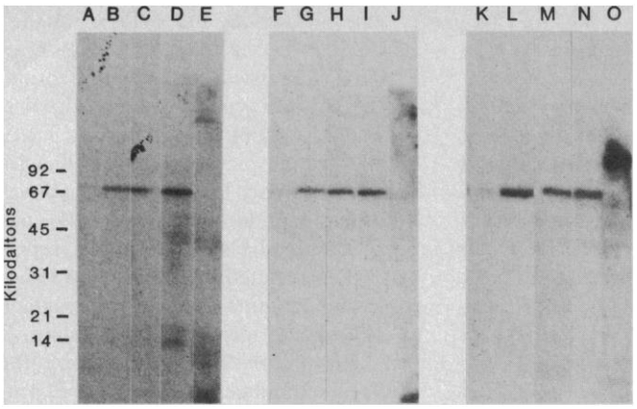

Fig. 3. Detection of a 70-kD protein in three cell lines. An extract of approximately 10^6 SSV-infected 3T3 cells (lanes A to E), TRD1 cells (lanes F to J), or MSTF cells (lanes K to O) per lane were transferred to nitrocellulose (*5*). Antisera against PDGF-1-(1–12) (lanes A to C, F to H, and K to M) or PDGF-2-(73–89) (lanes D, E, I, J, N, and O) were incubated with 100 μg of PDGF-1-(1–12) (lanes A, D, F, I, K, and N), PDGF-2-(1–17) (lanes B, G, and L), or PDGF-2-(73–89) (lanes C, E, H, J, M, and O) before being used to probe the extract. The minor 100-kD band detected in lanes G to I is more readily apparent in overexposed autoradiographs.

served with these antisera suggests that the amino end of PDGF-1 may not be readily accessible for antibody binding. In contrast, antiserum against the amino end of PDGF-2 (lane C) readily detected protein migrating at 18 and 14 kD, consistent with sequence analysis of PDGF-2 (*7*). Since this antiserum was directed against the amino terminus of PDGF-2, proteins of this size would be consistent with cleavages in the carboxyl portion of the molecule. The antisera against PDGF-2-(73–89) produced the same activities (lanes D and E) as seen in lane C. In contrast, antisera against PDGF-2-(126–145) did not have detectable activity against purified PDGF. Since the sequence of the peptide differs from c-*sis* at position 145 (*8*), it is possible that this amino acid change is contained in the binding site. This is unlikely, however, because the peptide is 20 amino acids long and the change is only in the carboxyl terminal position used to couple the peptide to the KLH carrier protein. The lack of activity is not due to generation of only peptide-specific antibodies because this antiserum reacts with $p20^{sis}$ (as described below). The 14- to 18-kD size of the detected PDGF in purified preparations suggests that most of this material is missing the carboxyl end of the predicted sequence of $p28^{sis}$.

To further analyze the presentation requirements of the antisera, purified PDGF was tested under nonreducing conditions (lanes G to K in Fig. 1). None of the antisera recognized PDGF under these conditions, suggesting that these three regions in the nonreduced molecule are not readily accessible or recognizable by the antisera.

Reactivities of the antisera with reduced proteins of SSV-infected normal rat kidney cells (SSV-NRK cells) are shown in Fig. 2. An antiserum against PDGF-2-(1–17) readily detected a 20-kD protein (lane A). The activity was specifically blocked by first incubating the antiserum with PDGF-2-(1–17) (lane B). Others have also detected a 20-kD protein in SSV-infected cells (*3*, *4*). This protein is thought to be generated by cleavage of $p28^{sis}$ at residue 67. In addition, minor reactivities with proteins of 31, 45, and 56 kD were blocked by the peptide while a stronger activity at 34 kD was only partially blocked. Probing this extract with an antiserum against PDGF-2-(73–89) produced similar results (lanes C and D). Antiserum against PDGF-2-(126–145) also readily detected the 20-kD protein (lanes E and F), indicating that the carboxyl terminus of $p20^{sis}$ is not readily cleaved in the cell extracts, in contrast to PDGF isolated from platelets. It is not known whether isolation procedures (*16*) or the amino acid change at residue 145 of PDGF-2 is responsible for this difference.

Incubation of the antiserum against PDGF-2(73–89), the peptide that contains this cysteine residue, resulted in blocking of the activity for the 20-kD protein and visualization of a new prominent band of about 54-kD (lane F in Fig. 2). The conditions under which this band was seen suggest that it may represent a protein that can bind to the peptide PDGF-2-(126–145). The cysteine residue may be important in this interaction because binding of the antisera to this region was less if the extract was not reduced before electrophoresis. Probing the reduced extract with antisera against PDGF-2-(1–12), which contains sequences not found in the predicted *sis* gene product, did not detect the 20-kD protein (lanes G and H in Fig. 2). Instead, only a minor band of 70 kD was detected by the antisera and blocked by the immunizing peptide.

To determine whether PDGF-like proteins might be synthesized in other transformed cell lines, extracts were made and probed with various antisera against PDGF. SSV-transformed NIH cells were probed with an antiserum to PDGF-1-(1–12) and PDGF-2-(73–89) (Fig. 3). Of the two antisera against PDGF-2-(73–89) (lanes D and E in Fig. 1), the serum used in lane D produced a weaker activity with purified PDGF, but, as seen in lane D of Fig. 3, a protein of approximately 70 kD was seen that was blocked with the immunizing peptide, PDGF-2-(73–89) (lane E in Fig. 3), and not blocked by prior incubation of the antiserum with PDGF-1-(1–12). The protein was also detected by antiserum against PDGF-1-(1–12). Recognition was blocked by incubating the antiserum with the immunizing peptide, but not with PDGF-2-(73–89) or PDGF-2-(1–17). Thus the specific reactivity with these proteins by both antisera demonstrates that this is not a fortuitous cross-reactivity with a small region of PDGF but that the molecule contains sequences homologous to at least the amino end of PDGF-1 and the central region of PDGF-2. The amounts of $p28^{sis}$ and $p20^{sis}$ were below the level of detection with antiserum to PDGF-2-(73–89). Similar results were obtained with other antisera, although overexposure did occasionally show a 20-kD band that was specifically detected.

Analysis of extracts of two other unrelated transformed cells with the antisera gave similar results. The TRD1 cell line is a spontaneously transformed BALB 3T3 cell line (*18*). This line also expresses a 70-kD protein and a minor immunologically related protein of approximately 100 kD (lanes G to I in Fig. 3). A third cell line (MSTF), a mink lung line (CCL64) productively infected with feline leukemia virus subtype B, and the Snyder-Theilen strain of feline sarcoma virus, also expressed a protein of the same size (lanes K to O in Fig. 3). Another antiserum against PDGF-1-(1–12) detected a protein of approximately 53 kD. These proteins are not serum contaminants because they are detected in extracts of cells that have been grown for 1 month in the absence of serum and are found in serum-free media conditioned by the TRD1 cell line. All cell lines tested contain these two PDGF-like proteins (*19*). Initial Northern blots of some of these cells, including those that secrete low levels of PDGF receptor–binding material, reveal the presence of messenger RNA that hybridizes to a *sis* complementary DNA probe (*19*, *20*).

Expression of PDGF-like molecules in a broad spectrum of cells, including cells

that are not oncogenically transformed (normal diploid rat smooth muscle and human lung fibroblasts), indicates that other processes are involved in transformation. Although all the cell lines contained 70- and 53-kD proteins detected with antisera against PDGF-1, the cells were heterogeneous with respect to size and intensity of other proteins detected with antisera to determinants predicted by the sequence of the PDGF-2 region. The nature of these differences is unknown. Generation of monoclonal antibodies against defined regions of PDGF, combined with pulse chase and partial digestion experiments, should help to resolve structural questions about the higher molecular weight forms of the molecule. Since these cells differ greatly in the rate of secretion of material that competes for binding to the PDGF receptor (*18, 21*), there may be cell-specific differences in the processing of intracellular PDGF-like molecules to activate secreted PDGF. Alternatively, the larger intracellular proteins reported here, although antigenically related to PDGF, may have functions other than as precursors to low molecular weight forms of PDGF (*22*).

HENRY L. NIMAN
RICHARD A. HOUGHTEN
Department of Molecular Biology, Research Institute of Scripps Clinic, La Jolla, California 92037
DANIEL F. BOWEN-POPE
Department of Pathology, University of Washington, Seattle 98195

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19. The following cell lines were found to express the 70-kD PDGF-like protein: mouse lines NIH 3T3, SSV-3T3, Ki-3T3, TRD1, Ha-3T3, C3H/MCAcl 15, and SP2/0; rat lines NRK, SSV-NRK, PR-SMC*, and SD-SM*; marmoset line NP5/SSV; mink lines CC164, 64F3cl 7, and MSTF; and human lines CALU-6, UCLA-P3, A427, CALU-1, SW900, SW271, T24*, HEP G2, HL-60, WIL2, M14, WI38, and WI38-VA13 [lines with asterisks contain poly A–selected RNA that hybridized to a *sis* complementary DNA probe (*20*)].
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22. A preliminary report of these findings was presented at the Hybridoma Conference, San Diego, California (20 February 1984).
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Pavoninins: Shark-Repelling Ichthyotoxins from the Defense Secretion of the Pacific Sole

Abstract. *A series of ichthyotoxic and hemolytic steroid aminoglycosides, pavoninins-1 to -6, has been isolated from the defense secretion of the sole* Pardachirus pavoninus, *and their respective chemical structures have been established by spectroscopic studies and chemical conversions. The pavoninins exert repellent activity against sharks and are considered to be the factors responsible for the predator-repelling property of the sole.*

Certain fishes, called ichthyocrinotoxic fishes, secrete toxic substances that repel their predators (*1*). Among them, the Red Sea Moses sole *Pardachirus marmoratus* repels sharks presumably by emission of its toxic secretion at the moment when it is about to be bitten (*2*). Although isolation of an ichthyotoxic protein, pardaxin, from the sole has been reported (*3*), the actual source of the shark-repelling property is vague. We report here isolation and full characterization of six shark-repelling ichthyotoxins, pavoninins-1 to -6, from *P. pavoninus*, a kin of Moses sole, in the Western Pacific; this fish is also ichthyocrinotoxic (*4*).

Seven individuals of *P. pavoninus* (20 to 30 cm long) were captured at the sandy bottom of a coral reef coast of Ishigaki Island, Ryukyu Archipelago, Japan; they were milked, on two successive days, by simply disturbing them. The secretion, collected along with some seawater, was lyophilized to yield 30 g of powder, which on resuspension in 0.1*M* aqueous ammonia and dilution with 10 volumes of acetone gave 10.7 g of a precipitate consisting mainly of proteinaceous substances. The solvent was removed from the filtrate, and the residue was partitioned between ethyl acetate and water; the toxicity resided in the ethyl acetate layer. Removal of the ethyl acetate yielded an oil (1.3 g), which was chromatographed on silica gel with a methanol-chloroform gradient (10:25) to yield 992 mg of mixed ichthyotoxins. This mixture was lethal to Japanese killifish *Oryzias latipes*; the lethal dose (killing 50 percent in 1 hour) was 8.5 μg/ml; and its hemolytic activity was comparable to that of saponin on rabbit erythrocytes. The total activity of the mixture accounted for 40 percent of the ichthyotoxicity and 80 percent of the hemolytic activity of the original lyophilized secretion, the remaining activity being present in the proteinaceous precipitate. Gel filtration of the precipitate has indicated that the activity is contained in the fraction having molecular weights of more than 10,000, possibly including pardaxin or related compounds.

Further silica-gel chromatography of the lipophilic ichthyotoxins yielded two