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A Small v-*sis*/Platelet-Derived Growth Factor (PDGF) B-Protein Domain in Which Subtle Conformational Changes Abrogate PDGF Receptor Interaction and Transforming Activity

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Deletion scanning mutagenesis within the transforming region of the v-*sis* oncogene was used to dissect structure-function relationships. Mutations affecting codons within a domain encoding amino acids 136 through 148 had no effect upon homodimer formation or recognition by antisera which detect determinants dependent upon native intrachain disulfide linkages, yet the same mutations completely abolished transforming activity. A platelet-derived growth factor B (PDGF B) monoclonal antibody that prevents its interaction with PDGF receptors recognized v-*sis*, Δ142 (deletion of codon 142), and Δ148 but not Δ136, Δ137, or Δ139 mutants. These findings mapped the epitope recognized by this monoclonal antibody to include amino acid residues 136 to 139. Furthermore, mutations in the codon 136 to 148 domain caused markedly impaired ability to induce PDGF receptor tyrosine phosphorylation. Thus, subtle conformational alterations in this small domain critically affect PDGF receptor recognition and/or functional activation.

Human platelet-derived growth factor (PDGF) is the major serum mitogen for cells of mesenchymal origin (29). PDGF has been directly or indirectly implicated in a number of pathological states, including neoplasia, arthritis, arteriosclerosis, and bone marrow fibrosis (29). This growth factor is a disulfide-linked dimer composed of two related polypeptides, designated A and B, which can be assembled as the heterodimer PDGF AB or as homodimers of PDGF A or B chains. PDGF BB has been identified as the human homolog of the v-*sis* oncogene product (7, 26, 33). The minimal v-*sis* transforming domain spans 89 amino acids identical in sequence to human PDGF B (12, 17) and contains eight cysteine residues, four of which are involved in intrachain disulfide linkages essential for PDGF function (8, 31).

Knowledge of regions within the molecule specifically involved in PDGF receptor binding and activation might be useful in efforts to develop specific PDGF antagonists. Recently, we used immunochemical approaches to localize a PDGF B surface subdomain and the site of interaction with a monoclonal antibody that neutralizes PDGF mitogenic activity and blocks ligand-receptor interaction (21). The present studies were undertaken in an effort to combine molecular genetic approaches with available immunologic probes to map the subdomain within the v-*sis*/PDGF B molecule critical to PDGF receptor interaction.

MATERIALS AND METHODS

Construction of v-*sis* mutants. Genetic manipulations of v-*sis* were accomplished by site-directed mutagenesis. A 1.2-kilobase-pair (kbp) fragment containing v-*sis* was removed from the pSSVSV2 plasmid and subcloned into M13mp19 as described previously (8). By means of 30-mer oligonucleotides, individual colons were deleted by the method of Zoller and Smith (35) as modified by Kunkel (19) to allow phenotypic selection of phage containing the desired mutation. Mutants were identified and their DNA sequence was verified by the dideoxynucleotide method (30). Each mutant was then transferred back into pSSVSV2 for further analysis.

Transfection analysis. Plasmid DNA from each variant was introduced into NIH 3T3 cells by the calcium phosphate precipitation technique (9, 34). Morphologically transformed foci were scored 2 to 3 weeks after transfection. Colony formation following selection in hypoxanthine-aminopterin-thymidine (HAT) medium containing mycophenolic acid was used as an internal marker of transfection efficiency (25). For transfection of COS-1 cells, in which pSV2 replicates as an episome to high copy number, cells were plated at a density of 2×10^6 per 10-cm petri dish 24 h prior to DNA transfection by the calcium phosphate precipitation method.

Immunoprecipitation. At 48 h after COS cell transfection, cells were metabolically labeled for 3 h with [^{35}S]methionine (125 μCi/ml) and [^{35}S]cysteine (125 μCi/ml) (1,200 Ci/mmol; Amersham) in methionine- and cysteine-free Dulbecco modified Eagle minimal essential medium (DMEM). Membrane fractions were prepared by hypotonically lysing cells in 1 mM phosphate buffer (pH 7.4). Nuclei were removed by centrifugation at 1,000 × *g* for 10 min. Supernatants were centrifuged at 100,000 × *g* for 90 min to precipitate membranes. The membrane pellet was suspended in 300 μl of 10 mM phosphate buffer (pH 7.4) and incubated at 95°C for 5 min to release *sis* protein. Membranes were pelleted at 100,000 × *g* for 45 min, and the supernatant, designated P100S, was used for immunoprecipitations. Samples (50 μl) of P100S were incubated with 3 μl of antiserum for 1 h at 4°C. Immunoprecipitates were recovered with the aid of *Staphylococcus aureus* protein A bound to Sepharose beads (Pharmacia, Inc.). After solubilization by boiling in 4% sodium dodecyl sulfate (SDS) with or without 1.4 M β-mercaptoethanol, proteins were analyzed by SDS-polyacryl-

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amide gel electrophoresis (PAGE) (14% polyacrylamide). Mutant v-*sis* proteins were quantitated in NIH 3T3 cells as described above except that the metallothionein vector was induced with DMEM containing 25 μM zinc chloride (20).

Phosphotyrosine analysis of PDGF receptors. NIH 3T3 cells or transfectants expressing v-*sis* constructs were washed with phosphate-buffered saline–1.0 mM sodium orthovanadate and lysed as described before (24). Protein extracts were immunoprecipitated with antipeptide antibodies specific for the α or β PDGF receptor, blotted to Immobilon-P, and probed with an antiphosphotyrosine-specific antibody (24). The filters were treated with ^{125}I-protein A and subjected to autoradiography.

Antibodies. Peptide antiserum against the carboxy terminus (anti-sis C) of the v-*sis* protein has been described previously (15, 26). Native human PDGF, isolated from platelets, was used to generate anti-PDGF polyclonal serum (15, 27). A neutralizing monoclonal antibody directed against the PDGF B homodimer designated MAb sis 1 has been reported (21). Antibodies recognizing either the α or β PDGF receptor were recently described (24).

RESULTS

Biological effects of single-codon deletions within the v-*sis* minimum transforming region. The v-*sis* oncogene encodes a 271-amino-acid polypeptide whose amino-terminal 52 residues are derived from the simian sarcoma-associated virus envelope gene (6). The *env* gene signal peptide directs the v-*sis* protein into the secretory pathway, a requirement for transforming activity (11). Sequences encoding the remaining 219 amino acids are derived from the woolly monkey PDGF B gene (6). Site-directed mutagenesis has identified the minimum transforming region of v-*sis* as an 89-codon stretch (12, 17). More recent studies have further localized this region to 84 codons bound by cysteine codons (unpublished). This stretch is identical to the analogous region of the human PDGF B coding sequence but differs at 42% of predicted human PDGF A residues (2), yet PDGF AA and PDGF BB homodimers both efficiently interact with and activate the α PDGF receptor, the product of one of two independent PDGF receptor genes (4, 24). Because of this allowable diversity in PDGF sequence, we reasoned that mutations more drastic than substitutions would be required to probe v-sis/PDGF B structure and function. Thus, single-codon deletions were introduced within its minimum transforming domain at approximately every third codon to identify amino acids critical for receptor interactions.

To examine the effect of such mutations on v-sis biologic activity, mutants were transferred into the pSSVSV2 expression vector and transfected into NIH 3T3 cells. The pSSVSV2 expression vector contains the dominant selectable *gpt* gene as a marker, making it possible to compare focus formation and killer HAT-resistant colony formation for each construct. This allowed an accurate determination of the specific transforming efficiency of each mutant. As shown in Table 1, each of 20 mutants was fully active in induction of colony formation in killer HAT medium. Deletion of codon 188 (Δ188), 189, or 192 had no effect upon specific transforming efficiency, whereas deletion of either codon 195 or 198 resulted in reduced but detectable focus-forming activity. However, each of the other mutants lacked transforming activity (Table 1). Thus, codon deletions which spared transforming function were clustered toward the distal end of the minimum transforming region.

TABLE 1. Analysis of v-sis/PDGF B deletion mutants by NIH 3T3 transfection assay[a]

Codon deleted	Transforming activity (FFU/pmol)	Killer HAT-resistant colony formation (CFU/pmol)	Specific transforming efficiency (FFU/CFU)
None	2×10^4	2×10^4	10^0
130	$<10^1$	2×10^4	$<10^{-3}$
133	$<10^1$	4×10^3	$<10^{-3}$
136	$<10^1$	2×10^4	$<10^{-3}$
137	$<10^1$	1×10^4	$<10^{-3}$
139	$<10^1$	1×10^4	$<10^{-3}$
142	$<10^1$	1×10^4	$<10^{-3}$
145	$<10^1$	2×10^4	$<10^{-3}$
148	$<10^1$	2×10^4	$<10^{-3}$
151	$<10^1$	2×10^4	$<10^{-3}$
154	$<10^1$	5×10^3	$<2 \times 10^{-3}$
157	$<10^1$	2×10^4	$<10^{-3}$
177	$<10^1$	5×10^3	$<2 \times 10^{-3}$
183	$<10^1$	5×10^3	$<2 \times 10^{-3}$
186	$<10^1$	1×10^4	$<10^{-3}$
188	2×10^4	2×10^4	10^0
189	2×10^4	2×10^4	10^0
192	2×10^4	2×10^4	10^0
195	2×10^2	1×10^4	2×10^{-2}
198	3×10^1	1×10^4	3×10^{-3}
201	$<10^1$	2×10^4	$<10^{-3}$

[a] Transfection of NIH 3T3 cells was performed as described before (9, 34). Cell transformation was scored at 2 to 3 weeks. Colonies selected by resistance to HAT medium containing mycophenolic acid were scored at 2 weeks. Abbreviations: FFU, focus-forming units. Values are per picomole of PDGF B coding sequence.

Identification of mutant v-*sis* gene products that efficiently dimerize but lack transforming activity. In mammalian cells, the primary v-*sis* translational product, p28$^{v\text{-}sis}$, undergoes a series of rapid processing steps (26, 28). These include formation of a disulfide-linked homodimer, p56$^{v\text{-}sis}$, followed by sequential amino- and carboxy-terminal cleavages to yield p35$^{v\text{-}sis}$ and p24$^{v\text{-}sis}$, respectively (28). The importance of disulfide bridge formation for PDGF function is underscored by observations that reduction abolishes biological activity of the molecule (1, 14). Moreover, nontransforming v-*sis* mutants so far described encode proteins that do not form dimers. To determine the ability of the mutant gene products to undergo proper processing, each mutant was transfected into COS cells. Cell lysates were then subjected to immunoprecipitation analysis with an antiserum which recognizes monomeric as well as dimeric forms of the v-sis/PDGF B protein (15, 27).

Three distinct phenotypes were observed, and representives of each class are shown in Fig. 1. Mutations introduced farthest from essential cysteine codons, as shown for Δ136, Δ148, Δ188, and Δ192, had no detectable effect upon dimerization of the v-*sis* protein encoded (Fig. 1). Moreover, such dimeric forms were processed in a manner indistinguishable from that of the wild-type protein (Fig. 1). More than 50% of the mutant proteins examined were in this group. Deletions nearer essential cysteine codons affected the efficiency of dimer formation. Thus, monomeric forms as well as dimers were observed for the products of Δ151 and Δ195 (Fig. 1). The third class of mutants consisted of deletions nearest the essential cysteine codons. The products of these mutants were unable to dimerize. Cells expressing the representative nondimerizing mutants Δ133 and Δ183 revealed only a faint p28$^{v\text{-}sis}$ monomer band (Fig. 1). The consistently lower levels of detection of such nondimerizing proteins may reflect reduced stability of improperly folded molecules. The effects of each mutation on v-*sis* transforming activity as

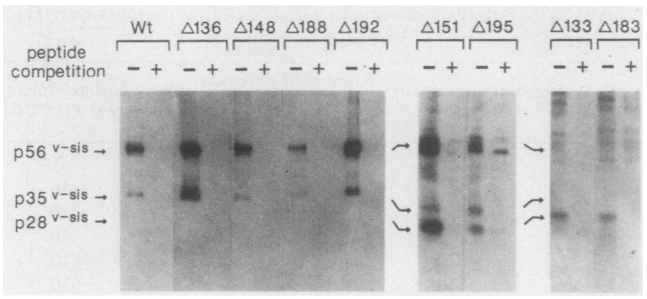

FIG. 1. Characterization of mutant gene products by immunoprecipitation. COS-1 cells were transfected with v-*sis* mutant DNA constructs and 48 h later were metabolically labeled as described in Materials and Methods. Cell lysates were incubated with anti-sis C serum, and immunoprecipitates were analyzed by fractionation in SDS-PAGE. Peptide competition symbols indicate whether anti-sis C was preincubated with homologous peptide. Protein products of parental v-*sis* as well as the representative deletion mutants indicated were analyzed. Bands representing $p56^{v\text{-}sis}$, $p35^{v\text{-}sis}$, and $p28^{v\text{-}sis}$ are indicated. Wt, Wild type.

well as on the ability to form dimers are summarized in Table 2. Lesions localized to the subdomain comprising amino acid residues 136 through 148 did not affect dimerization yet completely abolished the biologic activity of the molecule. Thus, minor conformational alterations in this subdomain appeared to profoundly affect transforming function.

Genetic mapping of the v-sis/PDGF B epitope recognized by a neutralizing monoclonal antibody. We have recently described a neutralizing monoclonal antibody, MAb sis 1, directed against PDGF B. Immunochemical studies have mapped its recognition site to the amino-terminal half of the v-sis/PDGF B product's transforming domain (21). The availability of our series of v-*sis* deletion mutants made it possible to combine molecular genetic and immunologic approaches to define the epitope recognized by this monoclonal antibody and to determine whether its recognition site overlapped with the codon 136 to 148 subdomain. Previous studies have shown that MAb sis 1 (21) as well as a polyclonal anti-PDGF serum (8) possess potent neutralizing activity but do not readily detect the fully reduced v-sis/PDGF B product. In contrast, anti-sis C peptide serum recognizes nonreduced and reduced forms with equal efficiency (26). Thus, we subjected the products of mutant v-*sis* genes to immunoprecipitation analysis with this panel of antibodies.

TABLE 2. Summary of biologic properties of v-*sis* deletion mutants

Codon deleted	Focus formation[a]	Dimer formation[b]	MAb Sis 1 recognition[c]
None	+	+	+
130	−	−	−
133	−	−	−
136	−	+	−
137	−	+	−
139	−	+	−
142	−	+	+
145	−	+	+
148	−	+	+
151	−	±	−
154	−	−	−
157	−	−	−
177	−	−	−
183	−	−	−
186	−	−	−
188	+	+	+
189	+	+	+
192	+	+	+
195	±	±	−
198	±	ND[d]	ND
201	−	−	−

[a] Quantitative results are shown in Table 1.
[b] Criteria for dimer formation are described in the text and shown in Fig. 1.
[c] For MAb sis 1 recognition, + and − signify detection at the levels shown in Fig. 2 for Δ142 and Δ136, respectively, and ± signifies intermediate levels.
[d] ND, Not determined.

Three different patterns of antibody recognition were observed. The wild-type v-sis/PDGF B protein was detected equally well by anti-sis C, anti-PDGF, and MAb sis 1 (Fig. 2). The same pattern was observed for six mutant gene products. A representative of this class, Δ142, is shown in Fig. 2. Like the wild-type molecule, Δ188, Δ189, and Δ192 retained the ability to induce focus formation (Table 2). In contrast, Δ142, Δ145, and Δ148 lacked detectable transforming activity (Table 2) while retaining a pattern of immunologic reactivity indistinguishable from that of the PDGF B wild-type molecule. The fact that MAb sis 1 detected these mutant proteins as efficiently as the wild-type protein argues further that the epitope recognized by this monoclonal antibody was not altered by deletion of codon 142, 145, or 148. Each nondimerizing mutant gene product, as represented by Δ133, was detected only by anti-sis C (Fig. 2), consistent with the known impaired recognition of reduced forms of the v-sis/PDGF B product by either anti-PDGF or MAb sis 1 (8, 21).

A novel pattern of antibody recognition was observed for the proteins encoded by Δ136, Δ137, and Δ139. Each was readily detected by anti-sis C and anti-PDGF but was poorly recognized by MAb sis 1. A representative mutant with this phenotype, Δ136, is shown in Fig. 2. Thus, subtle conformational alterations within this small domain of the v-sis/PDGF B protein not only abolished biological activity but specifically inhibited recognition by the neutralizing monoclonal antibody as well. These findings localized the epitope recognized by this monoclonal antibody (amino acids 136 to 139) to the small domain determined by molecular genetic analysis to be critical for ligand-induced transformation.

Involvement of codons 136 to 148 in PDGF receptor interaction. Transforming activity of the v-sis/PDGF B molecule is known to be mediated by interaction with PDGF α and β receptors (22, 24). To investigate the effects of conformational alterations induced by mutations in codons 136 to 148 on PDGF receptor interaction, we analyzed the level of steady-state PDGF receptor tyrosine phosphorylation, a marker of receptor kinase activity (24), in transfectants containing such mutants. Figure 3 shows that NIH 3T3 cells transformed by the parental v-sis/PDGF B gene under control of either the metallothionein or Moloney murine leukemia virus long terminal repeat (Mo-LTR) promoter, but not control NIH 3T3 cells, demonstrated readily detectable tyrosine-phosphorylated 180-kilodalton (kDa) α and β PDGF receptors, as reported previously (24). In contrast, Δ133, a mutant whose product exhibited gross conformational alterations that prevented intrachain disulfide linkages, showed no detectable receptor tyrosine phosphorylation. We also analyzed nontransforming mutants (Δ139 and Δ148) that either were not detected or were recognized by MAb sis 1, respectively. As shown in Fig. 3, neither mutant gene product caused detectable β receptor activation, and there was little if any tyrosine phosphorylation of α receptors (Fig.

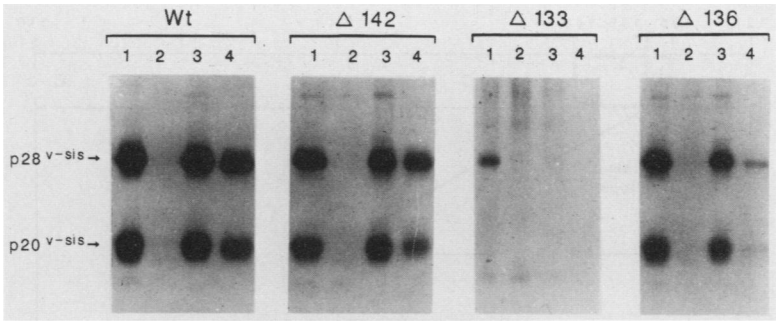

FIG. 2. Recognition of wild-type (wt) and mutant gene products by a panel of antibodies. COS-1 cells were transfected and metabolically labeled as described in the legend to Fig. 1. Cell lysates were immunoprecipitated with anti-sis C (lanes 1 and 2), anti-PDGF (lanes 3), or MAb sis 1 (lanes 4) and analyzed under reducing conditions. In some cases, antiserum was preincubated with excess homologous peptide (lanes 2). Bands representing $p28^{v\text{-}sis}$ and $p20^{v\text{-}sis}$ are indicated.

3). When the same cell lines were analyzed for expression of the v-*sis* product (Fig. 3), each of the mutants expressed $p28^{v\text{-}sis}$ at levels far in excess of those required to readily observe receptor activation by the wild-type v-*sis* gene under Mo-LTR control. The similar levels of steady-state receptor tyrosine phosphorylation observed with Mo-LTR and MMT Wt v-*sis* vectors (Fig. 3) were consistent with their comparable high transforming activities as determined by transfection analysis (data not shown). These findings indicate that efficient transformation can be achieved by low levels of steady-state v-sis protein expression. Thus, we conclude that mutations in this small domain of the v-sis/PDGF B molecule drastically impair its ability to interact with and/or functionally activate both PDGF receptors.

DISCUSSION

Our present studies have identified a critical subdomain within the v-sis/PDGF B molecule. Site-directed mutagenesis of the 84-codon minimum transforming region revealed a stretch of 13 amino acids in which single-codon deletions completely abolished biologic activity but did not impair proper disulfide-linked dimer formation. In contrast, biologic activity correlated with the extent of dimer formation for analogous deletions throughout the rest of the molecule. Using site-specific antisera directed against predicted v-sis peptides, we have recently shown that residues 131 to 154 represent a surface domain of PDGF BB (21). Moreover, v-sis/PDGF BB recognition by a neutralizing monoclonal antibody, MAb sis 1, has been immunochemically mapped to this domain, since peptide antisera recognizing the surface domain compete for MAb sis 1 recognition of PDGF BB (21). By combining molecular genetic and immunochemical approaches, we directly localized at least a portion of the MAb sis 1 binding site to amino acids 136 to 139. We further demonstrated that such mutations as well as those affecting codons 140 to 148 caused marked impairment of PDGF receptor activation. A schematic summary of these findings is shown in Fig. 4. All of these results establish that subtle conformational alterations affecting this small domain play a critical role in functional PDGF-receptor interactions.

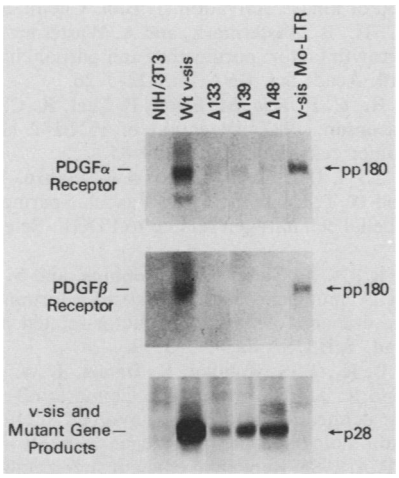

FIG. 3. Activation of PDGF receptors by wild-type (wt) and mutant v-*sis* gene products. NIH 3T3 cell transfectants expressing the v-*sis* genes indicated were lysed and subjected to immunoprecipitation with antisera specific for the α or β PDGF receptor as described before (24). Immunoprecipitates were fractionated by SDS-PAGE and blotted onto nitrocellulose filters. Tyrosine-phosphorylated proteins were visualized by sequential treatment of filters with antiphosphotyrosine antiserum and ^{125}I-protein A. In the case of v-*sis* or mutant v-*sis* proteins, NIH 3T3 cells were radiolabeled and immunoprecipitated as described in the text. Bands representing v-*sis* gene products and α and β PDGF receptors are indicated.

There is a high degree of evolutionary sequence conservation between PDGF A and PDGF B polypeptide chains (2, 10). Complete conservation of all eight cysteine residues as well as the natural occurrence of both heterodimeric and homodimeric PDGF ligands strongly suggest that folding and disulfide pairing are very similar for each molecule (2, 10). In spite of their similarities, PDGF BB activates both α and β PDGF receptors, whereas PDGF AA only activates the α receptor (24). Recent studies have demonstrated that functional domains within polypeptide hormones (5) or neurotransmitter receptors (18) can be identified by expression and characterization of chimeric proteins, taking advantage of structural similarities that exist between these related polypeptides. By construction of chimeric proteins between PDGF A and PDGF B, it has been possible to identify a 40-amino-acid subdomain of PDGF B that is responsible for its ability to activate β PDGF receptors (20). The amino acid stretch shown in our present study to be critical for PDGF receptor activation is encompassed within this 40-amino-acid subdomain.

The recently cloned vascular endothelial cell growth factor (VEGF) structurally resembles the PDGF ligands in conserved cysteine residues but is otherwise divergent (16, 23). In particular, comparison of VEGF within the region corresponding to amino acids 136 to 148 of the v-*sis* gene product reveals a high degree of dissimilarity. Whether

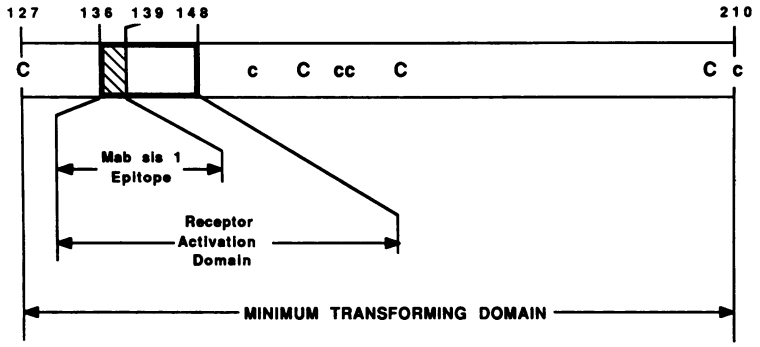

FIG. 4. Schematic summary of mutagenesis and MAb sis 1 recognition results. Only the minimum transforming domain (codons 127 to 210) of v-*sis* is shown; locations of essential (C) and nonessential (c) cysteine codons are shown. Codons 127 to 210 of the v-*sis* gene product correspond to codons 97 to 180 of c-sis/PDGF B.

chimeras between PDGF and VEGF can be used to map important functional domains of these molecules remains to be determined.

Recent reports have indicated that PDGF binding leads to receptor dimerization coincident with the induction of tyrosine kinase activity (3, 13, 32). These findings have suggested that PDGF receptor dimers may be cross-linked by a single bifunctional PDGF ligand (13, 32). According to this model, PDGF heterodimers comprising a deletion in codons 136 to 148 and a wild-type PDGF B chain might be expected to compete with the native PDGF molecule for receptor dimer formation and functional activation. If so, the mutants described here may provide a novel approach toward the generation of antagonists of PDGF action.

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