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The Role of Individual Cysteine Residues in the Structure and Function of the v-*sis* Gene Product

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The v-*sis* oncogene encodes a platelet-derived growth factor (PDGF)–related product whose transforming activity is mediated by its functional interaction with the PDGF receptor. PDGF, as well as processed forms of the v-*sis* gene product, is a disulfide-linked dimer with eight conserved cysteine residues in the minimum region necessary for biologic activity. Site-directed mutagenesis of the v-*sis* gene revealed that each conserved cysteine residue was required directly or indirectly for disulfide-linked dimer formation. However, substitution of serine for cysteine codons at any of four positions had no detrimental effect on transforming activity of the encoded v-*sis* protein. These results establish that interchain disulfide bonds are not essential in order for this protein to act as a functional ligand for the PDGF receptor. The remaining four substitutions of serine for cysteine each inactivated transforming function of the molecule. In each case this was associated with loss of a conformation shown to involve intramolecular disulfide bonds. These studies provide insight into the role of individual cysteine residues in determining the structure of the *sis*/PDGF molecule critical for biological activity.

THE PRODUCT OF THE SIMIAN SARCOMA virus (SSV) transforming gene, v-*sis*, exhibits all of the known structural and functional properties of human platelet-derived growth factor (PDGF), a potent mitogen for connective tissue cells (*1–4*). Moreover, v-*sis* alters growth properties only of cells that express PDGF receptors (*4*), implying that the transforming activity of v-*sis* is mediated directly by interaction of its PDGF-like product with the PDGF receptor. Since the minimal region of the v-*sis* gene product required for transforming activity is homologous to one of the two peptide chains comprising PDGF (*5–7*), genetic manipulations of v-*sis* that impair its transforming function provide a means of studying the ability of both its products and PDGF to act as ligands for the PDGF receptor.

PDGF as well as processed forms of the v-*sis* product are disulfide-linked dimers, whose mitogenic activities are abolished upon reduction (*8–10*). Despite evolutionary divergence (*1, 2*), PDGF-1, PDGF-2, and the v-*sis* product, which is the monkey homologue of PDGF-2, all contain eight identically spaced cysteine residues within a core sequence required for biological activity. In v-*sis* (Fig. 1), the core region contains 84 codons; five of the eight cysteines are clustered in a span of only 18 residues (amino acids 154 to 171). In the present study, we have investigated the role of individual cysteine residues on the structure and function of the v-*sis* transforming protein.

Genetic manipulations of v-*sis* were accomplished by site-directed mutagenesis. A 1.2-kbp fragment containing v-*sis* was removed from the pSSVSV2 plasmid (*5*) and subcloned into M13mp19. By means of a 17-mer oligonucleotide with the appropriate mismatch, a serine codon was substituted for each cysteine codon according to the methods of Zoller and Smith (*11*) as modified by Kunkel to allow for phenotypic selection of phage containing the desired mutation (*12*). Mutants were identified and their DNA sequences verified by the dideoxynucleotide method (*13*). Each mutant v-*sis* was then transferred back into pSSVSV2 for analysis of biological activity upon transfection of NIH 3T3 cells. Since the plasmid vector containing the v-*sis* mutants also had a dominant selectable *gpt* marker gene, it was possible to score focus-forming activity as well as colony formation for each plasmid.

Four of the eight mutants demonstrated transforming activities equivalent to that of wild-type SSV, whereas the other four mutants lacked any detectable transforming activity (Table 1). Each of the eight constructs was fully active with respect to the induction of colony formation in HAT (hypoxanthine, aminopterin, thymidine) medium. These results established that cysteine residues 127, 160, 171, and 208 were essential for v-*sis* transforming activity, whereas the loss of any one of the remaining cysteines had no

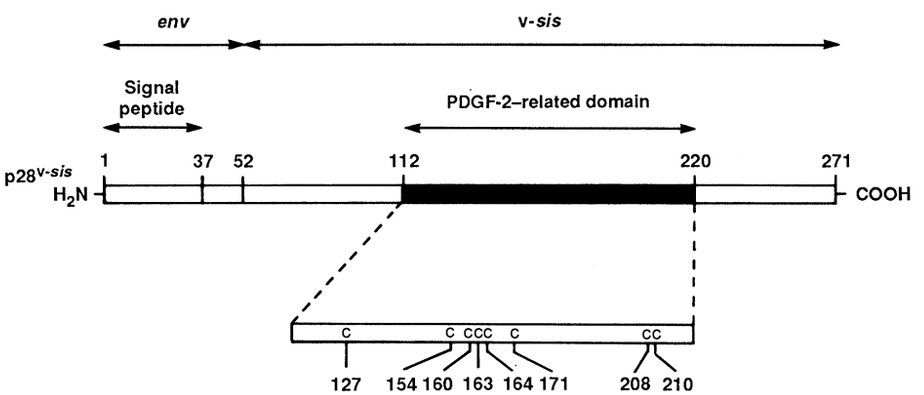

Fig. 1. Structural features of p28$^{v\text{-}sis}$ and distribution of cysteine residues within its PDGF-2–related domain. The initiation codon for p28$^{v\text{-}sis}$ synthesis is assigned position 1. The region spanning residues 1 through 51 is derived from the simian sarcoma–associated virus *env* gene, and residues 1 through 36 represent the p28$^{v\text{-}sis}$ signal peptide. The region that is homologous to human PDGF-2 spans residues 112 through 220.

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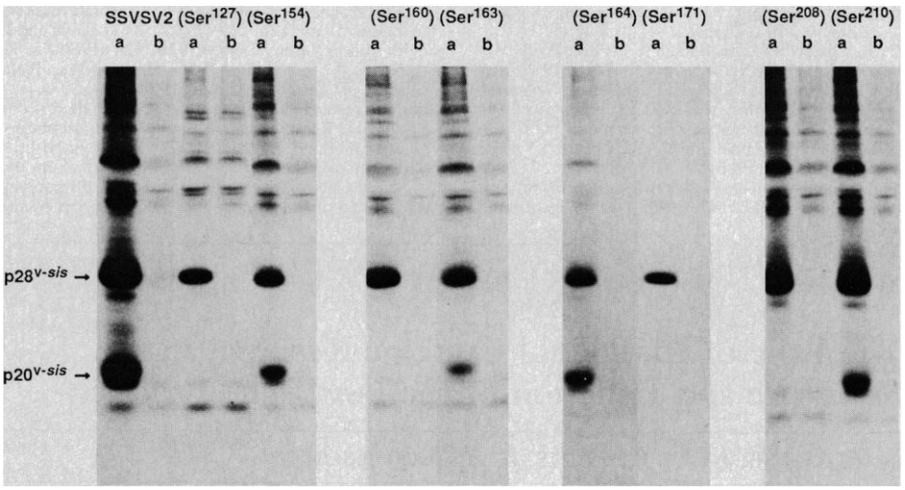

Fig. 2. Characterization of the in vivo translational products of wild-type and v-*sis* mutants with cysteine to serine codon substitutions. COS-1 cells in which pSV2 replicates as an episome to a high copy number (*23, 24*) were plated at a density of 2×10^6 per 10-cm petri dish 24 hours prior to DNA transfection by the calcium phosphate precipitation method (*21, 22*). At 48 hours after transfection, cells were metabolically labeled for 3 hours with [^{35}S]methionine (125 μCi/ml) and [^{35}S]cysteine (125 μCi/ml) (1200 Ci/mmol; Amersham) in methionine- and cysteine-free Dulbecco's modified Eagle's normal essential medium (DMEM). Labeled cells were lysed with 1 ml of a buffer containing 10 m*M* sodium phosphate, *p*H 7.5, 100 m*M* NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, and 0.1 m*M* phenylmethylsulfonyl fluoride and clarified at 100,000*g* for 30 minutes. Aliquots (200 μl) were incubated with 3 μl of anti-*sis* C (lanes a) or anti-*sis* C preincubated with the homologous peptide (lanes b) as described (*3*). Immunoprecipitates were recovered with the aid of *Staphylococcus aureus* protein A bound to Sepharose beads (Pharmacia). After solubilization by boiling in the presence of 4% SDS and 1.4*M* β-mercaptoethanol, proteins were analyzed by electrophoresis in 14% polyacrylamide gels (PAGE) containing SDS. Lysates from COS-1 cells transfected with either 10 μg of wild-type (SSVSV2) or mutant DNAs are specified above the lanes. Bands representing p28$^{v\text{-}sis}$ as well as its COOH-terminal cleavage product, p20$^{v\text{-}sis}$, are as indicated.

detrimental effect on v-*sis* transforming function.

The primary p28$^{v\text{-}sis}$ translational product undergoes a complex series of processing steps that involve rapid formation of a disulfide-linked homodimer, p56$^{v\text{-}sis}$, followed by NH_2-terminal and COOH-terminal cleavages to yield p35$^{v\text{-}sis}$ and p24$^{v\text{-}sis}$ dimer forms, respectively (*3*). These forms have been specifically identified by use of a variety of antisera, including those directed against NH_2- (*sis* N) and COOH- (*sis* C) terminal v-*sis* peptides as well as against PDGF (anti-PDGF) (*3, 14*).

To identify any alterations in protein processing that might accompany the substitution of individual cysteine residues, COS-1 cells transfected with wild-type or mutant v-*sis* genes were metabolically labeled, lysed, and examined by immunoprecipitation under reducing gel conditions. Anti-*sis* C detected wild-type p28$^{v\text{-}sis}$ as well as each of the mutant v-*sis* gene products (Fig. 2). The apparent molecular weight of each of these mutant proteins was indistinguishable from that of p28$^{v\text{-}sis}$, further establishing the intact nature of each mutant coding sequence.

NH_2-terminal processing of the wild-type v-*sis*/PDGF-2 protein occurs by proteolytic cleavage to yield a 20-kD monomer, p20$^{v\text{-}sis}$, observed under reducing conditions (*3*). When we analyzed the reduced forms of molecules synthesized by each v-*sis* mutant, a 20-kD form was detected only in those lysates of COS-1 cells transfected with transforming mutants. The inability to detect these same products with anti-*sis* N serum implied normal NH_2-terminal processing of each of the transforming mutant proteins. In contrast, under reducing as well as nonreducing conditions COS-1 cells transfected with each of the nontransforming mutants lacked a detectable 20-kD cleavage product despite readily observable levels of the 28-kD primary translational product (Figs. 2 and 3). Thus, the inability of certain v-*sis* mutants to induce transformation correlated with the lack of NH_2-terminal processing of their encoded products.

Previous studies have strongly suggested that a disulfide-linked dimer form is required for the biological functions both of PDGF and the v-*sis* gene product (*8–10*). To investigate the effect of individual cysteine substitutions on the ability of v-*sis* proteins to form disulfide-linked dimers, we examined v-*sis* mutant products expressed by transfected COS-1 cells under nonreducing conditions. The proteins specified by three of the transforming mutants, pv-*sis* (Ser^{154}), pv-*sis* (Ser^{164}), and pv-*sis* (Ser^{210}), migrated as monomers indistinguishable in their mobilities from wild-type p28$^{v\text{-}sis}$ and p20$^{v\text{-}sis}$, respectively (Fig. 3). The proteins expressed by the other transforming mutant, pv-*sis* (Ser^{163}), had altered electrophoretic mobilities. The larger species migrated faster than p28$^{v\text{-}sis}$, and its processed form migrated more slowly than p20$^{v\text{-}sis}$. As these altered mobilities were observed only under nonreducing conditions, it seems likely that the product encoded by pv-*sis* (Ser^{163}) exhibited aberrant intrachain disulfide bridge formation. Proteins specified by each of the nontransforming v-*sis* mutants also migrated as monomers indistinguishable from p28$^{v\text{-}sis}$. All of these findings demonstrated that each

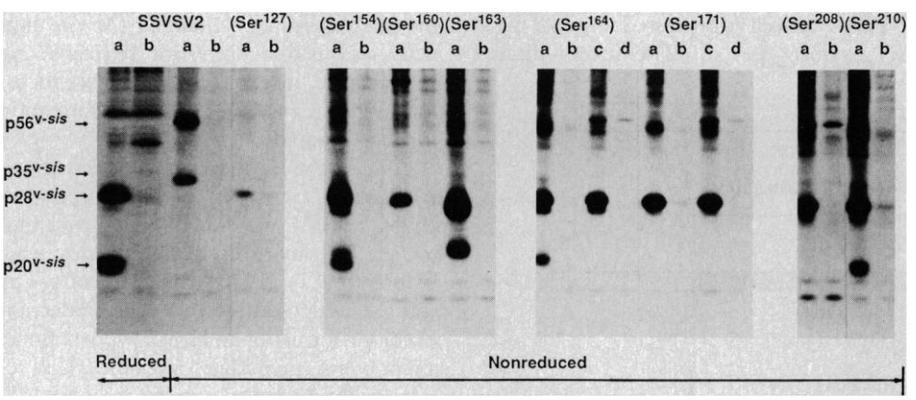

Fig. 3. Comparison of v-*sis* mutant translational products under nonreducing conditions. Lysates of COS-1 cells transfected with SSVSV2 or indicated mutant DNAs were prepared as described in the legend to Fig. 2. Extracts were immunoprecipitated with anti-*sis* C (lanes a), anti-*sis* C preincubated with homologous peptide (lanes b), anti-*sis* N (lanes c), or anti-*sis* N preincubated with homologous peptide (lanes d). Immunoprecipitates were solubilized prior to electrophoresis in the presence (reduced) or absence (nonreduced) of β-mercaptoethanol. Proteins in the 50- to 60-kD range detected with anti-*sis* C in extracts of cells transfected with pv-*sis* (Ser^{164}) and pv-*sis* (Ser^{171}) were not detected with anti-*sis* N serum. Thus, they do not represent p56$^{v\text{-}sis}$ dimers. Bands representing p28$^{v\text{-}sis}$ and p20$^{v\text{-}sis}$ monomers as well as p56$^{v\text{-}sis}$ and p35$^{v\text{-}sis}$ dimers are as indicated.

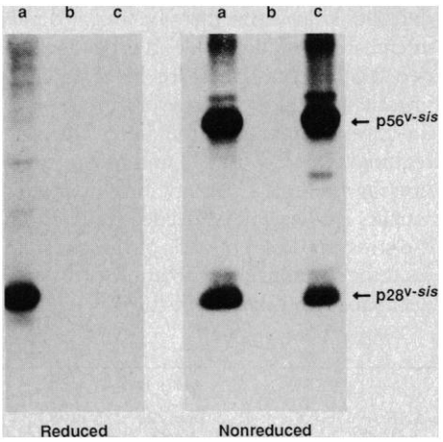

Fig. 4. Anti-PDGF serum detects the nonreduced but not the reduced p28$^{v\text{-}sis}$ monomer. HF/SSV cells were metabolically labeled for 15 minutes with [^{35}S]cysteine and [^{35}S]methionine as described (*3*). Cells were hypotonically lysed, and postnuclear supernatants were centrifuged at 100,000*g* for 90 minutes. Membrane-containing pellets were resuspended in 10 m*M* sodium phosphate at *p*H 7.5, boiled for 10 minutes, and clarified by centrifugation at 57,000*g*. The supernatant was divided into equal portions, one of which was reduced in the presence of 20 mmol of dithiothreitol and was blocked by alkylation with 40 m*M* iodoacetamide for 15 minutes. Each portion was dialyzed and immunoprecipitated with anti-*sis* C (lanes a), anti-*sis* C preincubated with the homologous peptide (lanes b), or anti-PDGF serum (lanes c). Immunoprecipitates were analyzed under nonreducing conditions by SDS-PAGE. Bands representing newly synthesized p28$^{v\text{-}sis}$ as well as its dimeric form p56$^{v\text{-}sis}$ are as indicated.

of the eight cysteine residues was required for intermolecular disulfide bridge formation, but that covalent linkage between monomers was not essential for transforming activity of the v-*sis* protein.

Polyclonal PDGF antibodies potently inhibit the mitogenic activities of both PDGF and the v-*sis* gene product (*15, 16*). Moreover, such antisera recognize the nonreduced dimeric forms of the *sis*/PDGF-2 gene product expressed in mammalian cells (*3*) but not the monomeric form of the same gene product synthesized in the reducing environment of a prokaryotic expression system (*17*). Thus, we reasoned that a conformation of the *sis*/PDGF-2 gene product dependent on inter- and/or intrachain disulfide bonds might be required for recognition by anti-PDGF serum.

Earlier studies have shown that although processing of the primary v-*sis* translational product to its p56$^{v\text{-}sis}$ dimeric form is rapid, short labeling periods allow detection of newly synthesized p28$^{v\text{-}sis}$ monomers under nonreducing conditions (*3*). When mammalian cells expressing wild-type v-*sis* were pulse-labeled for 15 minutes and lysates analyzed under such conditions, our anti-PDGF serum recognized not only the p56$^{v\text{-}sis}$ dimer but detected the p28$^{v\text{-}sis}$ monomer as well (Fig. 4). These results implied that the anti-PDGF serum recognized a conformation of the p28$^{v\text{-}sis}$ molecule that was independent of interchain disulfide bonds. To further investigate the contribution of intrachain disulfide bridges to recognition of the v-*sis*/PDGF-2 monomer by the PDGF antiserum, we reduced and blocked sulfhydral groups on the molecule. Under these conditions, anti-*sis* C readily recognized p28$^{v\text{-}sis}$, while the anti-PDGF serum failed to detect the same protein (Fig. 4). These results indicate that the anti-PDGF serum recognized a conformation of the v-*sis* product that required intrachain but not interchain disulfide bridges.

To address the question of which cysteines might be involved in intrachain disulfide linkages, we analyzed the ability of the anti-PDGF serum to recognize the nonreduced products of each v-*sis* mutant. The proteins specified by each of the four transforming mutants were readily detected, whereas the same antiserum failed to detect any of the nontransforming v-*sis*/PDGF-2 mutant proteins as is shown for several representatives (Fig. 5). Since the conformation of the p28$^{v\text{-}sis}$ monomer essential for detection by anti-PDGF was dependent on intramolecular disulfide bonds, these findings established that cysteines 127, 160, 171, and 208 must be involved in intrachain disulfide linkages essential for anti-PDGF recognition. Thus, intrachain disulfide bonds involving these four cysteine residues must provide a conformation to the v-*sis* encoded monomer sufficient for its functional interaction with the PDGF receptor and the subsequent cascade of events associated with v-*sis* transformation.

Table 1. Biologic activity of v-*sis* cysteine mutants. Transfection of NIH 3T3 cells was performed with the use of the calcium phosphate precipitation technique (*20, 21*). Cell transformation was scored at 2 to 3 weeks. Colonies, selected by their resistance to HAT medium containing mycophenolic acid (*22*), were scored at 2 weeks. Abbreviations: FFU, focus-forming units; CFU, colony-forming units, per picomole of PDGF-2 coding sequence; ND, not detectable.

DNA clone	Log focus formation (FFU/pmol)	Log colony formation (CFU/pmol)
pSSVSV2	4.9	4.0
pv-*sis* (Ser^{127})	ND	3.5
pv-*sis* (Ser^{154})	4.7	3.7
pv-*sis* (Ser^{160})	ND	3.9
pv-*sis* (Ser^{163})	4.2	3.4
pv-*sis* (Ser^{164})	4.1	3.6
pv-*sis* (Ser^{171})	ND	3.6
pv-*sis* (Ser^{208})	ND	4.0
pv-*sis* (Ser^{210})	4.7	3.6

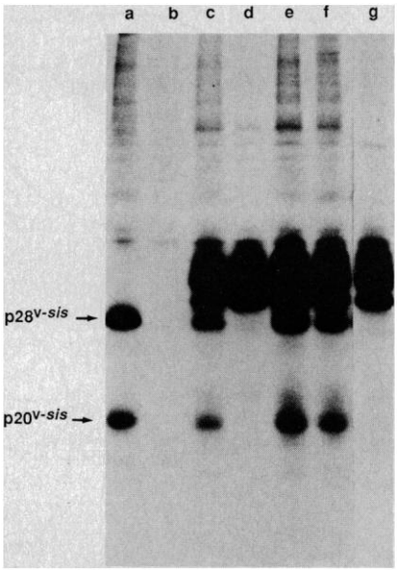

Fig. 5. Specific recognization of the translational products of transforming v-*sis* mutants by anti-PDGF serum. Lysates were prepared from COS-1 cells transfected with SSVSV2 (lanes a through c), pv-*sis* (Ser^{127}) (lane d), pv-*sis* (Ser^{154}) (lane e), pv-*sis* (Ser^{163}) (lane f), or pv-*sis* (Ser^{171}) (lane g) as described in the legend to Fig. 2. Extracts were immunoprecipitated with anti-*sis* C (lane a), anti-*sis* C preincubated with the homologous peptide (lane b), or anti-PDGF serum (lanes d through g). Immunoprecipitates were analyzed under reducing conditions by SDS-PAGE.

The present studies demonstrate that each of the eight cysteine residues encoded within the v-*sis* transforming region is required directly or indirectly for proper folding of the molecule necessary for formation of stable disulfide-linked dimers. Yet, serine substitutions for four of these cysteine codons were found to have no detrimental effect on the transforming activity of the encoded protein. Since substantial evidence indicates that v-*sis* transforming activity is directly mediated through activation of the PDGF receptor (*4*), our findings strongly imply that interchain disulfide bonds are not essential for the v-*sis* gene product to act as a functional ligand for the PDGF receptor.

The absence of covalent linkages suggests either that the monomer itself exhibits a conformation required for receptor interaction or that the molecule can form a biologically active noncovalently linked dimer structure as has been shown for nerve growth factor β (*18, 19*). One of the cysteine mutants with transforming activity, the pv-*sis* (Ser^{163}), encoded a protein with an aberrant monomeric structure. This mutant protein would not be expected to attain a dimer conformation analogous to that of the wild-type v-*sis* protein, which is more consistent with the concept that the monomer is able to act as a functional ligand. If this concept is correct, it will greatly simplify

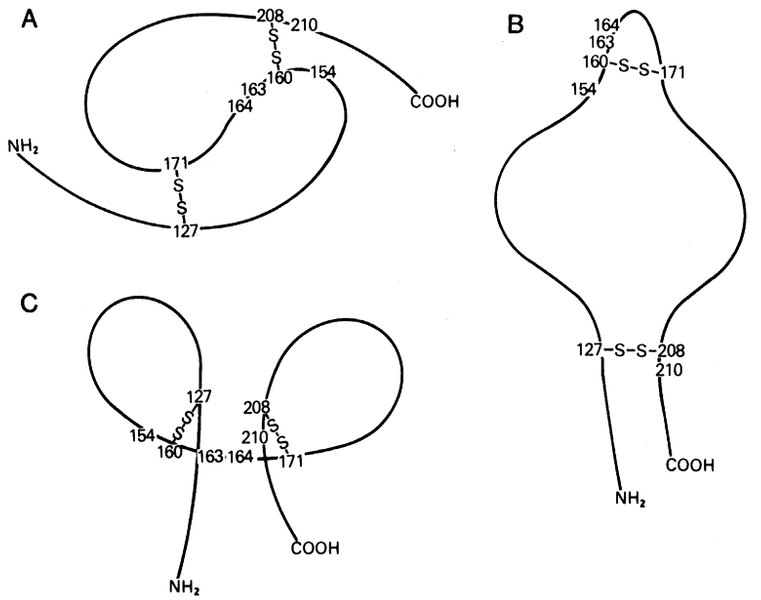

Fig. 6. Proposed models (**A** to **C**) for intrachain disulfide linkages essential for $p28^{v\text{-}sis}$ transforming activity. Cysteine residues are numbered.

efforts to determine the conformation of the PDGF molecule required for receptor interaction.

Individual serine substitutions for the remaining four cysteines completely inactivated biological activity of the v-*sis* gene. The encoded products of these mutants showed other abnormalities in processing in addition to their inability to form disulfide-linked dimers. Each failed to undergo normal NH_2-terminal cleavage, implying a conformational alteration that inhibited this processing step as well. The wild-type v-*sis*–encoded $p56^{v\text{-}sis}$ dimer and its processed forms, are mitogenic (*4*). Thus, the absence of NH_2-terminal cleavage of these mutant proteins is probably not responsible for their lack of biological activity but rather reflects primary conformational alterations that change both the processing and transforming properties of the protein.

Our findings also indicated that the maintenance of a PDGF antibody–reactive conformation must be dependent on at least one intrachain disulfide bridge. Those mutant v-*sis* proteins that retained transforming activity exhibited a conformation that was recognized by the PDGF antiserum; this conformation was not observed in the four nontransforming mutant proteins. A cysteine to serine substitution that only replaces a sulfur atom with an oxygen atom would be expected to exert only a subtle effect on protein secondary structure and would not be expected to so dramatically alter both the conformation and biological activity of the molecule. Thus, we conclude that cysteines 127, 160, 171, and 208 are likely to be involved in intrachain disulfide bridges critical to antibody recognition and biologic function of the molecule. These bridges could be organized in one of three different conformations (Fig. 6).

Structural assignments for the four cysteine residues that are not essential for transforming activity of the protein are more difficult. At least one must be involved in an interchain disulfide bridge. It is possible that two of these cysteines are involved in an intrachain disulfide bridge that is required for covalent linkage of the dimer but is not required either for PDGF antibody recognition or biological activity of the protein. Further studies will be needed to determine the structural roles of each of these cysteine residues in inducing formation of a stable disulfide-linked dimer structure.

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