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Identification of Nonessential Disulfide Bonds and Altered Conformations in the v-*sis* Protein, a Homolog of the B Chain of Platelet-Derived Growth Factor

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The protein encoded by v-*sis*, the oncogene of simian sarcoma virus, is homologous to the B chain of platelet-derived growth factor (PDGF). There are eight conserved Cys residues between PDGF-B and the v-*sis* protein. Both native PDGF and the v-*sis* protein occur as disulfide-bonded dimers, probably containing both intramolecular and intermolecular disulfide bonds. Oligonucleotide-directed mutagenesis was used to change the Cys codons to Ser codons in the v-*sis* gene. Four single mutants lacked detectable biological activity, indicating that Cys-127, Cys-160, Cys-171, and Cys-208 are required for formation of a biologically active v-*sis* protein. The other four single mutants retained biological activity as determined in transformation assays, indicating that Cys-154, Cys-163, Cys-164, and Cys-210 are dispensable for biological activity. Double and triple mutants containing three of these altered sites were constructed, some of which were transforming as well. The v-*sis* proteins encoded by biologically active mutants displayed significantly reduced levels of dimeric protein compared with the wild-type v-*sis* protein, which dimerized very efficiently. Furthermore, a mutant with a termination codon at residue 209 exhibited partial transforming activity. This study thus suggests that the minimal region required for transformation consists of residues 127 to 208. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis indicated that the v-*sis* proteins encoded by some of the biologically active mutants exhibited an altered conformation when compared with the wild-type v-*sis* protein, and suggested that Cys-154 and Cys-163 participate in a nonessential disulfide bond.

The v-*sis* gene is the transforming gene of simian sarcoma virus, an acutely transforming, replication-defective retrovirus which was isolated from a naturally occurring fibrosarcoma in a woolly monkey (11, 35). It arose through a recombinational event between the simian sarcoma-associated virus and a cellular gene termed c-*sis* (11). This event resulted in the creation of an 813-nucleotide fused gene encoding a protein of 271 amino acids in which the first 51 amino acids are encoded by the viral envelope (*env*) gene and the carboxy-terminal 220 amino acids are encoded by the c-*sis* gene. Biological activity is dependent upon an N-terminal, *env*-derived signal peptide which directs the translocation of the nascent polypeptide across the membrane of the rough endoplasmic reticulum (17, 18, 24, 30). The signal peptide is removed, and the protein is glycosylated at the consensus site for N-linked glycosylation, Asn-Met-Thr, residues 93 to 95. (Note that here, as throughout the text, amino acids are numbered beginning with the initial Met of the predicted v-*sis* gene product.) The protein undergoes dimerization and is proteolytically processed at the basic dipeptide, Lys-Arg, residues 110 and 111, resulting in the formation of a 42-kilodalton (kDa) dimer composed of two 20-kDa monomers (29).

Protein sequence data of human platelet-derived growth factor (PDGF) first indicated a relationship between the v-*sis* gene product and the B chain of PDGF (12, 22, 23, 36). PDGF is a heat-stable, cationic dimer of 25 to 35 kDa which consists of two related monomers, the A chain and the B chain (2, 22, 36). It is a potent mitogen for cells of mesenchymal origin (32), binding to the PDGF receptor and resulting in the stimulation of Tyr protein kinase activity (27). Both the intracellular and the secreted forms of the v-*sis* protein are also mitogenic (9, 13, 21, 28).

Disulfide bonds may stabilize the structure of the mature protein or provide transient bonds during folding as exemplified by bovine pancreatic trypsin inhibitor (5, 6). They may also be intramolecular or intermolecular, as for multisubunit proteins. Some disulfide bonds may not be strictly essential for a biologically active conformation. The serine proteases, for example, have different numbers of disulfide bonds, even though their three-dimensional structures are similar (8).

All the Cys residues in PDGF are involved in covalent bonds (10), and reduction of either PDGF or the v-*sis* protein abolishes its mitogenic activity (9, 13, 21, 28), indicating a crucial role for disulfide bonds in stabilization of a biologically active conformation. Such a large number of disulfide bonds may also account for the stability of PDGF to heat and acid. Analysis of the disulfide bonds of PDGF or of the v-*sis* protein is crucial for understanding their structures, given the importance of their disulfide bonds for biological activity.

The v-*sis* gene encodes 10 Cys residues, of which 8 are conserved in the PDGF B chain as well as the PDGF A chain (2). Previous studies (19, 33) with deletion mutants demonstrated that the transforming region of the v-*sis* protein is completely contained within residues 127 to 214, which includes all of the conserved Cys residues. In the present study, we used the technique of oligonucleotide-directed

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mutagenesis to probe the importance of these Cys residues for biological activity and dimerization.

MATERIALS AND METHODS

Preparation of template for mutagenesis. The starting plasmid for the mutagenesis was M13mp19*sis*-6. This contains the v-*sis*^{wt} gene inserted into a unique *Xho*I site in an M13mp19 derivative (constructed by converting the *Sal*I site in the multiple cloning site sequence of M13mp19 to an *Xho*I site). After plaque purification, a bacteriophage suspension was used to infect an RZ1032 culture. RZ1032 is an *Escherichia coli* strain derived from strain BW313 (HfrKL16 Po/45 *lysA61–62 thi-1 relA1 spoT1 dut-1 ung-1*) transduced with P1 grown on a tetracycline-resistant derivative of *E. coli* JM101 (JM101 *supE44, zbd-279*::Tn*10*). The template DNA containing uracil was prepared as described previously (25). Growth in the RZ1032 *dut ung* strain resulted in the incorporation of uracil residues into the M13 genome, rendering it biologically inactive in an *ung*$^+$ host, in our case *E. coli* 7118. However, in the *ung* strain it remained biologically active. This was demonstrated by comparing the survival of the phage in the *ung* and the *ung*$^+$ strains. Before any replication of the phage in RZ1032, the ratio of virus titers in strains 7118 and RZ1032 was 6.1, whereas after several cycles in RZ1032 the ratio was 4×10^{-5}.

Oligonucleotide-directed mutagenesis. The oligonucleotides used to construct the mutants are given in Table 1. Each one encodes a Ser residue (which is similar in size and polarity to a Cys residue) in place of the indicated Cys residue and also results in the creation of a new restriction enzyme site. The mutagenesis, however, did not change any of the surrounding amino acids. The oligonucleotides were 5′ phosphorylated with T4 polynucleotide kinase. The phosphorylated oligonucleotides were either used directly or purified on a 24% denaturing acrylamide gel before use. Conditions for hybridization and DNA synthesis were as described by Zoller and Smith (37). A 10-fold molar excess of each of the oligonucleotides was annealed with the template. Extension was done with high-pressure liquid chromatography-grade deoxynucleoside triphosphates (free of uridine) at 100 μM. T4 DNA ligase (6 U) and the Klenow fragment of *E. coli* DNA polymerase I (1 U) were added in a total volume of 40 μl, and the sample was incubated at room temperature for at least 6 h. We found that incubation for 6 h at room temperature was sufficient to convert the majority of the single-stranded DNA to the double-stranded form as shown by electrophoresis on a 0.7% agarose gel (data not shown). The DNA was used to transform $CaCl_2$-treated *E. coli* 7118. Individual plaques were picked into growing cultures of 7118 for analysis of the replicative forms by the appropriate restriction enzyme digests.

Reconstruction of mutant genes into expression vectors. The mutant genes were subcloned into an expression vector derived from simian virus 40 (SV40). The SV40-derived expression vector pJC119 (34) has a deleted copy of the SV40 genome with an *Xho*I site replacing most of the VP1 gene. A derivative of pJC119, pMH29, was constructed by inserting the v-*sis*^{wt} gene into the unique *Xho*I site of pJC119. For all the mutants except v-*sis*^{S127}, a *Bgl*II-to-*Bst*EII fragment encompassing the altered site was excised from the M13-derived replicative form and swapped with the corresponding fragment of pMH29. For v-*sis*^{S127}, a *Sst*I-to-*Bgl*II fragment encompassing the mutant site was swapped with pMH29. These plasmids were used for assays of transient protein expression in Cos-1 cells. The plasmid names are given in Table 1.

The mutants were sequenced in the SV40-derived expression vectors by using a modification of the Sanger sequencing procedure in which supercoiled plasmid DNA was used (4). Plasmid DNA (2 μg) was denatured in 200 mM NaOH–200 μM EDTA and then annealed with gel-purified, phosphorylated oligonucleotides encoding either Ser-127 or Ser-170 (Table 1) as primers. To confirm the sequence across

TABLE 1. v-*sis* mutants and their biological activities

Mutant	Oligonucleotide[a]	New site[b]	M13 clone	SV40 clone	M-MuLV clone	Focus assay (FFU/pmol)[c]
v-*sis*^{wt}	NA[d]	NA	mp19*sis*-6	pMH29	pDD120	1.4×10^5
v-*sis*^{S127}	CATGATTGCCGAG{TCC}AAGACACG	*Hin*fI	pMS066	pMS077	pMS088	$<1.0 \times 10^1$
v-*sis*^{S154}	CTGGTGTGGCCCCCG{AGC}GTGGAGG	*Ava*I	pMS063	pMS074	pMS080	9.2×10^4
v-*sis*^{S160}	GGAGGTGCAGCGG{AGC}TCCGGC	*Sst*I	pKM5	pMS073	pMS086	$<1.0 \times 10^1$
v-*sis*^{S163}	CGCTGCTCCGGA{TCC}TGCAACAACCGC	*Bam*HI	pMS094	pMS096	pMS098	1.2×10^5
v-*sis*^{S164}	CTGCTCCGGCTGC{AGC}AAGAACCG	*Pst*I	pMS062	pMS075	pMS081	3.8×10^4
v-*sis*^{S171}	CGCAACGTGCAG{TCG}CGACCCACC	*Nru*I	pMS065	pMS076	pMS087	$<1.0 \times 10^1$
v-*sis*^{S208}	GACCACCTGGCC{AGC}AAGTGTGA	*Hae*III	pMS070	pMS078	pMS082	$<1.0 \times 10^1$
v-*sis*^{S210}	GGCATGCAAG{TCT}GAGATAGTG	*Dde*I	pMS071	pMS079	pMS083	7.9×10^4
v-*sis*$^{S154/163}$	CTGGTGTGGCCCCCG{AGC}GTGGAGG CGCTGCTCCGGA{TCC}TGCAACAACCGC	*Ava*I *Bam*HI	pMS100	pMS103	pMS106	1.0×10^5
v-*sis*$^{S154/164}$	CTGGTGTGGCCCCCG{AGC}GTGGAGG CTGCTCCGGCTGC{AGC}AACAACCG	*Ava*I *Pst*I	pMS101	pMS104	pMS107	2.4×10^3
v-*sis*$^{S163/164}$	CGCTGCTCCGGA{TCC}{AGC}AACAACCGC	BamHI	pMS095	pMS097	pMS099	$<1.0 \times 10^1$
v-*sis*$^{S154/163/164}$	CTGGTGTGGCCCCCG{AGC}GTGGAGG CGCTGCTCCGGA{TCC}{AGC}AACAACCGC	*Ava*I *Bam*HI	pMS102	pMS105	pMS108	$<1.0 \times 10^1$
v-*sis*^{TAG209}	NA	*Nhe*I	NA	pMS179	pMS180	5.7×10^2

[a] Mutagenic oligonucleotide(s). Unmatched nucleotides are in large print. New Ser codons are enclosed in curly brackets.
[b] Newly created restriction enzyme sites.
[c] FFU, Focus-forming units. Assays were performed as described in Materials and Methods.
[d] NA, Not applicable.

and upstream of the codon at position 127, the v-*sis*^{S127} mutant was sequenced by the method of Maxam and Gilbert (26). All mutants were also confirmed by mapping with several restriction enzymes.

For focus assays with NIH 3T3 cells, the mutant genes were subcloned into a Moloney murine leukemia virus (M-MuLV)-derived expression vector, pDD102 (3). This vector was derived from the proviral clone of M-MuLV, p836 (20), by making deletions in the *gag* and *pol* genes and replacing most of the *env* gene with a unique *Xho*I site. The *Xho*I fragments containing the mutant v-*sis* genes in the SV40-derived vectors were inserted into this *Xho*I site in pDD102. Colony hybridization (16) was used to detect inserts, and restriction mapping was used to confirm the correct orientation of the inserts for expression. These plasmids are given in Table 1.

Construction of the mutant with a termination codon at position 209. The termination codon TAG was introduced into the wild-type v-*sis* gene by the following two-fragment ligation. One fragment was prepared by digestion of pMH29 with *Sph*I, removal of the overhang with Klenow fragment, ligation of the non-5'-phosphorylated linker CATGCTAG CATG, and subsequent digestion with *Bgl*II. The second fragment was prepared by digestion of pMH29 with *Bst*EII, repair of the overhang with Klenow fragment, ligation of the same linker as above, and subsequent digestion with *Bgl*II. The two fragments, each terminated at one end by the *Bgl*II overhang and at the other end by the overhang of the linker, were then ligated. This procedure recreated the Cys-208 codon TGC and introduced the termination codon TAG at position 209. The resultant mutant was confirmed by appropriate restriction digestions and nucleotide sequencing. As above, the mutant was used for transfection into Cos-1 cells for transient expression of v-*sis* protein and was also subcloned into an M-MuLV-derived expression vector for focus assays.

Biological assays. The protocol used for focus assays was as described previously (1, 15). NIH 3T3 cells were grown in Dulbecco modified Eagle medium supplemented with 10% calf serum. The DNA-calcium phosphate coprecipitates were made with 1 μg of each mutant in a M-MuLV vector plus 1 μg of a replication-competent clone of M-MuLV (p836 [20]) and 20 μg of sheared calf thymus DNA; they were added directly onto semiconfluent monolayers of NIH 3T3 cells in 60-mm dishes. The cells were refed after 12 to 18 h, and 2 h later the contents of each plate were divided onto four 10-cm plates. They were refed at 3-day intervals and scored for focus formation 7 days posttransfection. Focus assays were performed at least three times.

Transient protein expression and immunoprecipitations. For transient expression of high levels of the mutant gene products, the SV40-derived plasmids were transfected into Cos-1 cells by the DEAE-dextran method. Cells grown in 60-mm dishes were washed twice with TS (10 mM Tris [pH 7.4], 150 mM NaCl) and then incubated with a solution of TS containing 5 μg of the plasmid DNA plus 500 μg of DEAE-dextran (molecular weight, 2.0×10^6) per ml for 10 min at 37°C. After aspiration of the DNA solution, the cells were shocked with 10% dimethyl sulfoxide in TS for 1 min and washed twice with TS before being refed with medium containing 100 μM chloroquine. The cells were refed with fresh medium after 2 h and allowed to grow for 48 h to begin expressing the transfected genes. They were then metabolically labeled for 2 h with 100 μCi each of [^{35}S]Met and [^{35}S]Cys in Dulbecco modified Eagle medium lacking Met and Cys.

The immunoprecipitations were done as previously described (19, 33). The cells were washed five times with Versene (0.5 mM EDTA, 137 mM NaCl, 2.7 mM KCl, 5.1 mM Na_2HPO_4, 1.3 mM NaH_2PO_4) and then lysed in 600 μl of RIPA buffer (10 mM sodium phosphate [pH 7.0], 150 mM NaCl, 1% Nonidet P-40, 1% sodium dodecyl sulfate [SDS], 1% Trasylol [Mobay Chemical Corp.]) in the presence of 100 μM iodoacetamide. The lysates were centrifuged to remove cellular debris, and the supernatants were precleared by incubation with *Staphylococcus aureus* before they were used for immunoprecipitation. The antiserum used was raised against bacterially synthesized p28^{sis}. The specificity of this antiserum was previously confirmed by comparison with immunoprecipitations using a *sis* specific anti-peptide serum with and without the peptide block (data not shown). Lysates from mock transfections were also used as controls for the immunoprecipitations. After a 2-h incubation of the lysate with antiserum, the immunoglobulin G was collected with fresh *S. aureus*. The pellets were washed several times and then split into two aliquots. One aliquot was suspended in reducing sample buffer (50 mM Tris [pH 6.8], 2% SDS, 20% 2-mercaptoethanol, 10% glycerol) with 100 μM iodoacetamide, and the other suspended in nonreducing sample buffer which lacked the 2-mercaptoethanol. The samples were boiled for 2 min, clarified by centrifugation, and electrophoresed on SDS–15% polyacrylamide gels.

Construction of double and triple mutants. For the v-*sis*$^{S163/S164}$ double mutant, a new oligonucleotide was designed which encoded both altered residues (Table 1). The other double mutants, as well as the triple mutant, were constructed by annealing two oligonucleotides to the template concurrently (Table 1). The mutagenesis procedure used was the same as that for the single mutants, and the mutants were subcloned into SV40-derived and M-MuLV-derived expression vectors as above. The mutants were used in focus assays and for immunoprecipitations along with the single mutants.

RESULTS

Oligonucleotide-directed mutagenesis of Cys codons in the v-*sis* protein. The predicted v-*sis* protein is a 271-amino-acid protein, of which residues 112 to 220 are homologous to the B chain of PDGF. Previous studies from this laboratory have demonstrated that the transforming region of the v-*sis* protein is contained within residues 127 to 214, a region smaller than the PDGF B chain homologous region. Our previous work has also suggested a correlation between dimerization of the v-*sis* protein and biological activity, in that only mutants which were transforming also encoded proteins capable of dimerization (19, 33). There are eight Cys residues within residues 127 to 214. These eight Cys residues are Cys-127, Cys-154, Cys-160, Cys-163, Cys-164, Cys-171, Cys-208, and Cys-210 (Fig. 1). Using oligonucleotide-directed mutagenesis, we have changed each of the Cys codons, in turn, to a Ser codon. The oligonucleotides used are given in Table 1. They were designed such that a new restriction enzyme site was created simultaneously with the alteration of each Cys codon, allowing the mutants to be screened by restriction enzyme digests.

The mutants were subcloned from the M13-derived replicative form into a derivative of the SV40 expression vector pJC119 (34), and each mutant sequence was confirmed by nucleotide sequencing.

Retention of biological activity in four of the mutant v-*sis* proteins. To examine their biological activity, mutant genes

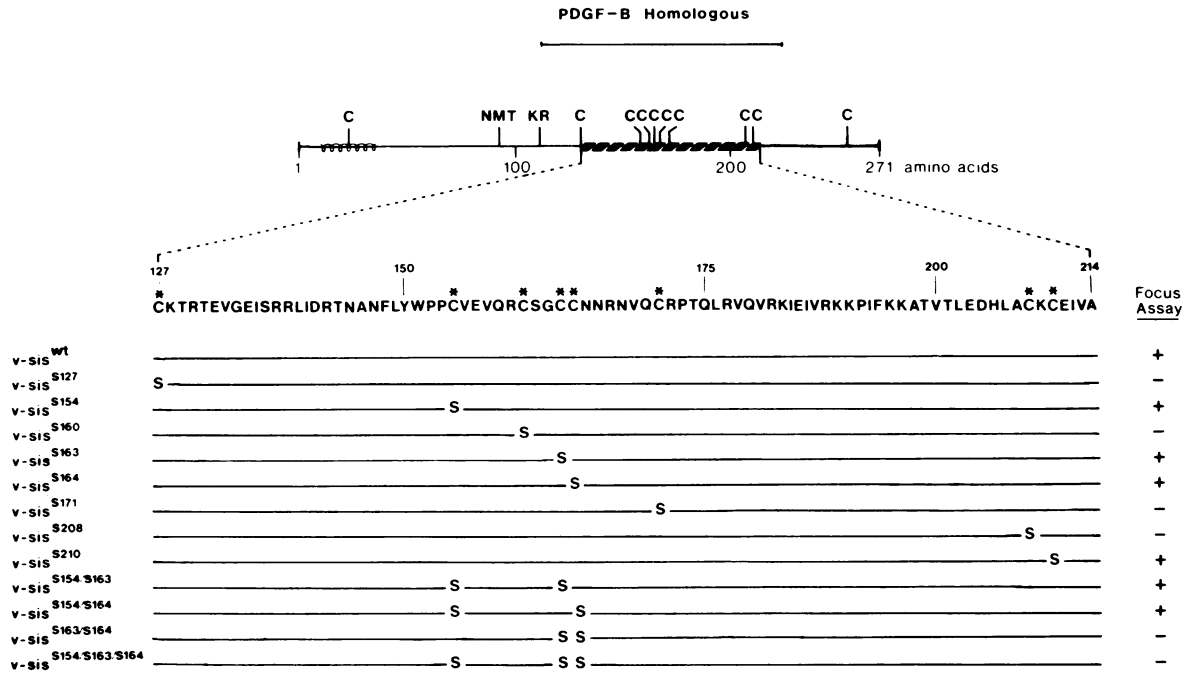

FIG. 1. Oligonucleotide-directed mutagenesis of Cys codons in the v-*sis* gene. A map of the v-*sis* gene product is given at the top. The predicted full-length polypeptide is 271 amino acids long. The Cys (C) residues are indicated, as are the N-linked glycosylation site Asn-Met-Thr (NMT), residues 93 to 95, and the proteolytic cleavage site Lys-Arg (KR), residues 110 and 111. (Note that the numbering begins with the initiating methionine of the predicted protein.) ▭, Signal sequence. The PDGF B chain corresponds to residues 112 to 220 of the v-*sis* protein. ▬, Region previously shown to be required for transformation, residues 127 through 214. Amino acids in this region are shown below, with Cys residues highlighted by asterisks. Below are given schematic maps of the v-*sis* wild-type and mutant proteins. All residues are identical, except for the substitution of Ser residues at the indicated positions. The mutants are named according to the position(s) of the newly created Ser residue(s). Focus assay results are given at the right: +, minimum value of 2.4×10^3 focus-forming units/pmol of transfected DNA; −, less than 1.0×10^1 focus-forming units/pmol.

were subcloned from the SV40-derived expression vector into a M-MuLV-derived expression vector, pDD102 (3), and assayed for focus formation in NIH 3T3 cells. The M-MuLV *gag* and *pol* genes have been deleted in pDD102, and most of the *env* gene has been replaced by a unique *Xho*I site into which foreign genes may be inserted. This vector retains the 5′ and 3′ splice sites, but the inserted gene must provide its own signal for initiation and termination of translation. Expression of foreign genes is through the *env* transcription pathway. A restriction fragment encompassing the entire v-*sis* gene from each of the mutants was inserted into the *Xho*I site of pDD102, and the correct orientation of the inserted gene was confirmed. Each resultant DNA was transfected into NIH 3T3 cells, along with a replication-competent clone of M-MuLV, by the DNA-calcium phosphate coprecipitate method (1, 15). The results of the focus assays are given in Table 1 and Fig. 1. The single mutants, v-*sis*^{S127}, v-*sis*^{S160}, v-*sis*^{S171}, and v-*sis*^{S208}, had no detectable transformation activity. The other mutants, v-*sis*^{S154}, v-*sis*^{S163}, v-*sis*^{S164}, and v-*sis*^{S210}, exhibited biological activities comparable to that of the v-*sis*^{wt} gene. These results suggest that the PDGF-like v-*sis* protein may fold into an active conformation even in the absence of certain disulfide bonds.

The fact that Cys-208 is required for biological activity, but Cys-210 is not, suggested that residues 209 to 271 might be dispensable for transformation. To test this directly, we constructed a mutant v-*sis* gene containing the termination codon TAG in place of the wild-type codon for Lys-209 by ligation of a linker containing the termination codon at a conveniently located restriction site (see Materials and Methods). When we assayed this mutant v-*sis*^{TAG209} gene for biological activity by using an M-MuLV-derived expression vector, it was transforming with an efficiency of 5.7×10^2 focus-forming units/pmol (Table 1). This transformation efficiency was approximately 250-fold lower than that of the wild type, but was still significantly above background. This result demonstrates that residues 209 to 271 are, strictly speaking, dispensable for transformation, although they clearly contribute to either the stability or the receptor-binding properties of the biologically active v-*sis* protein. This also suggests that the minimal region of v-*sis* protein required for transformation consists of residues 127 to 208.

Analysis of proteins encoded by mutant v-*sis* genes. For the analysis of proteins encoded by mutant v-*sis* genes, we examined mutants which affect Cys residues within the minimal transforming region, residues 127 to 208. For this analysis, we performed transient expression assays with SV40-derived expression plasmids. The plasmids were derived from pJC119, which has a deleted copy of the SV40 genome in a pBR322 derivative (34). This vector contains an SV40 origin of replication, but most of the VP1 open reading frame has been replaced by a unique *Xho*I site. It retains the late promoter, the 5′ and 3′ splice sites, and the polyadenylation site, such that foreign genes inserted at the *Xho*I site are expressed by using the late transcription pathway. The parental clone, pMH29, consists of the v-*sis*^{wt} gene in the *Xho*I site of pJC119 in the correct orientation for expression. The mutant genes were subcloned into the SV40-derived vector by swapping restriction fragments from each mutant in the M13-derived replicative form with the corresponding

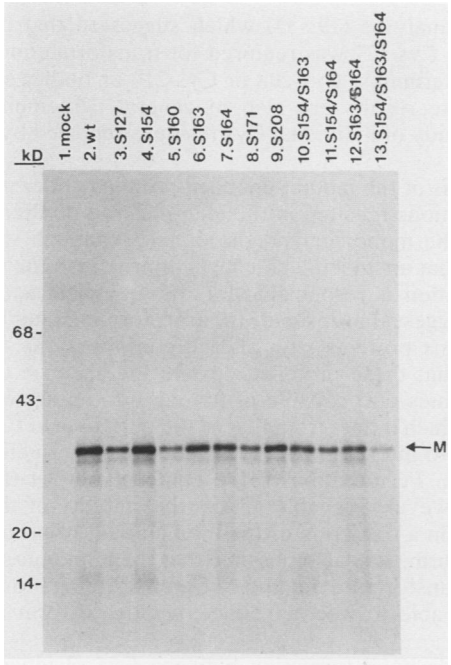

FIG. 2. Reducing SDS-PAGE analysis of v-*sis* proteins immunoprecipitated with anti-p28^{sis} serum. Cells were transfected either with the vector pJC119 alone (mock) or with the v-*sis* gene inserted into the expression vector as described in the text. Lysates were prepared in the presence of 100 μM iodoacetamide. The samples were analyzed in the presence of 2-mercaptoethanol as described in the text. Lanes: 1, mock; 2, wild type (wt); 3, v-*sis*^{S127}; 4, v-*sis*^{S154}; 5, v-*sis*^{S160}; 6, v-*sis*^{S163}; 7, v-*sis*^{S164}; 8, v-*sis*^{S171}; 9, v-*sis*^{S208}; 10, v-*sis*$^{S154/S163}$; 11, v-*sis*$^{S154/S164}$; 12, v-*sis*$^{S163/S164}$; 13, v-*sis*$^{S154/S163/S164}$. The lanes with samples from the mutant genes are labeled with the number of the newly created Ser residue. M indicates the reduced monomers at 32 kDa. Molecular mass markers are given at the left.

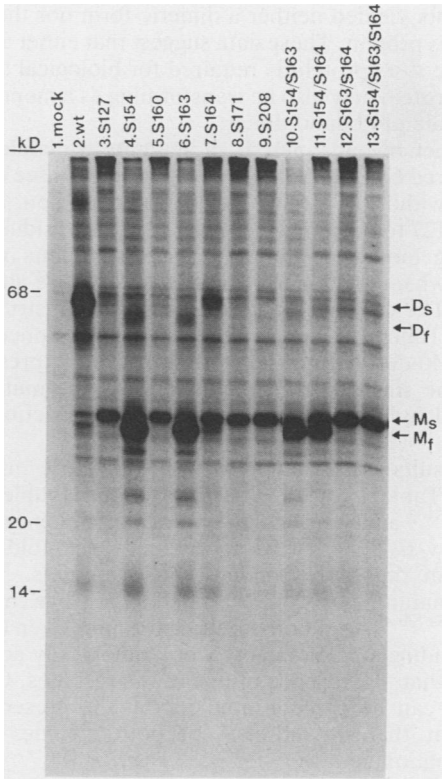

FIG. 3. Nonreducing SDS-PAGE analysis of v-*sis* proteins immunoprecipitated with anti-p28^{sis} serum. Aliquots of the immunoprecipitates described in the legend to Fig. 2 were analyzed in sample buffer without 2-mercaptoethanol. Lanes: 1, mock; 2, wild type (wt); 3, v-*sis*^{S127}; 4, v-*sis*^{S154}; 5, v-*sis*^{S160}; 6, v-*sis*^{S163}; 7, v-*sis*^{S164}; 8, v-*sis*^{S171}; 9, v-*sis*^{S208}; 10, v-*sis*$^{S154/S163}$; 11, v-*sis*$^{S154/S164}$; 12, v-*sis*$^{S163/S164}$; 13, v-*sis*$^{S154/S163/S164}$. The lanes with samples from the mutant genes are labeled with the number of the newly created Ser residue. M_f indicates the fast monomer with apparent molecular mass 30 kDa. M_s indicates the slow monomer with apparent molecular mass of 32 kDa. D_f indicates the fast dimer with apparent molecular mass of 52 kDa. D_s indicates the slow dimer with apparent molecular mass of 56 kDa. Molecular mass markers are at the left.

fragment of the wild-type gene in pMH29. The plasmids were transfected into Cos-1 cells, and at 48 h posttransfection the cells were metabolically labeled. Cell lysates were immunoprecipitated with antiserum raised against bacterially synthesized p28^{sis}, and the immunoprecipitated proteins were analyzed by SDS polyacrylamide gel electrophoresis (PAGE) under reducing conditions. Cells transfected with the v-*sis*^{wt} gene, as well as those transfected with the mutant v-*sis* genes, expressed a 32-kDa protein (Fig. 2, lanes 2 to 9). This has been designated the M (monomer) form.

When analyzed under nonreducing conditions, most of the v-*sis*^{wt} protein migrated as a dimer of about 56 kDa (Fig. 3, lane 2). The transforming single mutants, v-*sis*^{S154}, v-*sis*^{S163}, and v-*sis*^{S164}, also showed evidence of dimer formation (Fig. 3, lanes 4, 6, and 7). In contrast, none of the nontransforming mutant proteins showed a significant level of the dimeric form (Fig. 3, lanes 3, 5, 8, and 9). Two electrophoretic forms of dimer were noted, designated as D_f (dimer, fast) and D_s (dimer, slow). The mutants v-*sis*^{S154} and v-*sis*^{S163} gave rise to detectable amounts of the D_f protein (Fig. 3, lanes 4 and 6), whereas v-*sis*^{S164} gave rise to the D_s protein which comigrated with the wild type dimer (Fig. 3, lane 7). These results are generally consistent with earlier work indicating the correlation of dimerization of the v-*sis* proteins and transforming ability of the v-*sis* mutants (19, 24, 33).

All of the mutants also yielded significant amounts of protein migrating as monomers of approximately 32 kDa. This is probably due to altered stability of the proteins, resulting in a reduction of the proportion of total protein in the dimeric form. However, the possibility that an active protein exists as a monomer, or as a noncovalently bonded dimer, cannot be excluded at this time. Two different electrophoretic forms of the monomer were noted. These are designated M_f (monomer, fast) and M_s (monomer, slow). It should be emphasized that these proteins were from the same immunoprecipitated lysates as were used for the SDS-PAGE under reducing conditions (Fig. 2) and therefore represent the same protein existing in two different conformations. All of the nontransforming proteins were in the M_s form (Fig. 3, lanes 3, 5, 8, and 9). However, the transforming mutants v-*sis*^{S154} and v-*sis*^{S163} gave rise to the M_f form of the monomer (Fig. 3, lanes 4 and 6). Thus, the v-*sis* protein may exist in an altered conformation, yet still be biologically active. The third transforming mutant, v-*sis*^{S164}, also gave rise to some of the M_f protein (Fig. 3, lane 7), suggesting an identical conformation as the v-*sis*^{S154}- or v-*sis*^{S163}-encoded proteins. However, most of the v-*sis*^{S164} protein migrates as the M_s conformation. A small amount of both the M_f and M_s forms of the v-*sis*^{wt} protein also could be detected under nonreducing conditions (Fig. 3, lane 2). Unlike the wild type and the transforming mutants, however, the nontransform-

ing mutants yielded neither a dimeric form nor the M_f form of the v-*sis* protein. These data suggest that either a low level of dimeric v-*sis* protein is required for biological activity or that the protein may also be transforming as a monomer or as a noncovalently bound dimer.

Construction and analysis of mutants containing two or three altered Cys residues. As shown above, three of the Cys residues within the minimal transforming region, defined as residues 127 to 208, can be replaced by Ser residues without abolishing biological activity. Further mutants were constructed which contained two or three of those altered sites (Fig. 1). The oligonucleotides used in the constructions are given in Table 1. These mutants were subcloned into the same SV40-derived and M-MuLV-derived expression vectors as the single mutants were, and all the mutants were confirmed by sequencing as well as by restriction enzyme analysis.

The results of the focus assays with these mutants are given in Table 1 and Fig. 1. Two of the double mutants, v-$sis^{S154/S163}$ and v-$sis^{S154/S164}$, were still biologically active, although v-$sis^{S154/S164}$ had approximately 20-fold-lower activity than the corresponding single mutants. The other double mutant, v-$sis^{S163/S164}$, and the triple mutant, v-$sis^{S154/S163/S164}$, were both nontransforming, even though the corresponding single mutants were biologically active. This suggests that the absence of one of the residues, Cys-163 or Cys-164, can be compensated for by the presence of the other, but that the absence of both residues abolishes transformation activity.

When the proteins encoded by the mutants were analyzed under reducing conditions, they exhibited a mobility identical to that of the wild-type protein, M form (Fig. 2, lanes 10 to 13). However, under nonreducing conditions the majority of the protein encoded by the v-$sis^{S154/S163}$ gene is of the M_f conformation (Fig. 3, lane 10), previously observed for the single mutants v-sis^{S154} and v-sis^{S163}. This suggests that the absence of one or both of Cys-154 and Cys-163 results in a protein with the same active, although non-wild-type, conformation. The v-$sis^{S154/S164}$ double mutant is unique in that the two forms of the monomer, M_s and M_f, occur approximately equally (Fig. 3, lane 11). Once again, the extremely low level of dimeric protein observed raises the possibility that the monomeric form of the protein may be active alone or may be able to dimerize noncovalently. The monomers encoded by the nontransforming double and triple mutants, v-$sis^{S163/S164}$ and v-$sis^{S154/S163/S164}$, are of the M_s conformation (Fig. 3, lanes 12 and 13).

DISCUSSION

In the work described in this report we used oligonucleotide-directed mutagenesis to probe the importance of individual Cys residues within the v-*sis* protein. Mutant genes, encoding Ser residues in place of Cys residues, were assayed for focus formation in NIH 3T3 cells by using an M-MuLV-derived expression vector. Mutants which encode Ser residues in place of Cys-127, Cys-160, Cys-171, or Cys-208 were nontransforming, whereas mutants which encode Ser residues in place of Cys-154, Cys-163, Cys-164, or Cys-210 were transforming. Two double mutants which encode Ser residues in place of both Cys-154 and Cys-163 or Cys-154 and Cys-164 retained their biological activity. However, the double mutant which encodes Ser residues in place of Cys-163 and Cys-164 was nontransforming, as was the triple mutant which encodes Ser residues for Cys-154, Cys-163, and Cys-164. These results are consistent with previous deletion analyses (19, 33) which suggested that at the N-terminus, Cys-127 was required for transformation and that at the C-terminus, Cys-208 or Cys-210 or both were essential. These results are also in general agreement with a similar study of individual Cys residues reported by Giese et al. (14).

Analysis of the mutant-encoded proteins under nonreducing conditions resulted in the identification of altered forms of both the monomer and the dimer. Although we cannot rule out that an undetectable level of protein in the wild-type conformation is responsible for the biological activity, we would suggest that instead, the transforming activity is due to the v-*sis* proteins with altered conformations. The data suggest that these proteins, despite the absence of certain Cys residues, are capable of folding into an active conformation which differs from that of the normal wild type, yet is still able to interact with the PDGF receptor to elicit transformation. Perturbation of the conformation of the monomers, however, probably affects the stability of the dimer, resulting in a reduction of the total amount of protein in the dimeric form. It is also possible that the monomers encoded by the transforming mutants are transforming alone or that they are able to interact noncovalently to form an active complex.

Disulfide bond formation between Cys residues is often important for protein folding into an active tertiary conformation, as well as for covalently stabilizing a quaternary structure. Many proteins depend on disulfide bonds for stability, and their reduced forms are unfolded (5, 6). Precedent exists for the dispensability of some disulfide bonds, as exemplified by the serine protease family. Different members have between two and six disulfide bonds, yet their three-dimensional conformations are very similar (8). Disulfide bond formation in secretory proteins occurs as part of the secretory pathway through the endoplasmic reticulum and appears to be catalyzed by the microsomal enzyme protein disulfide isomerase (7). Disulfide bond formation and protein folding occur concomitantly and interdependently, and certain disulfide bonds may occur only transiently in intermediates during the folding pathway, as has been shown for bovine pancreatic trypsin inhibitor (5, 6).

Disulfide bonds which are dispensable probably form late during folding when the protein is already in a stable conformation close to that of the native protein. The protein must be able to adopt and retain an active conformation without the stabilization brought about by those disulfide bonds. If two Cys residues are involved in a nonessential disulfide bond with each other, then either or both residues should be dispensable. The mutants v-sis^{S154}, v-sis^{S163}, and v-$sis^{S154/S163}$ are all transforming with an efficiency equal to that of v-sis^{wt}. Moreover, the proteins they encode exhibited the same altered mobility when analyzed by SDS-PAGE under nonreducing conditions. The identical phenotypes exhibited by these mutants strongly suggest that Cys-154 is disulfide bonded to Cys-163. Thus, either or both residues can be altered to Ser, yielding proteins which are still in an active, although altered, conformation.

Both Cys-164 and Cys-210 appear to be dispensable for transformation. This suggests that Cys-164 and Cys-210 may participate in a nonessential disulfide bond, which could be either intramolecular or intermolecular. Another possibility, however, would be the existence of Cys-164–Cys-164 and Cys-210–Cys-210 intermolecular bonds in the wild-type dimeric protein. Unfortunately, resolution of this issue must await further examination of the disulfide bonds by peptide mapping techniques. The dispensability of Cys-210 indicates

that the minimal region required for transformation should be considered to be residues 127 to 208. Consistent with this, a mutant with a TAG termination codon at position 209 was biologically active in transformation assays, although at a significantly lower level than the v-*sis*^{S210} mutant. The reason for the difference in biological activity between v-*sis*^{TAG209} and v-*sis*^{S210} is not clear, but suggests that the disulfide bond involving Cys-208 does not form efficiently when this residue is the C-terminal residue of the polypeptide chain.

If Cys-154 and Cys-163 participate in a single disulfide bond, as suggested above, then mutation of either of these residues should be independent of further mutation at Cys-164. Surprisingly, however, the mutant v-*sis*$^{S154/S164}$ is transforming, whereas the mutants v-*sis*$^{S163/S164}$ and v-*sis*$^{S154/S163/S164}$ are not. These data suggest that Cys-163 or Cys-164, but not both, must be present for biological activity. As above, the explanation of this observation must await a complete description of the disulfide bonds by peptide mapping techniques.

Robson et al. (31) used computer simulations to predict the conformation of the v-*sis* protein. According to their predictions, intramolecular disulfide bonds occur either between the pairs Cys-127–Cys-171, Cys-160–Cys-163, and Cys-164–Cys-208, or between Cys-127–Cys-160, Cys-154–Cys-171, and Cys-164–Cys-208. Our results do not agree with these models. We find that Cys-163 is dispensable, but Cys-160 is not. Similarly, Cys-164 is nonessential, whereas Cys-208 is essential, and Cys-154 is nonessential, but Cys-171 is required. The only explanation possible to correlate our data with their predictions is to suggest that those indispensable residues are involved in essential transient disulfide bonds which differ from the final predicted pairs. The incompatability of our results with the structure predicted by Robson et al. suggests that three-dimensional structures based on computer-simulated algorithms for protein folding should be viewed tentatively in the absence of confirming biochemical evidence.

Our results demonstrate that Cys-127, Cys-160, Cys-171, and Cys-208 are all essential. They may be involved in transient disulfide bonds, or they may be necessary to hold the mature protein in an active conformation. In the absence of any of these disulfide bonds, the protein is likely to be in an unfolded conformation. Consistent with this, we would suggest that the M_s electrophoretic form, exhibited by their gene products under nonreducing conditions, corresponds to the random-coil form. An unfolded protein should exist in a random-coil formation with a larger radius of gyration than a globular protein, resulting in a slower mobility.

We have shown that four of the Cys residues in the v-*sis* protein are dispensable for biological activity. It should be noted that the elimination of any of these Cys residues drastically reduces the formation of dimeric v-*sis* protein, raising the possibility that monomeric forms of mutant v-*sis* proteins may be biologically active. Our data clearly suggest a nonessential Cys-154–Cys-163 disulfide bond, whose elimination results in v-*sis* proteins of altered conformation. In future experiments, the mutants described here will be used in conjunction with peptide mapping to find exactly which Cys residues are disulfide bonded to each other and whether these bonds are intramolecular or intermolecular.

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