effects resulting from dysregulated growth factor production in vivo.

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22. We would like to thank T. Deuel for suggesting the use of the KDEL signal in our experiments and D. Bodine for many helpful discussions.

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Transformation by v-*sis* Occurs by an Internal Autoactivation Mechanism

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Transformation by the v-*sis* oncogene appears to require an interaction of its protein product, p28$^{v\text{-}sis}$, with the receptor for the platelet-derived growth factor (PDGF). However, this interaction may not occur at the cell surface as predicted by the autocrine hypothesis because phenotypic transformation was not reversed by incubation of SSV-NRK cells with antisera to PDGF and because morphological transformation did not occur when nontransformed NRK cells were cultured continuously with p28$^{v\text{-}sis}$. A mutant of the wild-type v-*sis* gene was constructed that encodes a v-*sis* protein targeted for retention within the endoplasmic reticulum and Golgi. NRK cells expressing the mutant v-*sis* gene did not secrete any detectable v-*sis* protein but were as fully transformed as wild-type v-*sis* transfectants. The results support a mechanism of transformation by v-*sis* in which internal activation of the PDGF receptor occurs before expression of either p28$^{v\text{-}sis}$ or the PDGF receptor at the cell surface.

THE ONCOGENE OF THE SIMIAN SARcoma virus (SSV), v-*sis*, encodes a polypeptide that is 92% homologous to the nonglycosylated B chain of PDGF, a potent mitogen and chemoattractant for cells of mesenchymal origin (*1, 2*). According to the autocrine hypothesis (*3*), unregulated growth of transformed cells occurs through the secretion of a growth factor and its subsequent productive interaction with its own cell surface receptor. Evidence for an autocrine mechanism in SSV stimulation of cell growth was that SSV-transformed NIH 3T3 and NRK cells secrete a potent PDGF-like mitogen that interacted with the PDGF receptor (*2, 4*) and that [^{3}H]thymidine incorporation into DNA was reduced by 50% when SSV-NRK cells were grown in the presence of antisera to PDGF (*4*). However, the autocrine hypothesis was inadequate to fully explain the mechanism by which SSV induced transformation because addition of high concentrations of antiserum to PDGF to the medium of SSV-transformed cells did not reverse the transformed phenotype, and high levels of exogenous p28$^{v\text{-}sis}$ continuously added to the medium of nontransformed NRK cells did not induce morphological transformation. Furthermore, the SSV-transformed marmoset fibroblast cell line SSV-NP1 did not secrete detectable levels of growth factor, nor was the growth of SSV-NP1 cells inhibited by antisera to PDGF. These results led to the hypothesis that the mechanism of transformation by the v-*sis* gene product may involve activation of the PDGF receptor through an internal activation mechanism before the receptor is expressed at the cell surface (*4, 5*).

A COOH-terminal amino acid sequence causes the retention of soluble proteins within the endoplasmic reticulum and Golgi apparatus (*6*). This six–amino acid sequence Ser-Glu-Lys-Asp-Glu-Leu (SEKDEL) is specific, as a change in the last two amino acids Ser-Glu-Lys-Asp-Ala-Ser (SEKDAS) is sufficient to allow proteins to be secreted. We therefore cloned a synthetic DNA oligomer that encoded the SEKDEL retention sequence at the 3′ end of a truncated but fully active v-*sis* gene. NRK fibroblasts transfected with the v-*sis* gene containing the SEKDEL retention sequence (v-*sis* SEKDEL) were compared to cells transfected with constructs that secreted p28$^{v\text{-}sis}$ for their transforming capabilities.

Clones pSEB3, pSEB12, and pSEB34 (Fig. 1) (*7*), as well as the vector pSVFVXM, were integrated into the host chromosome at nearly identical rates, as determined by the number of G418-resistant colonies per microgram of DNA transfected (*8*). Although the pSVFVXM plasmid, which lacked the v-*sis* gene, was unable to induce foci of NRK cells, each of these plasmids that contained a v-*sis* gene construct readily and equally induced foci (Fig. 2). Similarly, cells that were transfected with each of the v-*sis* constructs had increased saturation densities at confluence, formed colonies in soft agarose, and established tumors in nude mice (Table 1). Thus, the v-*sis* SEKDEL mutant is fully transforming. RNA blot analyses and mitogenic assays of cell lysates established that all NRK cell lines containing the v-*sis* gene transcribed the v-*sis* gene at high levels, and each gene expressed a protein product that was mitogenically active (Table 1). The v-*sis* SEKDEL protein was also fully active when analyzed in fractions rich in endoplasmic reticulum and Golgi; nearly three times as much growth-promoting activity was observed per milligram of protein as was detected in preparations from v-*sis* SEKDAS. These results establish that the v-*sis* SEKDEL protein is an active mitogen and is highly enriched in

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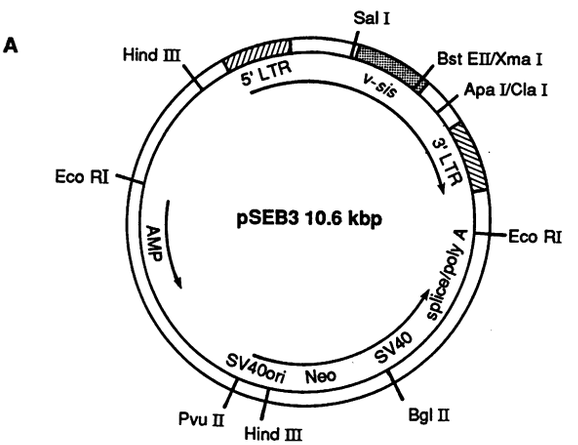
endoplasmic reticulum and Golgi fractions, as predicted by the retention sequence in the v-*sis* SEKDEL gene.

As it had been previously demonstrated that proteins containing the SEKDEL mutation may be secreted in very low levels (*6*), ^{35}S-labeled conditioned medium was prepared and immunoprecipitated with antisera to PDGF (*9*). Protamine sulfate, a polyionic compound that releases PDGF bound to the PDGF receptor or nonspecifically bound to the cell surface (*10*), was included to displace any v-*sis* protein potentially associated with the cell surface. Analysis of ^{35}S-labeled immunoprecipitates by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography (overnight exposure) showed a molecule of 33 kD in the medium of the positive control v-*sis* SEKDAS-expressing cells but not in conditioned medium of v-*sis* SEKDEL-expressing cells (Fig. 3). After additional exposure for 14 days (Fig. 3, bottom), the v-*sis* SEKDEL protein could not be detected. The 33-kD protein identified in v-*sis* SEKDAS-conditioned medium (Fig. 3) was competitively displaced from antisera by excess PDGF, and control cells transfected with the plasmid alone lacked detectable levels (*11*).

To estimate more quantitatively the level of v-*sis* SEKDAS protein detected and thus to obtain an estimate of sensitivity of the failure to detect v-*sis* SEKDEL, we analyzed medium from protamine sulfate-treated cell cultures by solid-phase immunoassay. No detectable levels of PDGF-like protein were found in medium from v-*sis* SEKDEL transfectants, whereas a PDGF-like protein (about 0.4 ng/ml) was detected in medium from v-*sis* SEKDAS-containing mutants. Conditioned medium was collected after a 3-day exposure to cells expressing the v-*sis* SEKDEL mutant and was concentrated (more than tenfold). No stimulation of incorporation of [^{3}H]thymidine into NRK cell DNA was detected, whereas v-*sis* SEKDAS medium was active (*12*). Therefore, the mutant v-*sis* SEKDEL protein was not delayed in secretion and did not, with time, achieve levels in conditioned medium sufficient to generate a mitogenic response. The results thus establish that the SEKDEL sequence is highly effective in retaining the mutant v-*sis* gene product within the endoplasmic reticulum or Golgi of NRK cells and in preventing its secretion at levels detectable by assays sensitive to much less than 0.1 ng/ml (~3 p*M*). Furthermore, by performing the assays with media conditioned by ^{35}S-labeled v-*sis* SEKDEL cells grown in the presence of protamine sulfate, it was possible to release any ^{35}S-labeled v-*sis* SEKDEL that may have been interacting with the PDGF receptor at the cell surface. No ^{35}S-labeled v-*sis* SEKDEL was found after cells were grown with protamine sulfate.

The first demonstrated and most clearly defined function for any oncogene product was the potent growth factor activity identified with the protein product of the v-*sis* gene (*2*). The autocrine mechanism (*3*) initially seemed to explain the mechanism of transformation of NRK cells by SSV when a PDGF-like growth factor was identified in medium from SSV-transformed cells and because [^{3}H]thymidine incorporation was blocked by ~50% when antiserum to PDGF was added to the culture medium (*4*). However, the autocrine mechanism



B

Mutant	Oligonucleotide sequence								
pSEB3	P CCG	TGA							
pSEB12	P CCG	A GCG	S AGT	E GAG	K AAG	D GAC	E GAG	L TTG	TGA
pSEB34	P CCG	A GCG	S AGT	E GAG	K AAG	D GAC	A GCC	S TCG	TGA
Wild-type v-*sis*	P CCG	G GGG	G GGT	S TCC	Q CAG	E GAG	Q CAG	R GAG	.....

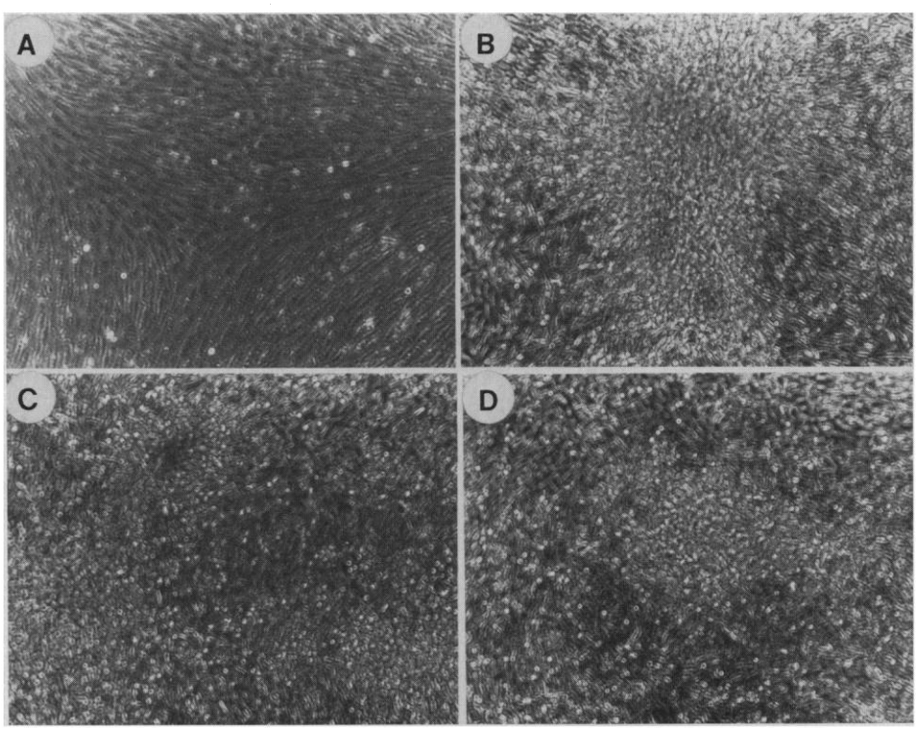


Fig. 2. Photomicrographs of the transfected cell lines. (**A**) Cells transfected with the expression vector pSVFVXM. (**B**) Cells transfected with the pSEB3 plasmid. (**C**) Cells transfected with pSEB34 that contains the SEKDAS mutation. (**D**) Cells transfected with pSEB12 that contains the SEKDEL mutation.

Table 1. Summary of characteristics of the cell lines used.

Plasmid used in transfection	Tumor formation*	Focus formation†	Colony formation†	Mitogenic activity‡	
				Secreted (ng/ml)	Cell lysate (dpm/μg protein)
pSVFVXM	0/10	−	−	ND	2
pSEB12	10/10	+	+	ND	127
pSEB34	10/10	+	+	~0.4	175

*The first number refers to the number of nude mice that formed tumors after being subcutaneously injected with 1×10^6 cells. The second number refers to the total number of mice injected. †The minus sign indicates that the cells transfected with this DNA could not form colonies in soft agarose. The plus sign indicates that colonies were detected. ‡ND, not detected. The cell lysate was measured per microgram of total protein (average of triplicate values).

could not account for the observations that the v-*sis* protein failed to induce phenotypic transformation when added regularly and in high concentrations to cultures of nontransformed NRK cells and that antiserum to PDGF did not reverse morphological transformation of stably transformed SSV-NRK cells. A possible mechanism to account for the failure of exogenous $p28^{v\text{-}sis}$ to induce morphological transformation of NRK cells may be the continued downregulation of the PDGF-receptor in the presence of $p28^{v\text{-}sis}$ Such a mechanism may preclude transformation by exogenous or secreted v-*sis* protein through the autocrine mechanism (*3*). However, the levels of PDGF receptors within the endoplasmic reticulum or Golgi in turn may be determined by the rates of synthesis and vesicular transport and thus this receptor population may lack the potential for downregulation until expressed at the cell surface. Our results indicate that the full signal transduction pathway and the substrates required for its activation are available to the activated PDGF receptor before it is expressed at the cell surface in v-*sis* SEKDEL-transformed cells.

Keating and Williams (*13*) and Huang and Huang (*14*) demonstrated that limited phosphorylation and apparent increased turnover of the PDGF receptor in SSV-transformed cells may occur within subcellular compartments. Although phosphorylation is not sufficient in itself to induce mitogenesis (*15*), these experiments support an internal activation mechanism of transformation. However, the earlier results and our present results are in apparent contrast to experiments (*16*) in which the ionophore monensin inhibited an increase in c-*fos* message induced by newly synthesized intracellular v-*sis*, suggesting that secretion may be required for transformation. However, monensin is believed to block processing in the proximal Golgi and has significant other effects on cellular function. Thus, interpretation of these results is difficult as it is with related experiments (*17*) in which the v-*sis* gene was expressed as a fusion product with the vesicular stomatitis virus G protein. The

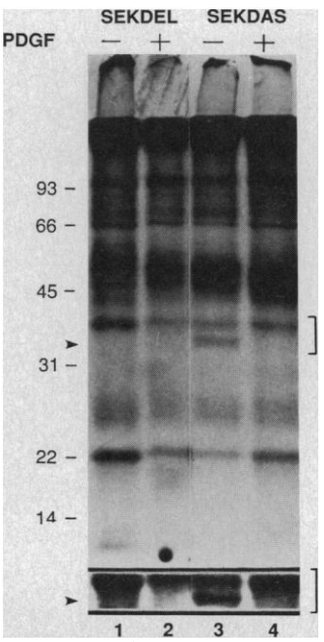


Fig. 3. Autoradiography of SDS-polyacrylamide gels of immunoprecipitates of conditioned media. Lanes marked with plus sign were immunoprecipitated in the presence of 1 μg of PDGF. Lanes 1 and 2 are immunoprecipitates of conditioned media from SEKDEL-expressing cells. Lanes 3 and 4 are immunoprecipitates of conditioned media from SEKDAS-expressing cells. The bracketed portion on the bottom is a portion from an autoradiograph exposed for 14 days. Arrowhead, 33-kD protein.

fusion protein dimerized but was not shown to functionally interact with the PDGF receptor. Although transformation appeared to occur only with fusion proteins expressed at the cell surface, it is possible that the v-*sis* protein interacted with receptors on neighboring cells or that the fusion protein was proteolytically processed to release v-*sis*.

Our experiments show that clones expressing a v-*sis* mutant that forced the retention of the v-*sis* gene product before expression of the cell surface induced transformation in NRK cells. These data, coupled with the failure of either exogenous v-*sis* protein to induce morphological transformation of NRK cells and of anti-PDGF antisera in media to reverse transformation in SSV-NRK cells directly support the internal activation hypothesis (*4*) and suggest that this internal activation mechanism may be the principal mechanism of transformation by v-*sis*.

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7. The plasmid pSEB3 was constructed by cloning the M-MuLV long terminal repeats as an Eco RI fragment from pFVXM into the Eco RI site of pSV2neo to create pSVFVXM. Mutant v-*sis* genes were cloned into the vector by digestion of the vector with Cla I, blunting of the ends with the Klenow fragment of DNA polymerase I, and redigestion with Sal I. The v-*sis* mutations were cloned as a Sal I–Xba I fragment that had its Xba I sites filled in with Klenow fragments. The mutation in pSEB3 was created by its digestion of the v-*sis* gene with Sma I and Bst EII. The digested DNA was treated with Klenow fragments before religation. The oligonucleotides that coded for the pSEB12 and pSEB34 mutations were cloned by digestion of v-*sis* with Xma I and Bst EII and ligation of the oligonucleotides. All mutations were sequenced either by the chemical degradation [A. M. Maxam and W. Gilbert, *Methods Enzymol.* **65**, 499 (1980)] or by the dideoxy method [F. Sanger, S. Nicklen, A. R. Coulson, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463 (1973)]. The mutations all occur three amino acids downstream from the threonine found as the COOH-terminal amino acid in the mature PDGF B chain.
8. Normal rat kidney fibroblasts (NRK 49F) were purchased from the American Type Culture Collection (ATCC). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) according to the recommendations of the ATCC. Transfections were performed essentially as described [F. L. Graham and A. J. Van der Eb, *Virology* **52**, 456 (1973)]. Briefly, cells were trypsinized and seeded at a density of 2×10^5 to 4×10^5 cells per 60-mm dish. After 16 to 24 hours, the medium was replaced with DMEM with 10% FCS and, after 3 hours, 0.5 ml of the transfection medium was added, which contained 137 m*M* NaCl, 10 m*M* KCl, 12 m*M* dextrose, 40 m*M* Hepes, 1.4 m*M* Na_2HPO_4, 134 m*M* $CaCl_2$, salmon sperm DNA (final concentration 20 μg/ml), and the appropriate plasmid DNA. This mixture was allowed to stand at room temperature for 20 min before it was added to the cells. After 3 to 4 hours, the medium was changed to DMEM with 10% FCS. At 16 to 24 hours after the last medium change, the cultures were finally placed into DMEM with 10% FCS along with 400 μg of geneticin sulfate per milliliter (G418; Gibco). Medium was then changed two to three times per week for 2 to 3 weeks. Cells were stained with Geimsa and counted or dispersed with trypsin, grown to confluency in DMEM with 10% FCS, and then grown for 2 to 3 weeks, in DMEM with 2% FCS.

9. To confluent 100 m*M* dishes, 5 ml of DMEM with 250 μCi of [^{35}S]cysteine per milliliter, with or without protamine sulfate (100 μg/ml), was added and allowed to incubate for 3 hours. Media were collected and centrifuged for 10 min at 4500*g*. Triton X-100 (1%), SDS (0.1%), sodium deoxycholate (0.5%), NaCl (200 m*M*), and phenylmethylsulfonyl fluoride (40 μg/ml) were added to the supernatant to the final concentrations indicated. Aliquots of 1 ml were used for immunoprecipitations. Antibody-antigen complexes were recovered with protein A Sepharose, and the beads were washed three times in 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 1*M* NaCl, and 10 m*M* Hepes (*p*H 7.4). Three secondary washes were performed in a low-salt buffer (200 m*M* NaCl) with the same detergent concentrations, as indicated, before samples were loaded onto a 12% nonreduced SDS-polyacrylamide gel [U. K. Laemmli, *Nature* **227**, 680 (1970)]. After electrophoresis the gels were stained with Coomassie G250 and treated with Amplify (Amersham) before drying and autoradiography.
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18. We thank F. Wong-Staal and M. Botchan for their gifts of pC60 and pFVXM, respectively. We also thank A. Thomason for performing the radioimmunoassay, M. Chu and B. B. Kennedy for their technical assistance, and B. Greene and D. Horn for their help in the preparation of this manuscript. Supported by NIH grants HL31102, HL14147, and CA49712 and by a grant from the Monsanto Company (T.F.D.), NIH training grant T32 HL07088 (D.Y.L.), and NIH National Research Service Award from the NCI-NRSA CA08229 (B.E.B.).

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Mechanism of Membrane Anchoring Affects Polarized Expression of Two Proteins in MDCK Cells

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The signals that direct membrane proteins to the apical or basolateral plasma membrane domains of polarized epithelial cells are not known. Several of the class of proteins anchored in the membrane by glycosyl-phosphatidylinositol (GPI) are expressed on the apical surface of such cells. However, it is not known whether the mechanism of membrane anchorage or the polypeptide sequence provides the sorting information. The conversion of the normally basolateral vesicular stomatitis virus glycoprotein (VSV G) to a GPI-anchored protein led to its apical expression. Conversely, replacement of the GPI anchor of placental alkaline phosphatase with the transmembrane and cytoplasmic domains of VSV G shifted its expression from the apical to the basolateral surface. Thus, the mechanism of membrane anchorage can determine the sorting of proteins to the apical or basolateral surface, and the GPI anchor itself may provide an apical transport signal.

CELLS IN EPITHELIAL TISSUE ARE joined by tight junctions (*1*). These junctions encircle each cell, dividing the surface into apical and basolateral domains. The two membrane regions contain different sets of proteins, which are not free to intermix. Madin-Darby canine kidney (MDCK) cells, which form polarized monolayers at confluency, are a useful model system for studying the sorting of membrane proteins [reviewed in (*1*)]. In MDCK cells, newly synthesized proteins destined for each surface are delivered there directly, without passage through the other membrane (*2*). Proteins are present in the same membrane compartment as far as the late Golgi (*3*) and are probably sorted from each other in the trans Golgi network (*4*). The location of sorting signals in membrane proteins is controversial. Several studies with truncated and hybrid proteins suggest a role for the extracellular domain in sorting. However, some work suggests that the cytoplasmic domain can also contain sorting signals [reviewed in (*5*)]. Interpretation of all these experiments is complicated by the fact that the default pathway for membrane proteins without sorting signals is not known.

A class of proteins, the members of which are anchored in the plasma membrane by a glycosyl-phosphatidylinositol (GPI) moiety instead of a classical proteinaceous transmembrane domain, has been described (*6*). These GPI-anchored proteins are synthesized with hydrophobic COOH-terminal domains that are removed almost immediately after translation. The new COOH-terminal amino acid is then covalently linked to presynthesized GPI (*7*).

The expression of several GPI-linked proteins in MDCK cells has been examined (*8*) by a procedure that should detect any such proteins on either the apical or basolateral surface. Though several GPI-anchored proteins were found on the apical surface, none could be detected on the basolateral membrane. Thus, it is possible that GPI itself might act as an apical sorting signal, although it cannot be the only signal for apical transport, as not all proteins on this surface

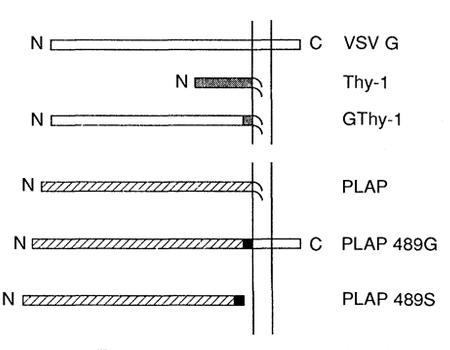

Fig. 1. Schematic diagram of the proteins used in this study. GThy-1 (*9*) contains amino acids 1 to 463 (the extracellular domain) of VSV G and amino acids 110 to 131 and the GPI moiety from Thy-1, assuming that cleavage and addition of GPI occur at Cys^{131} as in Thy-1. For expression in strain II MDCK cells, the GThy-1 cDNA was cloned into the unique Xho I site of pRSVΔSX (*11*). VSV G was expressed from pSV2*neo*-SVGΔSX (*11*). The hybrid protein PLAP 489G contains the transmembrane and cytoplasmic domains of VSV G attached to the extracellular domain of PLAP (*16*). PLAP 489S (*18*) is a truncated form of PLAP that terminates five amino acids COOH-terminal to the normal site of cleavage. It is secreted and does not acquire GPI; it is not known whether the five COOH-terminal amino acids are removed. All constructs were transfected as described (*11*). All plasmids except pSV2*neo*SVGΔSX were coexpressed with pSV2*neo* (*27*). G418-resistant clones were screened for high expression of transfected proteins by metabolic labeling and immunoprecipitation with antisera to VSV or PLAP. Open boxes, VSV G sequence; stippled boxes, Thy-1 sequence; hatched boxes, mature PLAP sequence; solid boxes, PLAP sequence normally removed after addition of GPI (five residues) and linker-encoded sequence (three residues) (*16*). GPI anchorage is symbolized by two short curved lines.

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