The v-sis Protein Retains Biological Activity as a Type II Membrane Protein When Anchored by Various Signal–Anchor Domains, Including the Hydrophobic Domain of the Bovine Papilloma Virus E5 Oncoprotein

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Abstract. Membrane-anchored forms of the v-sis oncoprotein have been previously described which are oriented as type I transmembrane proteins and which efficiently induce autocrine transformation. Several examples of naturally occurring membrane-anchored growth factors have been identified, but all exhibit a type I orientation. In this work, we wished to construct and characterize membrane-anchored growth factors with a type II orientation. These experiments were designed to determine whether type II membrane-anchored growth factors would in fact exhibit biological activity. Additionally, we wished to determine whether the hydrophobic domain of the E5 oncoprotein of bovine papilloma virus (BPV) can function as a signal-anchor domain to direct type II membrane insertion.

Type II derivatives of the v-sis oncoprotein were constructed, with the NH_2 terminus intracellular and the COOH terminus extracellular, by substituting the NH_2 terminal signal sequence with the signal–anchor domain of a known type II membrane protein. The signal–anchor domains of neuraminidase (NA), asialoglycoprotein receptor (ASGPR) and transferrin receptor (TR) all yielded biologically active type II derivatives of the v-sis oncoprotein. Although transforming all of the type II signal/anchor-sis proteins exhibited a very short half-life. The short half-life exhibited by the signal/anchor-sis constructs suggests that, in some cases, cellular transformation may result from the synthesis of growth factors so labile that they activate undetectable autocrine loops.

The E5 oncoprotein encoded by BPV exhibits amino acid sequence similarity with PDGF, activates the PDGF β-receptor, and thus resembles a miniature membrane-anchored growth factor with a putative type II orientation. The hydrophobic domain of the E5 oncoprotein, when substituted in place of the signal sequence of v-sis, was indistinguishable compared with the signal-anchor domains of NA, TR, and ASGPR, demonstrating its ability to function as a signal–anchor domain. NIH 3T3 cells transformed by the signal/anchor-sis constructs exhibited morphological reversion upon treatment with suramin, indicating a requirement for ligand/receptor interactions in a suramin-sensitive compartment, most likely the cell surface. In contrast, NIH 3T3 cells transformed by the E5 oncoprotein did not exhibit morphological reversion in response to suramin.

T HE v-sis oncoprotein, closely related to the B chain of PDGF, is synthesized with an NH_2-terminal signal sequence to direct its translocation across the membrane of the RER and into the secretory pathway (Hannink and Donoghue, 1984). The v-sis oncoprotein and PDGF are usually viewed as examples of secreted growth factors which are released from cellular membranes prior to binding and activation of receptors. This view remains fundamentally unchanged by the recent observation that some forms of PDGF contain a basic amino acid sequence leading to association with the extracellular matrix (LaRochelle et al., 1991; Raines and Ross, 1992), which may restrict their ability to diffuse to other cells.

PDGF belongs to a larger group of growth factors defined, in part, by homology among the receptors which they activate. The PDGF receptors, including the α receptor (Matsui et al., 1989) and the β receptor (Yarden et al., 1986), exhibit a "split-kinase" domain and define a family of receptors which includes: the stem cell factor (SCF)[1] receptor or c-kit (Qiu et al., 1988); the colony stimulating factor-1 (CSF-

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1. *Abbreviations used in this paper:* ASGPR, asialoglycoprotein receptor; BPV, bovine papilloma virus; CSF-1, colony stimulating factor-1; MLV, Moloney murine leukemia virus; NA, neuraminidase; RSV, Rous sarcoma virus; SCF, stem cell factor; TGF-α, transforming growth factor-α; TR, transferrin receptor; VSV-G, vesicular stomatitis virus glycoprotein.

1) receptor or c-*fms* (Coussens et al., 1986); and the vascular endothelial growth factor (VEGF) receptor or c-*flt* (De Vries et al., 1992; Shibuya et al., 1990). Several of the growth factors for the "split-kinase" receptors are synthesized as membrane-anchored precursors. For instance, the growth factor CSF-1 is synthesized as two different membrane-anchored precursors which are released by proteolysis (Kawasaki et al., 1985; Rettenmier and Roussel, 1988; Wong et al., 1987). The ligand of the c-*kit* receptor, referred to variously as SCF, mast cell growth factor, or *steel* factor, is also synthesized as a membrane-anchored precursor which undergoes rapid proteolysis to release mature SCF (Anderson et al., 1990; Huang et al., 1990; Martin et al., 1990). In these cases, the membrane-anchored precursors are type I proteins in which the NH_2 terminus is topologically extracellular and the COOH terminus remains within the cytoplasm ("N-out, C-in"). This orientation requires the presence of a conventional signal sequence located near the NH_2 terminus of the nascent polypeptide, which is removed during translocation across the membrane, coupled with a stop–transfer domain or membrane anchor located near the COOH terminus.

Although naturally occurring type I membrane-anchored forms of PDGF have not been identified, our laboratory has designed and described such constructs previously (Hannink and Donoghue, 1986a; Lee and Donoghue, 1991, 1992), using the membrane anchor of the vesicular stomatitis virus glycoprotein (VSV-G) to provide for membrane anchoring (Rose and Gallione, 1981; Adams and Rose, 1985; Guan et al., 1985). These prior studies demonstrated that membrane-anchored v-*sis*-G can still induce autocrine transformation (Hannink and Donoghue, 1986a), although its ability to induce PDGF receptor autophosphorylation is significantly reduced (Lee and Donoghue, 1991).

Petti and DiMaio (1992) demonstrated that the E5 oncoprotein, encoded by bovine papillomavirus (BPV), can be recovered in a complex with activated PDGF β receptors. The E5 oncoprotein is unusual due to its small size, only 44 amino acids, with a very hydrophobic NH_2-terminal region and a hydrophilic COOH-terminal region (Horwitz et al., 1988, 1989). Petti et al. (1991) first noted slight amino acid similarity between E5 and PDGF-B. The region of similarity includes the last two Cys residues of the minimal transforming region of v-*sis*, previously identified by deletion analysis (Giese et al., 1987; Sauer and Donoghue, 1988). In addition, the tripeptide $F^{148}L^{149}V^{150}$ in a putative receptor activating domain of v-*sis* (LaRochelle et al., 1989), also occurs in the E5 oncoprotein (Maher et al., 1993).

Previous studies localized the E5 oncoprotein predominantly to Golgi membranes and/or the cell surface, with the COOH terminus topologically extracellular (Burkhardt et al., 1989). The presence of an NH_2-terminal hydrophobic domain, together with a COOH-terminal hydrophilic domain exhibiting amino acid similarity to PDGF, suggests that E5 may function as a "miniature" membrane-anchored version of PDGF. By this model, E5 would exhibit a type II orientation, i.e., "N-in, C-out," allowing the COOH-terminal hydrophilic domain to be extracellular and available for PDGF receptor activation.

Several type II membrane-anchored proteins have been extensively characterized, including neuraminidase (NA) (Fields et al., 1982; Sivasubramanian and Nayak, 1987;

Brown et al., 1988; Nayak and Jabbar, 1989; Kundu et al., 1991), asialoglycoprotein receptor (ASGPR) (Spiess et al., 1985; Spiess and Lodish, 1985, 1986), and transferrin receptor (TR) (Schneider et al., 1984; Zerial et al., 1986; Kundu et al., 1991). These proteins possess a "signal–anchor" sequence located near the NH_2 terminus. The signal–anchor sequence provides the dual function of a signal sequence, directing translocation across the membrane of the rough ER, as well as a membrane anchor, resulting in the topology "N-in, C-out" (Hartmann et al., 1989; High et al., 1991).

In this study, we sought to determine whether the v-*sis* oncoprotein retains its biological activity when membrane anchored as a type II protein. To accomplish this, the DNA sequence encoding the signal sequence of v-*sis* was replaced with a sequence encoding the signal–anchor domain of a known type II membrane protein. We also wished to examine whether the hydrophobic domain of the E5 oncoprotein, when substituted in place of the normal signal sequence of the v-*sis* oncoprotein, could in fact function as a signal–anchor domain.

Our results indicate that the signal-anchor domains of NA, ASGPR and TR, as well as the hydrophobic domain of E5, all yield biologically active type II membrane-anchored derivatives of the v-*sis* oncoprotein. Although transforming, all of the type II signal/anchor-*sis* derivatives exhibited rapid turnover. These results indicate that there is, in principle, no reason to preclude the existence of naturally occurring membrane-anchored growth factors exhibiting a type II orientation.

Materials and Methods

Construction of Plasmids Encoding Signal/Anchor-Sis Fusions

The signal/anchor-*sis* constructs were derived by substitution of the v-*sis* signal sequence by a heterologous signal–anchor domain. The parental plasmid contained a mutant v-*sis* gene in which the dibasic proteolytic processing site Lys^{110}-Arg^{111} was previously mutagenized to Asn^{110}-Ser^{111} (Hannink and Donoghue, 1986b). Cleavage at this dibasic processing site occurs as a late event in the secretory pathway, probably between the *trans*-Golgi compartment and the plasma membrane (Robbins et al., 1985; Lokeshwar et al., 1990; Lee and Donoghue, 1992). This cleavage removes the propeptide sequence and generates the NH_2 terminus of the mature PDGF. To prevent proteolytic separation of the growth factor domain from the signal-anchor domain in the constructs reported here, it was essential to include the $Lys^{110}\rightarrow Asn$, $Arg^{111}\rightarrow Ser$ mutations in all clones. The parental plasmid, designated pRSV-*sis*^{N110S111}, contains the mutant v-*sis* gene as a HindIII-ClaI restriction fragment in a standard plasmid vector under control of the Rous sarcoma virus (RSV) promoter. The original pRSV-A_n plasmid (obtained from S. Gould and S. Subramani, University of California, San Diego, La Jolla, CA) contains the RSV long terminal repeat to drive transcription of inserted genes followed by the SV-40 poly A addition site.

The DNA sequence encoding the signal sequence of v-*sis* is easily removed from the parental plasmid pRSV-*sis*^{N110S111} as a HindIII-SstI restriction fragment, where the SstI site corresponds to nucleotide 3828 in the sequence of simian sarcoma virus (Devare et al., 1983). Removal of this restriction fragment removes the codons for amino acids 1-59 of wild type v-*sis* protein. Synthetic restriction fragments encoding heterologous signal–anchor domains were prepared using two long complementary oligonucleotides, designed to produce HindIII and SstI overhangs when annealed. The oligonucleotides ranged in length from 83-124 bases, and were prepared using a DNA synthesizer (381A; Applied Biosystems, Foster City, CA) with customized coupling times, reagent delivery times, and column configurations to reduce cost and synthesis time. Approximately 25 μg of each crude oligonucleotide was applied to a 6% denaturing polyacrylamide gel, separated electrophoretically, and the band corresponding to each full-length oligonucleotide was excised. Oligonucleotides were recovered by

overnight elution into 0.75 ml of elution buffer (containing 0.1% SDS, 0.5 M NH₄Ac, 10 mM MgAc₂), and ethanol precipitation. Resuspended oligonucleotides were then ligated with 0.15 μg of the vector DNA, pRSV-sis^N110s111, which was previously cleaved with HindIII and SstI and purified by agarose gel electrophoresis. Recombinant clones were recovered by standard techniques and the sequence of the signal–anchor domains encoded by the HindIII–SstI fragments were confirmed by nucleotide sequencing. All synthetic oligonucleotides also encoded a XhoI restriction site adjacent to the HindIII site, so that the entirety of the coding region for each signal/anchor-*sis* construct could be swapped into other vectors as either XhoI–ClaI or as HindIII–ClaI restriction fragments.

As an example, the NA-*sis* construct required the synthesis of two long oligonucleotides, designated D319 and D320, representing the sense strand and antisense strand, respectively. The sequence of the sense strand D319 oligonucleotide is: 5' AGCTTCTCGAGACC. ATG. AAT. CCA. AAT. CAG. AAA. ATA. ATA. ACC. ATT. GGA. TCA. ATC. TGT. CTG. GTA. GTC. GGA. CTA. ATT. AGC. CTA. ATA. CTG. CAG. ATA. GGG. AAT. ATA. ATC. TCA. ATA. TGG. ATT. AGC. GAG. CT³'. This oligonucleotide encodes the amino acid sequence MNPNQKIITIGSICLVVGLISLIL-QIGNIISIWISEL. The amino acids encoded by oligonucleotides D319/D320 represent amino acids 1-35 of the NA protein of human influenza virus (Fields et al., 1982), including the signal–anchor domain defined as amino acids 7-35 (Fields et al., 1982; Sivasubramanian and Nayak, 1987; Brown et al., 1988; Nayak and Jabbar, 1989; Kundu et al., 1991). It should be noted that the last two amino acids encoded by the D319/D320, as well as for the other constructs described below, correspond to E⁵⁸ and L⁵⁹ of wild type v-*sis* protein encoded at the unique SstI restriction site. In all constructs, the sequence surrounding the initiation codon was designed to provide for optimal initiation of translation (Kozak, 1986).

The E5-*sis* construct was similarly designed using a pair of oligonucleotides designated D341/D342, which encode the amino acid sequence MPNLWFLLFLGLVAAMQLLLLLFLLLFFLVEL. The first 30 residues correspond to amino acids 1-30 of the BPV E5 oncoprotein (DiMaio et al., 1986). The TR-*sis* construct was designed using a pair of oligonucleotides designated D383/D384. These oligonucleotides encode the amino acid sequence MKRCSGSICYGTIAVIVFFLIGFMIGYLGYCKEL. Amino acids 2-32 in this sequence correspond to amino acids 60-90 of the human TR protein (Schneider et al., 1984), which includes the signal–anchor domain located at amino acids 65-88. The first Met residue encoded by D383/D384 was added to provide for initiation of translation. The ASGPR-*sis* construct was designed using a pair of oligonucleotides designated D385/D386. These oligonucleotides encode the amino acid sequence MPRLLLLSLGLSLLL-LVVVCVIGSEL. Amino acids 2-24 in this sequence correspond to amino acids 39-61 of the human ASGPR H1 protein (Spiess et al., 1985), which includes the signal–anchor domain located at amino acids 41-59. As for the preceding construct, the first Met residue encoded by D385/D386 was added to provide for translational initiation.

Preparatory to DNA transfections into NIH 3T3 cells, DNA fragments encoding NA-*sis*, E5-*sis*, TR-*sis*, and ASGPR-*sis* were subcloned as XhoI–ClaI restriction fragments into the murine leukemia virus (MLV) expression vector pDM85, which was derived from the previously described retroviral vector pDD102 (Bold and Donoghue, 1985).

In Vitro Transcription and Translation of Signal/Anchor-Sis Constructs

DNA fragments encoding the signal/anchor-*sis* were also subcloned from the constructs described above into a vector derived from pSP64(polyA) (Promega Biotec, Madison, WI), designated pDD431, which contains an SP6 promoter for in vitro transcription. As controls, other *sis*-related genes were subcloned into the SP6-promoter vector, including wild type v-*sis* and v-*sis*²³⁹-G (Hannink and Donoghue, 1986a) which will be designated simply as v-*sis*-G throughout this work. The 5'-capped and polyadenylated RNAs were transcribed in vitro as described (Melton, 1987). RNAs were analyzed by gel electrophoresis and subjected to in vitro translation in rabbit reticulocyte lysates containing 50 μCi [³⁵S]Cys (1,000 Ci/mmol). Translation products were resolved by SDS-PAGE using 15% polyacrylamide in the separating gel, and detected by autoradiography of the dried gel.

Cell Culture, Focus Assays, and Transient Expression Assays

NIH 3T3 cells and CV-1 cells were cultured at 37°C in DME containing 10% calf serum. For focus assays, NIH 3T3 cells were transfected with the signal/anchor-*sis* constructs described above, under MLV retroviral promoter control, together with a replication competent helper provirus, pZAP (Hoffman et al., 1982). DNA transfections were carried out using a modified calcium phosphate transfection protocol (Chen and Okayama, 1987), as described previously (Maher et al., 1993). For some experiments, plates of transfected cells were allowed to overgrow to select for transformed cells by splitting cells weekly at a density of 1:6. For transient expression assays of protein expression, transfections into monkey CV-1 cells were carried out as described above using 12 μg DNA for each signal/anchor-*sis* construct under RSV promoter control.

Metabolic Labeling and Immunoprecipitations

NIH 3T3 or CV-1 cells expressing the signal/anchor-*sis* proteins were used as described in the text for immunoprecipitation of radiolabeled protein. Before labeling, cells were rinsed and incubated for 15 min in MEM lacking Cys and Met. For pulse–chase analyses, cells were labeled with 100 μCi/ml each [³⁵S]Cys and [³⁵S]Met for 30 min, rinsed twice with TBS, and chased for 10 min, 30 min, or 2 h in fresh DME. Labeled cells were subsequently lysed in 1.0 ml RIPA buffer (10 mM sodium phosphate [pH 7.0], 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 1% Aprotinin) and clarified lysates were prepared. Immune complexes were formed as previously described (Hannink and Donoghue, 1986a), using a rabbit antiserum directed against bacterially synthesized v-*sis* protein generously provided by Ray Sweet and Keith Deen (Smith, Kline and French, King of Prussia, PA). Immune complexes were collected using protein A Sepharose (Sigma Immunochemicals, St. Louis, MO). Samples were run on a 12.5% SDS–polyacrylamide gel and processed for fluorography to visualize proteins.

Indirect Immunofluorescence

To detect intracellular v-*sis* fusion proteins, cells were fixed in 3% paraformaldehyde/PBS for 10 min, followed by permeabilization in 1% Triton/PBS for 5 min. Cells were then incubated with a rabbit antiserum directed against the v-*sis* protein, followed by a rhodamine-conjugated goat anti-rabbit antibody. To detect cell surface v-*sis* fusion proteins, cells were fixed with paraformaldehyde and incubated with antibodies without permeabilization, as described previously (Lee and Donoghue, 1992; Hannink and Donoghue, 1986a).

Results

Construction of Signal/Anchor-Sis Proteins

The constructs used in this work were prepared by replacing the conventional signal sequence of v-*sis* with various signal–anchor domains. Signal–anchor domains provide the dual purpose of initiating protein translocation across the membrane of the ER, and also provide for membrane anchoring of the protein. In general, signal–anchor domains provide for a type II membrane orientation, in which the NH₂ terminus is located intracellularly and the COOH terminus is located extracellularly ("N-in, C-out"). As described in the Materials and Methods, a restriction fragment encoding amino acids 1-59 of the v-*sis* protein was substituted with a synthetic restriction fragment encoding the desired signal–anchor domain. The parental gene used, designated v-*sis*^N110s111 also contains a pair of mutations to destroy the proteolytic processing site, Lys¹¹⁰–Arg¹¹¹, which is used to cleave the propeptide sequence from the mature growth factor. Otherwise, the predicted cleavage following Lys¹¹⁰–Arg¹¹¹ would cleave the growth factor domain from its signal–anchor domain in the signal/anchor-*sis* constructs.

The signal–anchor domains used in this study were derived from three well-characterized type II membrane proteins: the influenza virus NA protein (Fields et al., 1982), the human TR protein (Schneider et al., 1984), and the human ASGPR H1 protein (Spiess et al., 1985). In addition, the hydrophobic domain of the BPV E5 protein (DiMaio et al., 1986), was used in these studies to determine if it could
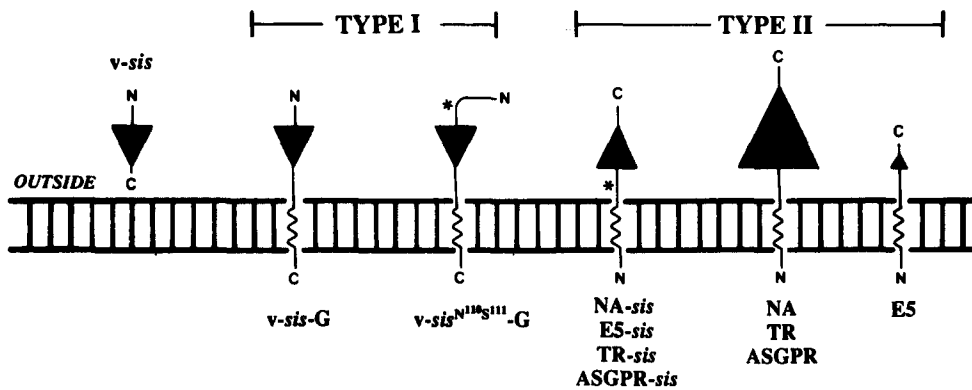
Figure 1. Structure of type II membrane-anchored *sis* proteins with respect to the membrane. The structure of the signal/anchor-*sis* constructs with respect to the membrane is shown. Also shown are the parental proteins for these constructs, including the type II proteins NA, TR, ASGPR, and also the papillomavirus E5 oncoprotein. The wild type v-*sis* protein and its type I membrane-anchored derivatives, v-*sis*-G and v-*sis*^{N110S111}-G, are also shown. The extracellular domain is indicated by a solid triangle, pointing towards the COOH terminus, whose size roughly corresponds to the relative size of the extracellular domain. The asterisk indicates the site of the $N^{110}S^{111}$ mutation in the dibasic processing site, which results in retention of the propeptide sequence. The location of the NH_2 terminus (*N*) and COOH terminus (*C*) is indicated for each protein. Note that many of these proteins exist as dimers or higher order oligomers, which is not shown in this figure.

also function as a signal–anchor domain. The resulting constructs were designated NA-*sis*, TR-*sis*, ASGPR-*sis*, and E5-*sis*, and were prepared under MLV promoter control and RSV promoter control.

The general structure of the signal/anchor-*sis* constructs with respect to the membrane is shown diagrammatically in Fig. 1, together with the parent proteins. In previous studies, we also made use of a type I membrane-anchored derivative of v-*sis*, designated v-*sis*-G, in which the transmembrane domain of VSV-G was appended near the COOH terminus of the PDGF-related domain (Hannink and Donoghue, 1986*a*; Lee and Donoghue, 1992).

The Signal/Anchor-Sis Constructs Transform NIH 3T3 Cells

The three different signal/anchor-*sis* constructs described here, NA-*sis*, TR-*sis*, and ASGPR-*sis*, all transformed cells with efficiencies similar to the type I construct, v-*sis*-G, ranging from 25–46% compared with wild type v-*sis* (see Table I, and also Fig. 6). Importantly, the construct E5-*sis* also transformed NIH 3T3 cells with an efficiency comparable to the signal/anchor-*sis* constructs. The ability of E5-*sis* to induce morphological transformation demonstrates that the hydrophobic domain of the BPV E5 oncoprotein can indeed initiate translocation of the PDGF-related domain across the membrane.

Table I. Transformation Efficiencies of Signal/Anchor-sis Constructs

Construct	Relative transformation*
v-*sis*	100%
E5	83%
mock	<1%
E5-*sis*	32%
NA-*sis*	30%
TR-*sis*	46%
ASGPR-*sis*	25%
v-*sis*-G	26%

* Transformation relative to that produced by v-*sis* (set to 100%).

In earlier work (Lee and Donoghue, 1992), we observed that the foci of NIH 3T3 cells transformed by type I v-*sis*-G are smaller and "tighter" than the foci generated by wild type v-*sis*, presumably due to the inability of the membrane-anchored growth factor to diffuse in the medium. Similarly, the foci generated by the four novel constructs described here, NA-*sis*, TR-*sis*, ASGPR-*sis*, and E5-*sis*, all resembled v-*sis*-G in this respect, producing foci that were in general about one third the size of those produced by wild type v-*sis*.

The Signal/Anchor-Sis Proteins Exhibit Rapid Turnover

We next attempted to detect the various signal/anchor-*sis* proteins by immunoprecipitation and SDS-PAGE analysis of radiolabeled cell lysates. In preliminary experiments, under conditions readily allowing detection of control proteins such as wild type v-*sis* or v-*sis*-G, we occasionally detected faint bands of radiolabeled proteins corresponding to the signal/anchor-*sis* proteins (data not shown). To demonstrate that the constructs encoded the expected proteins, we turned to an in vitro transcription/translation system. Results of a typical experiment are presented in Fig. 2, which shows proteins translated in vitro, using rabbit reticulocyte lysate, from the constructs E5-*sis* (Fig. 2, lane *1*) and NA-*sis* (lane *2*), in comparison with the control proteins wild type v-*sis* (lane *3*) and v-*sis*-G (lane *4*). In the experiment shown, the in vitro translations were subjected to immunoprecipitation with an antiserum against the v-*sis* protein before SDS-PAGE analysis. However, essentially identical bands were observed if the translations were analyzed by SDS-PAGE directly without immunoprecipitation (data not shown). The observed molecular weights were in general agreement with the predicted molecular weights given that the signal sequence would not be cleaved from the wild type v-*sis* and v-*sis*-G proteins under the conditions used for in vitro translation, nor would any of these polypeptides undergo N-linked oligosaccharide addition. Note that in Fig. 2 (lanes *3* and *4*) the lower band of the observed doublet is due to initiation during in vitro translation at a second AUG codon in the signal sequence of v-*sis*, shown previously to result in a functional protein (Hannink and Donoghue, 1984).

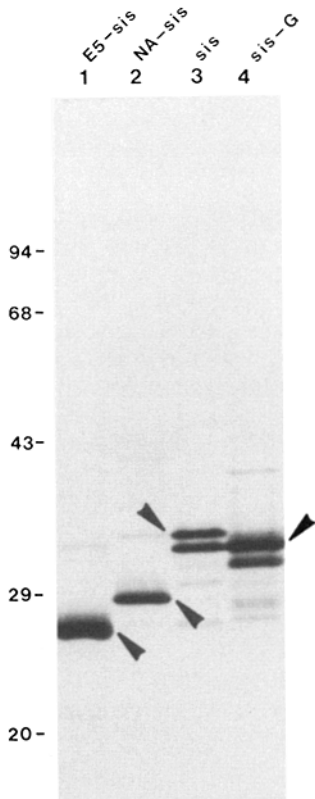This experiment demonstrates that in rabbit reticulocyte

Figure 2. In vitro translations of sis-related proteins. RNAs transcribed in vitro using SP6-promoter constructs were translated using rabbit reticulocyte lysate, labeled with [^{35}S]Cys, and the resulting protein products were resolved by 15% SDS-PAGE and detected by fluorography of the dried gel. In the experiment shown, translation products were immunoprecipitated prior to SDS-PAGE using a polyclonal antibody directed against v-sis. Lane *1*, E5-sis; lane *2*, NA-sis; lane *3*, wild type v-sis; and lane *4*, v-sis-G. Arrows indicate primary translation products which correspond to predicted molecular weights for the different constructs. Note that in lanes *3* and *4* the lower band of the observed doublet is due to the usage of an internal AUG initiation codon in the v-sis gene during in vitro translation. Autoradiogram was exposed for 2 h at −70°C.

lysate, the signal/anchor-*sis* proteins were translated as efficiently as the control proteins v-*sis* and v-*sis*-G. Fig. 2 also demonstrates that the recovery of the signal/anchor-*sis* proteins after immunoprecipitation was comparable to the recovery of the control proteins v-*sis* and v-*sis*-G. This indicates that the presence of the signal–anchor domain in the signal/anchor-*sis* proteins did not interfere with the ability of the antiserum to immunoprecipitate the protein.

Unfortunately, these results provided no explanation for the inability to detect significant expression of the signal/anchor-*sis* proteins in either transformed cells or transiently expressing cells. Suspecting that the signal/anchor-*sis* proteins might be quite unstable, a pulse–chase analysis was undertaken to determine the rate of turnover of the signal/anchor-*sis* proteins. A short labeling period (30 min) was used to incorporate [^{35}S]Cys and [^{35}S]Met, followed by various chase times during which cells were incubated in nonradioactive media. Using these conditions, radioactively labeled proteins could be detected following immunoprecipitation and SDS-PAGE, as shown in Fig. 3, and which comigrated with the protein products obtained by in vitro transcription/translation (data not shown). Data for the E5-*sis* (Fig. 3, lanes *5–8*) and for the Na-*sis* (Fig. 3, lanes *9–12*) constructs are shown. Quantitation of the bands shown in Fig. 3 revealed a half-life of ~19 min (±5 min) for the E5-*sis* and NA-*sis* constructs, compared with the much longer half-life of ~2 h for the control protein v-*sis*-G (Fig. 3, lanes *13–16*; and previously determined by Lee and Donoghue,
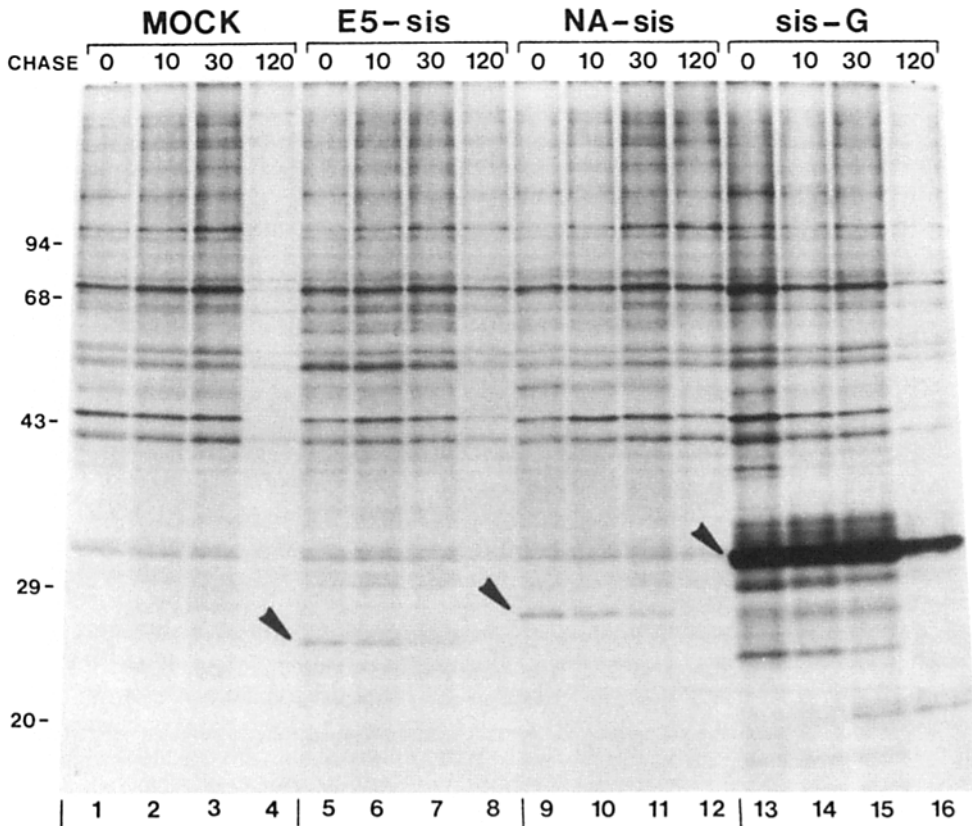

Figure 3. Pulse–chase analysis of E5-*sis* and NA-*sis* proteins in comparison with v-*sis*-G. Cells expressing different *sis*-related proteins were labeled with [^{35}S]Cys and [^{35}S]Met for 30 min, and then shifted into fresh DME for chase periods of 10 min, 30 min, or 2 h. Lanes *1–4*, mock-transfected cells; lanes *5–8*, cells expressing E5-*sis*; lanes *9–12*, cells expressing NA-*sis*; lanes *13–16*, cells expressing v-*sis*-G. Proteins were recovered by immunoprecipitation, separated by 15% SDS-PAGE, and detected by fluorography. Exposure time was 11 d at −70°.

1992). Pulse–chase analysis also indicated that the TR-*sis* and ASGPR-*sis* were similarly unstable, comparable to the E5-*sis* and NA-*sis* proteins (data not shown). These results indicate that the signal/anchor-*sis* proteins turn over very rapidly and explained the earlier difficulty in their detection.

We wished to confirm that the signal/anchor-*sis* proteins were not being detectably released from the cell surface. Therefore, we immunoprecipitated radiolabeled proteins released into the media in comparison with proteins recovered from lysates of the same cells. Although this experiment was conducted using a variety of conditions, the highest recovery of radiolabeled protein was obtained using RSV-promoter constructs in transient assays in CV-1 monkey cells. In these experiments, no secreted protein could be detected for any of the signal/anchor-*sis* proteins, nor for the type I membrane-anchored derivative, v-*sis*-G (data not shown), although secreted v-*sis* protein was easily detected in the media consistent with previous reports (Robbins et al., 1985; LaRochelle et al., 1991). This experiment was performed near the limit of detection for the signal/anchor-*sis* proteins, due to their rapid turnover. Since the amount of signal/anchor-*sis* proteins detected in the lysates never exceeded 5% of the amount of v-*sis* protein, it would have been difficult to detect proteolytic cleavage and/or secretion of a small fraction of the signal/anchor-*sis* proteins. Despite this

reservation, we conclude that in contrast to the efficient cleavage and secretion of v-*sis* protein, little or no detectable secretion occurs for the signal/anchor-*sis* proteins.

Immunofluorescence Localization of Signal/Anchor-Sis Proteins

Indirect immunofluorescence was used in an effort to determine the subcellular localization of the various signal/anchor-*sis* proteins. We first examined NIH 3T3 cells permeabilized by treatment with 1% Triton to allow detection of intracellular proteins. Proteins were detected using an antiserum against the v-*sis* protein, followed by a secondary rhodamine-conjugated goat anti–rabbit antiserum. Fig. 4 demonstrates that intracellular protein was readily detected for two control proteins, wild type v-*sis* (Fig. 4, *B*) and v-*sis*-G (*C*) protein. Similarly, the signal/anchor-*sis* proteins all demonstrated reticular ER/Golgi staining, as shown for E5-*sis* (Fig. 4, *D* and *E*) and NA-*sis* (*F*). The constructs TR-*sis* and ASGPR-*sis* also exhibited similar staining patterns in permeabilized cells (data not shown).

We previously demonstrated cell surface expression of the type I derivatives, v-*sis*-G, and v-*sis*^{N110S111}-G, whereas cell surface staining is essentially undetectable for wild type v-*sis* (Hannink and Donoghue, 1986a). We therefore exam-
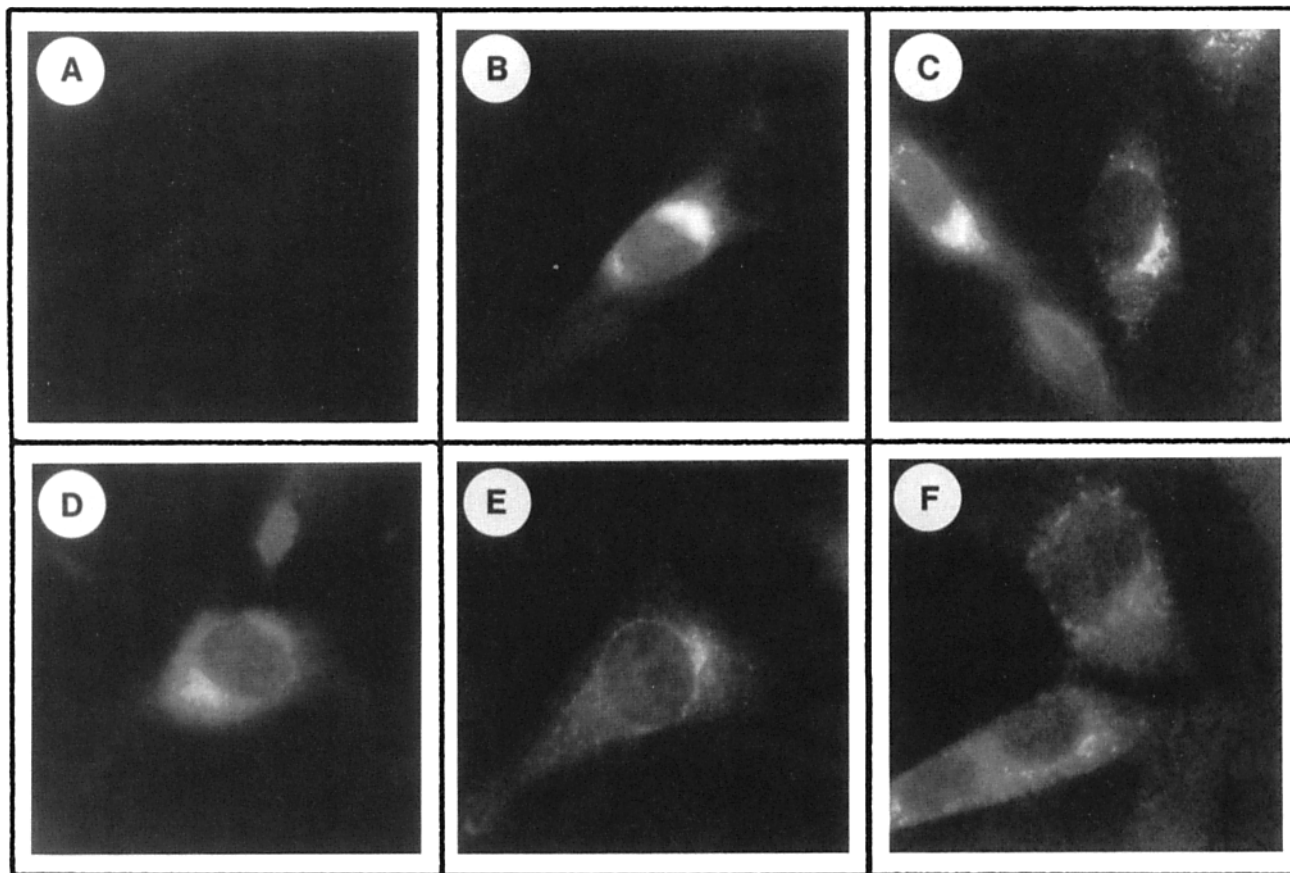

Figure 4. Intracellular localization of *sis*-related proteins by immunofluorescence microscopy. An antibody directed against the v-*sis* protein was used to detect the intracellular localization of *sis*-related proteins in transformed NIH 3T3 cells by indirect immunofluorescence. (*A*) Control NIH 3T3 cells; (*B*) wild type v-*sis*; (*C*) v-*sis*-G; (*D* and *E*) E5-*sis*; and (*F*) NA-*sis*. Except for the control cells, all the other cells exhibit reticular ER/Golgi staining characteristic of *sis*-related proteins.
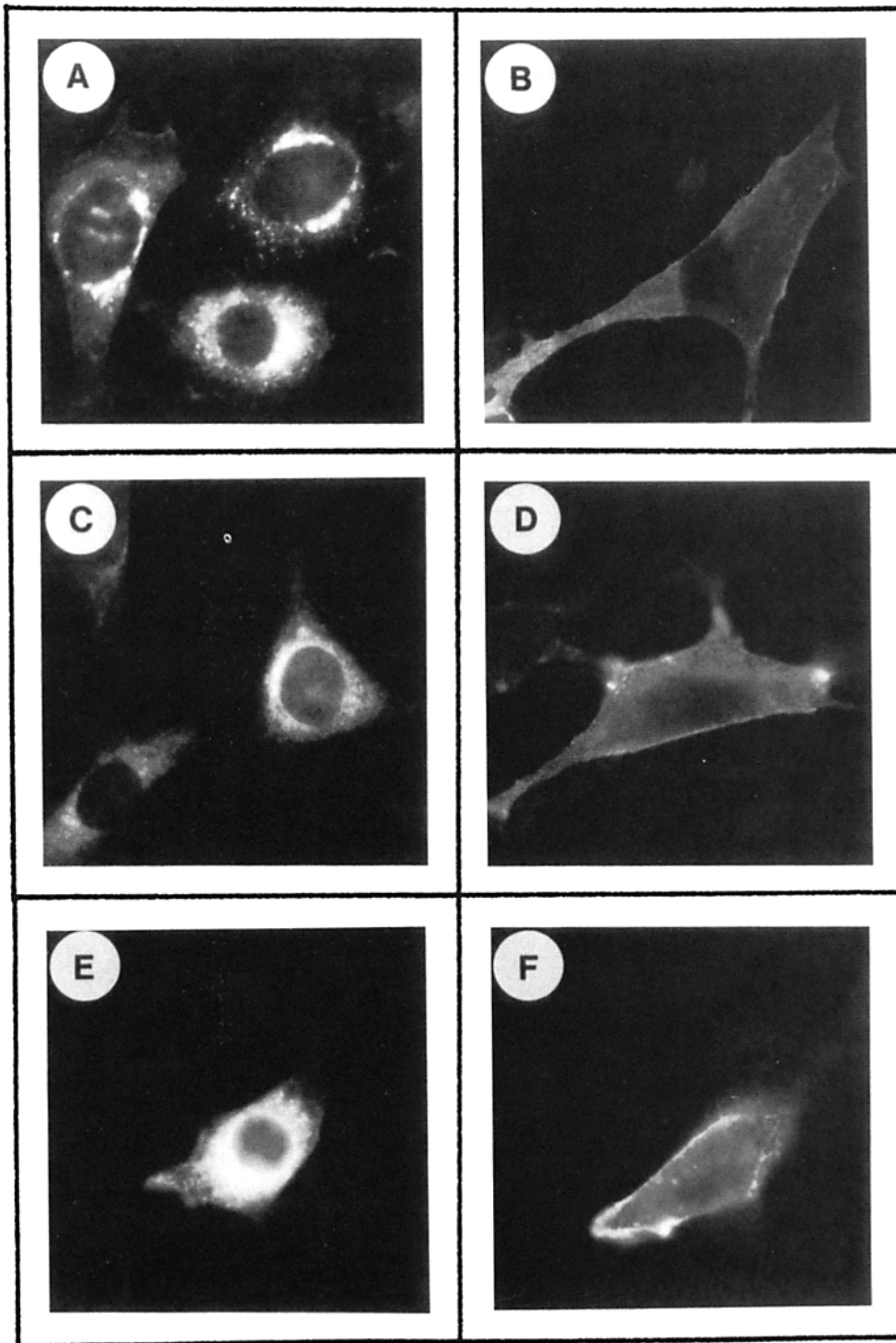
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Figure 5. Intracellular and cell surface localization of *sis*-related proteins by immunofluorescence microscopy. Indirect immunofluorescence with an antibody against v-*sis* was used to detect protein expression in either cells permeabilized with 1% Triton to detect intracellular proteins, or nonpermeabilized cells to detect cell surface expression. (*A*) Intracellular expression of v-*sis*^{N110S111}-G; (*B*) cell surface expression of v-*sis*^{N110S111}-G; (*C*) intracellular expression of ASGPR-*sis*; (*D*) cell surface expression of ASGPR-*sis*. (*E*) intracellular expression of E5-*sis*; (*F*) cell surface expression of E5-*sis*.

ined nonpermeabilized NIH 3T3 cells expressing each of the signal/anchor-*sis* constructs for cell surface staining using an antiserum against v-*sis* (Fig. 5). As a control, the staining of cells expressing v-*sis*^{N110S111}-G is shown for both permeabilized cells (Fig. 5 *A*) and nonpermeabilized cells (*B*). In general, cells expressing the signal/anchor-*sis* proteins exhibited much lighter staining than these control cells. However, in populations of acutely infected NIH 3T3 cells, it was possible to identify cells exhibiting similar staining patterns. For example, Fig. 5 (*C* and *D*) show intracellular and cell

surface staining for ASGPR-*sis*, and *E* and *F* show intracellular and cell surface staining for E5-*sis*.

Suramin Reverts Transformation by the Signal/Anchor-Sis Proteins, but Not by BPV E5

Transformation by PDGF-related growth factors has been shown previously to be sensitive to suramin, a polysulfonated naphthylurea derivative, which results in morphological reversion to a "flat" phenotype (Fleming et al., 1989; Lee
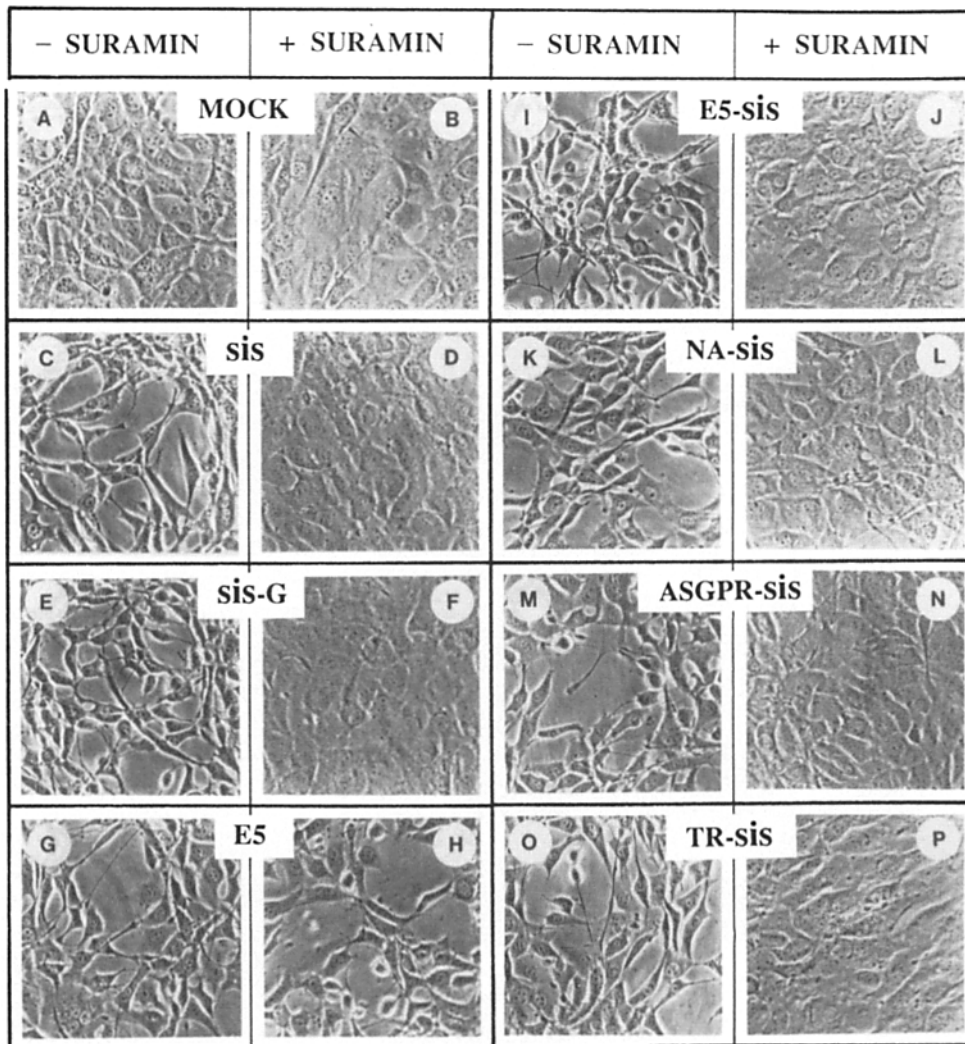
Figure 6. Photomicrographs of cells from transformation assay. For each construct, the left panel shows cells in the absence of suramin (*A, C, E, G, I, K, M,* and *O*), while the right panel shows cells in the presence of 100 μM suramin (*B, D, F, H, J, L, N,* and *P*). (*A* and *B*) Mock-transfected NIH 3T3 cells. (*C* and *D*) Cells transformed by wild type v-*sis*. (*E* and *F*) Cells transformed by v-*sis*-G. (*G* and *H*) Cells transformed by wild type E5. (*I* and *J*) Cells transformed by E5-*sis*. (*K* and *L*) Cells transformed by NA-*sis*. (*M* and *N*) Cells transformed by ASGPR-*sis*. (*O* and *P*) Cells transformed by TR-*sis*.

and Donoghue, 1992). To the extent that cells transformed by the signal/anchor-*sis* constructs might exhibit morphological reversion upon suramin treatment, this would provide clear evidence that the PDGF-related domain was translocated across the membrane and transported to a suramin-accessible compartment of the cell, such as the plasma membrane. As shown in Fig. 6, cells transformed by each of the signal/anchor-*sis* constructs were examined in the absence or presence of suramin (overnight at 100 μM). Cells transformed by all of these constructs, including E5-*sis*, exhibited morphological reversion as did cells transformed by wild type v-*sis*. These results clearly demonstrate that the hydrophobic domain of E5 can target the PDGF-related domain of the E5-*sis* construct to the same subcellular compartment as achieved by the well-characterized signal–anchor domains of NA, TR, and ASGPR.

In contrast to the signal/anchor-*sis* constructs, cells transformed by expression of the native BPV E5 oncoprotein did not exhibit morphological reversion in response to suramin. The E5 oncoprotein has previously been localized to intracellular membranes of the cell, and the suramin insensitivity of this transforming interaction might suggest a Golgi localization of the transforming interaction between the E5 oncoprotein and PDGF receptors. This would be consistent with a recently proposed model for PDGF receptor activation by E5 occurring in the Golgi (Petti and DiMaio, 1992).

Discussion

Sis Is Biologically Active As a Type II Membrane-anchored Protein

Previously, we demonstrated that v-*sis* can be tethered to the membrane as a type I protein using the membrane anchor and cytoplasmic tail of VSV-G (Hannink and Donoghue, 1986*a*; Lee and Donoghue, 1992). In the study presented here, we have extended our earlier results to describe biologically active derivatives which are membrane anchored as type II proteins.

Membrane insertion of type I proteins, which display an "N-out, C-in" orientation, has been extensively characterized (Walter and Lingappa, 1986; Rapoport and Wiedmann, 1985). Translocation across the membrane for type I proteins is initiated by a cleavable signal sequence, and membrane anchoring is provided by a separate domain located nearer to the COOH terminus. In contrast, type II proteins insert into the membrane using a signal–anchor domain that initiates translocation across the membrane and also serves

to anchor the protein (Spiess and Lodish, 1986; Zerial et al., 1986; Lipp and Dobberstein, 1988). Despite the difference in the final orientation between type I and II proteins, the basic mechanism of insertion may be quite similar as both require signal recognition particle (SRP) for membrane insertion (High et al., 1991). Previous comparisons of types I and II transmembrane domains suggest that the one fundamental difference may be the relative charge distribution within 15 residues flanking the transmembrane segment (Hartmann et al., 1989). In general, the extracellular segment exhibits a greater net negative charge compared to the intracellular segment.

The proteins NA, TR, and ASGPR represent classic type II proteins which have been well characterized. For example, the signal–anchor domain of NA has been extensively mutagenized to define a minimal region comprising the redundant functions of both signal sequence and membrane anchor (Brown et al., 1988; Nayak and Jabbar, 1989). The TR signal–anchor domain has been used to direct the membrane insertion of heterologous proteins such as dihydrofolate reductase and α-globin (Zerial et al., 1986). A recent study also demonstrated interchangeability of the NA and TR signal–anchor domains, with the resulting chimeric molecules transported to the cell surface (Kundu et al., 1991).

The results presented here demonstrate that the signal–anchor domains of the type II proteins NA, TR and ASGPR can all function to direct membrane insertion of a biologically active derivative of the v-sis oncoprotein.

The Hydrophobic Domain of the E5 Oncoprotein Functions as a Signal Anchor

Previous studies have demonstrated that the BPV E5 oncoprotein, only 44 amino acids, can be divided into two general domains. The NH$_2$-terminal two-thirds is very hydrophobic and may represent a membrane-spanning segment, whereas the COOH-terminal domain is hydrophilic. Previous studies have yielded conflicting results concerning the subcellular localization of the E5 protein. Studies in which synthetic E5-derived peptides were microinjected directly into cells suggested that its site of action might be cytoplasmic or even nuclear (Rawls et al., 1989; Green and Loewenstein, 1988). Other studies, using indirect immunofluorescence, identified E5 in cellular membrane fractions presumably derived from the Golgi or plasma membrane. E5 has been identified in a complex with the 16-kD subunit of the vacuolar ATPase (Goldstein et al., 1991, 1992) and more recently with PDGF β-receptor (Petti and DiMaio, 1992), suggesting a membrane localization for E5. Petti and DiMaio (1991) first observed amino acid sequence similarity between the hydrophilic domain of E5 and PDGF. Since PDGF acts from the extracellular side of the membrane to activate PDGF receptors, these observations suggest that the hydrophilic (COOH-terminal) domain of E5 would also be located extracellularly. This inferred orientation as a type II membrane protein ("N-in, C-out") for the E5 oncoprotein would place a net positive charge on the inner face and a net negative charge on the outside of the membrane, consistent with the other transmembrane segments (Hartmann et al., 1989).

The results presented here clearly demonstrate that the E5 oncoprotein possesses a functional signal–anchor domain, similar to type II proteins such as NA, TR, and ASGPR.

Three separate results support this conclusion. First, the E5-sis construct results in cellular transformation, indicating that the PDGF-related domain has been translocated across the membrane. Second, the ability of suramin to revert transformation by E5-sis demonstrates functional interactions with PDGF receptors at the cell surface, consistent with prior studies localizing the site of ligand/receptor interactions for PDGF (Hannink and Donoghue, 1988; Fleming et al., 1989; Lee and Donoghue, 1992). Third, the ability to detect cell surface localization of E5-sis by indirect immunofluorescence directly confirms the ability of the E5 hydrophobic domain to provide for membrane anchoring.

Comparison with Other Membrane-anchored Growth Factors

PDGF occurs naturally as three different dimeric forms: AA and BB homodimers, and AB heterodimers. Although none of these forms contains any transmembrane domains, recent work suggests a stable association with the extracellular matrix due to a basic amino acid sequence within the COOH-terminal domain (LaRochelle et al., 1990, 1991; Raines and Ross, 1992). Thus, although not a classical transmembrane protein, PDGF-BB may exert some autocrine or paracrine effects by virtue of remaining associated with the extracellular matrix. In contrast, the v-sis-G and the signal/anchor-sis proteins are clearly membrane anchored, as is the E5 oncoprotein which may be viewed as a miniature membrane-anchored analog of PDGF.

Table II also presents other growth factors which are membrane anchored, all with a type I orientation except for the E5 oncoprotein. For most of these, differences in biological activity as a result of membrane anchoring have not yet been demonstrated. However, recent studies have examined the mitogenic properties of the membrane-anchored form of SCF in comparison with an exclusively secreted form. Although both forms are active in proliferation assays using mast cells, only the membrane-anchored form of SCF stimulates the survival of primordial germ cells (Dolci et al., 1991; Godin et al., 1991). Transforming growth factor-α (TGF-α) provides another interesting case, and studies have shown that an "obligate" membrane-anchored form of TGF-α stimulates EGF receptors of heterologous cells in a paracrine interaction (Brachmann et al., 1989; Wong et al., 1989).

Reduced Receptor Autophosphorylation by Membrane-anchored Growth Factors

Previously, we demonstrated that the type I protein v-sis-G, although transforming in NIH 3T3 cells, induces little or no detectable tyrosine phosphorylation of PDGF receptors (Lee and Donoghue, 1992). Similarly, previous studies of membrane-anchored proTGF-α demonstrated a 50–100-fold reduction, compared with secreted TGF-α, in stimulation of EGF receptor tyrosine phosphorylation (Brachmann et al., 1989). We therefore wished to examine the signal/anchor-sis constructs described here for their ability to induce tyrosine phosphorylation of PDGF β-receptors. We were consistently unable to detect any phosphorylation of PDGF receptors in cells expressing the signal/anchor-sis proteins, either in stably transformed cell lines or transiently expressing cells (data not shown). This observation allows for two possible interpretations. First, the signal/anchor-sis proteins may in-

Table II. Membrane-anchored Growth Factors

Growth factor	Transmembrane form?		Reference
PDGF	no	secreted; some isoforms extracellular matrix associated	(LaRochelle, 1990, 1991; Raines and Ross, 1992)
v-sis-G	yes	artificial construct; VSV-G membrane anchor	(Hannink and Donoghue, 1986a; Lee and Donoghue, 1992)
BPV-E5	yes	44 amino acid bovine papillomavirus oncoprotein; homology with PDGF	(Petti et al., 1991)
CSF-1	yes	alternative splicing yields two transmembrane forms, released by proteolysis	(Kawasaki et al., 1985; Rettenmier and Roussel, 1988; Wong et al., 1987)
SCF	yes	proteolytic release from transmembrane precursor	(Anderson et al., 1990; Huang et al., 1990; Martin et al., 1990)
TGF-α	yes	proteolytic release from transmembrane precursor	(Brachmann et al., 1989; Wong et al., 1989)
EGF	yes	proteolytic release from enormous transmembrane precursor	(Gray et al., 1983; Scott et al., 1983)
VVGF	yes	vaccinia virus growth factor; homolog of EGF	(Stroobant et al., 1985)

duce receptor autophosphorylation which is undetectable due to the small number of molecules involved. Second, the signal/anchor-sis proteins, like v-sis-G and membrane-anchored pro-TGF-α, may be deficient in their ability to induce receptor autophosphorylation. At the present time, we are unable to distinguish between these two alternative views.

Why Are the Signal/Anchor-Sis Proteins So Unstable?

The short half-life exhibited by the signal/anchor-sis proteins might conceivably be dependent upon interaction with PDGF receptors. To examine this possibility, transient expression assays were conducted using canine epithelial cell lines either lacking PDGF receptors or specifically expressing the PDGF β-receptor (Kazlauskas and Cooper, 1989). These experiments demonstrated that the signal/anchor-sis proteins were equally short-lived in both cell lines (data not shown), indicating that the presence or absence of PDGF receptors did not influence their turnover. The reasons for the intrinsic instability of the signal/anchor-sis proteins will require further investigation.

The instability of the signal/anchor-sis proteins made their detection and characterization in this study quite difficult. Although no examples of naturally occurring type II membrane-anchored growth factors have been reported, there is no reason a priori that type II growth factors should not exist in nature. It is impossible to predict whether the instability of the signal/anchor-sis proteins will represent a general feature of type II growth factors.

Unstable Growth Factors May Result in "Ephemeral" Autocrine Loops

The short half-life of the signal/anchor-sis constructs deserves one further comment, as it seems surprising that such labile growth factors should result in autocrine transformation. This suggests that in some cases cellular transformation may result from the synthesis of growth factors which are so transitory as to be undetectable, and that some transformed cells may exhibit autocrine loops of such an ephemeral nature as to preclude their identification.

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