The v-*sis* Oncoprotein Loses Transforming Activity When Targeted to the Early Golgi Complex

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Abstract. The location of autocrine interactions between the v-*sis* protein and PDGF receptors remains uncertain and controversial. To examine whether receptor-ligand interactions can occur intracellularly, we have constructed fusion proteins that anchor v-*sis* to specific intracellular membranes. Fusion of a *cis*-Golgi retention signal from a coronavirus E1 glycoprotein to v-*sis* protein completely abolished its transforming ability when transfected into NIH3T3 cells. Fusion proteins incorporating mutations in this retention signal were not retained within the Golgi complex but instead were transported to the cell surface, resulting in efficient transformation. All chimeric proteins were shown to dimerize properly. Derivatives of some of these constructs were also constructed bearing the cytoplasmic tail from the glycoprotein of vesicular stomatitis virus (VSV-G). These constructs allowed examination of subcellular localization by double-label immunofluorescence, using antibodies that distinguish between the extracellular PDGF-related domain and the VSV-G cytoplasmic tail. Colocalization of *sis*-E1–G with Golgi markers confirmed its targeting to the early Golgi complex. The *sis*-E1 constructs, targeted to the early Golgi complex, exhibited no proteolytic processing whereas the mutant forms of *sis*-E1 exhibited normal proteolytic processing. Treatment with suramin, a polyanionic compound that disrupts ligand/receptor interactions at the cell surface, was able to revert the transformed phenotype induced by the mutant *sis*-E1 constructs described here. Our results demonstrate that autocrine interactions between the v-*sis* oncoprotein and PDGF receptors within the early Golgi complex do not result in functional signal transduction.

Another v-*sis* fusion protein was constructed by attaching the transmembrane domain and COOH-terminus of TGN38, a protein that localizes to the *trans*-Golgi network (TGN). This construct was primarily retained intracellularly, although some of the fusion protein reached the surface. Deletion of the COOH-terminal region of the TGN38 retention signal abrogated the TGN-localization, as evidenced by very prominent cell surface localization, and resulted in increased transforming activity. The behavior of the *sis*-TGN38 derivatives is discussed within the context of the properties of TGN38 itself, which is known to recycle from the cell surface to the TGN.

TRANSFORMATION and tumorigenesis are frequently associated with the abnormal expression of growth factors and their receptors. Many oncogenes have been shown to be homologues of normal cellular proteins, as in the case of the retroviral oncogene v-*sis* (Devare et al., 1983), which is homologous with the B chain of platelet-derived growth factor (PDGF) (Doolittle et al., 1983; Waterfield et al., 1983). Expression of the v-*sis* protein activates cellular PDGF receptors, resulting in the stimulation of signal transduction pathways that ultimately leads to cellular transformation.

Autocrine transformation (Sporn and Todaro, 1980) occurs when the same cell expresses PDGF receptors as well as the v-*sis* protein. In this situation, there also exists the possibility of intracellular ligand/receptor interactions within the secretory pathway (Betsholtz et al., 1986). The secretory pathway consists of functionally distinct compartments, including the endoplasmic reticulum (ER) and the entire Golgi apparatus, consisting of the *cis*-Golgi network, the *cis*-, *medial*-, and *trans*-Golgi cisternae, as well as the *trans*-Golgi network (TGN)[1] (Machamer, 1993; Pelham, 1991). Determination of the site of autocrine ligand/receptor interactions has distinct implications for the treatment of human cancers that exhibit autocrine activation of signal transduction pathways. If autocrine interactions only occur on the cell surface,

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1. *Abbreviations used in this paper*: E1, avian coronavirus E1 glycoprotein; MLV, Moloney murine leukemia virus; PDGF, platelet-derived growth factor; TGN, *trans*-Golgi network; VSV-G, vesicular stomatitis virus glycoprotein.

then transformed cells should be responsive to treatment with exogenous substances that disrupt these interactions. However, if such interactions occur intracellularly, such as in the ER or the Golgi complex, then addition of such antagonists will not be sufficient to revert transformation. Thus, it is of vital importance, both from a clinical and a molecular standpoint, to understand fully the mechanism and cellular location of autocrine interactions between ligands and receptors.

There exists considerable controversy over the biological significance of intracellular interactions between v-*sis* and the PDGF-receptor. It has been demonstrated that mitogenesis can be blocked in some *sis*-transformed cells by treating them with antibodies against PDGF (Huang et al., 1984). While these results indicate that cell surface interactions between the v-*sis* protein and PDGF receptors are important in the transformation process, these same researchers demonstrated that some *sis*-transformed cells did not detectably secrete v-*sis* protein, and anti-PDGF antibody did not affect transformation. This implies that an intracellular mechanism of autocrine transformation may also exist. Other researchers have reported that in normal cells, PDGF only activates receptors present on the cell surface, but that in *sis*-transformed cells, intracellular receptors are activated and undergo autophosphorylation in an autocrine fashion (Keating and Williams, 1988; Bejcek et al., 1992). There is also evidence that E5, the transforming protein of bovine papillomavirus, can interact with immature, intracellular forms of PDGF receptors, stimulating their autophosphorylation activity (Goldstein et al., 1992; Petti and DiMaio, 1992; Cohen et al., 1993).

Since receptor autophosphorylation represents a key event in activating PDGF-mediated signaling pathways (Williams, 1989), these intracellular forms of the receptors may be able to transmit signals that lead to autocrine transformation of cells. However, it is not clear whether the downstream effectors of PDGF-stimulated signaling are accessible from intracellular compartments. Bejcek et al. (1992) have demonstrated that internally activated receptors may associate with PI-3 kinase in a manner similar to mature cell surface PDGF receptors, suggesting that these receptors may indeed be capable of signaling from within the cell.

Indirect methods that rely upon pharmacologic agents have been used by some researchers in attempts to identify the site of ligand/receptor interactions. Monensin has been shown to block transport of proteins through the *trans*-Golgi portion of the secretory pathway (Tartakoff, 1983). Treatment of v-*sis*-expressing cells with monensin prevents autophosphorylation of mature PDGF receptors and expression of c-*fos* (a nuclear protein involved in cellular growth regulation), suggesting that v-*sis* must be transported beyond the point of monensin's inhibitory activity (past the *trans*-Golgi portion) in order to activate signal transduction pathways (Hannink and Donoghue, 1988). However, monensin exerts pleiotropic effects on cations within cells, so other cellular events may have been affected in these experiments. Suramin, a potent inhibitor of proliferation of cells expressing v-*sis* and PDGF receptors, seems to interfere only with ligand/receptor interactions at the cell surface, reducing the level of tyrosine-phosphorylated receptors. Suramin has little or no effect on intracellular phosphorylated receptors (Fleming et al., 1989). This suggests a requirement for cell-surface interactions between receptor and ligand for expression of a transformed phenotype. However, the mechanism of action of suramin is unclear, and there is evidence that it accumulates within endosomes (Hawking, 1978). Thus, experiments utilizing agents such as monensin or suramin have been viewed as problematic by some researchers.

Therefore, we have recently exploited more direct approaches to address this issue of intracellular ligand/receptor interactions. The recent identification of specific targeting and retention signals makes it possible to localize v-*sis* protein to specific intracellular compartments. This potentially allows one to scan the secretory pathway for compartments that allow functional transforming interactions between v-*sis* and PDGF receptors. This represents a powerful approach for examining autocrine interactions, and can be applied to other autocrine growth factors or systems as well. ER-anchored forms of v-*sis* were previously constructed by this lab, using an adenovirus transmembrane protein E3/19K retention signal, DEKKMP (Nilsson et al., 1989). These constructs prevented cell surface expression of v-*sis* protein as determined by immunofluorescence, and transformation was inhibited by retention of the fusion protein in the ER (Lee and Donoghue, 1992). In this report, we continued our analysis of autocrine transformation from within secretory pathway compartments by creating novel v-*sis* fusion proteins targeted to unique subcellular compartments. One signal that we chose was the *cis*-Golgi localization signal represented by the first transmembrane domain of the avian coronavirus E1 glycoprotein (E1) of infectious bronchitis virus, which has been shown by others to target heterologous proteins such as VSV-G and αm (a derivative of the human chorionic gonadotropin-α subunit) to the *cis*-Golgi complex (Swift and Machamer, 1991). In addition, we chose to exploit the TGN-localization signal of the protein TGN38, contained within its transmembrane domain and cytoplasmic tail, which has been shown to retarget heterologous proteins, such as the LDL receptor and the Tac antigen, to the TGN (Bos et al., 1993; Humphrey et al., 1993). The resulting *sis* fusion proteins, referred to as *sis*-E1 and *sis*-TGN38, allowed a further characterization of the site of autocrine interactions between the v-*sis* oncoprotein and PDGF receptors.

Materials and Methods

Construction of v-sis Fusion Proteins

Plasmid pMS150, encoding the v-*sis* gene under control of the Rous sarcoma virus promoter, was used as a parental clone to make the fusion proteins. The DNA sequence encoding amino acids 238–271 of v-*sis* is easily removed from pMS150 as a BstEII-ClaI fragment, allowing for insertion of novel sequences to create various fusion constructs. Optimized oligonucleotide synthesis and purification were as previously described (Xu et al., 1993). The complementary oligonucleotides for each fusion protein were designed so that, when annealed, 5′ BstEII and 3′ ClaI overhangs were produced. Oligonucleotides were then ligated with vector DNA (pMS150) previously digested with BstEII and ClaI and purified by agarose gel electrophoresis. Recombinant clones were recovered and the sequences of the oligonucleotides were confirmed by dideoxy nucleotide sequencing.

For example, the *sis*-E1 contsruct required synthesis of sense and antisense oligonucleotides, designated D369 and D370, respectively. The sequence of D369 is: 5′GTG.ACC.TAT.AAC.CTG.TTC.ATC.ACC.GCC.TTC.CTG.CTG.TTC.CTG.ACC.ATC.ATC.CTG.CAG.TAT.GGC.TAT.GCC.ACC.CGG.AGC.AAG.TAA.T3′. This oligonucleotide encodes the amino acid sequence VTYNLFITAFLLFLTIILQYGYATRSK*, where the first two amino acids correspond to amino acids 238 and 239 of v-*sis*, which lie at the BstEII restriction site. The remaining sequence corresponds to amino acids 21–45 of E1, which encompasses the first transmembrane domain (Swift and Machamer, 1991). Mutant versions of *sis*-E1, designated *sis*-E1(*QI*) and *sis*-

El(*ins*), were also constructed in the same manner. The complementary oligonucleotides encoding *sis*-El(*ins*) are designated D419 and D420 and code for the sequence VTYNLFITAFL*IIL*FLTIILQYGYATRSK*, which contains an insertion of two Ile residues between amino acids 29 and 30 of El (shown in bold italics). The oligonucleotides encoding the other mutant, *sis*-El(*QI*) are designated D421 and D422 and code for the sequence VTYNLFITAFLLFLTIIL*I*YGYATRSK*, which contains a mutation of Glu37 to Ile (shown in bold italics).

Similar constructs were also designed to encode an extended cytoplasmic tail derived from VSV-G. The oligonucleotides encoding *sis*-El-G were designated D455 and D456. The sense strand oligonucleotide D455 encodes the amino acid sequence VTYNLFITAFLLFLTIILQYGYATRVGIHLCIKLKHTKKRQIYTDIEMNRLGK*. The first 25 amino acids are the same as in the *sis*-El construct, but the final 28 originate from the COOH-terminus of the VSV-G protein. *sis*-El(*QI*)-G and *sis*-El(*ins*)-G were constructed in the same manner.

The final constructs utilize the transmembrane domain and cytoplasmic tail of TGN38. *sis*-TGN38 was encoded by oligonucleotides D496 and D497. The sense strand oligonucleotide D496 encodes the amino acid sequence VTESSHFFAYLVTAAVLVAVLYIAYHNKRKIIAFALEGKRSKVTRRPKASDYQRLNLKI*. Again the first two amino acids correspond to 238 and 239 of v-*sis*, and the remaining 57 amino acids correspond to residues 284–340 of TGN38, encoding the transmembrane domain and cytoplasmic tail of this protein. A mutant version, *sis*-TGN38Δ, was also constructed using oligonucleotides D498 and D499. These oligonucleotides encode a truncated version of the sequence shown above: VTESSHFFAYLVTAAVLVAVLYIAYHNKRS*, and with the final amino acid changed from a lysine to a serine.

The parental vector contains an XhoI restriction site just upstream of the v-*sis* coding sequence. For use in transfection and infection of NIH3T3 cells, DNA fragments encoding the above described v-*sis*–fusion proteins were subcloned as XhoI-ClaI restriction fragments into the murine leukemia virus (MLV) expression vector pMS177, which was derived from the previously described retroviral vector pDD102 (Bold and Donoghue, 1985).

Focus Formation Assays and Infection Protocol

NIH3T3 cells were maintained at 37°C, 10% CO_2 in DME containing 10% calf serum, fed every 3 d with fresh medium, and passaged when 70–80% confluent. For focus assays, cells were split at a density of 2×10^5 cells per 60-mm plate and transfected the following day with 50 ng of expressing plasmid, 50 ng pZAP helper virus (Hoffman et al., 1982), and 9.9 μg carrier plasmid DNA using the calcium phosphate precipitation protocol (Chen and Okayama, 1987). Cells were incubated with precipitate for 18–20 h at 37°C, 3% CO_2, then refed and transferred to 10% CO_2. The cells from each 60-mm plate were split 1:12 the following day and scored for foci 4–5 d later.

For infections, NIH3T3 cells were transfected as described above with 9 μg of MLV DNA plus 1 μg pZAP helper virus. Transfected cells were split as described above, and allowed to grow for 4 d without refeeding. The supernatant media, containing viral particles, were then collected, centrifuged to pellet any cells, and used to infect monolayers of fresh NIH3T3 cells, split $1\text{–}2 \times 10^5$ cells per 60-mm plate one day earlier. Polybrene (4 μg/ml) was added to the newly infected cells to increase the efficiency of infection. Infected cells were refed the following day with fresh DME media, and were used 2 or 3 d later for labeling and immunoprecipitations or immunofluorescence. This protocol results in a very high percentage of cells expressing the desired protein.

Metabolic Labeling and Immunoprecipitations

NIH3T3 cells were subjected to the infection protocol described above. 3 or 4 d after infection with viral supernatants, cells were incubated for 15 min in MEM lacking cysteine and methionine. Each plate was labeled with 100 μCi [^{35}S]Met and [^{35}S]Cys in 0.5 ml MEM minus cysteine and methionine for 2 h. Cells were lysed with 1.0 ml radioimmunoprecipitation assay buffer (10 mM sodium phosphate, pH 7.2, 150 mM NaCl, 1% NP-40, 1% DOC, 0.1% SDS, 10 μg/ml Aprotinin), clarified by centrifugation, and incubated with 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) for 2 h at 4°C with rotation. Protein A-Sepharose beads (Sigma Chemical Co., St. Louis, MO) preincubated with unlabeled NIH3T3 cell lysate were used to isolate immune complexes. After separation on a sucrose gradient and extensive washing in radioimmunoprecipitation assay buffer, the beads were treated with 2× sample buffer, and immunoprecipitates were separated on a 15% SDS-PAGE gel and detected by fluorography. For analysis of dimer formation, half of each sample was treated with reducing sample buffer (50 mM Tris, pH 6.8, 2% SDS, 20% 2-mercaptoethanol, 10% glycerol), while the other half was resuspended in nonreducing sample buffer which lacked the 2-mercaptoethanol. The samples were run on the same 15% SDS-PAGE gel and detected as described above.

Indirect Immunofluorescence

NIH3T3 cells were grown on coverslips and transfected as described above with 10 μg of Rous sarcoma virus constructs, or subjected to infection with viral supernatants (see above). 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 (Hannink and Donoghue, 1986*a*; Lee and Donoghue, 1992).

For double-labeling experiments to detect expression of both intracellular and cell surface fusion proteins, cells were fixed as described above, then treated with a rabbit antibody to v-*sis*, then with rhodamine-conjugated goat anti-rabbit antibody. The same cells were then permeabilized and treated with 10 μg/ml of mAb P5D4 (Kries and Lodish, 1986) against the COOH terminus of VSV-G (kindly made available by William Balch, Scripps Clinic and Research Foundation, La Jolla, CA). These intracellular proteins were detected with a biotin-conjugated goat anti-mouse antibody which was in turn detected by FITC-conjugated streptavidin. For colocalization experiments, NIH3T3 cells grown on coverslips were infected with viral supernatants derived from *sis*-El-G–expressing cells. Coverslips were fixed and permeabilized as described above, and *sis*-El-G was detected with α-*sis* antibody and rhodamine-conjugated goat anti-rabbit IgG. For colocalization with the lectin, cells were then incubated with FITC-conjugated *Lens culinaris* lectin (Sigma). For colocalization with mAb 10E6, cells were then incubated with 10E6 mAb, which was detected with FITC-conjugated goat anti-mouse IgG (kindly provided by V. Malhotra [Univ. California, Davis, CA] and W. J. Brown [Cornell Univ., Ithaca, NY]).

Suramin Treatment of Transformed Cells

NIH3T3 cells were transfected as described above with 9 μg of MLV expression constructs plus 1 μg pZAP helper virus. Cells from individual foci were isolated with cloning rings and transferred to 24-well plates with trypsin, and allowed to grow for 2–3 d, refeeding after 1 d. The cells were trypsinized, transferred to 60-mm plates, then 1 d later split 1:4 to two 10-cm plates, and the following day treated with media with or without 100 μM suramin. Cells were examined for morphological changes after 24 h.

In Vitro Kinase Assay

NIH3T3 cells were transfected with 9 μg of MLV constructs plus 1 μg pZAP, and supernatants collected and used for infection of fresh NIH3T3 cells. 2 d after infection, the 60 mm plates of cells were serum starved in DME for 24 h, then treated for 5 min with 100 ng/ml PDGF-BB (Amgen, Thousand Oaks, CA) or left untreated. Cells were lysed in NP-40 lysis buffer (20 mM Tris, pH 7.5, 137 mM NaCl, 1% NP-40, 1 mM sodium orthovanadate, 5 mM EDTA, 10 μg/ml Aprotinin, and 10% glycerol) and scraped from plates with rubber policemen. The lysates were clarified by centrifugation, then incubated for 2 h with a rabbit antiserum specific for the mouse PDGF-β receptor (Upstate Biotechnology Inc., Lake Placid, NY). Immune complexes were collected with protein A-Sepharose beads, spun through a 10% sucrose in NP-40 lysis buffer solution, washed twice with NP-40 lysis buffer, and once with 20 mM Tris, pH 7.5. 40 μl of kinase buffer (20 mM Tris, pH 7.5, 10 mM $MnCl_2$, 10 mM $MgCl_2$) containing 5 μCi [γ-^{32}P]ATP was then added to the beads, and reactions were incubated for 10 min at 37°C. Reaction products were separated by SDS-PAGE (7.5%) and visualized by autoradiography.

Results

Construction of Plasmids Encoding Golgi-localized Derivatives of v-sis

Fig. 1 depicts the orientation and organization of the fusion

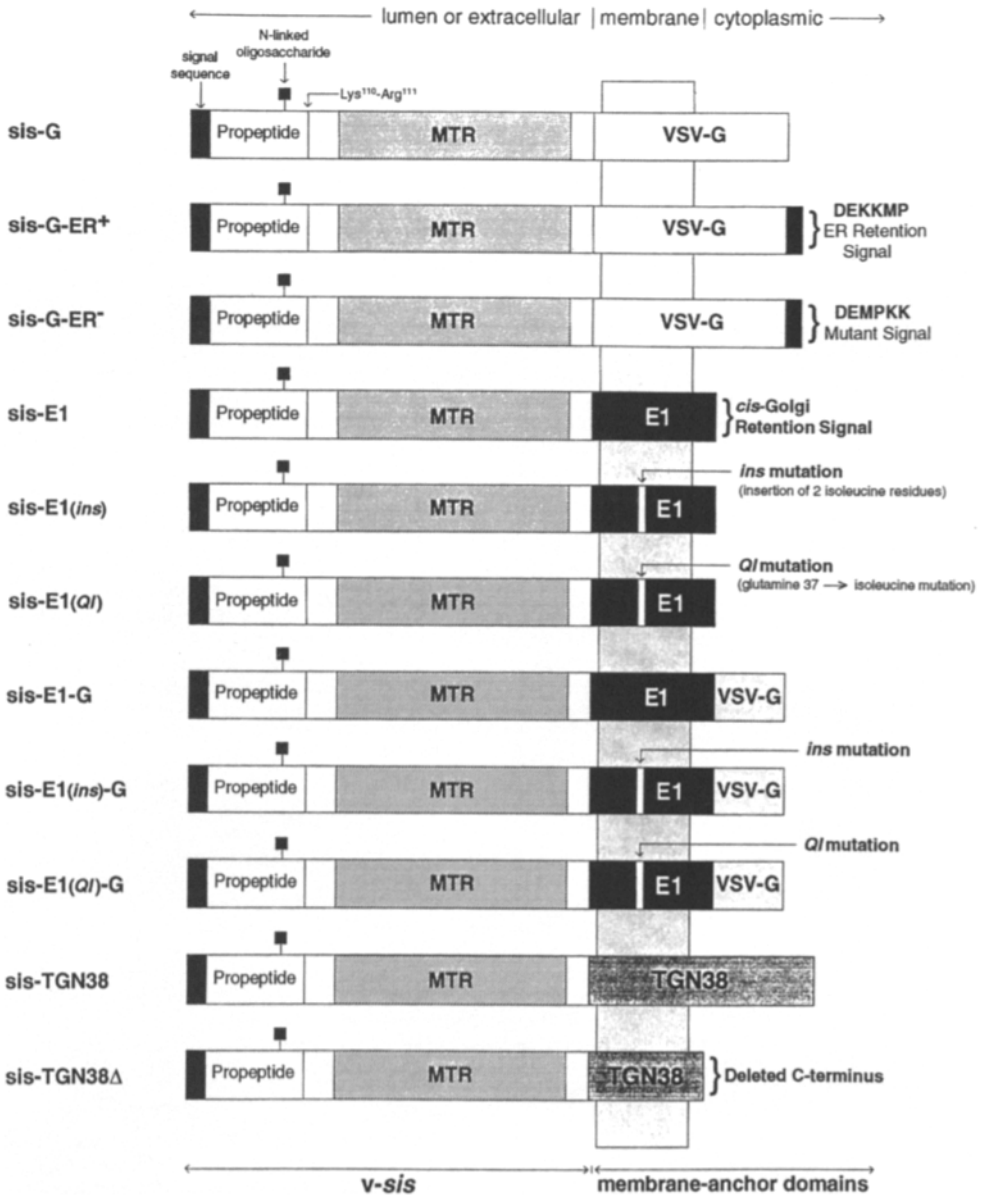


Figure 1. Structure of Golgi-localized v-*sis* derivatives. All constructs used the first 239 amino acids of the v-*sis* protein, which includes: a signal sequence; a propeptide with N-linked oligosaccharide addition site and dibasic proteolytic processing site; and the 82-amino acid MTR. The *sis*-G-ER$^+$ and *sis*-G-ER$^-$ constructs were produced by fusing a known ER retention signal (DEKKMP) or a scrambled signal (DEMPKK) from an adenoviral protein, E3/19K, onto the cytoplasmic end of *sis*-G fusions (Lee and Donoghue, 1992). The next six constructs contain residues 21–45 of E1, a glycoprotein from an avian coronavirus. These residues encode the first transmembrane domain of E1, which has been shown to confer *cis*-Golgi localization. The last three constructs in the E1 set have a 39-amino acid section of the glycoprotein G from the vesicular stomatitis virus (VSV-G) fused to the cytoplasmic tail. The mutant forms of these constructs, *sis*-E1(*QI*) and *sis*-E1(*ins*), should abolish the signal's Golgi-retention capabilities. The final two constructs have a section of TGN38, a TGN-localized protein, encoding the transmembrane domain and C-terminal tail (amino acids 284–340) fused to v-*sis*. A mutant form was made by truncating the cytoplasmic tail region, and thus uses only amino acids 284–311 of TGN38. This construct should not be retained intracellularly.

proteins constructed for these experiments. The first three constructs, *sis*-G, *sis*-G-ER$^+$, and *sis*G-ER$^-$, were described previously (Lee and Donoghue, 1992), and served as a basic model for the construction of these new fusion proteins. In all cases, the first 239 amino acids of v-*sis* are included in the fusion proteins. This portion of v-*sis* provides a signal sequence, a dibasic proteolytic processing site, and the 82-amino acid minimal transforming region (Hannink and Donoghue, 1986*b*; Sauer et al., 1986; Giese et al., 1987; Sauer and Donoghue, 1988). All constructs are in Type I orientation – the NH_2 terminus is "out", and the COOH terminus "in".

The *sis*-E1 fusion incorporates a 25-amino acid segment of the avian coronavirus E1 protein, containing the entire first transmembrane domain of E1 and a short cytoplasmic tail. This region has been shown to confer localization of E1 to the *cis*-Golgi cisternae, allowing for assembly of the coronavirus to occur at intracellular membranes (Swift and Machamer, 1991; Machamer et al., 1990). When incorporated into heterologous proteins, this E1 transmembrane domain can function as a *cis*-Golgi localization signal, as shown by incorporation of this transmembrane domain into fusion proteins with VSV-G and αm (a human chorionic gonadotropin/VSV-G fusion protein) (Swift and Machamer, 1991). The *sis*-E1(*QI*) construct incorporates a mutation that changes Gln 37 to Ile, and this mutation abolishes correct localization (Swift and Machamer, 1991). The *sis*-E1(*ins*) construct contains an insertion of two Ile residues in the transmembrane domain, which similarly abolishes *cis*-Golgi localization. The *sis*-E1-G constructs are quite similar to the above, but they have an extended COOH-terminal domain provided by a portion of the G protein from VSV-G (Rose and

Gallione, 1981). The addition of the G tail allows for double-label immunofluorescence experiments to be performed, and does not significantly alter the localization efficiency or function of the fusion proteins, as described below.

The membrane-anchor region of *sis*-TGN38 consists of 59 amino acids, containing the transmembrane domain and cytoplasmic tail of TGN38, a Type I-oriented protein which was isolated from rat liver Golgi membranes (Luzio et al., 1990) and is normally localized to the TGN (Ladinsky and Howell, 1992; Bos et al., 1993; Reaves et al., 1993). A tyrosine-containing motif, Tyr-Gln-Arg-Leu (YQRL), in the COOH-terminal domain of TGN38, has been shown to be essential for TGN localization (Bos et al., 1993; Humphrey et al., 1993; Wong and Hong, 1993). This motif has also been shown to be sufficient for localizing heterologous proteins, such as the LDL receptor and the Tac antigen (interleukin-2 receptor A-chain) to the TGN (Bos et al., 1993; Humphrey et al., 1993). A mutant version of the *sis*-TGN38 construct, referred to as *sis*-TGN38Δ, was constructed using only the 28 amino acids of TGN38 that encompass the transmembrane domain. Thus, the *sis*-TGN38Δ construct lacks the essential tyrosine-containing motif, and should not be retained in the TGN.

The sis-E1 Fusion Proteins Are Unable to Cause Cellular Transformation

The transforming ability of the various v-*sis* fusion proteins was assayed by transfection of NIH3T3 cells using MLV-based retroviral constructs (Bold and Donoghue, 1985). The relative ability to transform cells was based on the number of foci formed, with both positive and negative (mock) controls for comparison. The same amount of DNA was transfected for each construct, and the number of foci formed was normalized to the activity of the positive control, *sis*-G. As shown in Table I, cells transfected with *sis*-E1 or *sis*-E1-G exhibited negligible transforming activity, comparable to the mock-transfected cells. The presence of the VSV-G cytoplasmic tail in the *sis*-E1-G construct seemed to have little or no effect on its transforming activity. Thus, addition of the retention signal of the E1 protein abrogated the transforming potential of v-*sis*, presumably by localizing virtually all of the fusion protein to an intracellular location incapable of autocrine stimulation.

In contrast, all of the derivatives that incorporated mutations in the E1 localization signal, including *sis*-E1(*QI*), *sis*-E1(*QI*)-G, *sis*-E1(*ins*), and *sis*-E1(*ins*)-G, exhibited transformation with efficiencies ranging from ~25–50% of *sis*-G (Table I). These fusion proteins were consistently less transforming the *sis*-G, but this phenomenon has been observed before in our lab, with other membrane-anchored derivatives of v-*sis* (Hannink and Donoghue, 1986*a*; Lee and Donoghue, 1991; Xu et al., 1993). This may result from the fact that these membrane-anchored ligands are restricted in their ability to diffuse, and thus less likely to activate receptors as efficiently as the native secreted protein.

The sis-TGN38 Fusion Protein Causes Inefficient but Detectable Cellular Transformation

Focus assays were also performed by transfecting NIH3T3 cells with the *sis*-TGN38 and *sis*-TGN38Δ constructs. Table I shows that, when fused to a portion of TGN38 containing the TGN-localization signal, v-*sis* can interact with the PDGF receptor to result in autocrine transformation with an efficiency of 32% of the *sis*-G control. The mutant derivative, *sis*-TGN38Δ, which lacks the tyrosine-containing TGN localization signal, was consistently more active in transformation assays, exhibiting 55% as many foci as the *sis*-G control. Recently, it was demonstrated that TGN38 recycles from the TGN to the cell surface (Reaves et al., 1993). This observation complicates our results, in that we are at this point unable to determine if the transforming potential of the *sis*-TGN38 fusion protein is due to a subpopulation of protein molecules present on the cell surface at any given time, or whether it is truly mediated by ligand/receptor interactions occurring within the TGN. Further experiments will be needed to clarify this.

Dimerization of the Fusion Proteins

To verify that the constructs used in this study were capable of encoding the desired proteins, the coding regions from each construct were swapped into a pSP64(polyA) vector (Promega Biotec, Madison, WI) for in vitro transcription and translation experiments. Messenger RNA was synthesized (Melton, 1987) and translation reactions were performed in rabbit reticulate lysate in the presence of [^{35}S]Cys. SDS-PAGE analysis of the products of these reactions demonstrated proteins with molecular weights that correlated with the expected sizes of each of the fusion proteins (data not shown). This indicates that our constructs encode the desired fusion proteins.

Table 1. Properties of Golgi-localized sis-derivatives

Construct	Origin of TM domain	Origin of cyto tail	Predicted location	Localization by IF		Transformation
				Permeabilized	Non-perm.	
sis-E1	IBV E1	–	*cis*-Golgi	ER/early-Golgi	–	0.6%
sis-E1(*ins*)	IBV E1	–	Surface	ER/Golgi	Surface	27%
sis-E1(*QI*)	IBV E1	–	Surface	ER/Golgi	Surface	34%
sis-E1-G	IBV E1	VSV-G	*cis*-Golgi	ER/early-Golgi	–	0.7%
sis-E1(*ins*)-G	IBV E1	VSV-G	Surface	ER/Golgi	Surface	25%
sis-E1(*QI*)-G	IBV E1	VSV-G	Surface	ER/Golgi	Surface	49%
sis-TGN38	TGN38	TGN38	TGN	Golgi	Surface (weak)	32%
sis-TGN38Δ	TGN38	Δ	Surface	Golgi	Surface	55%

Transformation efficiency for each construct is shown as a percentage of the positive control *sis*-G. Data shown is from a single experiment, which was repeated at least three times with similar results.

The results summarized in Table I indicate that *sis*-E1 and *sis*-E1–G chimeric proteins are not able to cause transformation. The most likely explanation is that these proteins are anchored in a cellular compartment where they are unable to functionally interact with PDGF receptors. However, one might postulate other explanations for the lack of transforming activity exhibited by these proteins. For example, a critical post-translational event may be altered by forcing v-*sis* to remain intracellular, thereby abolishing its activity. N-linked glycosylation of v-*sis* is not required for its biological activity (Sauer et al., 1986), and bacterially-expressed PDGF-BB (which has no N- or O-linked glycosylation) is biologically active (Hoppe et al., 1989). Therefore, glycosylation of v-*sis*, although altered in these fusion proteins, does not constitute a critical processing event. However, v-*sis* must dimerize in order to function (Hannink et al., 1986), so perhaps these fusion proteins do not dimerize correctly. To address this, we infected NIH3T3 cells with viral supernatants collected from cells transfected with the indicated v-*sis* fusion constructs (see legend to Fig. 2). The fusion proteins were immunoprecipitated using the α-*sis* antibody, and one half of the sample was treated with nonreducing sample buffer to examine dimeric proteins, while the other half was treated with reducing sample buffer to examine monomeric forms. Fig. 2, lanes *3* and *9* shows that the *sis*-E1 chimera indeed dimerizes, yielding a dimer band of ~62–64 kD under nonreducing conditions, and a 32-kD monomer band under reducing conditions. Comparison of the left and right panels of Fig. 2 further illustrates that all of the chimeric proteins dimerize as expected, indicating that this critical processing event is not altered in the fusion proteins. Thus, these data suggest that the transforming activity of the v-*sis* fusion proteins described here most closely correlates with their subcellular localization within the cell, as verified further below, rather than resulting from differences in posttranslational processing events.

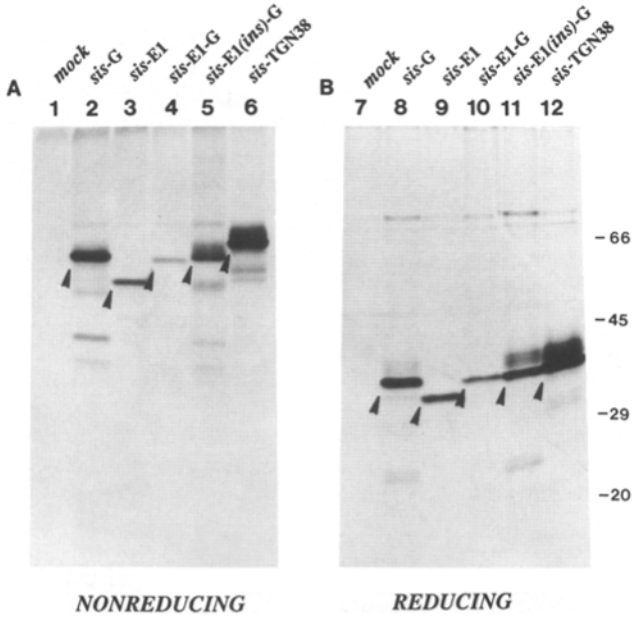


Figure 2. SDS-PAGE analysis of dimers of v-*sis* fusion proteins. Equivalent numbers of NIH3T3 cells expressing various v-*sis* fusion proteins were labeled for 2 h with 100 μCi each [^{35}S]Cys and [^{35}S]Met. Cell lysates were immunoprecipitated with an antibody directed against v-*sis*. One-half of the sample was treated with nonreducing sample buffer, to visualize dimers, while the remaining half was treated with reducing sample buffer (containing 2-mercaptoethanol), to visualize monomers. Samples were analyzed by 15% SDS-PAGE followed by fluorography. The arrows indicate the position of dimer and monomer bands, respectively, of v-*sis* fusion proteins. Molecular mass markers in kD are indicated at the right. Lanes *1* and *7*, mock-infected cells; lanes *2* and *8*, *sis*-G; lanes *3* and *9*, *sis*-E1; lanes *4* and *10*, *sis*-E1-G; lanes *5* and *11*, *sis*-E1(*ins*)-G; and lanes *6* and *12*, *sis*-TGN38. Exposure time was 3 d.

Fusion Proteins Are Synthesized and Are Processed According to Subcellular Location

Like most other secreted proteins, v-*sis* undergoes a variety of posttranslational modifications as it passes through the secretory pathway. One of these processing events is cleavage at a dibasic site to release the propeptide region of the protein. This is thought to occur late in the secretory pathway, between the *trans*-cisternae of the Golgi complex and the plasma membrane (Robbins et al., 1985; Lokeshwar et al., 1990). Thus, *sis*-E1 and *sis*-E1–G, if retained in the early Golgi complex, should not undergo this processing step. The mutant versions of these two fusion proteins, however, should reach the cell surface and thus should exhibit processing of this propeptide. Similarly, the *sis*-TGN38 and *sis*-TGN38Δ fusion proteins both should exhibit processing, since these should be either retained in a compartment that is past the site of this modification, or be present on the cell surface.

To examine proteolytic processing of our fusion proteins, NIH3T3 cells expressing the desired proteins were metabolically labeled with [^{35}S]Cys and [^{35}S]Met, and labeled proteins were immunoprecipitated and separated by SDS-PAGE. As shown in Fig. 3, the *sis*-E1–G protein showed no detectable processed forms (Fig. 3, lane *2*), implying retention in an early Golgi compartment. On the other hand, the mutant derivatives *sis*-E1(*QI*)–G and *sis*-E1(*ins*)–G both exhibited processing, which appears as a doublet of lower mo-

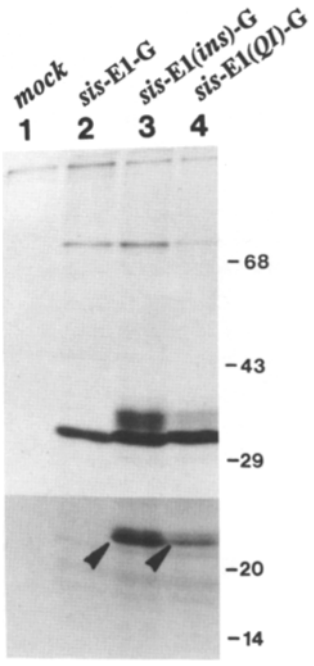


Figure 3. SDS-PAGE analysis of immunoprecipitated proteins encoded by Golgi-localized v-*sis*-derivatives. Equivalent numbers of NIH3T3 cells expressing various v-*sis* fusion proteins were labeled for 2 h with 100 μCi each [^{35}S]Cys and [^{35}S]Met. Cell lysates were immunoprecipitated with an antibody directed against v-*sis* and analyzed by 15% SDS-PAGE followed by fluorography. The arrows indicate the position of proteolytically processed v-*sis* fusion proteins. Molecular mass markers in kD are indicated at the right. Lane *1*, mock-infected cells; lane *2*, *sis*-E1–G; lane *3*, *sis*-E1(*ins*)–G; lane *4*, *sis*-E1(*QI*)–G. Exposure time to show the processed bands was 2 d for the top half of the figure, and 8 d for the bottom half.

lecular mass bands (Fig. 3, lanes *3* and *4*, indicated by arrows). Similar results were obtained using the constructs lacking the G tail (data not shown). The diffuse signal above the major bands most likely represents heterogeneity of O-linked oligosaccharides which, although previously observed (Lee and Donoghue, 1992), have not been extensively characterized. In summary, these results are consistent with localization of *sis*-E1 and *sis*-E1–G to the early Golgi complex, whereas the proteins encoded by the other constructs have clearly progressed through the secretory pathway beyond the *trans*-Golgi complex.

Cleavage of the propeptide region of v-*sis* is not required for its activity, as demonstrated initially in previous work from this laboratory in which the Lys-Arg cleavage site of v-*sis* was mutated to Asn-Ser with no change in biological activity (Sauer et al., 1986). In subsequent studies from our lab, the KR to NS mutation has been incorporated into a variety of membrane-anchored derivatives (Hannink and Donoghue, 1986*b*; Lee and Donoghue, 1992; Xu et al., 1993), including *sis*-G and *sis*-G–ER$^-$, with no effect on biological activity. This is an important point, as the constructs that are retained in the early Golgi, *sis*-E1 and *sis*-E1–G, would not be expected to undergo this cleavage process. Thus, we can conclude that the inactivity of these proteins is not due to their lack of propeptide cleavage.

Cell Surface Expression Is Detectable in sis-E1 Mutants and sis-TGN38 Derivatives

To examine the subcellular location of v-*sis* fusion proteins, NIH3T3 cells were transfected with MLV expression constructs and then processed for immunofluorescence. The proteins were detected by an antibody to the v-*sis* portion of the fusion proteins. As visualized in the permeabilized cells shown in Fig. 4, there was a high level of expression for all the fusion proteins presented in this figure (see Fig. 4, *A*, *C*, *E*, and *G*). Staining can be seen of the ER and Golgi of these cells, indicating the presence of these fusion proteins throughout the secretory compartment. The cells depicted in Fig. 4 are representative of the positive cells seen in these transient expression assays. The percentage of cells expressing the desired constructs typically ranged from ~1–5%.

Surface staining of nonpermeabilized cells was readily detectable for cells expressing *sis*-E1(*ins*), *sis*-E1(*QI*), and *sis*-TGN38Δ derivatives (see Fig. 4, *B*, *D*, and *H*), indicating that these fusion proteins are efficiently transported to the cell surface, as expected. The *sis*-TGN38 construct (*F*) displays decreased but detectable surface staining, indicating that a portion of the population of this protein reaches the cell surface. This is consistent with the behavior of native TGN38, which has been shown to recycle between the TGN and the cell surface (Reaves et al., 1993). Since a small amount of this *sis*-TGN38 fusion protein reaches the cell surface, this may explain the transforming activity of this construct in the focus formation assays (see Table I). However, at this time we have no way of determining if the TGN-retained population of the fusion protein contributes in any way to the transforming activity.

The sis-E1–G Fusion Protein Is Efficiently Retained within the Cell

While Fig. 4 clearly demonstrates that the *sis*-E1 mutants and the *sis*-TGN38 derivatives reach the cell surface, it does not answer the question of whether the *sis*-E1 or *sis*-E1–G fusions do not reach the surface. To address this question, double-label immunofluorescence was performed using the constructs bearing the VSV-G cytoplasmic tail. This allowed for simultaneous examination of both intracellular and cell surface populations of the various fusion proteins within the same cell. Cell surface protein was detected with an antibody against v-*sis*. The cells were then permeabilized, and intracellular fusion proteins were detected with a monoclonal antibody to the VSV-G protein. As positive controls, both the *sis*-E1(*ins*)–G and the *sis*-E1(*QI*)–G fusion proteins were included in this assay. As in Fig. 4, Fig. 5 shows that these proteins were readily detected within the cell (*E* and *G*) as well as on the cell surface (*F* and *H*). As another control, *sis*-G was included. This fusion protein was created in our lab for previous experiments, and it localizes to the cell surface (Hannink and Donoghue, 1986*a*). *A* and *B* of Fig. 5 clearly demonstrate both intracellular and surface staining for this construct. *C* demonstrates the reticular and perinuclear intracellular staining consistently seen for *sis*-E1–G, indicating presence of this protein within the ER and Golgi complex.

When looking for *sis*-E1–G protein on the surface of the same cell, *D* demonstrates that there is no detectable surface staining. As with Fig. 4, these were transient expression assays, and the typical percentage of cells expressing the transfected fusion constructs was ~1–5%. We deliberately examined cells expressing high levels of protein within the cell, so that even weak cell surface staining would be detectable. Although deliberate selection of high-expressing cells tended to obscure any detail present in the permeabilized cells, the issue of whether *sis*-E1 and *sis*-E1–G are in fact localized to the early Golgi complex is addressed in colocalization experiments in the subsequent section. The cell featured in Fig. 5 is representative of all *sis*-E1–G–expressing cells, in that we were never able to detect protein on the cell surface. Thus, the *cis*-Golgi retention signal of the E1 glycoprotein, when appended to the v-*sis* protein, results in efficient retention of the fusion protein to an intracellular compartment.

Colocalization with Golgi Markers Confirms the Targeting of sis-E1–G

To demonstrate that *sis*-E1 and *sis*-E1–G fusion proteins are indeed targeted to the early Golgi complex, we have used double-label immunofluorescence to colocalize these chimeric proteins with known Golgi markers. The Golgi markers used were (*a*) *Lens culinaris* lectin, a carbohydrate-binding protein that binds to terminal α-mannosyl and α-D-glucosyl residues (Kornfeld et al., 1981), and has been shown to stain primarily the Golgi complex of cells (Hsu et al., 1992; Machamer et al., 1993); and (*b*) a monoclonal antibody 10E6, described by Wood et al. (1991), which was localized to the *cis*-Golgi complex of NRK cells by immunoelectron microscopy. In these studies, the *sis*-E1–G chimera was expressed in NIH3T3 cells by infection with retroviral supernatants, and was detected in fixed and permeabilized cells with a polyclonal rabbit antisera to v-*sis*. This in turn was visualized with a rhodamine-conjugated goat anti-rabbit IgG. To visualize the Golgi complex, these same cells were treated with either a fluorescein-conjugated *Lens culinaris* lectin, or with the mouse mAb 10E6, which was visualized with a fluorescein-conjugated goat anti-mouse IgG. The two cells shown for each condition in Fig. 6 are representative

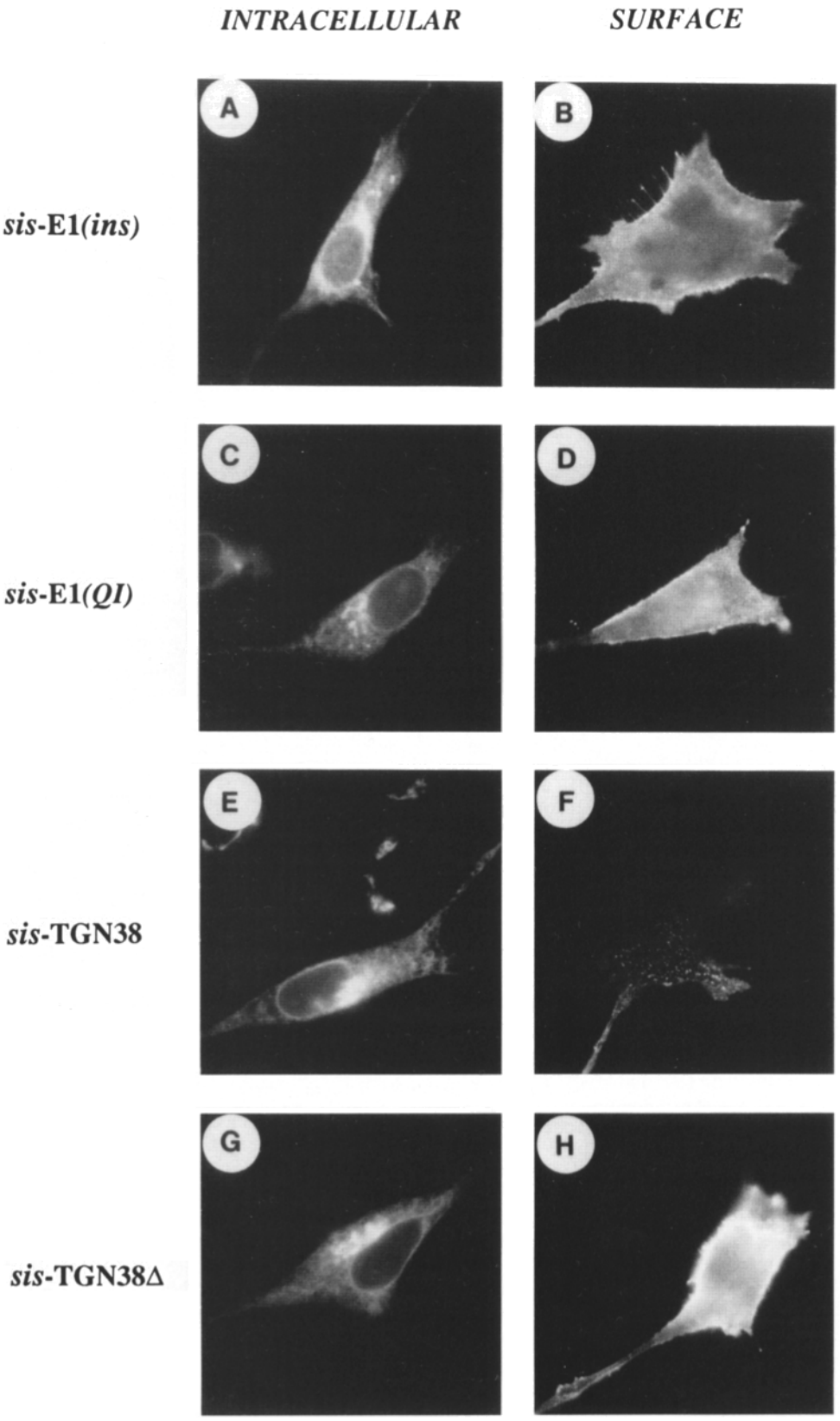


Figure 4. Intracellular and cell surface localization of v-*sis* fusion proteins. Immunofluorescence was performed on permeabilized cells (*A*, *C*, *E*, and *G*) and nonpermeabilized cells (*B*, *D*, *F*, and *H*) expressing various v-*sis* fusion proteins. Proteins were detected with a rabbit antiserum specific for the v-*sis* protein and a rhodamine-conjugated goat anti-rabbit secondary antibody. *A* and *B*, *sis*-E1(*ins*); *C* and *D*, *sis*-E1(*QI*); *E* and *F*, *sis*-TGN38; *G* and *H*, *sis*-TGN38Δ.

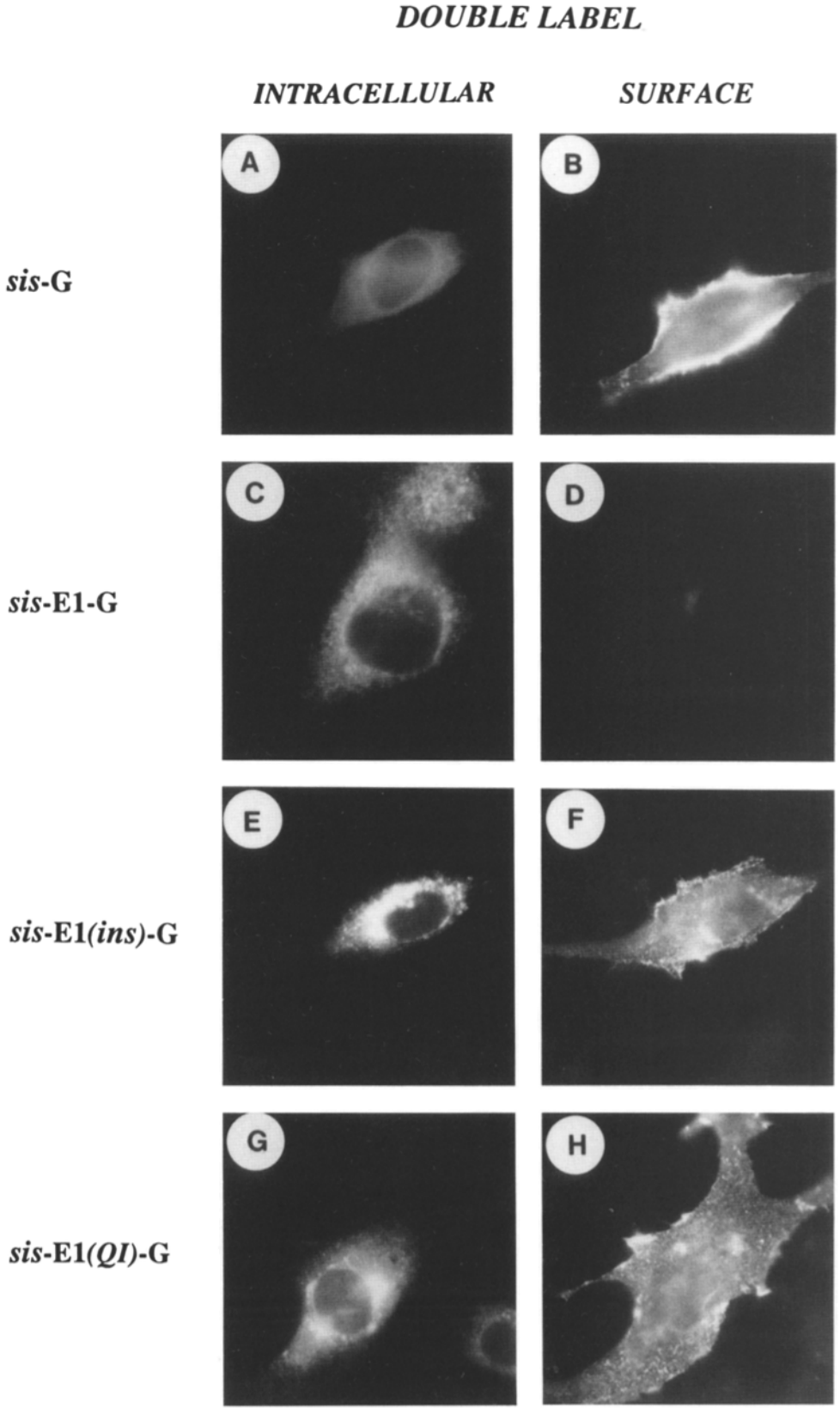


Figure 5. Double-label immunofluorescence allows for simultaneous examination of intracellular and cell surface expression of fusion proteins. Cells expressing various fusion proteins were processed for immunofluorescence. Surface proteins were detected by a rabbit serum directed against the v-*sis* protein, and rhodamine-conjugated goat anti-rabbit antibody (*B*, *D*, *F*, and *H*). Intracellular proteins were detected by a mouse mAb against the COOH-terminal portion of the VSV-G protein and a biotin-conjugated goat anti-mouse antibody, followed by FITC-conjugated streptavidin (*A*, *C*, *E*, and *G*). *A* and *B*, *sis*-G, *C* and *D*, *sis*-E1-G; *E* and *F*, *sis*-E1(*ins*)-G; *G* and *H*, *sis*-E1(*QI*)-G.

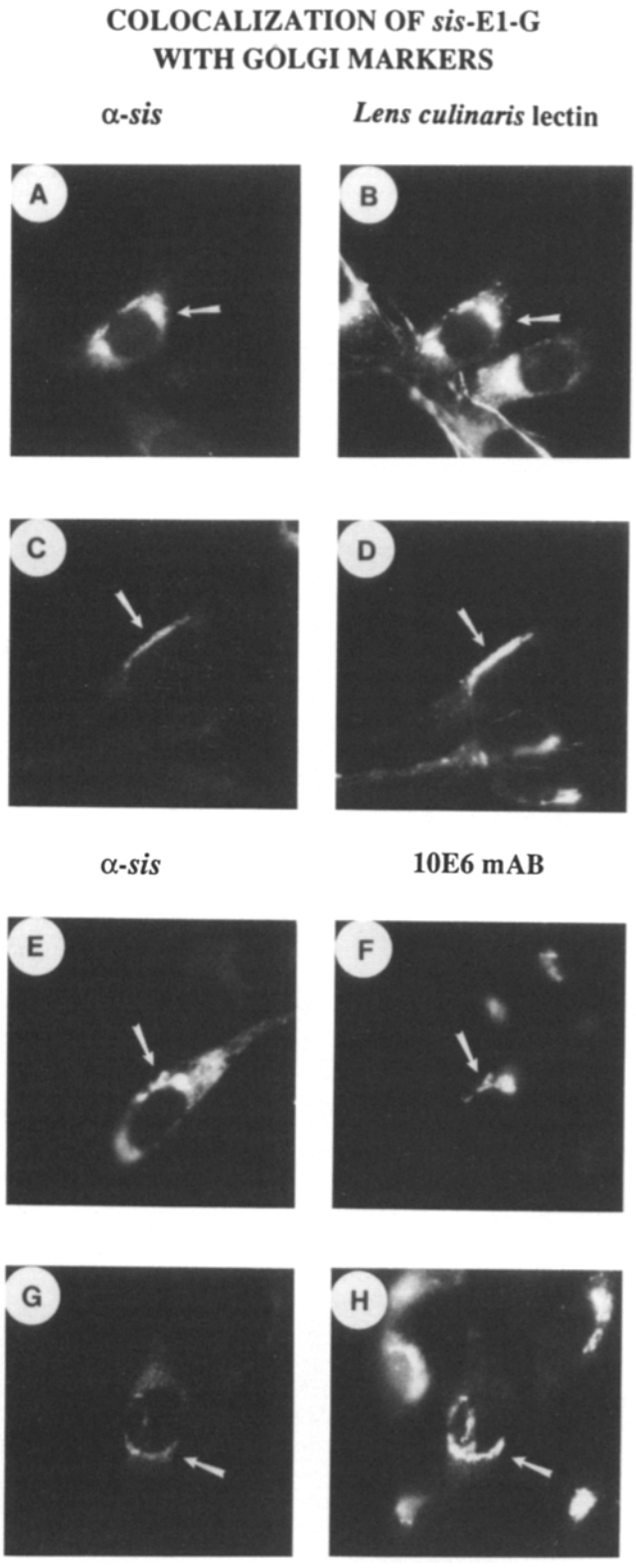


Figure 6. Colocalization of *sis*-El–G with Golgi markers. Cells expressing the *sis*-El–G fusion protein were processed for immunofluorescence. The fusion protein was detected by a rabbit serum directed against the v-*sis* protein, and rhodamine-conjugated goat anti-rabbit antibody (*A*, *C*, *E*, and *G*). To simultaneously detect the Golgi complex, cells were then incubated with either fluorescein-conjugated *Lens culinaris* lectin (*B* and *D*), or mAb 10E6 plus fluorescein-conjugated goat anti-mouse IgG (*F* and *H*). Arrows indicate regions of each cell that stain positively for both the *sis*-El-G fusion protein and the Golgi complex.

of the *sis*-El–G–expressing cells generated in these immunofluorescence assays. The percentage of cells expressing protein was higher using this infection protocol than that obtained by transient transfections. This percentage varied from ~10–15%. As Fig. 6 shows, the *sis*-El–G fusion protein clearly colocalizes with both the lectin (see *A* and *B*, *C* and *D*) and the mAb 10E6 (see *E* and *F*, *G* and *H*). Thus, the El *cis*-Golgi targeting signal functions correctly and targets v-*sis* to the early Golgi when incorporated into a fusion protein.

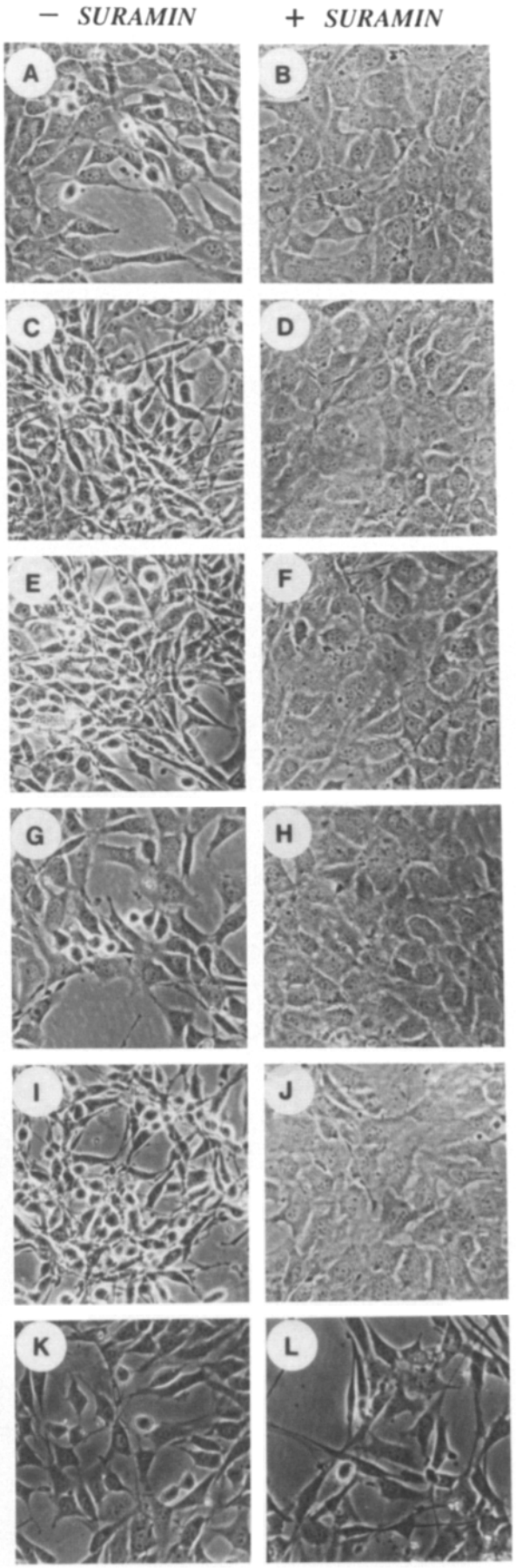
Suramin Reverts the Transformed Phenotype of sis-El Mutants and sis-TGN38 Constructs

Suramin is a polysulfonated naphthylurea derivative reported to inhibit PDGF mitogenic activity and to revert the v-*sis* transformed phenotype (Betsholtz et al., 1986). It is postulated to exert this effect by disruption of ligand-receptor interactions that occur on the cell surface. This mechanism of action is supported by the ability of suramin to reduce the level of tyrosine-phosphorylated cell surface PDGF receptors, while having no effect on the levels of tyrosine phosphorylation of the intracellular, immature forms of the receptors (Fleming et al., 1989). Since suramin has been shown to accumulate intracellularly (Hawking, 1978; LaRocca et al., 1990), it may also be possible for it to interfere with intracellular interactions between receptor and ligand (Huang and Huang, 1988).

To further examine the mechanism of transformation occurring in our cells, we treated NIH3T3 cells expressing the transforming constructs with suramin to see if the transformed phenotype would revert in its presence. Transformed cells expressing *sis*-El(*QI*), *sis*-El(*ins*), *sis*-TGN38, *sis*-TGN38Δ, and v-*sis* as a positive control, were examined in the absence of suramin (Fig. 7, *A*, *C*, *E*, *G*, and *I*), or in the presence of suramin (Fig. 7, *B*, *D*, *F*, *H*, and *J*). In all cases, suramin did indeed revert the phenotype. It has been shown that E5, an oncoprotein derived from the bovine papillomavirus, can interact with immature intracellular forms of PDGF receptors, and may stimulate their autophosphorylation activity (Goldstein et al., 1992; Petti and DiMaio, 1992; Cohen et al., 1993). It has also been shown by Xu et al. (1993) that BPV-E5–transformed cells do not revert in the presence of suramin. Thus, cells transformed by E5 were included as a negative control in the suramin reversion assay. As expected, the presence of suramin did not affect the transformed phenotype of NIH3T3 cells expressing E5 (Fig. 7, *K* and *L*). These results indicate that the productive transforming interactions between PDGF receptors and the v-*sis* fusion proteins described here are occurring in a suramin-sensitive site, most likely the cell surface.

Downregulation of PDGF-β Receptors Does Not Occur in Response to Intracellularly Retained Fusion Proteins

A normal response in cells that are chronically exposed to PDGF is the downregulation of PDGF receptors (Garrett et al., 1984). This downregulation occurs via endocytosis of



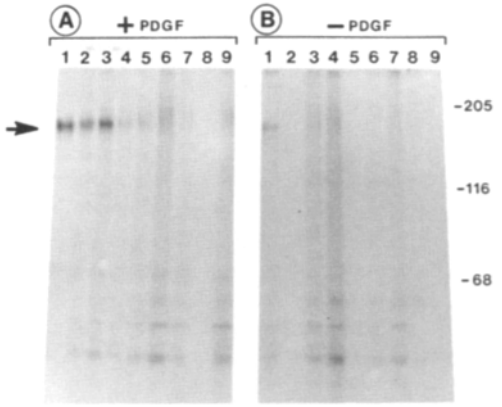


Figure 8. In vitro kinase analysis of surface PDGF-β receptors. Infected NIH3T3 cells expressing v-*sis* fusion proteins were starved for 24 h in DME media minus calf serum, then treated with 100 ng/ml PDGF-BB for 5 min (*A*, lanes 1–9) or left untreated (*B*, lanes 1–9). Receptors were immunoprecipitated with a rabbit antiserum directed against mouse PDGF-β receptors, and subjected to kinase reactions. Products were analyzed by SDS-PAGE and autoradiography. *A* and *B*, lane 1 uninfected NIH3T3 cells; *A* and *B*, lane *2*, mock-infected cells; *A* and *B*, lane *3*, *sis*-E1; *A* and *B*, lane *4*, *sis*-E1(*ins*); *A* and *B*, lane *5*, *sis*-E1(*QI*); *A* and *B*, lane *6*, *sis*-TGN38; *A* and *B*, lane *7*, *sis*-TGN38Δ; *A* and *B*, lane *8*, *sis*-G; lane 9, *sis*-G-ER$^+$. Molecular mass markers are indicated in kD. The arrow indicates the position of the PDGF-β receptor.

receptors from the cell surface and subsequent degradation. To analyze if such a process occurred in cells expressing any of the v-*sis* fusion proteins described in this paper, PDGF-β receptors were immunoprecipitated from serum-starved cells expressing the fusion proteins either before or after stimulation with PDGF-BB. These receptors were then subjected to an in vitro kinase assay, and incorporation of labeled phosphate from [γ-^{32}P]ATP into the receptor protein was visualized by SDS-PAGE. As seen in Fig. 8, in the absence of stimulation with PDGF, little or no activatable receptors were detected (*B*, lanes *1–9*). After stimulation with PDGF-BB, however, NIH3T3 cells, mock-transfected cells, and cells expressing *sis*-E1 all exhibited a significant level of cell surface activatable receptors (*A*, *1–3*), as demonstrated by the phosphorylation of PDGF-β receptors of ~180 kD. Expression of the mutant *sis*-E1(*QI*) and *sis*-E1(*ins*) constructs, which reach the cell surface, led to downregulation of cell surface activatable receptors, and thus there was little detectable kinase activity in these samples (Fig. 8*A*, lanes *4* and *5*). Similar results were obtained with *sis*-TGN38, *sis*-TGN38Δ and v-*sis*, which also reach the cell surface (Fig. 8*A*, lanes *6*, *7*, and *8*). These results demonstrate that when v-*sis* is forced to remain in an intracellular compartment, such as the early Golgi, it is unable to downregulate cell surface PDGF receptors.

Figure 7. Suramin treatment reverts the transformed phenotype of cells expressing cell-surface fusion proteins. For each construct, the left panel shows cells in the absence of suramin (*A*, *C*, *E*, *G*, *I*, and *K*), while the right panel shows cells after 24-h treatment with 100 μM suramin (*B*, *D*, *F*, *H*, *J*, and *L*). *A* and *B*, *sis*-E1(*ins*)-G; *C* and *D*, *sis*-E1(*QI*)-G; *E* and *F*, *sis*-TGN38; *G* and *H*, *sis*-TGN38Δ; *I* and *J*, v-*sis*; *K* and *L*, BPV-E5.

Discussion

Retention of the v-*sis* oncogene in the early Golgi complex by means of a transmembrane retention signal abolishes its transforming ability, as demonstrated by a dramatic decrease in focus forming activity. All fusion proteins constructed for these experiments dimerized properly, indicating that this critical post-translational modification of the v-*sis* portion of the fusions was not altered. The fusion proteins *sis*-E1 and *sis*-E1-G were efficiently retained intracellularly, as evidenced by the lack of proteolytic processing of the constructs and lack of downregulated cell surface PDGF receptors. Immunofluorescence data are consistent with Golgi localization of the *sis*-E1 and *sis*-E1-G constructs. Colocalization with *Lens culinaris* lectin and mAb 10E6 confirm targeting to the early Golgi complex of the *sis*-E1 and *sis*-E1-G constructs. Mutant derivatives of the these constructs, containing defects in the *cis*-Golgi localization signal, were not retained inside the cell and were transforming. These proteins were proteolytically processed as expected, and were detectable on the cell surface by immunofluorescence. Suramin reverted the transformation induced by these latter constructs, providing further evidence that functional interactions between v-*sis* and PDGF receptors occur primarily on the cell surface. The results obtained with the *sis*-E1 and *sis*-E1-G constructs indicate that the intracellular compartment of the early Golgi complex does not allow for autocrine activation of PDGF receptors.

Attempts to retain v-*sis* protein in a more distal Golgi region by attachment of a TGN retention signal yielded ambiguous results. While most of the *sis*-TGN38 fusion protein was retained intracellularly, some of the protein was able to reach the cell surface, as shown by immunofluorescence. Indeed, it has been demonstrated recently that TGN38 actually recycles from the cell surface and back to the TGN (Reaves et al., 1993). It is likely that this population of molecules that reached the cell surface was responsible for the transformation seen in the focus assays in cells expressing this fusion protein, since treatment with suramin reverted the transformed phenotype. However, we cannot conclusively rule out the possibility that functional autocrine interactions can occur in the TGN. Significantly, however, when the COOH-terminus of the TGN38-derived domain was truncated, the transforming efficiency of the derivative *sis*-TGN38Δ nearly doubled (Table I). This data certainly provides a correlation between transformation and increased cell surface localization, compared with TGN localization.

Previous Evidence for Intracellular Autocrine Interactions

Since v-*sis*-transformed cells express both v-*sis* protein and PDGF receptors, there exists the possibility that these two proteins can interact as they pass simultaneously through the secretory pathway. Keating and Williams (1988) reported the detection of PDGF receptors that are activated intracellularly in v-*sis*-transformed cells. These receptors were of an immature form, as determined by their molecular mass (160 kD) and lack of glycosylation (Huang and Huang, 1988; Keating and Williams, 1988), and are rapidly degraded after stimulation by v-*sis* (Keating and Williams, 1988; Bejcek et al., 1992). High concentrations of antisera to PDGF were shown to be unable to reverse transformation of NRK cells. Also, high levels of exogenously added v-*sis* protein have not been shown to cause transformation of NRK cells (Bejcek et al., 1989). Both of these observations suggest that an intracellular autocrine mechanism may exist. These same researchers attached a six-amino acid ER-retention signal, SEKDEL, to the v-*sis* protein and observed morphological transformation of cells expressing this fusion protein. No secreted fusion protein was detectable. Bejcek and coworkers (1992) also have shown that v-*sis*, but not endogenously expressed PDGF-A homodimers, can activate PDGF receptors intracellularly; thus the capacity of v-*sis* to act intracellularly may underlie its mechanism of transformation.

Evidence for a Post-endoplasmic Reticulum Location for Autocrine Interactions

The KDEL retention signal used by Bejcek et al. (1989) has since been shown to be a retrieval signal—not a true retention signal—allowing for return of escaped proteins to the ER (Pelham, 1991). There is also evidence that this signal allows some leakage of proteins to the cell surface (Zagouras and Rose, 1989). Thus, the finding that v-*sis* can transform cells with this KDEL signal attached most likely indicates that an undetectable amount of the fusion protein was able to escape the ER to a more distal location, such as the cell surface, where productive autocrine interactions occurred. If protein was secreted, it likely was rapidly internalized by receptor-mediated endocytosis, and thus escaped detection. Indeed, we have experienced difficulty in immunoprecipitating and detecting wild-type v-*sis* protein in transformed cells due to this rapid internalization (data not shown).

A different ER retention signal has been identified by Nilsson et al. (1989) from the adenovirus protein E3/19K. Lee and Donoghue (1992) appended this signal to the COOH terminus of the v-*sis* protein and demonstrated that (*a*) the retention signal effectively retained v-*sis* in the ER, with no leakage to the cell surface as confirmed by immunofluorescence, and (*b*) this ER-retained form of v-*sis* was no longer able to transform NIH3T3 cells in an autocrine fashion. This evidence suggests that v-*sis* cannot productively interact with the PDGF receptor within this compartment.

Support for Localization of Autocrine v-sis/PDGF Receptor Interactions Beyond the Trans-Golgi

Hannink and Donoghue (1988) constructed an inducible autocrine system in NIH3T3 cells by placing the v-*sis* gene under control of the hsp70 heat shock promoter, allowing for induction of v-*sis* expression by a short incubation at 45°C. With this system, they demonstrated that productive interactions between v-*sis* and PDGF receptors occur in a monensin-insensitive site. Since monensin acts by disrupting the structure and function of the *trans*-Golgi complex, and reduces the rate of transport of proteins to the cell surface (Tartakoff, 1983), these results indicate that transformation only results when v-*sis* interacts with receptors in a region past the *trans*-Golgi complex. However, since monensin is a pleiotropic agent, there may have been other effects on the cells that were not taken into account. Also, the temperature shock required for induction of the hsp70 promoter may have induced other endogenous heat shock proteins.

It has also been demonstrated that suramin treatment of v-*sis*-transformed cells reverts the transformed phenotype (Fleming et al., 1989), and that suramin decreases phosphorylation levels of cell-surface PDGF receptors with little effect on intracellular receptor phosphorylation. These experiments suggest that v-*sis* protein can interact with intracellular forms of the PDGF receptors in cells and stimulate autophosphorylation activity of these immature receptors, but that activated receptors must reach a suramin-sensitive, cell surface location in order to trigger the signal transduction cascade that leads to transformation.

Previous experiments by Lee and Donoghue (1992) suggested that the ER compartment of the secretory pathway does not support transforming interactions between v-*sis* and PDGF-R. One reason that the *sis*-G-ER$^+$ construct could not productively interact with PDGF-R could be that either the ligand or the receptors had not yet undergone critical posttranslational modifications required for functional interactions and signal transduction. Such modifications may occur in the Golgi portion of the secretory pathway, particularly modifications of N-linked oligosaccharide or the addition of O-linked oligosaccharide which are largely confined to the later Golgi compartments. We have previously demonstrated that, except for disulfide bond formation which occurs in the ER very shortly after translation, further posttranslational modifications are not required for the biological activity of the v-*sis* protein (Hannink et al., 1986; Hannink and Donoghue, 1986*b*; Sauer and Donoghue, 1988). However, extensive oligosaccharide addition and modification to PDGF-R occurs, and its importance is not clear (Keating and Williams, 1987). Use of an early Golgi retention signal—the first transmembrane domain of the E1 glycoprotein—allowed us to begin to investigate possible autocrine interactions within the Golgi region by retaining v-*sis* as a fusion protein in this compartment. This retention of the v-*sis* oncoprotein within the early Golgi complex completely abrogated its transforming ability, and thus we conclude that productive autocrine interactions cannot occur in the early secretory pathway.

Localization of v-*sis* to the TGN by means of a retention signal derived from TGN38 resulted in decreased levels of transforming ability. However, immunofluorescence data indicate that a small portion of this *sis*-TGN38 fusion protein was able to reach the cell surface, a finding that is consistent with reports by Reaves et al. (1993) that TGN38 recycles between the TGN and the cell surface. Suramin treatment of cells expressing *sis*-TGN38 leads to reversion of the transformed phenotype, further implicating a cell surface pool of *sis*-TGN38 in the transformation of these cells. However, we are unable to conclusively determine from these experiments that v-*sis* targeted to the TGN is not transforming. Currently, we are undertaking studies which should further clarify interactions within the late Golgi compartments. These new studies utilize a similar approach of constructing fusion proteins, this time using the transmembrane domains and cytoplasmic tails of well-characterized glycosyltransferases, which are resident Golgi enzymes. These membrane anchors should give tighter retention in the later Golgi compartments than seen with the TGN38-derived retention signal, and will hopefully provide a conclusive indication of whether v-*sis* is able to engage in productive autocrine interactions within the late Golgi complex.

Why Might sis Be Unable to Transform from an Intracellular Compartment?

While it has been established that immature forms of the PDGF-R can be stimulated by v-*sis* to undergo phosphorylation within the secretory pathway (Hannink and Donoghue, 1988; Keating and Williams, 1988; Bejcek et al., 1992), the question remains—are these interactions functional? That is, does this simple intracellular interaction between v-*sis* and PDGF-R contribute to acquisition of the transformed phenotype? One argument against this possibility is that the downstream effector molecules, such as PLC-γ (Kumjian et al., 1989; Meisenhelder et al., 1989; Wahl et al., 1989; Morrison et al., 1990), PI-3 kinase (Coughlin et al., 1989; Kazlauskas and Cooper, 1989), *ras*-GAP (Kaplan et al., 1990; Kazlauskas et al., 1990), and others, that normally interact with activated PDGF-R at the plasma membrane may not be available to the immature activated receptors present within the secretory pathway. The evidence from Bejcek et al. (1992) that indicates the intracellularly phosphorylated, immature forms of the receptor are capable of interacting with PI-3 kinase in 3T3 cells begins to address the functionality of intracellular v-*sis*/PDGF receptor interactions, but far from answers the question. Future studies in our lab will be aimed at examining the availability of such effector molecules within the secretory pathway, and whether they can indeed be activated intracellularly. These studies will include attempts to retarget some members of the signal transduction machinery to the early Golgi complex, to see if this will then allow *sis*-E1 or *sis*-E1–G to signal from this compartment. The results of these future studies should provide significant insight into the mechanisms of autocrine transformation.

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