Intracellular Retention of Membrane-anchored v-*sis* Protein Abrogates Autocrine Signal Transduction

Bruce A. Lee*§ and Daniel J. Donoghue‡§

Departments of ‡Chemistry/Division of Biochemistry, *Biology, and §Center for Molecular Genetics, University of California, San Diego, La Jolla, California 92093-0322

Abstract. An important question regarding autocrine transformation by v-*sis* is whether intracellularly activated PDGF receptors are sufficient to transform cells or whether activated receptor–ligand complexes are required at the cell surface. We have addressed this question by inhibiting cell surface transport of a membrane-anchored v-*sis* protein utilizing the ER retention signal of the adenoviral transmembrane protein E3/19K. A v-*sis* fusion protein containing this signal was retained within the cell and not transported to the cell surface as confirmed by immunofluorescent localization experiments. Also, proteolytic maturation of this protein was suppressed, indicating inefficient transport to post-Golgi compartments of the secretory pathway. When compared with v-*sis* proteins lacking a functional retention signal, the ER-retained protein showed a diminished ability to transform NIH 3T3 cells, as measured by the number and size of foci formed. In newly established cell lines, the ER-retained protein did not downregulate PDGF receptors. However, continued passage of these cells selected for a fully transformed phenotype exhibiting downregulated PDGF receptors and proteolytically processed v-*sis* protein. These results indicate that productive autocrine interactions occur in a post-ER compartment of the secretory pathway. Transport of v-*sis* protein beyond the Golgi correlated with acquisition of the transformed phenotype. Furthermore, suramin treatment reversed transformation and upregulated the expression of cell surface PDGF receptors, suggesting an important role for receptor–ligand complexes localized to the cell surface.

THE study of growth factors and growth factor receptors has led to a better understanding of the processes involved in the regulation of normal cell proliferation. Aberrant expression of such proteins is implicated in altered patterns of cell growth which can lead to cellular transformation and tumorigenesis (for reviews see Aaronson, 1991; Yarden and Ullrich, 1988; Deuel, 1987). The v-*sis* oncogene encodes the transforming protein of simian sarcoma virus (SSV)[1], an acutely transforming retrovirus originally isolated from a woolly monkey fibrosarcoma (Theilen et al., 1971; Devare et al., 1983). Sequence analysis indicated that v-*sis* encodes a protein structurally related to the B chain of platelet-derived growth factor (PDGF), thus indicating a role for this growth factor in tumorigenesis (Doolittle et al., 1983; Waterfield et al., 1983; Johnsson et al., 1984; Josephs et al., 1984). Only cells which express PDGF receptors can be transformed by v-*sis*, supporting a role for an autocrine loop in v-*sis* transformation (Deuel et al., 1983; Garret et al., 1984; Huang et al., 1984; Leal et al., 1985).

There has been some controversy as to where autocrine interactions take place between v-*sis* protein and PDGF receptors in v-*sis*-transformed cells. Antibodies directed against PDGF have been shown to inhibit both transformation and the incorporation of [^{3}H]thymidine into some SSV-transformed cells, suggesting the importance of an extracellular site of interaction (Huang et al., 1984; Johnsson et al., 1985). However, in some cases v-*sis* protein was not detectably secreted and such transformed cells were not affected by antibody addition (Huang et al., 184), indicating the possibility of intracellular interaction sites. The possibility of important intracellular interactions has also been supported by the observation that continuous growth of nontransformed cells in the presence of v-*sis* or c-*sis* protein does not result in cellular transformation (Huang and Huang, 1988). Others, however, have claimed no phenotypic difference between v-*sis* transformed cells and nontransformed cells treated externally with PDGF (Johnsson et al., 1986; Fleming et al., 1989), again supporting an extracellular site of interaction.

Since both the v-*sis* protein and PDGF receptors are synthesized through the secretory pathway of cells, the potential exists for growth factor–receptor interactions within various compartments of the ER and Golgi, as well as at the cell surface. Indeed, intracellular interactions, as detected by immature receptor autophosphorylation, have been observed in both SSV-transformed cells (Keating and Williams, 1988;

1. *Abbreviations used in this paper*: CSF-1, macrophage colony stimulating factor; CS, calf serum; M-MuLV, Molony murine leukemia virus; SCF, stem cell factor; SSV, simian sarcoma virus; VSV G, vesicular stomatitis virus glycoprotein.

Huang and Huang, 1988) and cells which inducibly express v-*sis* protein (Hannink and Donoghue, 1988). Rapid turnover of both v-*sis* protein and PDGF receptors was also noted in SSV-transformed cells and the majority of newly synthesized receptors in these cells failed to reach the cell surface (Keating and Williams, 1988; Huang and Huang, 1988; Lokeshwar et al., 1990). Thus, it seems quite apparent that PDGF receptors can and do interact intracellularly with v-*sis* protein. An important question remains, however, as to whether intracellularly activated PDGF receptors are sufficient to transform cells, or whether activated receptor–ligand complexes are required at the cell surface. This latter possibility encompasses two distinct models. In the first model, activated complexes may reach the cell surface after intracellular formation and subsequent cell surface transport. In the second model, cell surface transport may occur separately for both growth factor and receptor, after which point receptor–ligand complexes form leading to signal transduction. There is evidence supporting a model by which receptors are activated internally, but where mitogenic signalling requires the cell surface localization of these activated receptors (Fleming et al., 1989).

To further examine the question of where productive receptor–ligand interactions occur, a membrane-anchor form of the v-*sis* protein (Hannink and Donoghue, 1986*a*) was localized to the ER using the retention signal previously described for the adenoviral transmembrane protein E3/19K (Nilssan et al., 1989). This signal can function to retain heterologous transmembrane proteins such as human CD4 and CD8 within the ER. It should be noted that the E3/19K retention signal differs from the KDEL retention signal described for luminal ER proteins (Munro and Pelham, 1987). The KDEL signal presumably interacts with cellular retention machinery on the luminal side of the ER, while the E3/19K retention signal functions on the cytoplasmic side of the ER.

In this report, we demonstrate that cellular transformation is inhibited by ER retention of a membrane-anchored v-*sis* fusion protein. We also demonstrate that transport of this fusion protein beyond the ER and into the *trans*-Golgi network correlates with downregulation of cell surface PDGF receptors and acquisition of the transformed phenotype.

Materials and Methods

Cell Culture and Antibody Reagents

NIH 3T3 cells and their derivatives were cultured at 37°C in DME containing 10% calf serum (CS) in a humidified 10% CO_2 incubator. Cells were passaged every 4 d in culture. Goat antiserum directed against human PDGF-AB and rabbit antiserum directed against the human PDGF β receptor were purchased from Upstate Biotechnology Inc. (Lake Placid, NY). The human PDGF β receptor antibody cross reacts with murine PDGF β receptors. Rabbit antiserum directed against bacterially synthesized v-*sis* protein was generously provided by Ray Sween and Keith Deen (Smith, Kline, and French, King of Prussia, PA). Rabbit antiserum directed against the murine PDGF β receptor was kindly provided by Jung Huang (St. Louis University School of Medicine) (Huang and Huang, 1988). The mouse mAb P5D4 (Kreis and Lodish, 1986) directed against the COOH terminus of the vesicular stomatitis virus glycoprotein (VSV G) was made available by William Balch (Scripps Clinic and Research Foundation, La Jolla, CA).

Construction of v-sis Fusions

The v-sis^{239}-G gene, which encodes a fusion between v-*sis* and VSV G (Hannink and Donoghue, 1986*a*), was used as the parental clone to construct the v-*sis* fusions described in this report. Polymerase chain reaction (PCR) was utilized to fuse sequences encoding a functional (DEKKMP) or nonfunctional (DEMPKK) ER retention signal (Nilsson et al., 1989) onto the 3′ end of the v-sis^{239}-G gene. Three oligonucleotides were synthesized to amplify a DNA fragment between the v-*sis*/VSV G junction and the 3′ end of the VSV G gene (~190 bp). One oligonucleotide encompassed sequences surrounding the junction region between v-*sis* and the VSV G gene. This junction occurs at the BstEII site, nt 929, of v-*sis* (Devare et al., 1982) and an AluI site, nt 1418 of VSV G (Rose and Gallione, 1981), just upstream of sequences encoding the VSV G transmembrane domain. This oligonucleotide, ^{5}CC. CAA. AGT. CGG. GTG. ACC. TCT. ATT. GCC$^{3'}$, is sense strand sequence and encodes QSRVTSIA, where T is amino acid 239 of the v-*sis* protein, and the second S residue is amino acid 464 of VSV G. The other two oligonucleotides encompassed the extreme 3′ coding region of the v-sis^{239}-G gene including extra sequences encoding a functional or nonfunctional ER retention signal followed by an XhoI site for cloning purposes. The oligonucleotide encoding the functional ER retention signal, $^{5'}$GGCCCTCGAG. TTA. GGG. CAT. TTT. TTT. CTC. GTC. CTT. TCC. AAG. TCG. GTT. CAT. CTC. TAT$^{3'}$, is antisense sequence and encodes the amino acid sequence IEMNRLGKDEKKMP-term in the sense strand. The oligonucleotide encoding the nonfunctional ER retention signal, $^{5'}$GGCCCTCGAG. TTA. TTT. TTT. GGG. CAT. CTC. GTC. CTT. TCC. AAG. TCG. GTT. CAT. CTC. TAT$^{3'}$, is also antisense sequence and encodes IEMNRLGKDEMPKK-term in the sense strand. PCR was used to amplify appropriate DNA fragments using the v-sis^{239}-G gene as a template. PCR amplified products were cleaved with BstEII and XhoI, purified, and ligated into a BstEII/XhoI cleaved plasmid containing the v-sis^{239}-G gene to create the fusions v-sis^{239}-G-ER^{+} and v-sis^{239}-G-ER^{-} (Fig. 1 *B*). The v-sis^{239}-G-ER^{+} protein contains the functional ER retention signal, while the v-sis^{239}-G-ER^{-} protein contains the nonfunctional mutant signal. Appropriate addition of sequences encoding the two hexapeptides was confirmed by DNA sequencing. DNA fragments encoding v-sis^{239}-G, v-sis^{239}-G-ER^{+}, and v-sis^{239}-G-ER^{-} were subsequently cloned into retroviral expression vectors for transfection into NIH 3T3 cells (see below). Mutation of the dibasic proteolytic processing site of the v-*sis* protein from Lys^{110}-Arg^{111} to Asn^{110}-Ser^{111} has previously been reported (Hannink and Donoghue, 1986*b*). Sequences encoding this mutation were swapped into v-*sis* expression vectors using standard molecular cloning techniques.

Focus Formation Assay and Expression of v-sis Proteins

Genes encoding the membrane-anchored v-*sis* fusion proteins depicted in Fig. 1 *B* were placed under control of the Molony murine leukemia virus (M-MuLV) long terminal repeat in the retroviral expression vector, pDD102 (Bold and Donoghue, 1985). For focus assays, NIH 3T3 cells were transfected with these expression plasmids in the presence of a replication competent helper provirus, pZAP (Hoffman et al., 1982), using a modified calcium phosphate transfection protocol (Chen and Okayama, 1987). Briefly, 2×10^5 cells were split onto 60-mm dishes and the following day transfected with a mixture of 50 ng expression plasmid, 50 ng pZAP, 0.5 μg pRSVneo, and 9.4 μg carrier plasmid DNA. Cells were incubated with precipitate overnight, refed, and split the following day 1:4 onto 100-mm dishes. Half the cells were scored for focus formation after 5 d and the other half selected in G418 (400 μg/ml) for 10 d, after which time transfection efficiency was compared by scoring G418 resistant colonies.

To obtain pools of cells expressing the various v-*sis* fusion proteins, transfections were carried out as described above, except larger relative amounts of each plasmid were used (8 μg expression plasmid, 1 μg pZAP, and 1 μg pRSVneo). Infected virus-producing pools of cells resulting from G418 selection were carried in culture for extended periods of time, up to 10 wk, and used for immunofluorescence, metabolic labeling, and in vitro kinase assays. >80% of G418 resistant cells expressed the various fusion proteins as determined by immunofluorescence analysis.

Indirect Immunofluorescence

To detect only 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 both intracellular and cell surface expressed v-*sis* fusion proteins, cells were fixed with paraformaldehyde, and treated with the v-*sis* protein antibody before permeabilization. Cells were then permeabilized and treated with 10 μg/ml of a mAb directed against the COOH

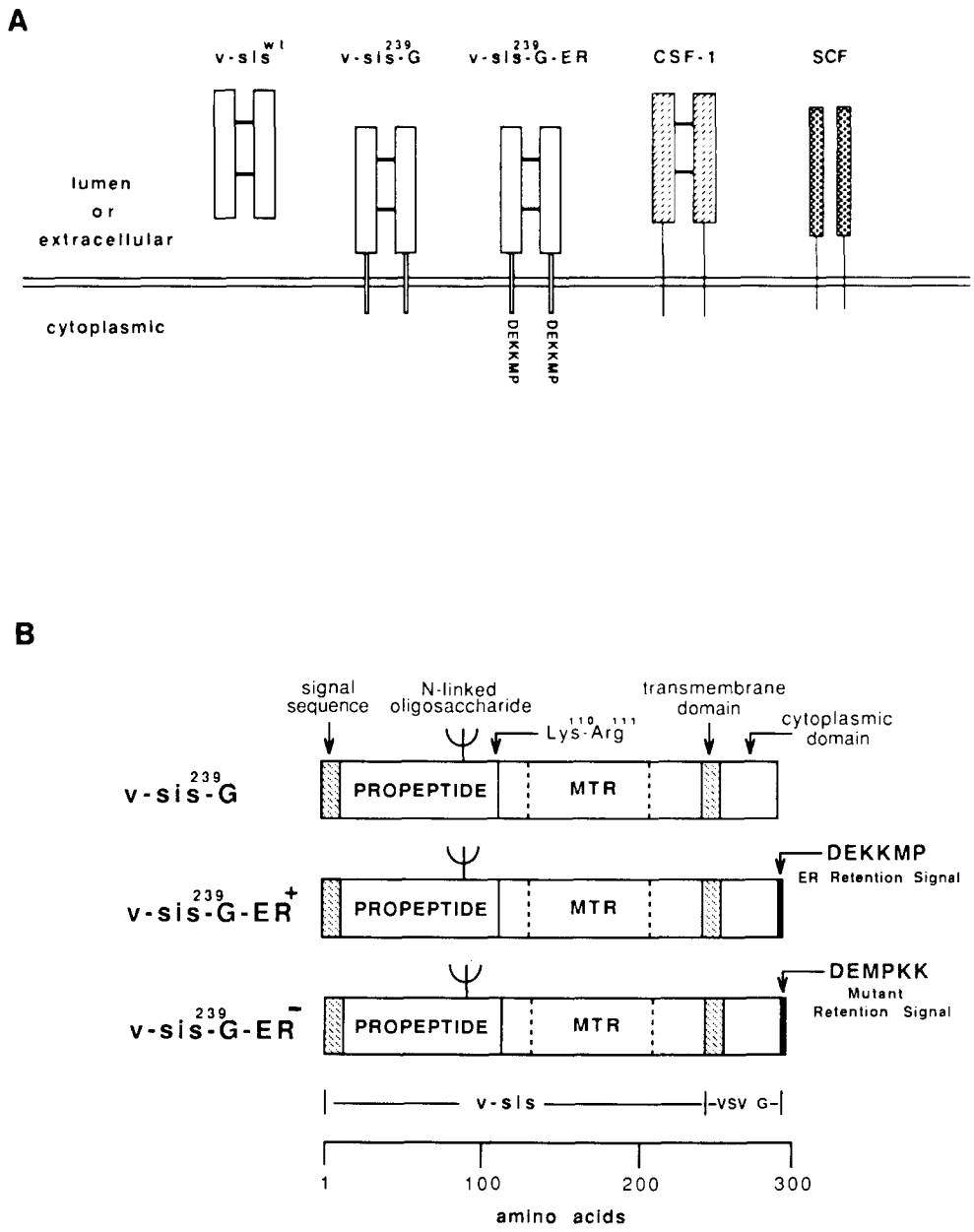

Figure 1. Membrane-anchored v-*sis* fusion proteins. *A* depicts the membrane orientation of various v-*sis* proteins as well as two other growth factors, CSF-1 and SCF, known to exist as membrane-anchored precursors. SCF is also known as mast cell growth factor, and is the ligand for the *kit* receptor. *B* shows a more detailed representation of various v-*sis* fusion proteins. The v-*sis*^{239}-G fusion was constructed by combining sequences encoding the first 239 amino acids of the v-*sis* protein with sequences encoding the COOH-terminal 49 amino acids of the VSV G protein (Hannink and Donoghue, 1986*a*). The v-*sis*^{239}-G-ER$^+$ and v-*sis*^{239}-G-ER$^-$ fusions contain COOH-terminal hexapeptides defined as a functional (*DEKKMP*) or mutant (*DEMPKK*) ER retention signal (Nilssan et al., 1989). Other significant features shown include: a signal sequence for secretion; an N-linked oligosaccharide addition site; and a dibasic proteolytic processing site (*Lys*^{110}-*Arg*^{111}). Also indicated are the v-*sis* minimal transforming region (*MTR*, enclosed by the *dotted lines*) as well as the transmembrane and cytoplasmic domains from VSV G.

terminus of the VSV G protein. Cell surface expressed v-*sis* fusion proteins were detected with a rhodamine-conjugated goat anti-rabbit antibody, while intracellular v-*sis* fusion proteins were detected with a biotin-conjugated goat anti-mouse antibody, followed by FITC-conjugated streptavidin.

Metabolic Labeling and Immunoprecipitations

NIH 3T3 cells expressing various v-*sis* fusion proteins were obtained as described above. Cells were split 5×10^5 on 60-mm plates and the following day rinsed and incubated for 15 min in MEM lacking cysteine and methionine. For the glycosidase digestion experiment, cells were labeled for 3 h with 200 μCi/ml each [^{35}S]cysteine and [^{35}S]methionine in MEM lacking cysteine and methionine. For pulse-chase analyses, cells were labeled with 150 μCi/ml each [^{35}S]cysteine and [^{35}S]methionine for 30 min, rinsed twice with TBS, and chased for various times in fresh DME. Labeled cells were subsequently lysed in 1.0 ml radioimmunoprecipitation assay (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 incubated for 2 h with either a goat antibody directed against human PDGF-AB or a rabbit antibody directed against the murine PDGF β receptor. Immune complexes were isolated with either rabbit anti-goat IgG coupled to agarose beads or with Protein A-Sepharose (Sigma Chemical Co., St. Louis, MO). For oligosaccharide analysis, immunoprecipitates were digested with endoglycosidase H, neuraminidase, and *O*-glycosidase (all from Boehringer Mannheim Corp., Indianapolis, IN) as described previously (Daniel et al., 1987). Immunoprecipitates were subsequently analyzed by SDS-PAGE and detected by fluorography.

Suramin Treatment and In Vitro Kinase Assays

NIH 3T3 cells expressing various v-*sis* fusion proteins were split 5×10^5 cells per 100-mm plate. The following day, medium was removed and replaced with DME containing 10% CS with or without 100 μM suramin. Cells were examined for morphological changes after 24 h in suramin.

For in vitro kinase analysis, cells were split 5×10^5 per 60-mm plate and the following day serum starved for 24 h in DME containing 0.5% CS with or without 100 μM suramin. Following serum starvation, cells were left untreated or incubated with 100 ng/ml PDGF-BB (Amgen, Thousand Oaks, CA) at 37°C for 5 min, after which cells were lysed in 1 ml NP-40 lysis buffer (20 mM Tris, pH 7.4, 137 mM NaCl, 1% NP-40, 1 mM sodium

orthovanadate, 5 mM EDTA, 1 mM PMSF, 10 μg/ml Aprotinin, 10% glycerol) on ice for 10 min. Cell lysates were scraped from plates and clarified for 10 min in a microcentrifuge to remove cellular debris. Clarified lysates were incubated for 2 h with a rabbit antiserum directed against the human PDGF β receptor and immune complexes isolated with Protein A–Sepharose. Immunoprecipitates were washed twice in NP-40 lysis buffer and once in 20 mM Tris, pH 7.4. Washed complexes were resuspended in 40 μl kinase (20 mM Tris, pH 7.4, 10 mM $MnCl_2$, 5 mM $MgCl_2$) containing 5 μCi γ-^{32}P ATP and incubated at 37°C for 10 min followed by 7.5% SDS-PAGE and autoradiography.

Results

Membrane-anchored v-sis Fusion Proteins

A biologically active form of the v-*sis* protein, v-*sis*^{239}-G, is anchored to the cell membrane via the transmembrane domain of the VSV G and has been described previously (Hannink and Donoghue, 1986*a*). Although PDGF has not been found as a naturally occurring transmembrane growth factor, macrophage colony stimulating factor (CSF-1) and stem cell factor (SCF) do exist in transmembrane forms and the receptors for these growth factors are members of the PDGF receptor family (Rettenmier et al., 1987; Anderson et al., 1990). Thus, the study of v-*sis*^{239}-G may serve as a model system for understanding how these naturally occurring membrane-anchored growth factors function. The membrane orientation of v-*sis*^{239}-G as well as CSF-1 and SCF is depicted in Fig. 1 *A*. Also depicted are v-*sis*^{wt} and the ER-retained form of v-*sis*^{239}-G described below.

The v-*sis*^{239}-G protein contains the first 239 amino acids of the v-*sis* protein fused to the COOH-terminal 49 amino acids of VSV G (Fig. 1 *B*). The v-*sis* portion of the v-*sis*^{239}-G fusion contributes: (*a*) a signal sequence for secretion; (*b*) the propeptide region containing a single site for N-linked oligosaccharide addition; (*c*) the dibasic proteolytic processing site (Lys^{110}-Arg^{111}); and (*d*) sequences which encompass the minimal transforming region (MTR) of v-*sis* (Hannink et al., 1986; Sauer et al., 1986; King et al., 1985). The VSV G portion of the fusion contributes the transmembrane domain and COOH-terminal cytoplasmic tail of the protein. Upon synthesis in mammalian cells, the v-*sis*^{239}-G protein enters the secretory pathway where it undergoes signal sequence cleavage, N-linked oligosaccharide addition, and protein dimerization. Following its transport through the Golgi complex, the protein is subsequently proteolyzed at the dibasic processing site to yield the mature membrane-anchored form of the v-*sis* protein. This protein retains the ability to interact productively with PDGF receptors as demonstrated by its ability to efficiently transform NIH 3T3 cells (Hannink and Donoghue, 1986*a*). Surprisingly, however, no autophosphorylation of PDGF receptors is detected in response to mitogenically active v-*sis*^{239}-G (Lee and Donoghue, 1991).

To address the question of whether cell surface expression of v-*sis* protein is required for efficient transformation of NIH 3T3 cells, the v-*sis*^{239}-G protein was retained within the cell and prevented from reaching the cell surface. This was accomplished by appending a signal for ER retention onto the COOH terminus of v-*sis*^{239}-G and subsequently examining the effects of intracellular retention on biological transformation. A hexapeptide sequence (DEKKMP), described as an ER retention signal for the adenoviral transmembrane protein E3/19K (Nilsson et al., 1989), was appended to the COOH terminus of the v-*sis*^{239}-G protein to create v-*sis*^{239}-G-ER$^+$ (Fig. 1 *B*). As a control, a permuted mutant signal (DEMPKK) was also added to the COOH terminus of v-*sis*^{239}-G to create v-*sis*^{239}-G-ER$^-$ (Fig. 1 *B*). This mutant signal does not function as an ER retention signal (Nilsson et al., 1989).

ER Retention Signal Prevents Cell Surface Expression of Membrane-anchored v-sis Protein

The v-*sis*^{239}-G-ER$^+$ and v-*sis*^{239}-G-ER$^-$ proteins were expressed in NIH 3T3 cells using a M-MuLV-based retroviral expression vector (Bold and Donoghue, 1985) and the subcellular localization of the proteins examined by indirect immunofluorescence using an antibody directed against the v-*sis* protein. As shown in Fig. 2, the v-*sis*^{239}-G-ER$^+$ protein accumulated to much higher levels within cells (Fig. 2 *A*) when compared with the v-*sis*^{239}-G-ER$^-$ protein (Fig. 2 *B*). More reticular staining was visible in cells expressing v-*sis*^{239}-G-ER$^+$, while distinct Golgi-staining was observed in cells expressing v-*sis*^{239}-G-ER$^-$. This pattern of expression is consistent with the v-*sis*^{239}-G-ER$^+$ protein being retained in the ER and the v-*sis*^{239}-G-ER$^-$ protein being transported beyond the ER, through the Golgi complex, and to the cell surface. Untransfected NIH 3T3 cells did not exhibit any specific staining using the antibody directed against the v-*sis* protein (data not shown).

To examine whether these v-*sis* fusion proteins were being transported to the plasma membrane, a double-label immunofluorescence experiment was performed. An antibody that recognizes the COOH terminus of the VSV G protein was used to visualize intracellular fusion proteins, while an antibody directed against the v-*sis* protein was used to examine fusion proteins expressed on the cell surface. To detect cell surface v-*sis* proteins with the v-*sis* protein antibody, it was necessary to use fusions that had the dibasic proteolytic processing site mutated from Lys^{110}-Arg^{111} to Asn^{110}-Ser^{111}. This mutation prevents proteolytic maturation of v-*sis*, but has no effect on the transforming ability of the protein (Hannink and Donoghue, 1986*a*,*b*). This manipulation was necessary since the v-*sis* protein antibody preferentially recognizes a determinant within the propeptide of v-*sis* (our own unpublished observation), and this determinant is missing in mature proteins expressed on the cell surface. As shown in Fig. 3, external staining of cells expressing the Asn^{110}-Ser^{111} mutants of v-*sis*^{239}-G (Fig. 3 *A*) and v-*sis*^{239}-G-ER$^-$ (Fig. 3 *E*) showed a general overall fluorescence pattern indicative of proteins expressed on the cell surface. However, little or no cell surface fluorescence was observed for cells expressing the Asn^{110}-Ser^{111} mutant of v-*sis*^{239}-G-ER$^+$ (Fig. 3 *C*), even though this protein was still being expressed intracellularly (Fig. 3 *D*). Untransfected NIH 3T3 cells did not exhibit specific staining using either the v-*sis* protein antibody or the antibody directed against the COOH terminus of VSV G (data not shown). These results indicate that appending an ER retention signal onto the COOH terminus of v-*sis*^{239}-G results in intracellular retention of the protein and an inhibition of detectable cell surface expression.

ER Retention Signal Prevents Proteolytic Maturation of Membrane-anchored v-sis Protein

The v-*sis* protein undergoes several posttranslational modi-

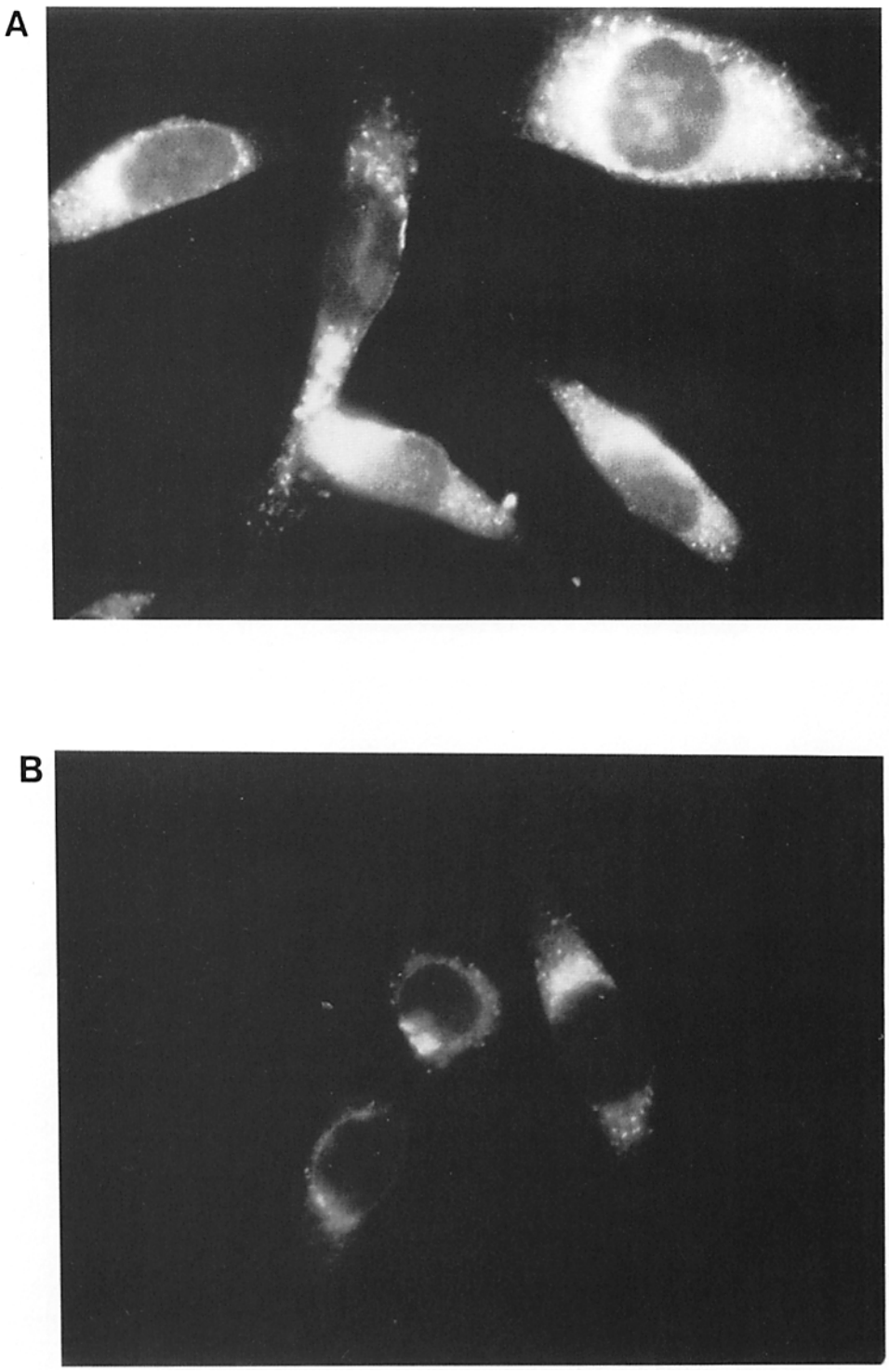

Figure 2. Intracellular accumulation of membrane-anchored v-*sis* protein containing an ER retention signal. Indirect immunofluorescence was performed on permeabilized NIH 3T3 cells expressing v-sis^{239}-G-ER$^+$ (*A*) or v-sis^{239}-G-ER$^-$ (*B*). Fusion proteins were detected using a rabbit serum directed against the v-*sis* protein followed by a rhodamine-conjugated goat anti-rabbit IgG. Note the increased reticular staining visible in cells of *A* compared with *B*.

fications during its transport through the secretory pathway. One of these, an NH_2-terminal proteolytic processing event, is believed to occur late in the secretory pathway somewhere between the *trans*-Golgi compartment and plasma membrane (Robbins et al., 1985; Lokeshwar et al., 1990). If the v-sis^{239}-G-ER$^+$ protein is retained in the ER and prevented from reaching later compartments of the secretory pathway, differences should be observed in the extent of proteolytic maturation of v-sis^{239}-G-ER$^+$ compared to v-sis^{239}-G and v-sis^{239}-G-ER$^-$. The extent of NH_2-terminal proteolytic processing was examined by immunoprecipitation using an antibody directed against human PDGF-AB. This antibody recognizes both the full length and mature form of the v-*sis* protein. Immunoprecipitates were also treated with various glycosidases to examine oligosaccharide content. As shown in Fig. 4, protein doublets of ~23 kD (Fig. 4, *arrow 3*) were detected from cells expressing v-sis^{239}-G and v-sis^{239}-G-ER$^-$, but were absent from cells expressing v-sis^{239}-G-ER$^+$.

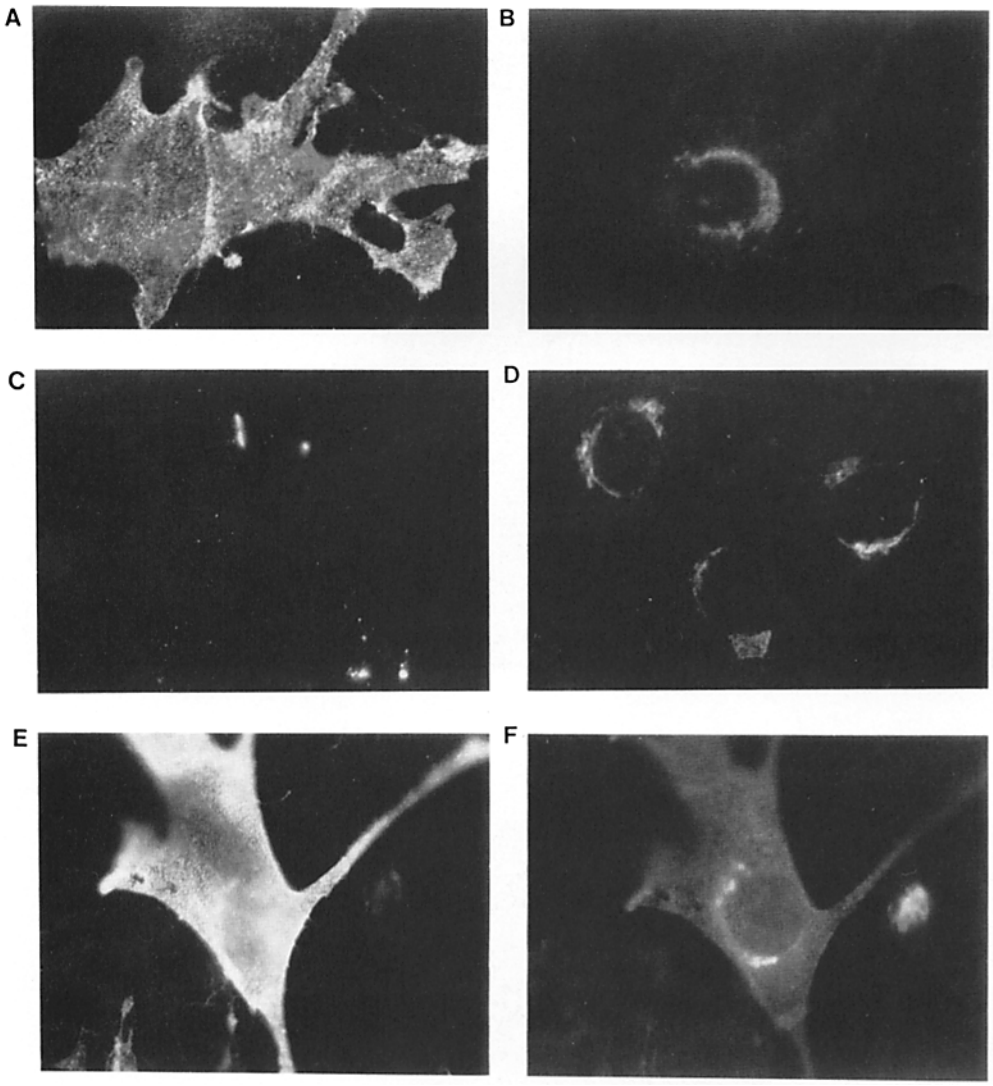

Figure 3. ER retention signal prevents cell surface expression of membrane-anchored v-*sis* protein. Cell surface and intracellular v-*sis* proteins were examined using double-label immunofluorescence as described in Materials and Methods. The extracellular domain of various fusion proteins was detected using a rabbit serum directed against the v-*sis* protein. The intracellular domain of the fusion proteins was detected using a mouse mAb directed against the COOH-terminus of the VSV G protein. (*A* and *B*) v-*sis*^{239NS}-G; (*C* and *D*) v-*sis*^{239NS}-G-ER^{+}; (*E* and *F*) v-*sis*^{239NS}-G-ER^{-}. *A*, *C*, and *E* are cell surface staining. *B*, *D*, and *F* are intracellular staining. "NS" indicates the Asn^{110}-Ser^{111} mutation of the Lys^{110}-Arg^{111} processing site, resulting in an uncleaved propeptide and increased immunofluorescent detection using the v-*sis* protein antibody.

These protein doublets were the predicted size for the proteolytically processed mature fusion proteins. Thus, the v-*sis*^{239}-G-ER^{+} protein did not appear to be proteolyzed to the mature form. The heterogeneity in size observed for the processed mature proteins was likely due to differences in *O*-linked glycosylation, since treatment with both neuraminidase and *O*-glycosidase slightly shifted the mobility of these proteins. The mobility shift seen after *O*-glycosidase treatment was not as pronounced as that seen after neuraminidase treatment, but it is real and experimentally reproducible. When immunoprecipitates were treated with endoglycosidase H, which cleaves high mannose N-linked oligosaccharide, mobility shifts occurred from 33-kD proteins (Fig. 4, *arrow 1*) to 30-kD proteins (Fig. 4, *arrow 2*). This indicates that the 33-kD proteins contain N-linked oligosaccharide and correspond to full length v-*sis* fusion proteins. Failure of the 33-kD proteins to be modified by either neuraminidase or *O*-glycosidase indicates that proteolytic processing occurs before the addition of sialic acid and O-linked oligosaccharides. This implies that sialic acid and O-linked oligosaccharide additions occur in a post-Golgi compartment of the secretory pathway, since previous studies have localized the v-*sis* proteolytic processing event to a post-Golgi compartment (Lokeshwar et al., 1990; Robbins et al., 1985). This should not be surprising, since glycosyltransferases have been detected in a variety of post-Golgi compartments (Taatjes et al., 1988), and evidence does exist for extra-Golgi or cell surface localized glycosyltransferases (Shur, 1982).

In addition to the above mentioned experiment which detects steady-state levels of v-*sis* fusion proteins, a pulse-chase analysis was performed to examine the conversion of full length v-*sis* fusion proteins to the corresponding mature forms. The turnover of full length v-*sis* fusions was used as a measure of the rate of proteolytic processing. As shown in Fig. 5, the full length v-*sis*^{239}-G and v-*sis*^{239}-G-ER^{-} proteins turned over and were converted to mature proteins with half-times of ~1.5 h, while the v-*sis*^{239}-G-ER^{+} protein showed little detectable conversion to mature protein after a 3-h chase. Further analysis with chase times out to 24 h detected a very small amount (<5% conversion) of mature v-*sis*^{239}-G-ER^{+} protein (data not shown), indicating a slight leakiness in the ER retention system of these cells. Nonetheless, these data indicate that v-*sis*^{239}-G-ER^{+} is overwhelmingly

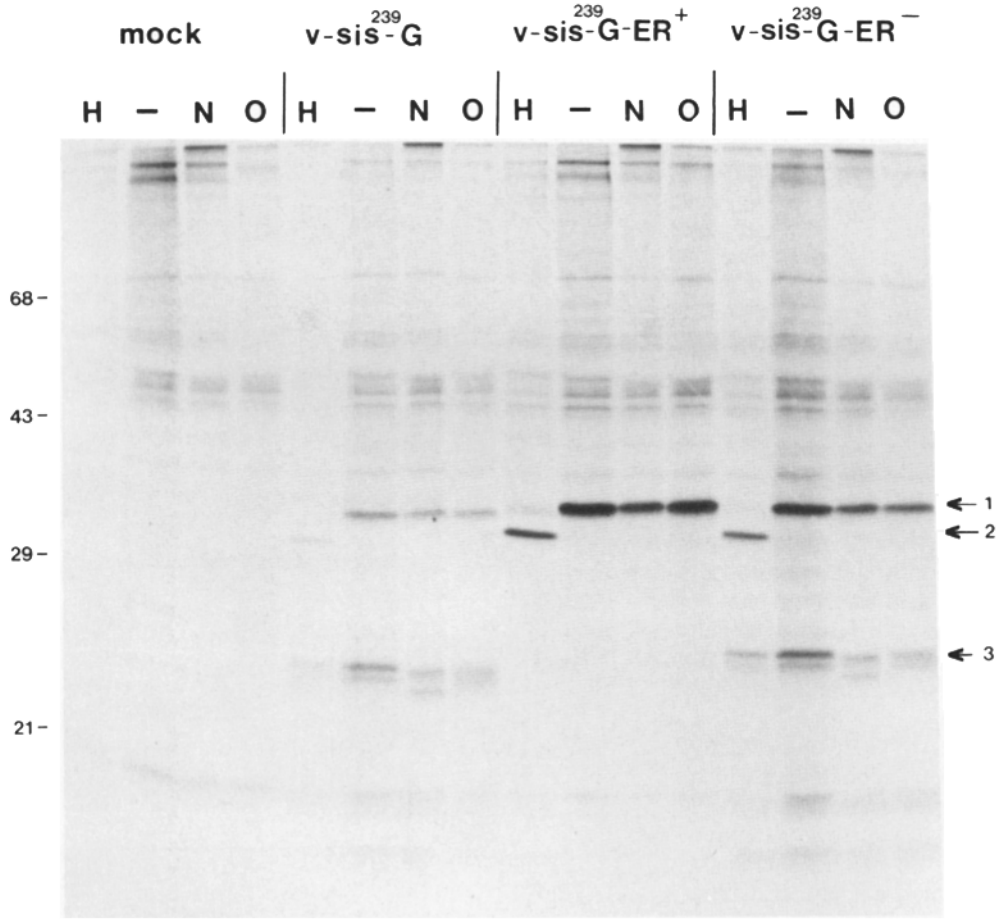

Figure 4. ER retention signal prevents proteolytic maturation of membrane-anchored v-*sis* protein. Equivalent numbers (5×10^5) of NIH 3T3 cells expressing various v-*sis* fusion proteins were labeled with [^{35}S]cysteine and [^{35}S]methionine for 3 h and immunoprecipitated with an antiserum directed against human PDGF-AB. Immunoprecipitated proteins were left untreated (–) or digested with endoglycosidase H (*H*), *O*-glycosidase (*O*), or neuraminidase (*N*), and subsequently analyzed by 15% SDS-PAGE followed by fluorography. Molecular mass markers in kilodaltons are indicated at the left. Numbered arrows indicate the following proteins: *1*, full length v-*sis* fusion proteins containing N-linked oligosaccharide; *2*, full length v-*sis* fusion proteins lacking N-linked oligosaccharide; *3*, proteolytically processed v-*sis* fusion proteins. Exposure time was 2 wk.

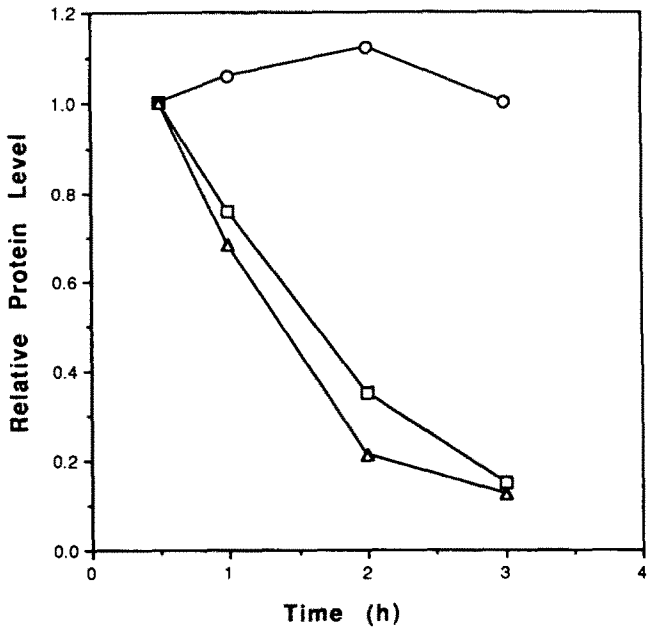

Figure 5. Turnover of full length membrane-anchored v-*sis* proteins. NIH 3T3 cells expressing various v-*sis* fusion proteins were pulse labeled for 30 min with [^{35}S]cysteine and [^{35}S]methionine and chased for 0, 1, 2, and 3 h. v-*sis* fusion proteins were immunoprecipitated with an antibody directed against human PDGF-AB and analyzed by SDS-PAGE and detected by fluorography. Relative protein level was determined by densitometric scanning of protein bands corresponding to each full length v-*sis* fusion protein and plotted against time to examine protein turnover. (–□–) v-*sis*^{239}-G; (–○–) v-*sis*^{239}-G-ER^{+}; (–△–) v-*sis*^{239}-G-ER^{-}.

retained in the ER with little transport to the *trans*-Golgi network where proteolytic maturation of v-*sis* protein is thought to occur.

ER-retained v-sis Protein Exhibits Reduced Transforming Activity

The effect of ER localization on the transforming activity of membrane-anchored v-*sis* protein was examined by assaying the ability of v-*sis*^{239}-G-ER^{+} to induce focus formation in NIH 3T3 cells. The v-*sis* fusions depicted in Fig. 1 *B* were expressed in NIH 3T3 cells using a M-MuLV-based retroviral vector and a replication competent helper provirus to provide viral spread (Bold and Donoghue, 1985; Hoffman et al., 1982). As shown in Table I and Fig. 6, the v-*sis*^{239}-G-ER^{+} protein was inhibited in its ability to transform NIH 3T3 cells. The v-*sis*^{239}-G-ER^{+} protein induced 10-fold fewer foci (Table I), which were also much smaller in size (Fig. 6 *D*) when compared with v-*sis*^{wt} (Fig. 6 *B*), v-*sis*^{239}-G (Fig. 6 *C*), and v-*sis*^{239}-G-ER^{-} (Fig. 6 *E*). The foci induced by v-*sis*^{wt} were much larger and diffuse compared with foci induced by membrane-anchored v-*sis* proteins. This was presumably due to secretion of v-*sis*^{wt} protein and its growth-

Table I. Transforming Activities of Membrane-anchored v-sis Fusions

v-*sis* Fusion	Focus assay	Relative transforming activity
	FFU/pmol	*Percent v-sis^{wt}*
mock	0	0
v-*sis*^{wt} (secreted)	3.7×10^4	100
v-*sis*^{239}-G	3.4×10^4	92
v-*sis*^{239}-G-ER$^+$	3.4×10^3	9.2
v-*sis*^{239}-G-ER$^-$	3.3×10^4	89

v-*sis* fusions were assayed for focus-forming activity as described in Materials and Methods. Results from a typical focus assay are presented and represent the average from two experiments. Focus assay results are given in focus-forming units per pmol of plasmid DNA transfected (*FFU/pmol*).

promoting effect on neighboring cells. Similar results were also obtained with the corresponding Asn^{110}-Ser^{111} processing site mutants (data not shown), confirming that propeptide cleavage is not required for efficient transformation of NIH 3T3 cells. The reduced level of transformation exhibited by v-*sis*^{239}-G-ER$^+$ demonstrates that inefficient transformation results when an otherwise fully transforming v-*sis* protein is retained in the ER.

The residual foci observed for v-*sis*^{239}-G-ER$^+$ most likely result from leaky retention in a subpopulation of cells which fail to retain the protein and therefore transport small amounts beyond the ER. The possibility of leaky retention is supported by the extended pulse–chase analysis mentioned earlier in which production of a small amount of mature v-*sis*^{239}-G-ER$^+$ protein was noted after a 24-h chase. Leaky retention is also supported by the following observation. When pools of cells expressing the v-*sis*^{239}-G-ER$^+$ protein were carried in culture for an extended period of time (>6 wk), the cells gradually acquired a transformed morphology which eventually become indistinguishable from cells expressing v-*sis*^{239}-G or v-*sis*^{239}-G-ER$^-$. This was due to selection of a population of cells exhibiting leaky retention of the v-*sis*^{239}-G-ER$^+$ protein. In cells carried for 8 wk in culture, transport of v-*sis*^{239}-G-ER$^+$ protein beyond the ER was noted and confirmed by detection of significant amounts of proteolytically processed protein (Fig. 7, lane *3*), while a newly established pool of v-*sis*^{239}-G-ER$^+$ expressing cells showed no detectable proteolytic processing (Fig. 7, lane *5*). Thus, a correlation exists between the appearance of proteolytically processed v-*sis*^{239}-G-ER$^+$ protein and the acquisition of the transformed phenotype. These results suggest that efficient transformation requires the transport of membrane-anchored v-*sis* protein beyond the ER, possibly up to or past the site where proteolytic maturation occurs.

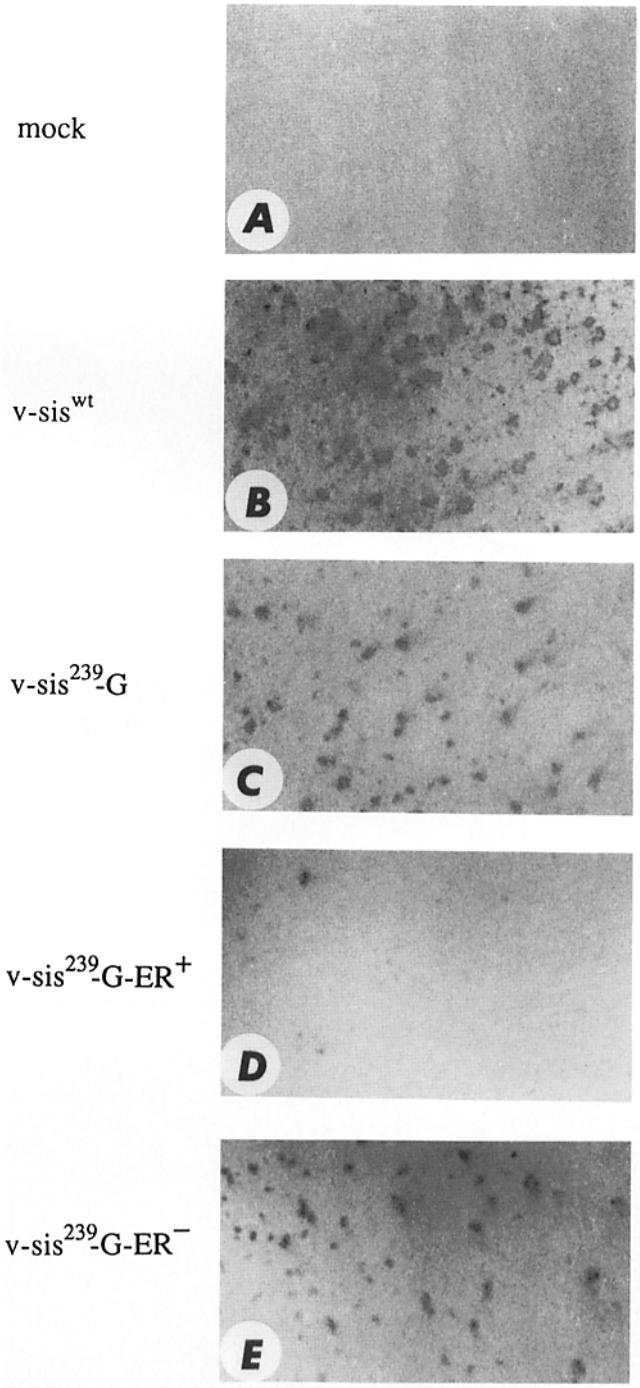

Figure 6. Focus-formation assay. Representative samples from a focus assay were photographed after fixing in methanol and subsequent staining with 0.4% Giemsa in 100 mM Tris, pH 6.8, methanol.

ER-retained v-sis Protein Does Not Downregulate PDGF β Receptors

Cell surface PDGF receptors are normally downregulated in cells transformed by the v-*sis*^{wt} protein. This is because of chronic interaction of v-*sis* protein with PDGF receptors followed by internalization and degradation via receptor-mediated endocytosis. The ability of various membrane-anchored v-*sis* proteins to downregulate PDGF β receptors was examined by measuring the level of cell surface activatable receptors and also by examining receptor turnover by pulse–chase analysis. Activatable cell surface receptors were examined by PDGF-BB treatment of serum-starved cells, followed by PDGF receptor immunoprecipitation and in vitro kinase analysis. As shown in Fig. 8, cells expressing transforming v-*sis* proteins (v-*sis*^{wt}, v-*sis*^{239}-G and v-*sis*^{239}-G-ER$^-$) all showed reduced levels of cell surface activatable receptors (Fig. 8, lanes *10*, *11*, and *13* respectively). An 8-wk culture of cells expressing the v-*sis*^{239}-G-ER$^+$ protein also showed reduced levels of activatable receptors, ~10-fold less

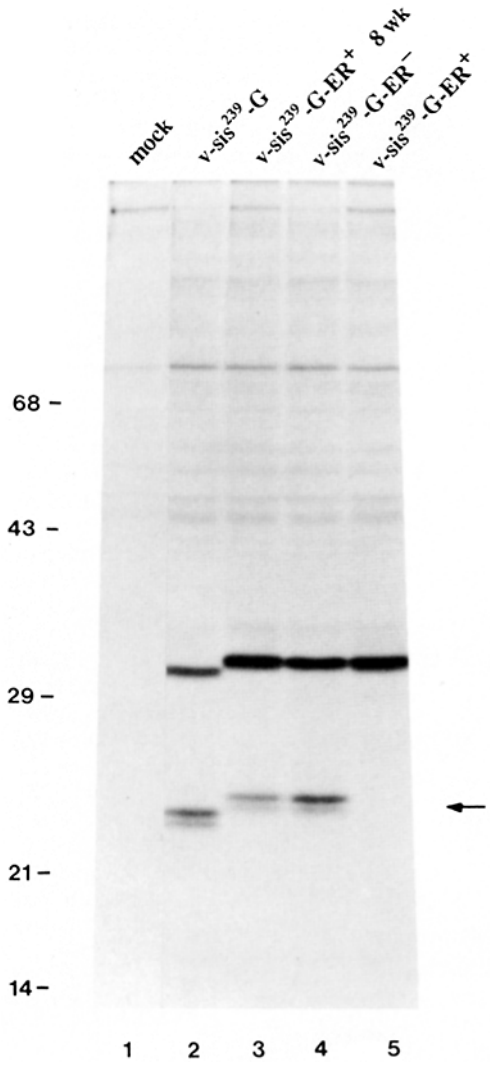

Figure 7. Proteolytic processing of v-*sis*^{239}-G-ER$^+$. Equivalent numbers (5×10^5 of NIH 3T3 cells expressing various v-*sis* proteins were labeled for 2.5 h with 200 μCi/ml each [^{35}S]cysteine and [^{35}S]methionine. Cell lysates were immunoprecipitated with an antibody directed against human PDGF-AB and analyzed by 15% SDS-PAGE followed by fluorography. The arrow indicates the position of proteolytically processed v-*sis* fusion proteins. Molecular mass markers in kilodaltons are indicated at the left. Lane *1*, mock; lane *2*, v-*sis*^{239}-G; lane *3*, v-*sis*^{239}-G-ER$^+$ from cells carried in culture for 8 wk; lane *4*, v-*sis*^{239}-G-ER$^-$; and lane *5*, v-*sis*^{239}-G-ER$^+$. Comparison of lanes *3* and *5* indicates that cells expressing v-*sis*^{239}-G-ER$^+$ exhibit detectable proteolytic processing in transformed cells passaged for 8 wks. Exposure time was 3 d.

compared with mock-infected cells (Fig. 8, lanes *9* and *12*). However, a newly established pool of cells expressing v-*sis*^{239}-G-ER$^+$ exhibited levels of activatable receptors comparable with mock-infected cells (Fig. 8, lanes *9* and *14*). These results indicate that the v-*sis*^{239}-G-ER$^+$ protein cannot downregulate PDGF β receptors when efficiently retained in the ER. Receptor downregulation does occur, however, when the v-*sis*^{239}-G-ER$^+$ protein is transported beyond the ER as with cells carried in culture for 8 wk.

Turnover of mature PDGF β receptors was also examined by pulse–chase analysis. As seen in Fig. 9, when compared with mock-infected NIH 3T3 cells, cells expressing v-*sis*^{wt} exhibited rapid turnover of PDGF β receptors, while cells expressing v-*sis*^{239}-G-ER$^+$ did not. For cells expressing v-*sis*^{239}-G or v-*sis*^{239}-G-ER$^-$, an intermediate rate of receptor turnover was observed indicating that these fusion proteins still induced receptor turnover, but did so less efficiently than secreted v-*sis*^{wt} protein. This slower rate of receptor turnover was likely the result of steric constraints placed upon the protein by its membrane anchor. These results indicate that membrane-anchored v-*sis* protein, when transported beyond the ER, can interact with PDGF β receptors and increase receptor turnover, albeit not as efficiently as secreted v-*sis*^{wt} protein.

Suramin Reverts Transformation and Upregulates PDGF β Receptors

Suramin is a polyanionic compound that has been reported to inhibit PDGF mitogenic activity and to revert the v-*sis* transformed phenotype (Betsholtz et al., 1986). Suramin is believed to exert this effect by disrupting receptor–ligand interactions at the cell surface as indicated by the ability of suramin to reduce the level of tyrosine-phosphorylated cell surface PDGF receptors, while not affecting the level of intracellular tyrosine-phosphorylated receptors (Fleming et al., 1989). However, since suramin also reaches intracellular sites, notably lysosomes at high concentrations (Hawking, 1978; La Rocca et al., 1990), it has been postulated that suramin may disrupt intracellular interactions between v-*sis* protein and PDGF receptors (Huang and Huang, 1988). Nevertheless, the work of Fleming et al. (1989) strongly indicates an extracellular site of action for suramin in the disruption of v-*sis*/PDGF receptor interactions.

Fig. 10 shows the effect of suramin on the morphology of various cell pools. Suramin reverted the transformed phenotype of cells expressing v-*sis*^{wt} (Fig. 10 *F*), v-*sis*^{239}-G (Fig. 10 *H*) and v-*sis*^{239}-G-ER$^-$ (Fig. 10 *J*), but had no effect on the morphology of cells transformed by a non-growth factor–related oncogene, v-*mos* (Fig. 10 *L*). Suramin also reverted the transformed morphology of v-*sis*^{239}-G-ER$^+$ expressing cells that had been carried in culture for over 8 wk (data not shown), consistent with transport of v-*sis*^{239}-G-ER$^+$ to a suramin-sensitive location. Suramin had no obvious effect on the morphology of early passage cells expressing v-*sis*^{239}-G-ER$^+$ (Fig. 10 *D*).

The effect of suramin on the expression of cell surface PDGF β receptors was also examined by in vitro kinase analysis. As described previously, activatable cell surface receptors were examined by PDGF-BB treatment of serum-starved cells, followed by PDGF receptor immunoprecipitation and in vitro kinase analysis. As shown in Fig. 11, suramin treatment led to the upregulation of activatable cell surface PDGF β receptors in cells expressing v-*sis*^{wt}, v-*sis*^{239}-G, and v-*sis*^{239}-G-ER$^-$ (Fig. 11, lanes *7*, *8*, and *10*, respectively). These results are consistent with suramin disrupting receptor–ligand interactions at the cell surface. In summary, these experiments suggest that there is an essential cell surface component to membrane-anchored v-*sis* transformation.

Discussion

Results presented in this report demonstrate that addition of an ER retention signal onto the COOH terminus of

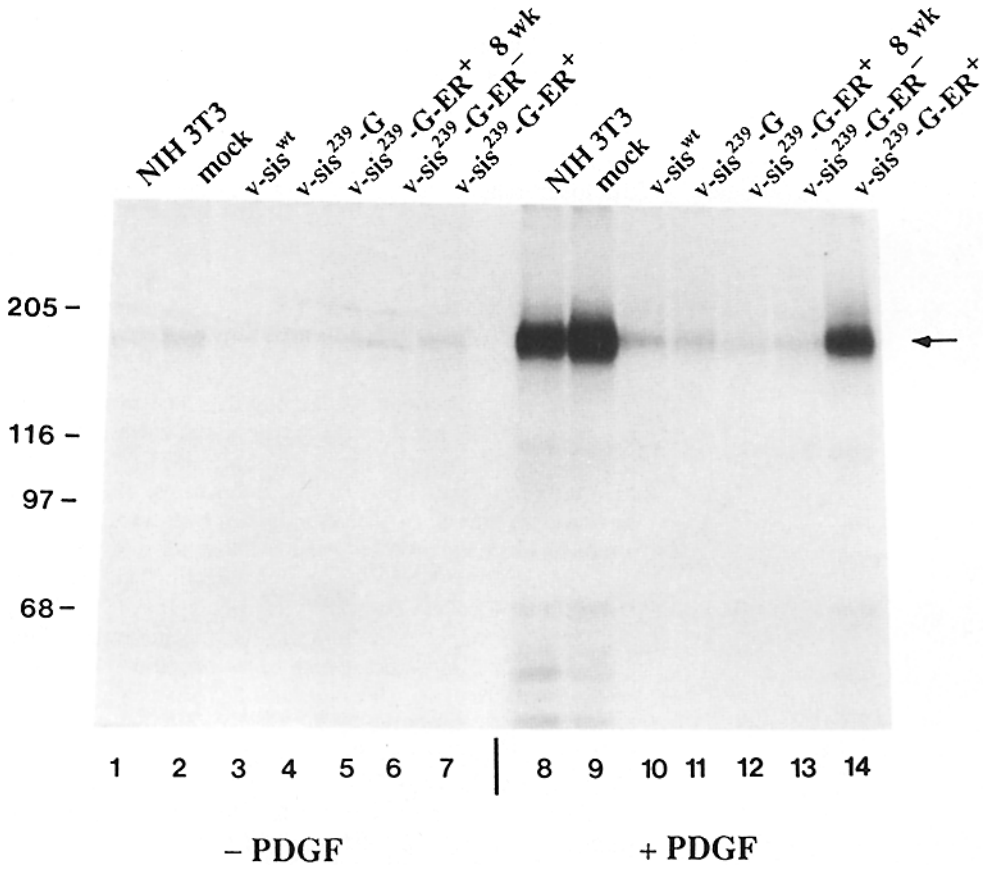

Figure 8. In vitro kinase analysis of cell surface PDGF β receptors. Equivalent numbers (5×10^5 of NIH 3T3 cells expressing various v-*sis* proteins were serum-starved for 24 h in DME containing 0.5% FCS. After serum starvation, cells were either left untreated or treated with 100 ng/ml PDGF-BB for 5 min at 37°C. PDGF receptors were subsequently immunoprecipitated and subjected to in vitro kinase assay as described in Materials and Methods. ^{32}P-labeled PDGF β receptors were detected by SDS-PAGE and autoradiography. The arrow indicates the position of the cell surface PDGF β receptor. Molecular mass markers in kilodaltons are indicated at the left. (Lanes *1* and *8*) untransfected NIH 3T3 cells; lanes *2* and *9*, mock transfected NIH 3T3 cells; lanes *3* and *10*, v-*sis*^{wt}; lanes *4* and *11*, v-*sis*^{239}-G; lanes *5* and *12*, v-*sis*^{239}-G-ER$^+$ from cells carried in culture for 8 wk; lanes *6* and *13*, v-*sis*^{239}-G-ER$^-$, and lanes *7* and *14*, v-*sis*^{239}-G-ER$^+$ from early passage cells. Exposure time was 3 h.

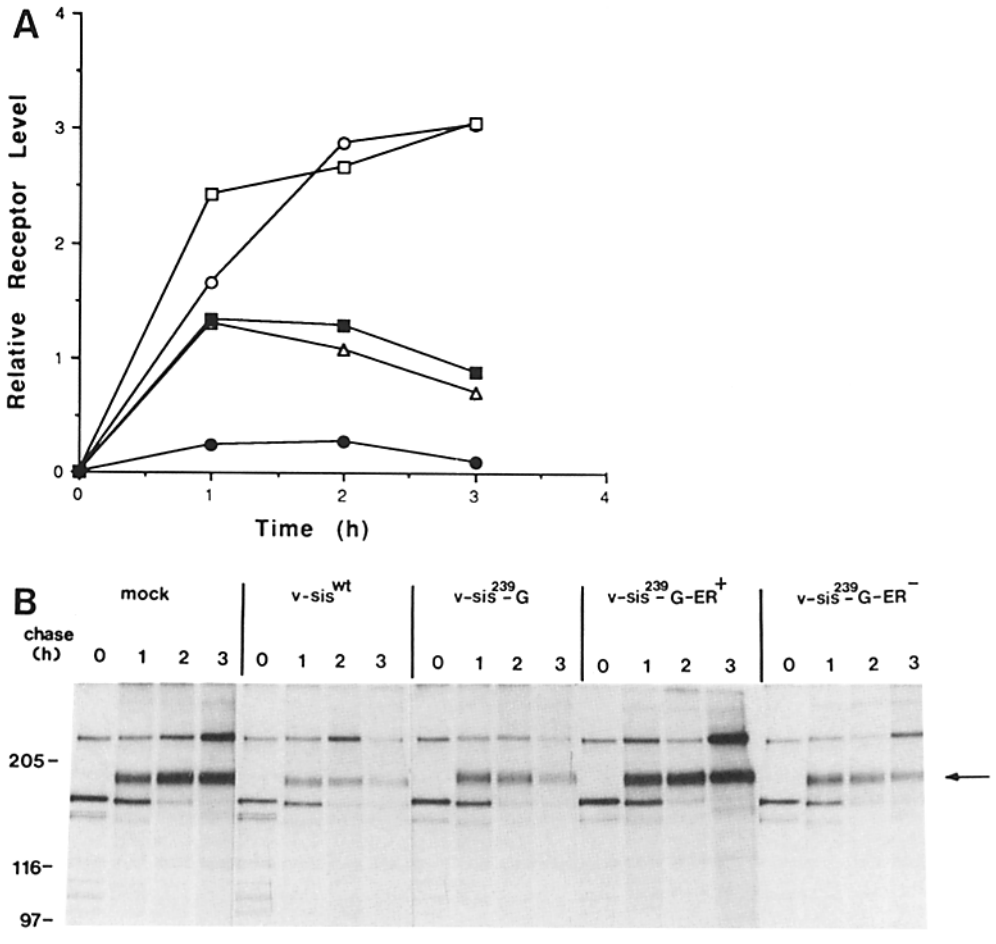

Figure 9. Turnover of PDGF β receptors. Equivalent numbers (5×10^5) of NIH 3T3 cells expressing various v-*sis* proteins were pulse labeled for 30 min with [^{35}S]cysteine and [^{35}S]methionine and chased for 0, 1, 2, and 3 h. Cell lysates were immunoprecipitated with an antibody directed against the murine PDGF β receptor and analyzed by 7.5% SDS-PAGE followed by fluorography. (*A*) Relative receptor level was determined by densitometric scanning of protein bands corresponding to the mature PDGF β receptor (p180) and plotted against time to examine receptor turnover. (-○-) mock; (-●-) v-*sis*^{wt}; (-△-) v-*sis*^{239}-G; (-□-) v-*sis*^{239}-G-ER$^+$; (-■-) v-*sis*^{239}-G-ER$^-$. (*B*) The arrow indicates the position of the mature PDGF β receptor. Molecular mass markers in kilodaltons are indicated at the left. Exposure time was 6 d.

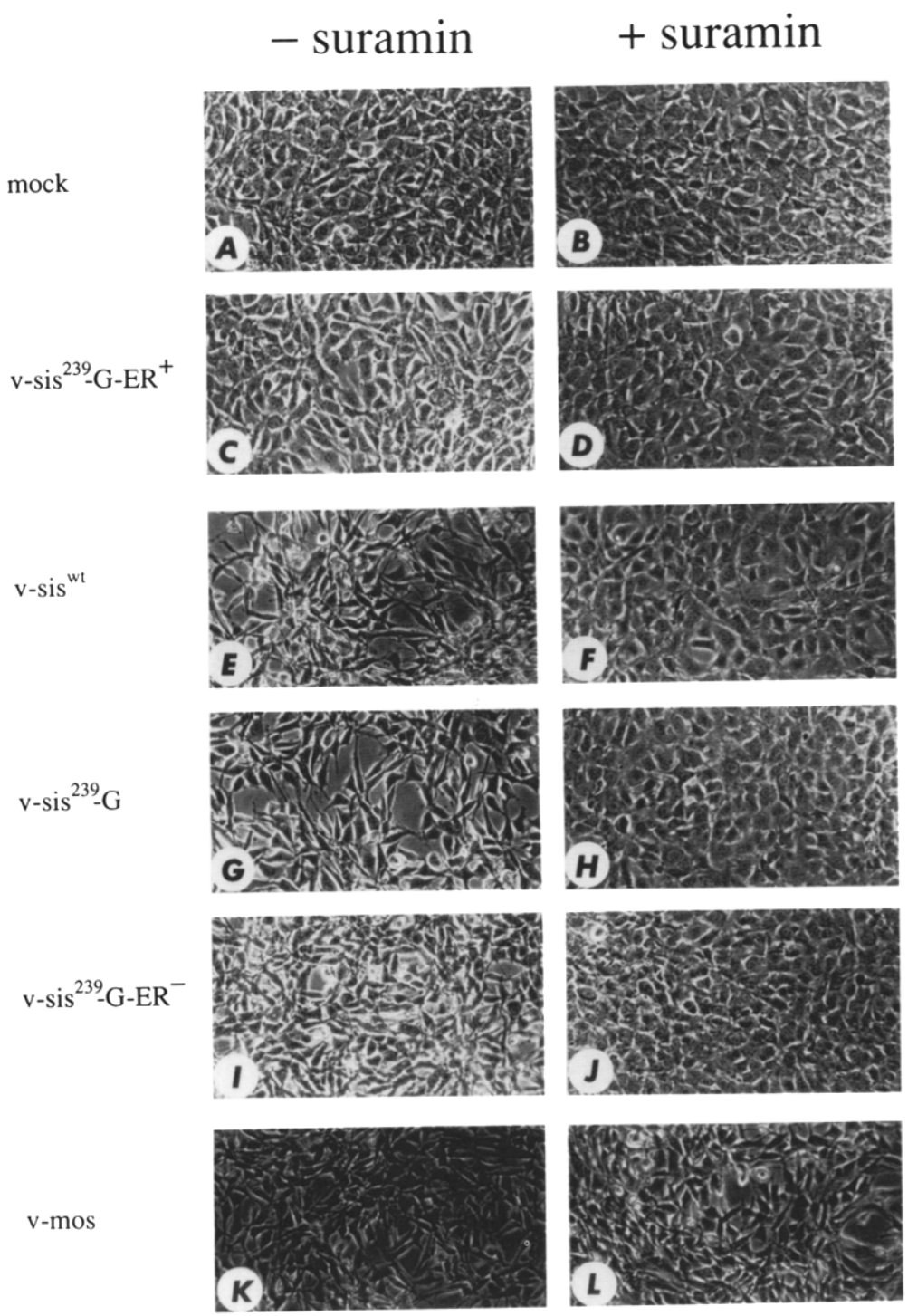

Figure 10. Suramin reversion of membrane-anchored v-*sis* transformation. NIH 3T3 cells expressing various v-*sis* proteins were treated with 100 μM suramin for 24 h at which time cell morphology was examined and compared with untreated cells. Representative photomicrographs are presented.

v-*sis*^{239}-G results in intracellular retention and inhibited proteolytic processing of the protein. This ER-retained protein exhibited reduced transforming activity and was unable to downregulate PDGF β receptors. Acquisition of the transformed phenotype correlated with transport of the protein beyond the ER to a site where proteolytic processing can occur. Finally, suramin treatment reverted transformation and upregulated cell surface PDGF β receptors. These results indicate that productive receptor–ligand interactions do not occur in the ER and suggest that signal-transducing interactions occur post-ER, most likely at the cell surface.

Previous Evidence Supporting "Intracrine" Interactions

The question of where productive receptor–ligand interactions take place in v-*sis*–transformed cells has been the subject of some debate. In v-*sis*–transformed cells, both v-*sis* protein and PDGF receptors are synthesized through the secretory pathway, and therefore the opportunity exists for growth factor–receptor interactions within the cell, as well as at the cell surface. Keating and Williams (1988) have detected activated receptors within intracellular compartments

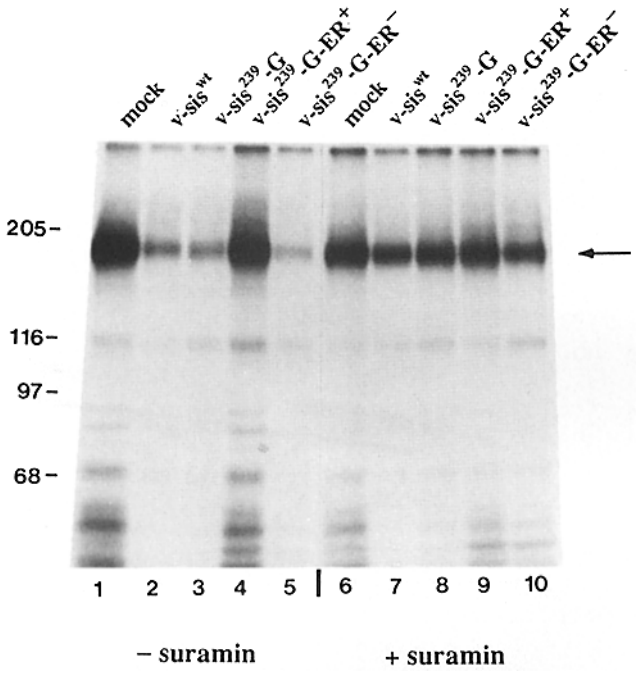

Figure 11. Upregulation of cell surface PDGF β receptors in response to suramin. Equivalent numbers (5×10^5) of NIH 3T3 cells expressing various v-*sis* proteins were serum-starved for 24 h in 0.5% CS or 0.5% CS containing 100 μM suramin. After serum starvation, cells were incubated with 100 ng/ml PDGF-BB for 5 min at 37°C. PDGF receptor immunoprecipitates were subsequently analyzed for in vitro kinase activity as described in Materials and Methods. ^{32}P-labeled PDGF β receptors were detected by SDS-PAGE and autoradiography. The arrow indicates the position of the mature cell surface PDGF β receptor. Molecular mass markers in kilodaltons are indicated at the left. Lanes *1* and *6*, mock transfected NIH 3T3 cells; lanes *2* and *7*, v-*sis*^{wt}; lanes *3* and *8*, v-*sis*^{239}-G; lanes *4* and *9*, v-*sis*^{239}-G-ER$^+$; and lanes *5* and *10*, v-*sis*^{239}-G-ER$^-$.

of SSV-transformed NRK cells. The majority of these receptors did not reach the cell surface and had altered processing compared with receptors stimulated by exogenous PDGF. Huang and co-workers demonstrated similar results in that most newly synthesized PDGF receptors in SSV-transformed cells were not detected at the cell surface and exhibited rapid turnover because of intracellular interaction with v-*sis* protein possibly within the ER and/or Golgi complex (Huang and Huang, 1988; Lokeshwar et al., 1990). Rapid turnover of v-*sis* protein was also noted and intracellular forms of the protein were shown to be mitogenic. These observations complemented the work of Keating and Williams (1988) and suggested that intracellular receptor activation, termed "intracrine" activation, was sufficient for autocrine transformation by v-*sis*.

Bejcek et al. (1989) have also addressed the question of where productive receptor–ligand interactions occur by appending an ER retention signal for luminal ER proteins (KDEL) onto the COOH terminus of a normally secreted v-*sis* protein. This v-*sis*–KDEL fusion was not detectably secreted into the medium and was assumed to be retained in the ER. When assayed for transforming ability, the v-*sis*–KDEL fusion was just as active as the secreted v-*sis* protein, implying that intracellular interactions were sufficient for autocrine stimulation of growth. At the time this experiment was performed, the available evidence suggested that the KDEL retention signal provided for efficient retention in the ER. However, subsequent work has shown that the KDEL retention signal retards, but does not prevent, the secretion of fusion proteins containing the signal (Zagouras and Rose, 1989). Thus, another interpretation of the results of Bejcek et al. (1989) would be that sufficient v-*sis*–KDEL protein reached the cell surface, resulting in morphological transformation, but that the fusion protein was rapidly internalized via receptor-mediated endocytosis and thus not detectably secreted.

Previous Evidence Supporting a Post-Golgi or Cell Surface Site for Productive Interactions

Hannink and Donoghue (1988), using an inducible autocrine system, have also examined the site of productive interaction by blocking cell surface transport of newly synthesized v-*sis* protein using the carboxylic ionophore monensin. Monensin blocks the transport of secretory proteins through the *trans*-Golgi complex (Tartakoff, 1983) and was shown to prevent both autophosphorylation of cell surface PDGF receptors and expression of c-*fos* in response to induced intracellular synthesis of v-*sis* protein. Monensin did not, however, prevent autophosphorylation of an intracellular ER–Golgi form of the receptor, indicating that signal transduction can be blocked even in the presence of activated intracellular receptors. It is important to note that the observation of internally activated PDGF receptors in this study is consistent with the work of Keating and Williams (1988), and Huang and Huang (1988), but this study additionally suggests that transport of v-*sis* protein to a compartment past the site of monensin action (i.e., the *trans*-Golgi complex or beyond) is required to functionally couple activated PDGF receptors to growth signalling pathways.

Fleming et al. (1989) have also addressed the site of productive interaction by examining autocrine-stimulated PDGF receptors in the presence of suramin. Suramin was shown to completly block the proliferation of v-*sis*–transformed cells and to markedly reduce the level of tyrosine-phosphorylated cell surface receptors. This treatment did not, however, affect the level of intracellular tyrosine-phosphorylated receptors, suggesting that the PDGF receptor species relevant to fibroblast transformation was an activated receptor–ligand complex located at the cell surface. These results are consistent with the work of Hannink and Donoghue (1988) described above, which identifies the location of autocrine signalling as being beyond the site of monensin action, i.e., in the *trans*-Golgi network or at the cell surface.

Autocrine Signal Transduction Requires v-sis Protein Transport Beyond the ER

In this report, we have examined the site of productive autocrine interactions by preventing cell surface expression of a membrane-anchored v-*sis* protein using the ER retention signal from the adenoviral transmembrane protein E3/19K. ER retention of this protein led to inefficient cellular transformation, in contrast to the results of Bejcek et al. (1989). These contradictory results may be because of differences in how

the two ER retention signals function or in the efficiency of retention provided by each signal. As mentioned earlier, it has since been shown that the KDEL retention signal retards, but does not prevent, secretion of fusion proteins containing the signal (Zasgouras and Rose, 1989). It is also possible that the cytoplasmically located E3/19K retention system exerts tighter control over retention, or may be less easily saturable, compared with the luminal KDEL retention system.

Our data demonstrate that transformation by membrane-anchored v-*sis* is not completely inhibited by addition of an ER retention signal. There is, however, a significant decrease in the number and size of foci formed. The residual transformation observed for v-*sis*^{239}-G-ER^{+} is likely because of leaky retention in a subpopulation of cells which fail to efficiently localize the protein to the ER. Failure to localize this protein in the ER may result from saturation of the retention system in cells which overexpress the protein, or may result from a subpopulation of cells which exhibit defective retention. A leaky retention system would allow some v-*sis*^{239}-G-ER^{+} protein to be transported past the ER to a site where growth signalling interactions can occur. This explanation for residual transformation is consistent with the ability to select, during 8 wk in culture, for a transformed population of cells which was able to proteolytically process v-*sis*^{239}-G-ER^{+} protein and which exhibited downregulated PDGF β receptors. The detection of significant amounts of proteolytically processed v-*sis*^{239}-G-ER^{+} protein (Fig. 7, lane *3*) indicated efficient transport of this protein to the *trans*-Golgi network, since proteolytic maturation of v-*sis* protein is known to occur in a post-Golgi compartment of the secretory pathway (Robbins et al., 1985; Lokeshwar et al., 1990). The escape of v-*sis*^{239}-G-ER^{+} protein from the ER might also have occurred by selection for cells expressing mutant forms of v-*sis*^{239}-G-ER^{+} lacking a retention signal. The appearance of proteins with altered molecular weight would have supported this model. However, as shown in Fig. 7, and also in several additional experiments (data not shown), we have seen no evidence that continued passage of cells selected for the expression of truncated forms of v-*sis*^{239}-G-ER^{+}.

Where Do Productive Receptor–Ligand Interactions Occur in the v-sis Autocrine System?

Our results suggest that membrane-anchored v-*sis* protein cannot productively interact with PDGF receptors in the ER. Productive interactions must therefore occur post-ER, either in the Golgi, *trans*-Golgi network, or at the cell surface. It is possible that interactions between growth factor and receptor still occur in the ER, but that receptor-mediated endocytosis and transmission of autocrine growth signals cannot occur until the complex is transported beyond the ER. This is consistent with our observation that transport of v-*sis*^{239}-G-ER^{+} past the ER correlated with acquisition of the transformed phenotype and the ability to downregulate PDGF receptors. It would be informative to determine whether ER-retained v-*sis* protein can activate PDGF receptors within the ER. However, since the membrane-anchored v-*sis* protein does not induce PDGF receptor autophosphorylation (Lee and Donoghue, 1991), activated receptors cannot be examined directly.

The previously mentioned results from Hannink and Donoghue (1988) demonstrate that monensin blocks v-*sis* autocrine signal transduction and suggest that productive receptor–ligand interactions occur in or past the *trans*-Golgi. Our observation demonstrating that acquisition of the transformed phenotype correlated with transport of v-*sis* protein beyond the Golgi, to the site where proteolytic processing occurs, also supports a potential post-Golgi site for autocrine signal transducing interactions. These results taken together suggest that productive autocrine interactions may occur in the *trans*-Golgi network or at the cell surface.

Results demonstrating that suramin can revert transformation by membrane-anchored v-*sis* indicate that productive receptor–ligand interactions occur at a site sensitive to the action of suramin. As mentioned above, the exact site of suramin action is not completely clear, however, strong evidence exists indicating that suramin may act by disrupting activated receptor–ligand complexes at the cell surface (Fleming et al., 1989). In our report we demonstrate that suramin reverts membrane-anchored v-*sis* transformation and upregulates the expression of cell surface PDGF receptors. This is consistent with the disruption of cell surface receptor–ligand interactions. However, it is also consistent with the disruption of intracellular receptor–ligand interactions followed by transport of unoccupied receptors to the cell surface. If suramin has an exclusive extracellular site of action, these results would indicate that productive growth signalling interactions are limited to the cell surface. However, since suramin is known to have intracellular effects (Hawking, 1978; La Rocca et al., 1990), we cannot completely rule out the possibility that some component of transformation may be because of internally activated PDGF receptors.

Implications for Perturbing Autocrine Signal Transduction

Determining the site where productive autocrine interactions occur has implications for the design and use of therapeutic agents employed in the treatment of human cancers which involve autocrine loops. Tumor cells which exhibit productive intracellular interactions would not likely be treatable with antagonists whose action is limited to the cell surface. However, if productive autocrine interactions occur exclusively at the cell surface, then autocrine transformed cells may be amenable to treatment with exogenous antagonists. The results presented in this paper as well as those of Fleming et al. (1989), suggest an important cell surface component to autocrine transformation by v-*sis*. Therefore, antagonists of ligand–receptor complex formation, such as suramin or future drugs exhibiting greater specificity, offer hope for the efficient perturbation of autocrine signal transduction loops.

In conclusion, our results indicate that productive autocrine interactions between membrane-anchored v-*sis* and PDGF receptors occur post-ER. Our results are consistent with productive interactions occurring in a post-Golgi, suramin-sensitive location, most likely at the cell surface. Cell surface expression of activated receptor/ligand complexes may be required for efficient signal transduction in the event that key intermediates involved in this process are also localized to the cell surface.

We wish to thank Ray Sweet and Keith Deen for their gift of antiserum raised against the bacterially synthesized v-*sis* protein. We also wish to thank William Balch for the VSV G mAb and Jung Huang for antiserum

directed against the murine PDGF β receptor. We thank Jon Singer and Immo Scheffler for the use of their microscope facilities.

This work was supported by grant CA 40573 from the National Institutes of Health. D. J. Donoghue also gratefully acknowledges support from an American Cancer Society Faculty Research Award.

Received for publication 28 February 1992 and in revised form 10 June 1992.

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