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The v-*sis*/PDGF-2 transforming gene product localizes to cell membranes but is not a secretory protein

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The v-*sis* transforming gene encodes the woolly monkey homologue of human platelet-derived growth factor (PDGF) polypeptide 2. After its synthesis on membrane bound polyribosomes, the glycosylated precursor dimerizes in the endoplasmic reticulum and travels through the Golgi apparatus. At the cell periphery, the precursor is processed to yield a dimer structurally analogous to biologically active PDGF. Small amounts of two incompletely processed forms are detectable in tissue culture fluids of simian sarcoma virus (SSV) transformants. However, the vast majority remains cell associated. Thus, this growth factor-related transforming gene product is not a classical secretory protein. These findings define possible cellular locations where the transforming activity of the *sis*-PDGF-2 protein may be exerted.

Key words: simian sarcoma virus/growth factor/biosynthesis/precursors

Introduction

Acute transforming retroviruses have arisen in nature from chronic retroviruses by transduction of a small set of highly conserved cellular genes. Such cell-derived *onc* sequences confer neoplastic properties to these viruses. Significant progress has recently been made in deciphering the normal functions of the cellular progenitors, or proto-oncogenes, that give rise to retroviral *onc* genes. The v-*sis* transforming gene of simian sarcoma virus (SSV) has been shown to encode a protein closely related to a major structural component of human platelet-derived growth factor (PDGF) (Doolittle *et al.*, 1983; Waterfield *et al.*, 1983), a potent mitogen for connective tissue cells (Ross *et al.*, 1974; Scher *et al.*, 1979; Heldin *et al.*, 1979). Moreover, v-*erb*B, an avian erythroblastosis virus *onc* gene, encodes a protein closely related in predicted amino acid sequences to the epidermal growth factor (EGF) receptor (Downward *et al.*, 1984). These and other findings imply that viral *onc* genes may often act to subvert the normal growth regulatory pathways mediated by growth factors and their receptors.

Our laboratory is investigating the mode of action of v-*sis*. We have established that the v-*sis* transforming gene product is immunologically related to PDGF and is processed to yield a PDGF-2-like dimer which is conformationally analogous to biologically active PDGF (Robbins *et al.*, 1983). Moreover, the human homologue of v-*sis* codes for a protein identical to human PDGF-2, firmly establishing that the v-*sis* arose from the PDGF-2 gene (Chiu *et al.*, 1984). Recently we showed that the normal human *sis*/PDGF-2 coding sequence has transforming activity if provided with necessary signals for initiation of transcription and translation (Gazit *et al.*, 1984). All these findings have implied that the normal growth factor polypeptide has transforming activity if expressed by an appropriate target cell. In an effort to define possible locations at which the activity of the *sis* protein is exerted, we have examined its biosynthetic pathway in SSV-transformed cells.

Results

p28^{sis} is synthesized on membrane-bound polyribosomes as a glycoprotein

Nucleotide sequence analysis of v-*sis* has revealed a potential site for dolicol N-linked glycosylation at position 42–44 of the v-*sis* coded domain of p28^{sis} (Devare *et al.*, 1983). To investigate whether this protein was glycosylated *in vivo*, HF/SSV, an SSV-transformed marmoset cell line, or uninfected HF cells were metabolically labeled with [^{3}H]mannose or glucosamine and analyzed by immunoprecipitation with anti-*sis* N serum. The v-*sis* gene product p28^{sis}, was detected in lysates of the SSV-transformed, but not uninfected cells (data not shown). To examine the nature of this carbohydrate linkage, HF/SSV cells were metabolically labeled with [^{35}S]methionine and [^{35}S]cysteine following treatment with tunicamycin, an inhibitor of N-linked, but not O-linked glycosylation (Gahmberg *et al.*, 1980).

As shown in Figure 1, p28^{sis} was immunoprecipitated with anti-*sis* N serum from extracts of untreated SSV-transformed cells (lane c). When the same cells were treated instead with tunicamycin prior to labeling, p28^{sis} migrated as a 26 000-dalton protein (lane f). The effectiveness of tunicamycin treatment was demonstrated by a similar analysis of SSAV gp70, which is known to contain N-linked oligosaccharides (Thiel *et al.*, 1981). Following tunicamycin exposure, the 70 00-dalton SSAV envelope glycoprotein migrated as a 60 000-dalton species (Figure 1). All of these findings demonstrated that p28^{sis} posessed an oligosaccharide moiety N-linked to its polypeptide chain.

To determine the time interval required for glycosylation of gp28^{sis}, HF/SSV cells were pulse labeled with [^{35}S]methionine and [^{35}S]cysteine for varying periods, and analyzed for the appearance of gp28^{sis}. The shortest time required for label to be detectably incorporated was 5 min. Under these conditions (Figure 2), gp28^{sis}, but not the 26 000-dalton non-glycosylated form was detected, indicating the rapid glycosylation of the primary v-*sis* translational product. Since cellular glycosyl transferases are known to be specifically associated with rough endoplasmic reticulum (see Hubbard and Ivatt, 1981), these results also provide strong evidence that gp28^{sis} is synthesized on membrane-bound polyribosomes.

Glycosylation is apparently not essential for processing of the sis/PDGF-2 product

gp28^{sis} is processed to amino- and carboxy-terminal peptides of 11 000 and 20 000 daltons, respectively (Robbins *et al.*, 1983). The larger cleavage product corresponds in size and amino acid sequence to a PDGF-2 monomer. To localize the site(s) of N-linked carbohydrate attachment within the gp28^{sis} molecule, HF/SSV cells were treated with tunicamycin, pulse labeled, and

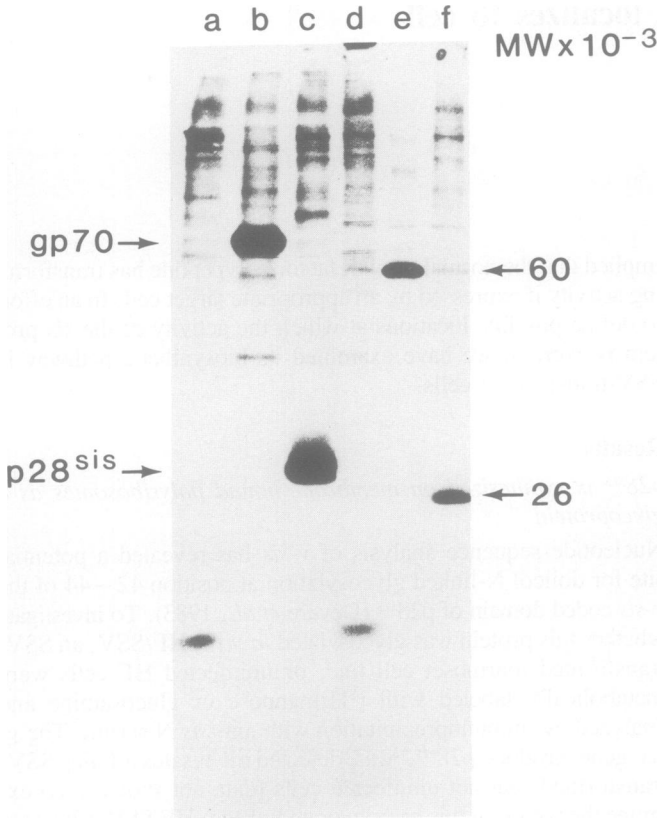

Fig. 1. Effect of tunicamycin on p28^{sis} synthesis. Subconfluent HF/SSV cultures (~10^7 cells/10 cm Petri dish) were pre-incubated for 4 h in methionine- and cysteine-free medium alone (**lanes a–c**) or containing 5 μg/ml tunicamycin (**lanes d–e**) and then metabolically labeled for 1 h, in the presence of 250 μCi of [^{35}S]methionine and cysteine per Petri dish. Cells were washed with PBS, and disrupted as described in Materials and methods. Lysates were analyzed by immunoprecipitation with normal rabbit (**lanes a** and **d**), anti-SSAV gp70 (**lanes b** and **e**) or anti-*sis* N (**lanes c** and **f**) sera.

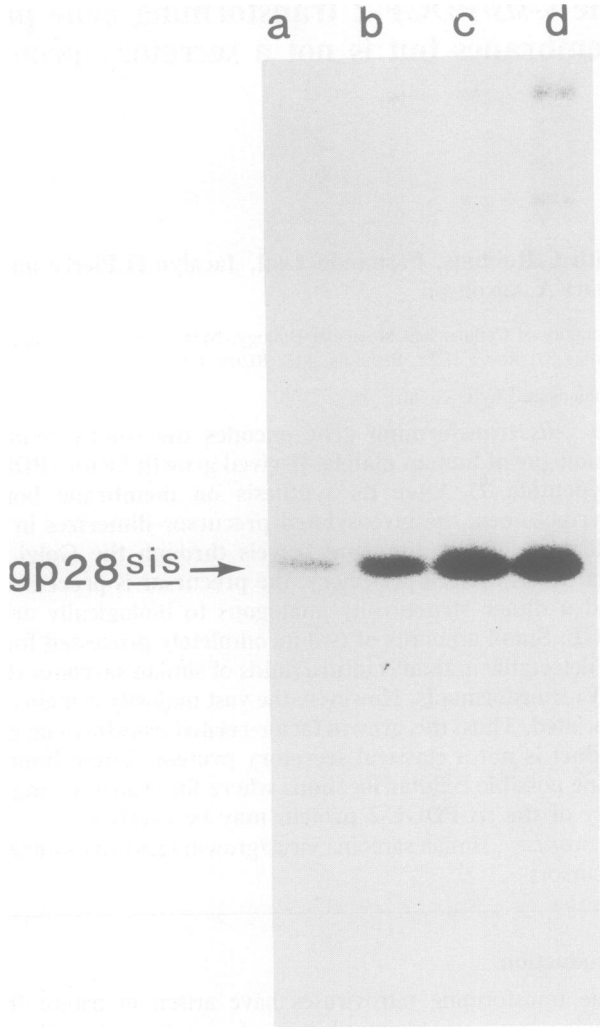

Fig. 2. Appearance of gp28^{sis} under short pulse labeling conditions. Subconfluent HF/SSV cultures were pre-incubated for 2 h in methionine- and cysteine-free medium, and then labeled in the presence of 250 μCi [^{35}S]methionine and cysteine for 5 (**lane a**), 10 (**lane b**), 15 (**lane c**) or 20 (**lane d**) min. Cells were washed in ice-cold PBS, and disrupted as described in Materials and methods. Lysates were analyzed by immunoprecipitation with anti-*sis* N serum.

analyzed by immunoprecipitation with either anti-*sis* N or anti-*sis* C serum. As shown in Figure 3, following tunicamycin treatment, p11^{sis} (lane a) migrated as a 6000-dalton species (lane e). In each case, immunoprecipitation was specifically blocked by competition with the *sis* N peptide (lanes b and f). In contrast, the electrophoretic mobility of p20^{sis} (lane c) was unaltered by tunicamycin treatment (lane g). As expected, gp28^{sis} migrated at 26 000 daltons under these conditions. These results localized the site of glycosylation to the p11^{sis} cleavage product and implied that cleavage of the primary v-*sis* translational product was independent of its glycosylation.

When analyzed under non-reducing conditions, gp28^{sis} rapidly forms a disulfide-linked dimer, gp56^{sis} (Robbins *et al.*, 1983). To assess whether glycosylation plays a role in dimer formation, SSV-transformed cells were treated with tunicamycin, pulse labeled and analyzed by immunoprecipitation using non-reducing conditions. As a positive control, anti-*sis* C serum detected the primary *sis* translational product, gp28^{sis}, and *sis* dimers gp56^{sis}, gp42^{sis} and p35^{sis} in untreated SSV-transformed cells (Figure 4). When the same cells were exposed to tunicamycin prior to labeling, proteins corresponding to gp28^{sis}, gp56^{sis} and gp42^{sis} were still detected but migrated instead as 26 000, 50 000 and 38 000 dalton species, respectively. The electrophoretic mobilities of other processed dimers corresponding to p35^{sis} and p24^{sis} were not affected by tunicamycin exposure. Thus, the apparent sizes only of those forms of the *sis* protein containing the amino terminus and, therefore, the oligosaccharide moiety of gp28^{sis}, were affected by tunicamycin exposure. Moreover, these findings demonstrated that N-linked oligosaccharide is not essential for the complex series of processing steps affecting the v-*sis* gene product.

Proteolytic processing of gp56^{sis} occurs in association with peripheral cell membrane components

Previous studies have shown that the gp28^{sis} dimer is rapidly processed to lower mol. wt. forms (Robbins *et al.*, 1983). In an effort to determine the site of these intracellular processing events, we fractionated metabolically labeled SSV-transformed cells and assayed each subcellular compartment for the presence of gp28^{sis} cleavage products. HF/SSV cells were labeled with [^{35}S]-methionine and cysteine for 3 h, sufficient to allow incorporation at steady-state levels into each of the v-*sis* translational product. The cells were then disrupted hypotonically, and fractionated into nuclear, cytosol and crude membrane components by differential centrifugation. Lactate dehydrogenase, NADH

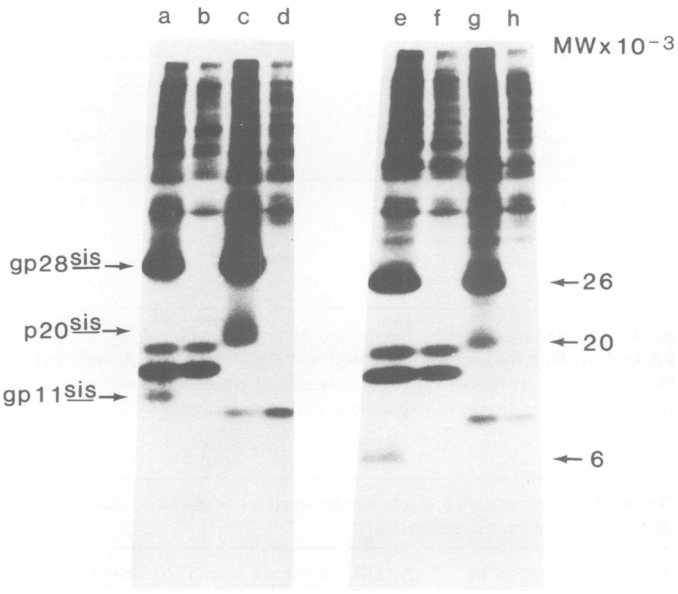

Fig. 3. Glycosylation of p11^{sis} is tunicamycin sensitive. Subconfluent HF/SSV cultures were metabolically labeled in the presence (**lanes e–h**) or absence (**lanes a** and **d**) of 5 μg/ml tunicamycin as described in the legend for Figure 1. Lyates were immunoprecipitated with anti-*sis* N (**lanes a, b, e** and **f**) or anti-*sis* C (**lanes c, d, g** and **b**) serum. In some cases, sera were pre-incubated with 3 μg of *sis* N (**lanes b** and **f**) or *sis* C (**lanes d** and **h**) peptides. Immune complexes were analyzed as described in Materials and methods.

diaphorase and 5′ nucleotidase were used as enzyme markers for cytosol, endoplasmic reticulum (ER) and plasma membrane, respectively. As shown in Table I, the cytosol fraction (S100) contained the vast majority of lactate dehydrogenase activity present in HF/SSV cells but was contaminated with a small amount of NADH diaphorase activity. The nuclear fraction was relatively pure as assessed by marker enzyme activity (Table I) as well as phase contrast microscopy. The P100 fraction was found to be significantly enriched for both NADH diaphorase and 5′ nucleotidase activity.

When the same subcellular fractions were assayed for *sis* gene products, such proteins were found only to be associated with the P100 fraction (Figure 5). These results demonstrated that v-*sis* coded proteins were membrane associated and that processing did not occur within either nuclear or soluble cytoplasmic compartments. In order to resolve whether *sis* protein cleavage occurred within the ER and/or was associated with other membrane components, we utilized a fractionation procedure which readily resolved enzyme markers for ER and plasma membrane (Table II). When these fractions were analyzed by immunoprecipitation, p20^{sis}, the most readily detected cleavage product was found in fractions specifically enriched for plasma membrane (Figure 6). The ratio of p20^{sis} and gp28^{sis} was >20-fold greater in plasma membrane compared with ER-enriched fractions, as judged by densitometry scanning. All of these results implied that *sis* protein cleavage occurred subsequent to its association with the ER at a membrane site nearer the cell periphery.

The oligosaccharide moieties of glycoproteins synthesized in

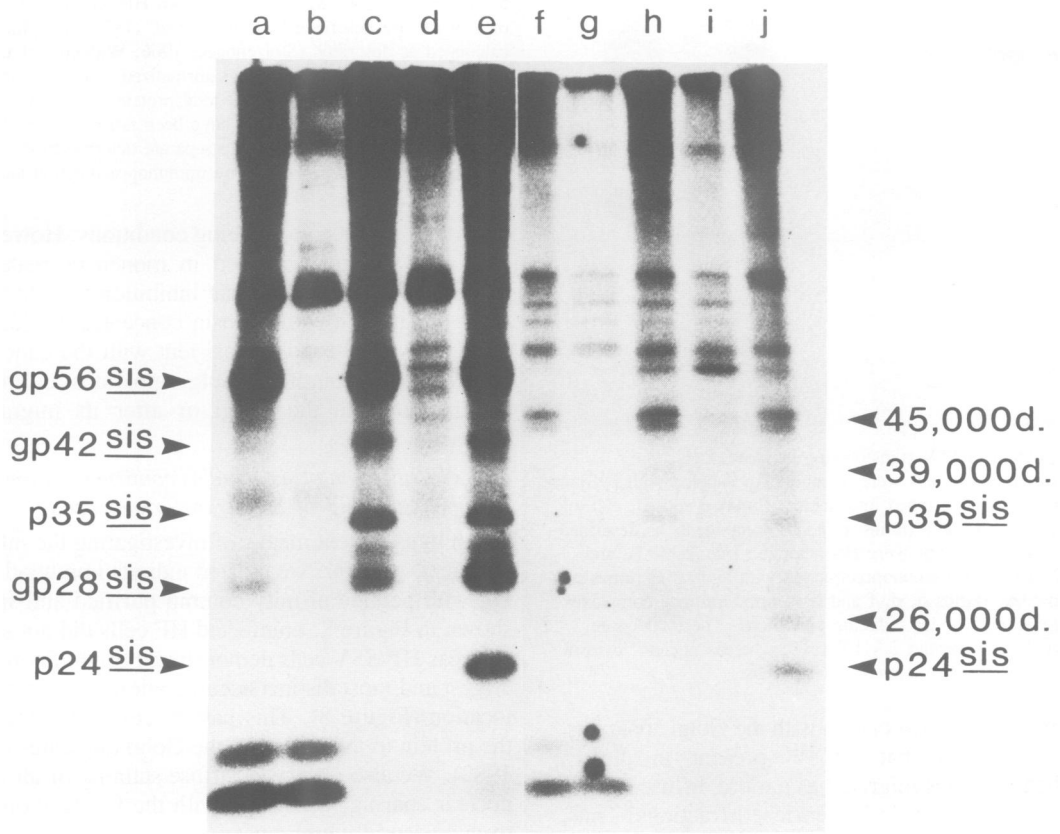

Fig. 4. Dimerization and processing of gp56^{sis} occurs in the presence of tunicamycin. Subconfluent HF/SSV cultures were metabolically labeled for 3 h in the presence (**lanes f–j**) or absence (**lanes a–c**) or 5 μg/ml tunicamycin. Cell lysates were immunoprecipitated with anti-*sis* N (**lanes, a, b, f** and **g**), anti-*sis* C (**lanes c, d, h** and **i**) or anti-PDGF (**lanes e** and **j**) sera. In some cases antisera were pre-incubated with 3 μg of *sis* N (**lanes b** and **g**) or *sis* C (**lanes d** and **i**) peptides. Immune complexes were analyzed under non-reducing conditions by SDS-PAGE.

Table I. Characterization of subcellular fractions prepared by differential centrifugation

Fraction	Percent of total protein (72)	Lactate dehydrogenase		NADH diaphorase		5′ Nucleotidase	
		Relative specific activity	Percent of total (53)	Relative specific activity	Percent of total (52)	Relative specific activity	Percent of total (85)
Total lysate	100	1.0	100	1.0	100	1.0	100
Nuclear	10	0.3	1	0.02	1	0.1	1
S100	42	1.6	90	0.24	14	0.08	2
P100	48	0.15	9	1.7	85	3.6	97

HF/SSV cells were disrupted and fractionated as described in Materials and methods. Protein was measured by the method of Lowry *et al.* (1951). Lactate dehydrogenase, NADH diaphorase and 5′ nucleotidase activities were calculated as described (Stolzenbach, 1966; Widnell and Unkeless, 1968; Avruch and Wallach, 1971), and normalized to that activity present in the total lysate. Actual recoveries from individual fractions, indicated in parentheses, were normalized to 100%. All results represent the mean values of two separate determinations. Samples from this experiment were also analyzed by immunoprecipitation (see Figure 5).

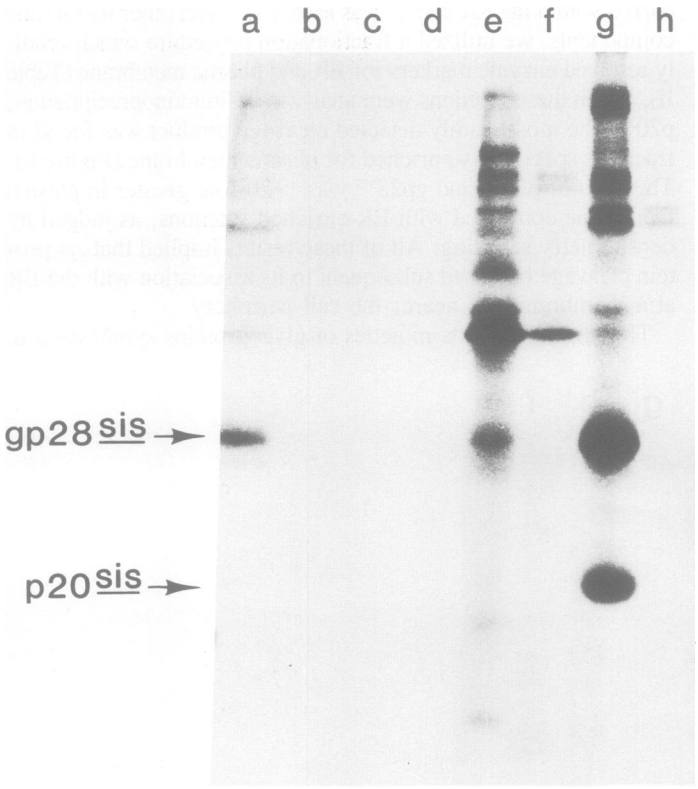

Fig. 5. Subcellular location of SSV transforming gene products. Exponentially growing HF/SSV cells were metabolically labeled with [^{35}S]-methionine plus cysteine and fractioned into nuclear (**lanes c** and **d**), cytosol (**lanes e** and **f**) or crude membrane (**lanes g** and **h**) components as described in Materials and methods. Fractions were characterized (see Table I), and identical volumes of each were immunoprecipitated with anti-*sis* C (**lanes a, c, e** and **g**) or pre-immune (**lanes b, d, f** and **h**) serum. Immune complexes were analyzed by SDS-PAGE under reducing conditions. The total lysate analyzed (**lanes a** and **b**) represented 2 x 10^6 cells, whereas nuclear, cytosol and crude membrane fractions represented 1 x 10^7 cells.

the ER are often modified in association with the Golgi apparatus. We consistently observed that gp28^{sis} present in plasma membrane-enriched fractions migrated as a more diffuse species than gp28^{sis} molecules present in ER-enriched fractions. Thus, it was possible that gp56^{sis} processing involved its association with this organelle. In an effort to address this question, we exposed SSV-transformed cells to monensin, a drug which is known to inhibit the export of glycoproteins from the Golgi apparatus (Ledger *et al.*, 1980). As shown in Figure 7, monensin treatment did not affect our ability to detect gp28^{sis} under reducing

Table II. Characterization of membrane fractions prepared by discontinuous density gradient centrifugation

Fraction (percent sucrose)	Percent of total protein	NADH diaphorase		5′ Nucleotidase	
		Relative specific activity	Percent of total	Relative specific activity	Percent of total
0/20	2	2.0	2	5.8	3
20/30	38	2.1	15	6.3	68
30/35	24	4.3	25	2.5	18
35/40	33	5.0	55	0.8	8
40/50	3	4.8	3	2.7	3

Membrane fractions were prepared from HF/SSV cells using a modification of a procedure described by Lutton *et al.* (1979). Enzymatic activities were calculated as described (Stolzenbach, 1966; Widnell and Unkeless, 1968; Arvuch and Wallach, 1971), and normalized to that activity present in the total lysate. Actual recoveries of total protein as well as enzyme activities were between 50 and 60% and have been normalized to 100%. Results represent the mean values of two separate determinations. Samples from this experiment were also analyzed by immunoprecipitation (see Figure 6).

or gp56^{sis} under non-reducing conditions. However, *sis* protein cleavage was not observed in monensin-treated cells. Dose-response studies showed that inhibition of gp56^{sis} cleavage was a direct function of monensin concentration (data not shown). These results are most consistent with the concept that gp56^{sis} is processed through the Golgi apparatus and that its cleavage occurs either in the Golgi or after its migration from this organelle.

Golgi localization of sis proteins confirmed by immunofluorescent antibody labeling of SSV-transformed cells

As an independent means of investigating the intracellular location of *sis* proteins, we utilized indirect immunofluorescent labeling with peptide affinity column purified anti-*sis* C serum. As shown in Figure 8, uninfected HF cells did not stain detectably, whereas HF/SSV cells demonstrated bright fluorescence with the largest and most distinct accumulation in an acentric perinuclear location (Figure 8). This pattern confirmed the localization of the protein to the region of the Golgi apparatus (Louvard *et al.*, 1982). We also observed diffuse staining of the cytoplasm with nuclear sparing, consistent with the localization of *sis* proteins to cyoplasmic membranes.

Some v-sis gene product becomes exposed on the transformed cell surface but is not actively secreted into tissue culture fluids

The involvement of peripheral membranes in the processing of *sis* proteins led us to inquire next whether any *sis* protein was exposed on the surface of SSV-transformed cell. Metabolically

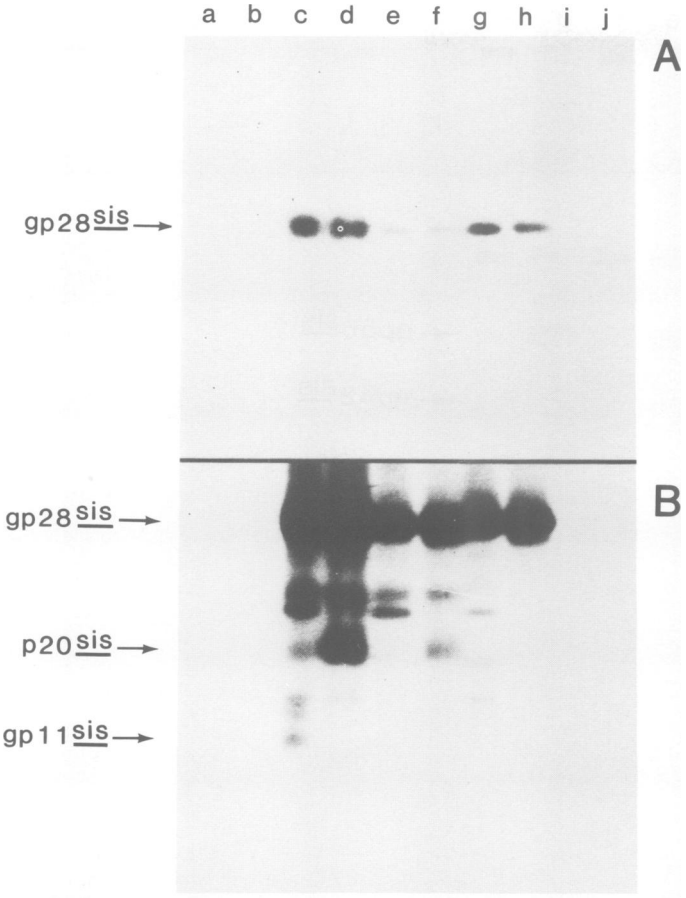

Fig. 6. Detection of *sis* gene products in membrane fractions prepared by discontinuous sucrose gradient centrifugation. Exponentially growing HF/SSV cells were metabolically labeled with [^{35}S]methionine plus cysteine and fractionated by the modified procedure of Lutton *et al.* (1979) as described in Materials and methods. Material sedimenting at the interfaces of 0/20 (**lanes a** and **b**), 20/35 (**lanes c** and **d**), 35/40 (**lanes e** and **f**), 40/50 (**lanes g** and **h**) or 50/60 (**lanes i** and **j**) % sucrose layers were characterized (see Table II), and identical portions of each were immunoprecipitated with anti-*sis* N (**lanes a–f**) or anti-*sis* C (**lanes g–i**) sera. Immune complexes were analyzed by SDS-PAGE under reducing conditions. Fluorograms were exposed to Kodak XAR film for 1 (**panel A**) or 4 (**panel B**) days.

labeled HF/SSV cells were treated with anti-*sis* C serum and washed extensively to remove any unbound antibody prior to cell lysis. There was no detectable cell toxicity associated with such treatment. To compare the amount of exposed cell surface *sis* protein detected by this approach with total cell-associated *sis* protein, cultures labeled in parallel were lysed prior to antibody addition. As shown in Figure 9, ~10% of the total cellular *sis* protein was present at a cell surface location accessible to the antibody. As a test of this method of analysis, we also examined cells transformed by Harvey murine sarcoma virus, whose *onc* gene product, p21^{ras}, resides at the inner surface of the plasma membrane (Willingham *et al.*, 1980). As shown in Figure 9, p21^{ras} was not detectable when antibody was added to live, intact cells, but was readily observed when the same cells were lysed prior to antibody addition. All of these findings demonstrated that some v-*sis* gene product is exposed on the surface of SSV-transformed cells.

To assess whether *sis* proteins were released from SSV transformants, we compared the time course of intracellular processing of *sis* proteins with their appearance in tissue culture fluids. HF/SSV cells were pulse-labeled for 15 min and subjected to chase periods of up to 4 h. As shown in Figure 10, cellular processing of the *sis* proteins occurred rapidly, culminating in the appearance of p24^{sis} which possessed a half-life of ~2 h. When cell culture fluids from the same cell were concentrated and similarly analyzed, no *sis* protein in any form was detectably released (Figure 10). As a test for our ability to recover *sis* proteins from cell culture medium, a hypotonic lysate of labeled HF/SSV cells was added to medium from unlabeled HF/SSV cells and treated in parallel. The recovery of all *sis* protein forms was nearly quantitative (data not shown).

We were able to observe very small amounts of gp42^{sis} and p35^{sis} in the cell culture medium, but only after relatively long labeling periods (Figure 11). A comparison of the intracellular levels of these two proteins with their levels in extracellular fluids revealed that only 1–2% of each of these species was released from the SSV transformant. Since the gp42^{sis} and p35^{sis} species represent only a small fraction of the cell-associated forms of the v-*sis* gene product, the actual fraction of total *sis* protein released must be considerably less.

To determine whether the subcellular localization of *sis* proteins observed with HF/SSV cells could be generalized to other SSV transformants, we analyzed SSV-transformed NRK and NIH/3T3 cell lines. As shown in Table III the vast majority of v-*sis* coded protein was found to be associated with the membrane fraction of these cell lines. Very low levels of gp42^{sis} and p35^{sis} species representing <1% of the total associated with SSV NRK cells were detected in tissue culture fluids, whereas no detectable *sis* products were present in supernatants of the SSV NIH/3T3 transformants (Table III). These findings generalize our conclusions that v-*sis* gene products localize to cell membrane components but are not actively secreted by SSV-transformed cells.

Discussion

The demonstration that the *sis* transforming gene product is closely related to PDGF (Doolittle *et al.*, 1983; Waterfield *et al.*, 1983) has suggested that a growth factor synthesized by a cell responsive to it can lead to growth alterations associated with malignancy. In the present studies, we have investigated the biosynthesis of the *sis* PDGF-2 gene product in efforts to learn where this protein may interact with its cellular target(s) and whether *sis* proteins are quantitatively released by SSV-transformed cells as are known secretory proteins. Our findings, summarized in Figure 12, document the biosynthetic pathway of the primary p28^{sis} product. This protein is synthesized as a glycosylated precursor which dimerizes rapidly to yield gp56^{sis}. The unprocessed precursor then passes through the Golgi apparatus where its N-linked oligosaccharides are modified. After reaching a distal membrane location, processing of its amino and then its carboxy termini occurs, leading finally to a 24 000-dalton disulfide-linked dimer, p24^{sis}.

All processing steps were shown to occur at membrane locations. The fact that the primary SSV transforming gene product, p28^{sis}, possessed N-linked oligosaccharide very soon after synthesis identified membrane-bound polyribosomes as the site of its synthesis. Enzymes capable of this post-translational modification are found only in the ER. Glycosylation of the v-*sis* translational product also implies translocation of the nascent v-*sis* translational product across the ER membrane, a process which is thought to be mediated by a peptide leader sequence. Although there appears to be no consensus for the amino acid sequence of leader peptides, features common to them have been describ-

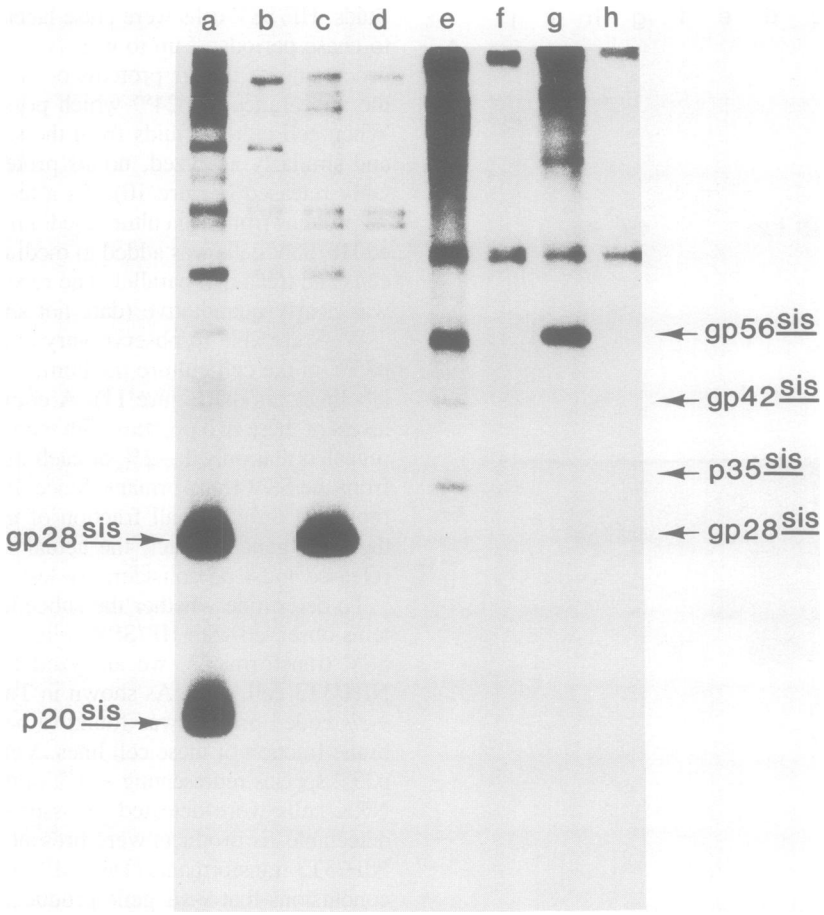

Fig. 7. Monensin inhibits gp56^{sis} processing. Subconfluent HF/SSV cultures were metabolically labeled following pre-incubation overnight in methionine- and cysteine-free medium alone (**lanes a, b, e** and **f**) or containing 10^{-6} M monensin (**lanes c, d, g** and **h**). Cell lysates were immunoprecipitated with anti-*sis* C serum alone (**lanes a, c, e** and **g**) or after pre-incubation with *sis* C peptide (**lanes b, d, f** and **h**). Immune complexes were analyzed by SDS-PAGE under reducing (**lanes a – d**) or non-reducing conditions (**lanes e – h**).

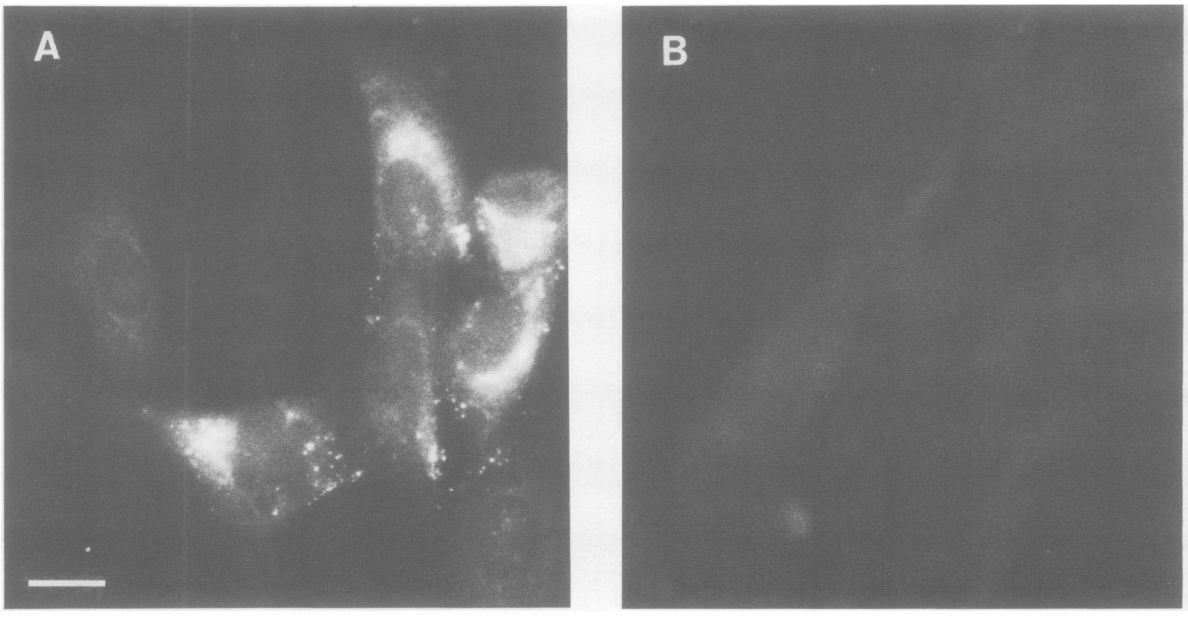

Fig. 8. Distribution of v-*sis* translational products in SSV-transformed cells using indirect immunofluorescence. Permeabilized HF/SSV-transformed (**panel A**) or HF control (**panel B**) cells were treated with peptide affinity column-purified anti-*sis* C serum and visualized by fluorescein epifluorescence microscopy after treatment with fluorescein-conjugated goat anti-rabbit IgG. Bar equals 20 μM.

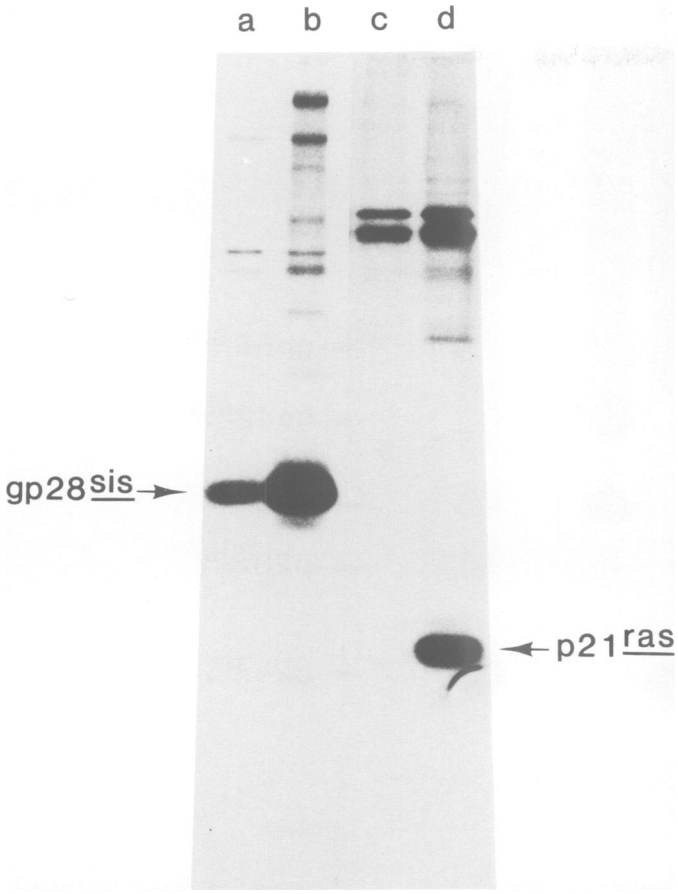

Fig. 9. Detection of v-*sis* coded protein on the surface of SSV-transformed cells. Subconfluent HF/SSV (**lanes a** and **b**) or Harvey murine sarcoma virus-transformed non-producer NRK (**lanes c** and **d**) cells were metabolically labeled with [^{35}S]methionine and cysteine. Cells were washed with PBS at 4°C (**lanes a** and **c**) or washed and disrupted with lysing buffer (**lanes b** and **d**), and incubated with identical volumes of anti-*sis* C (**lanes a** and **b**) or anti-p21^{ras} (**lanes c** and **d**) sera. Live cells incubated with antibody were extensively washed with PBS and subsequently disrupted with lysing buffer. Immunoprecipitates were analyzed by SDS-PAGE under reducing conditions.

ed (Kreil, 1981). Signal peptides are typically 15 – 26 amino acids in length and contain a charged amino acid (usually arginine or lysine) followed by a central hydrophobic region of at least nine residues. The open reading frame preceding v-*sis* in SSV contains three ATG codons which are theoretically capable of initiating synthesis of the v-*sis* gene product (Devare *et al.*, 1983). The sequence between the second and third ATG codons encodes a stretch of amino acids which possess features in common with previously described signal peptides. A recent report has indicated that this leader sequence is, in fact, necessary for v-*sis* transformation (Hannink and Donoghue, 1984). Thus, it is likely that a helper virus-derived leader peptide serves to direct the v-*sis* gene product to an intracellular location which provides a means for transport of gp56^{sis} toward the periphery of the transformed cell.

Normal PDGF biosynthesis culminates with its storage in the alpha granules of platelets. Current understanding indicates that the Golgi apparatus is the site at which molecules are sorted for packaging or release. Our present findings suggest that biosynthesis of the SSV transforming protein closely parallels that predicted for PDGF at least up to the point at which PDGF is packaged into granules. The peripheral membrane accumulation

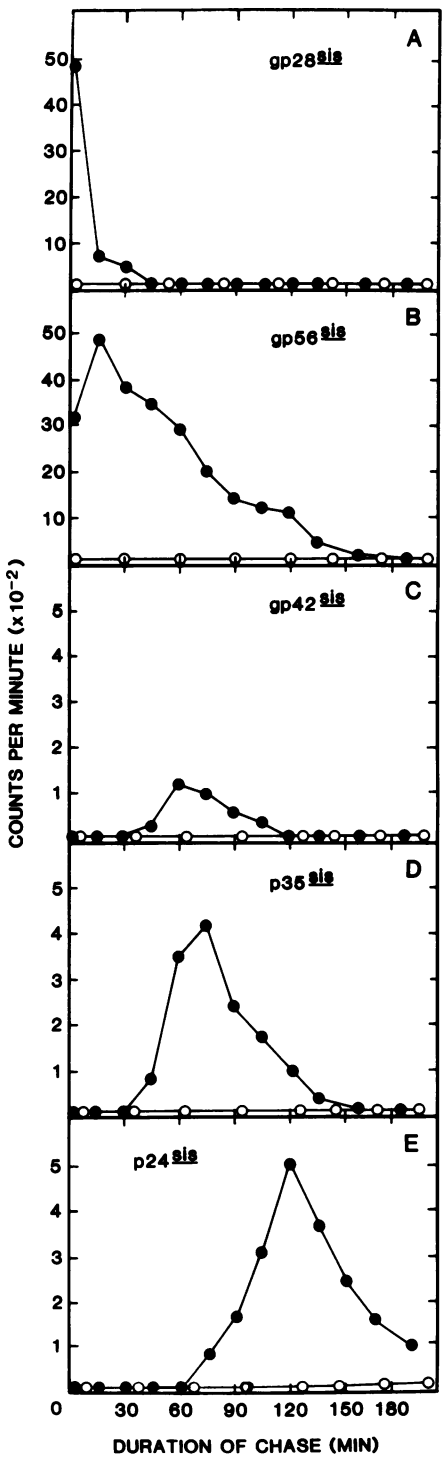

Fig. 10. Proteins encoded by v-*sis* are not quantitatively released from SSV-transformed cells. HF/SSV cells, pulse labeled for 15 min with [^{35}S]-methionine and cysteine, were chased with DMEM supplemented with 250 μg unlabeled methionine and cysteine per ml for periods up to 180 min. Immediately after each chase, culture medium was collected, lyophilized, resuspended in 10 mM Tris pH 7.5, 2 mg/ml BSA and dialyzed *versus* 10 mM Tris pH 7.5. Labeled cells were washed in ice-cold PBS immediately after the chase period and disrupted. Identical volumes of culture medium (○) or total cell lysate (●), each adjusted to represent 2 x 10^7 cells per ml, were immunoprecipitated with anti-*sis* C (**panels A – D**) or anti-PDGF (**panel E**) sera. Immune complexes were analyzed by SDS-PAGE using non-reducing conditions, fluorographed, dried and exposed to Kodak XAR film. Regions of the gel containing the labeled proteins indicated were excised using a scalpel. Radioactivity present in each band was determined by liquid scintillation.

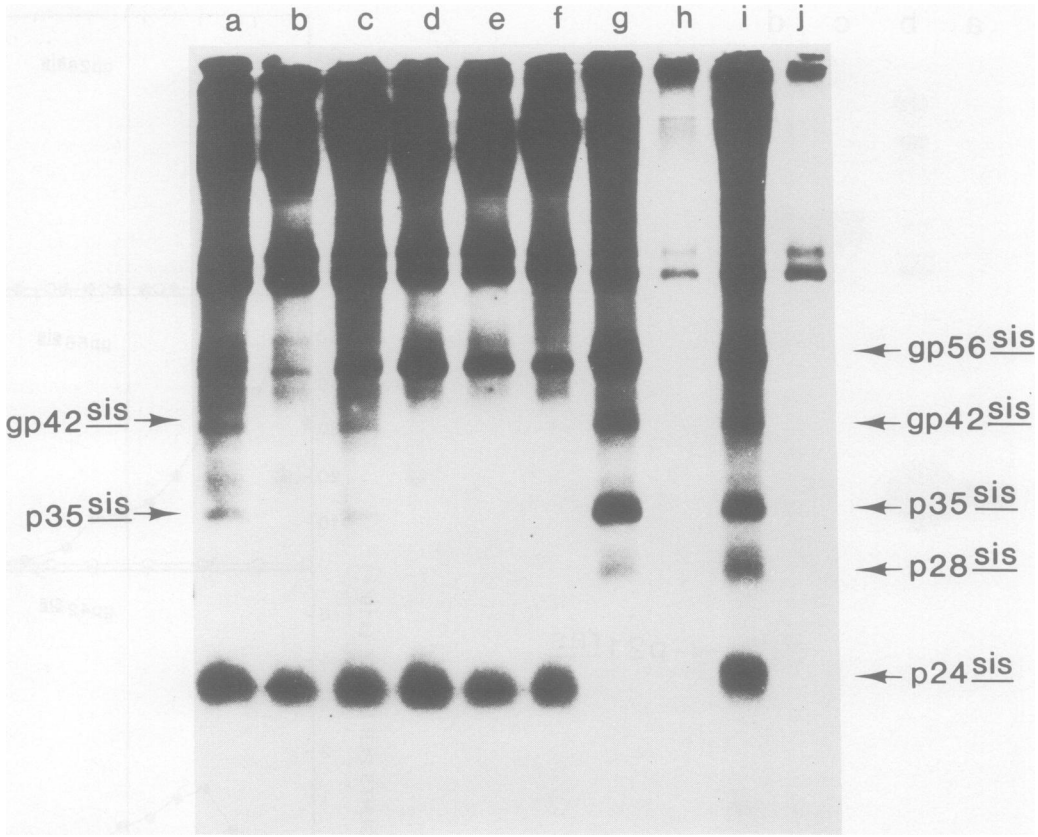

Fig. 11. Detection of low levels of gp42^{sis} and p35^{sis} tissue culture fluids of SSV transformants. Subconfluent HF/SSV or uninfected HF cultures were labeled for 4 h with [^{35}S]methionine and cysteine. Culture fluids were processed as indicated in the legend for Figure 10 so that fluids from 1 x 10^8 cells were concentrated to a final volume of 1 ml. Cells were suspended in lysing buffer at a concentration of 2 x 10^7 cells/ml. Identical volumes of SSV-transformed (**lanes a–c**) or uninfected (**lanes d–f**) cell culture medium, as well as total lysates of SSV-transformed (**lanes g–i**) or uninfected (**lane j**) cells were immunoprecipitated with anti-*sis* C (**lanes a, b, d, e, g, h** and **j**) or anti-PDGF (**lanes c, f** and **i**) sera. In some cases (**lanes b, e** and **h**) anti-*sis* C serum was incubated with 5 μg of *sis* C peptide before immunoprecipitation. Immune complexes were analyzed by SDS-PAGE.

of the v-*sis* gene products in SSV-transformed fibroblasts may reflect the fact that fibroblasts lack differentiated functions required for normal storage of the molecule in granules.

As much as 10% of the steady-state levels of the v-*sis* gene product was detectable in physical association with the outer surface of the SSV-transformed cell membrane, indicating that some of the unprocessed dimer reaches an extracellular location. We have not yet determined which processing steps occur in association with the outer cell surface, nor have we identified the cell surface components to which the *sis* protein is bound. It is possible that some *sis* protein is non-specifically extruded or released from the transformed cell and immediately sequestered at the cell surface. Another hypothesis derives from the novel synthesis of both the growth factor (*sis*/PDGF-2) and its receptor by the same cell. Known receptors for growth factors typically are synthesized in the ER and pass through the Golgi apparatus as they travel to the cell surface. Since the v-*sis* gene product exhibits this same biosynthetic pathway, this growth factor-like transforming protein may bind its receptor at a common intracellular location, with some of the complex reaching the cell surface.

Several recent reports have indicated that SSV transformants release high levels of PDGF-like activity (Bowen-Pope *et al.*, 1984; Owen *et al.*, 1984; Huang *et al.*, 1984; Garrett *et al.*, 1984; Johnsson *et al.*, 1985). The ability of such factors to bind and trigger autophosphorylation of the PDGF receptor as well as to stimulate DNA synthesis in quiescent fibroblasts has led to the suggestion that these activities represent the *sis* gene product (Huang *et al.*, 1984; Johnsson *et al.*, 1985). However, evidence that these secretory mitogens are coded by the v-*sis* gene relies on the fact that v-*sis* gene transcripts or products are also detected in these same cells and that PDGF antibody can neutralize a portion of the secreted activity. The v-*sis* gene product consists of only one component found in PDGF preparations and PDGF antibody might be expected to recognize related molecules capable of binding PDGF receptors. Thus, the evidence to date provides

Table III. Localization of *sis* gene products in cultures of SSV-transformed fibroblasts

Sample	Percent of total v-*sis* coded protein		
	HF/SSV	SSV.NRK	SSV.NIH
Lysate	100	100	100
Nuclear	3	2	3
Cytosol	0	9	0
Membrane	84	81	87
Culture medium	<1	<1	N.D.

Subconfluent cultures were metabolically labeled for 4 h in serum-free medium containing [^{35}S]methionine and cysteine. Culture fluids were concentrated while cells were hypotonically lysed and fractionated. All samples were resuspended in lysing buffer and were analyzed by immunoprecipitation using anti-PDGF serum. Levels detected were quantitated as described in the legend for Figure 10. That amount detected in aliquots of total lysates served as a standard, and is indicated as 100%. N.D., not detected.

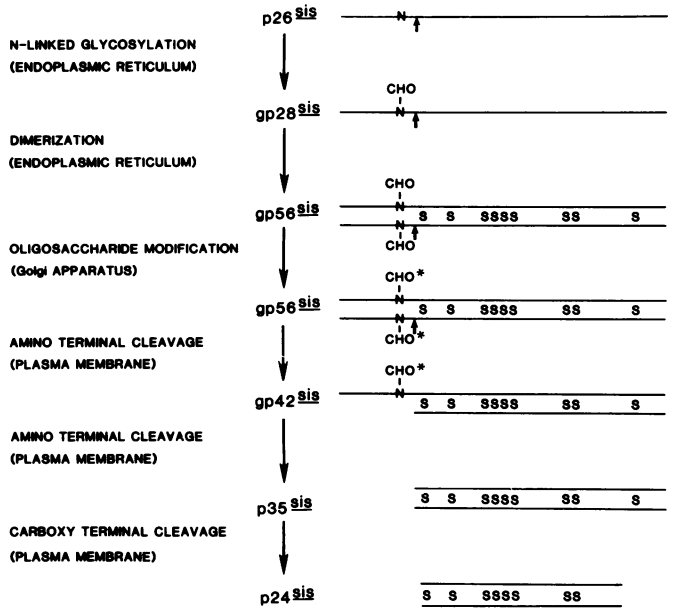

Fig. 12. Summary of processing events affecting the v-*sis* translational product. Solid lines represent single polypeptide chains. N or S represent asparagine or cysteine residues, respectively, present in the primary v-*sis* gene product. CHO and CHO* represent N-linked carbohydrate and modified N-linked carbohydrate, respectively.

no direct link between secreted PDGF-like activity and the v-*sis* gene product. The fact that *sis* proteins are not actively secreted does not exclude the possibility that the forms of the v-*sis*-coded protein released possess a very high specific activity for fibroblast mitogenesis. Alternatively, a growth factor (or factors) which possesses PDGF-like properties but is not *sis* coded may be actively secreted from tissue culture cells in response to transformation by SSV. Such a hypothesis is consistent with reports that cells transformed by agents other than SSV also release a PDGF-like mitogen (Bowen-Pope *et al.*, 1984; Bleiberg *et al.*, 1985). In fact, SV40- and Ab-MuLV-transformed cells, which are among the highest secretors of such activity, lack detectable *sis* transcripts (unpublished observations). Approaches which involve purification and amino acid sequence analysis of the PDGF-like factors released from transformed cells should aid in resolving this question.

We have recently shown that expression of the normal human PDGF-2 coding sequence causes morphologic transformation of appropriate target cells (Gazit *et al.*, 1984). Moreover, when incorporated by a retrovirus, the v-*sis*/PDGF-2 transforming gene induces fibrosarcoma and glioblastoma (Wolfe *et al.*, 1971; our unpublished observations). Many human glioblastomas and fibrosarcomas express *sis*/PDGF-2 transcripts, whereas normal fibroblasts and glial cells so far analyzed do not (Eva *et al.*, 1982; our unpublished observations). The cell membrane-associated *sis* products detected by us in the present studies on the outer surface of SSV-transformed cells may or may not play an important role in the SSV transformation process. In either case, if biosynthesis of the normal human PDGF-2 gene product parallels that shown for the v-*sis* gene product, this protein may serve as a specific cell surface marker for certain human tumors.

Materials and methods

Cell lines

Continuous NRK (Duc-Nguyen *et al.*, 1966) and SSV-transformed non-producer NRK (Aaronson *et al.*, 1975) cell lines have been described previously. Uninfected marmoset (HFϕTC12) and SSV (SSAV)-transformed producer marmoset (HF/SSV) cell lines were provided by H.-J.Thiel. NRK cells non-productively transformed by Harvey murine sarcoma virus have also been described (Willingham *et al.*, 1980).

Antisera

N-terminal (*sis* N) and C-terminal (*cis* C) peptides were synthesized on the basis of the nucleotide sequence of v-*sis* (Devare *et al.*, 1983) by the method described (Marglin and Merrifield, 1970). Rabbits were immunized with 100 μg of peptide conjugated with thyroglobulin according to Kagan and Glick (1979). Thereafter, 100 μg of conjugated peptide was administered i.p. at 14 day intervals. Animals were bled 1 week after each injection. Antisera against chromatographically purified SSAV gp70 and isopycnically banded SSAV were provided by the Office of Resources and Logistics of the National Cancer Institute. PDGF antibody reactivity with v-*sis* gene products has been described (Robbins *et al.*, 1983). Monoclonal antibody, Y13-259, which recognized the p21 product of the Harvey murine sarcoma virus transforming gene, was also utilized (Furth *et al.*, 1982). Rabbit anti-PDGF serum and Y13-259 rat monoclonal antibody to p21 were generously provided by H.Antoniades and E.Scolnick, respectively.

Metabolic labeling and immunoprecipitation

Subconfluent cultures containing ~2 x10^7 cells per 10-cm Petri dish were labeled for 3 h, at 37°C with 4 ml of methionine and cysteine-free Dulbecco's modified Eagle's minimal essential medium (DMEM) containing 100 μCi of [^{35}S]methionine and 100 μCi of [^{35}S]cysteine (~1200 Ci/mmol; Amersham) per ml, or glucose-free DMEM containing 1% dialyzed fetal calf serum and 200 μCi [^{3}H]glucosamine or [^{3}H]mannose/ml. For pulse-chase experiments, medium containing labeled amino acids was removed after the pulse and replaced with complete medium supplemented with 250 μg of unlabeled methionine and cysteine per ml.

Cell extracts were prepared by lysing with 1 ml of buffer A which contained 10 mM sodium phosphate, pH 7.5, 100 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS and 0.1 mM phenylmethylsulfonyl fluoride. Clarified extracts were aliquoted into 200 μl portions and then incubated with 4 μl of antiserum for 60 min, at 4°C. Blocking experiments were performed by pre-incubation of anti-*sis* N or anti-*sis* C with peptides as previously described (Robbins *et al.*, 1982). Immunoprecipitates were recovered with the aid of *Staphylococcus aureus* protein A bound to Sepharose beads (Pharmacia) and boiled for 3 min in sample buffer which contained 30% glycerol, 0.04% SDS, 0.1 M Tris-HCl, pH 6.8 and 8% 2-mercaptoethanol (reducing conditions). Alternatively, immunoprecipitates were boiled in sample buffer, but without 2-mercaptoethanol (non-reducing conditions). All samples were analyzed by electrophoresis in SDS-14% polyacrylamide gels (SDS-PAGE) as described (Barbacid *et al.*, 1980).

Subcellular fractionation

A modification of the procedure described by Hay (1974) was initially utilized. Exponentially growing SSV-transformed cells, metabolically labeled with [^{35}S]-methionine plus [^{35}S]cysteine were harvested with isotonic Tris-HCl-buffered saline containing 1 mM EDTA, washed wth the same buffer, and suspended in 10 mM Tris-hydrochloride (pH 7.5) containing 1 mM $MgCl_2$ at a final density of 10^7 cells/ml. Cells were incubated on ice for 15 min, and disrupted with 25 – 50 strokes in a Dounce homogenizer. Undisrupted cells, nuclei, and large cellular fragments were collected by centrifugation at 2000 r.p.m. for 5 min. The resulting pellet was suspended, with the aid of a Dounce homogenizer, in 0.5 volume of 20 mM Tris-hydrochloride, pH 7.5 containing 1 mM $MgCl_2$ (TM buffer), which also contained 0.5% Triton X-100, and 30% sucrose. This suspension was layered onto 10 ml of 60% sucrose in TM buffer and centrifuged at 100 000 *g* for 1 h. The pellet, designated nuclear fraction, was washed twice in TM buffer and stored at −70°C until used. The post-nuclear supernatant was centrifuged for 90 min, at 100 000 *g* to separate the soluble proteins (S-100) from the pelletable cellular membrane fraction (P-100). The P-100 fraction was washed once by resuspension in 0.5 volume of 20 mM Tris-HCl, pH 7.5, containing 1 mM EDTA (TE buffer) and re-sedimentation at 100 000 *g* for 60 min, while the S100 fraction was lyophilized. Both fractions were resuspended in 1 ml of TE buffer.

Alternatively, cellular membrane fractions were obtained using a modification of the procedure described by Lutton *et al.* (1970). Metabolically labeled cells were washed with isotonic Tris-HCl-buffered saline containing 1 mM EDTA, overlaid with 10 ml of ice-cold 1 mM Tris-HCl pH 7.2, and incubated for 15 min at 4°C. The buffer was replaced with 1 ml of 1 mM Tris-HCl, and cells were lysed by scraping. Lysates were placed in siliconized cellulose nitrate tubes, underlaid with 1.6 ml of 60%, 50%, 40%, 35% and 20% (w/v) sucrose in 1 mM Tris-HCl, pH 7.2 and centrifuged at 36 000 r.p.m. for 2 h at 4°C. Material accumulating at interfaces was harvested and washed once by resuspension in 5 ml TE buffer and re-sedimentation at 100 000 *g* for 60 min. Fractions were resuspended in 0.5 ml TE buffer and analyzed for enzymatic activities as described in the text. These same fractions were diluted 2-fold in 2 x buffer A, and identical portions of each were analyzed by immunoprecipitation.

Fluorescence microscopy

Cells were grown on printed slides, fixed for light microscopy with 80% acetone,

20% H_2O at 23°C for 5 min, and incubated with peptide affinity column purified rabbit anti-*sis* C or normal rabbit IgG diluted 1:40 in phosphate-buffered saline containing 2 mg/ml normal goat globulin, 2 mg/ml bovine serum albumin, PNB. After washing three times in PNB, cells were incubated for 30 min at 37°C with goat fluorescein-conjugated anti-rabbit IgG (Cappel) diluted 1:50 in PNB. Cells were then washed three times in PNB and three times in PBS before mounting in glycerol buffer medium. Fluorescence, observed using a Nikon fluorphot microscope equipped with epifluorescence optics and a 40 x fluor lens, was photographed with 3M black and white slide 1000 film (ASA 1000).

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