Membrane-anchored Form of v-*SIS*/PDGF-B Induces Mitogenesis Without Detectable PDGF Receptor Autophosphorylation

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Abstract. The v-*sis* protein is structurally and functionally related to PDGF. Forms of the v-*sis* protein which are anchored to the cell membrane via the transmembrane domain of the vesicular stomatitis virus G protein have been previously described (Hannink, M., and D. J. Donoghue. 1986. *J. Cell Biol.* 103:2311–2322). Several of these fusion proteins were shown to interact productively with the PDGF receptor (PDGFR) based on their ability to transform NIH 3T3 cells. In this report, we further characterized one of these membrane-anchored v-*sis* proteins, designated v-sis^{239}-G. The gene encoding v-sis^{239}-G was placed under control of the *Drosophila melanogaster hsp70* promoter and synthesis of this protein was shown to induce a mitogenic response in NIH 3T3 cells. Unexpectedly, v-sis^{239}-G did not induce detectable autophosphorylation of the PDGFR, in contrast to a similarly expressed secreted form of the v-*sis* protein. Thus, it appears that a PDGFR-mediated mitogenic response may be dissociated from detectable receptor autophosphorylation. Furthermore, induced synthesis of v-sis^{239}-G was shown to lead to c-*fos* expression even in the absence of detectable receptor autophosphorylation. Interestingly, a nonmitogenic membrane-anchored form of the v-*sis* protein, designated v-sis^{239}-G^{338}, also induced c-*fos* without receptor autophosphorylation. These results raise interesting questions regarding the roles of autophosphorylation and c-*fos* induction in PDGFR-mediated signal transduction and suggest the possibility of an autophosphorylation-independent signal transduction pathway.

GROWTH factor signal transduction occurs via the interaction of growth factors with specific cell surface receptors. The binding of growth factors to their receptors initiates a complex series of events which eventually lead to DNA replication and cell division (Ullrich and Schlessinger, 1990; Yarden and Ullrich, 1988; Rozengurt, 1986). Binding of PDGF to its receptor leads to the activation of second messenger systems which are important in initiating downstream effects of PDGF (Williams, 1989). Early events associated with the interaction of PDGF with the PDGF receptor (PDGFR)[1] include: stimulation of receptor tyrosine kinase activity; induction of receptor autophosphorylation; and receptor association with, and phosphorylation of, cellular substrates including PLC-gamma (Meisenhelder et al., 1989; Morrison et al., 1990; Kumjian et al., 1989; Wahl et al., 1989), PI-3 kinase (Kazlauskas and Cooper, 1989; Coughlin et al., 1989), Raf-1 (Morrison et al., 1988, 1989), and GAP (Kaplan et al., 1990; Kazlauskas et al., 1990). Other events initiated by PDGF include increased phosphoinositol turnover, calcium mobilization, protein kinase C activation, and the induction of a number of growth-related genes, including c-*fos* and c-*myc* (Kruijer et al., 1984; Kelly et al., 1983). All of these events are initiated by PDGF binding to its receptor, and receptor kinase activity is known to be essential (Williams, 1989; Escobedo et al., 1988). It is not clear, however, if all or a subset of these PDGF-induced events are necessary to stimulate cell proliferation. There may be multiple signal transduction pathways at work which use subsets of these events to mediate the different downstream effects of PDGF.

PDGF is a potent mitogen for cells of mesenchymal origin and consists of a disulfide-linked dimer of two related polypeptide chains designated A and B (Ross et al., 1986; Hammacher et al., 1988). In addition to the PDGF-AB heterodimer, both AA and BB homodimers have been identified (Heldin et al., 1986; Stroobant and Waterfield, 1984; Hart et al., 1990). The v-*sis* gene encodes a protein homologous to the PDGF-B chain (Doolittle et al., 1983; Waterfield et al., 1983; Johnsson et al., 1984; Josephs et al., 1984*a*; Deuel et al., 1983). The v-*sis* protein has been identified as a BB homodimer, and expression of v-*sis* in cells which express the PDGFR leads to autocrine stimulation of growth and cellular transformation (Deuel et al., 1983; Huang et al., 1984; Owen et al., 1984; Garrett et al., 1984; Josephs et al., 1984*b*).

Membrane-anchored v-*sis* fusion proteins have been previously described, and several were shown to retain the abil-

1. *Abbreviation used in this paper*: PDGFR, platelet-derived growth factor receptor.

ity to transform NIH 3T3 cells (Hannink and Donoghue, 1986). Thus, these membrane-anchored proteins can interact productively with the PDGFR. The fusion proteins were anchored to the cell membrane via the transmembrane domain contained within the COOH terminal portion of the vesicular stomatitis virus G protein. These membrane-anchored v-*sis* proteins were shown to be N-linked glycosylated, dimerized, and underwent proteolytic processing similar to the v-sis^{wt} protein. Transforming activity correlated with cell surface expression of these proteins, indicating that active growth factor/receptor complexes may need to reach the cell surface in order to be transforming.

A number of known growth factors, including EGF, TGF-alpha, and CSF-1, are synthesized as membrane-bound precursors (Mroczkowski, 1989; Teixido et al., 1987; Rettenmier et al., 1987). Membrane-bound TGF-alpha has been shown to interact productively with EGF receptors on adjacent cells to stimulate tyrosine kinase activity (Brachmann et al., 1989; Wong et al., 1989). This was shown to lead to autophosphorylation of the EGF receptor and to a rise in intracellular calcium levels. Thus, a precedent exists for biologically active membrane-bound growth factors. Membrane-anchored growth factors raise an interesting question regarding how they interact with their receptors. An active membrane-anchored form of the v-*sis* protein can serve as a model system to study the interaction of membrane-bound growth factors with their receptors.

In this paper, we have examined some of the properties of membrane-anchored v-*sis* proteins. Unexpectedly, a lack of detectable PDGFR autophosphorylation was observed in response to a mitogenic membrane-anchored form of the v-*sis* protein. Thus, it appears that a PDGFR-mediated mitogenic response can be dissociated from detectable PDGFR autophosphorylation. We also observed that PDGFR-mediated c-*fos* induction can occur in the absence of both a mitogenic response and detectable PDGFR autophosphorylation. These results suggest the possibility of an autophosphorylation-independent signal transduction pathway.

Materials and Methods

Construction of Plasmids and Cell Lines

Genes encoding the three v-*sis* proteins depicted in Fig. 1 were placed under transcriptional control of the *Drosophila melanogaster hsp70* heat shock promoter. Construction of pMH119, a plasmid with the *hsp70* promoter driving expression of the v-sis^{wt} gene, has been previously reported (Hannink and Donoghue, 1988). Similar plasmids for expression of v-sis^{239}-G and v-sis^{239}-G^{338} were constructed as follows. The *hsp70* promoter was isolated from pMH118 (Hannink and Donoghue, 1988) as a 640-bp HindIII(blunt)-ClaI fragment by linearizing the plasmid with HindIII, treating the 5′ overhang with the Klenow fragment of *Escherichia coli* DNA polymerase I in the presence of the four deoxynucleoside triphosphates, and then cutting with ClaI. Plasmids pMH113 and pMH85 contain the genes for v-sis^{239}-G and v-sis^{239}-G^{338}, respectively, in the SV-40 late expression vector pJC119 (Sprague et al., 1983). The 5′ portions of the genes encoding v-sis^{239}-G and v-sis^{239}-G^{338} were isolated as 750-bp BamHI(blunt)-BstEII fragments from pMH113 and pMH85 by cutting these plasmids with BamHI, treating with Klenow fragment and the deoxynucleoside triphosphates, and then cutting with BstEII. The 3′ portions of the genes encoding v-sis^{239}-G and v-sis^{239}-G^{338} were isolated from pMH113 and pMH85 as BstEII-ClaI fragments. Three piece ligations of the appropriate isolated fragments led to pAL128 and pAL129, which are heat-inducible expression plasmids for v-sis^{239}-G and v-sis^{239}-G^{338}, respectively.

Cell lines capable of expressing the proteins depicted in Fig. 1 were selected in NIH 3T3 cells by cotransfecting the heat-inducible expression plasmids described above with pFR400, a plasmid containing a mutant form of dhfr which allows for selection and amplification in $dhfr^+$ cells (Simonsen and Levinson, 1983). NIH 3T3 cells were grown in DME containing 10% calf serum in a humidified 10% CO_2 incubator. Subconfluent NIH 3T3 cells (2×10^5 per 60-mm plate) were transfected with 1 μg of pFR400 and 10 μg of pMH119, pAL128, or pAL129 using a modified calcium phosphate coprecipitation technique (Chen and Okayama, 1987). 2 d posttransfection, each 60-mm plate was split onto five 100-mm plates and the following day cells were selected in DME containing 10% dialyzed calf serum and 250 nM methotrexate. The cells were carried for 2 wk and individual colonies grown up as cell lines. Unless noted, selected cell lines were grown in DME containing 10% dialyzed calf serum and 250 nM methotrexate. Cell lines were screened for inducible protein expression by subjecting cells to a brief heat shock and recovery period followed by indirect immunofluorescence to identify cell lines expressing v-*sis* proteins.

Indirect Immunofluorescence

Cell lines were plated onto coverslips and 12–24 h later induced with a 43°C heat shock for 40 min. Heat induction was initiated with 43°C prewarmed DME and further incubation carried out in a 43°C humidified 10% CO_2 incubator. After the heat induction period, cells were transferred to a 37°C humidified 10% CO_2 incubator and allowed to recover for 2 h. Immediately after the recovery period, cells were fixed for 10 min with 3% paraformaldehyde in PBS, washed with 0.1 M glycine in PBS, and permeabilized for 5 min with 1% Triton X-100 in PBS. The v-*sis* proteins were detected using a primary rabbit antibody directed against bacterially expressed v-*sis* protein (kindly provided by Keith Deen, Smith Kline and French Laboratories, King of Prussia, PA) followed by a rhodamine-conjugated goat anti-rabbit secondary antibody.

Metabolic Labeling and Immunoprecipitations

Selected cell lines were split at 2×10^5 cells per 60-mm plate and grown for 3 d. Each plate was then refed with 37°C DME for 2 h, after which time the media was aspirated and cells heat induced with prewarmed 43°C DME. Cells were immediately placed in a 43°C incubator for 40 min. After this heat induction period, cells were rinsed once with MEM lacking cysteine and methionine and metabolically labeled for 2 h at 37°C with 100 μCi/ml of both [^{35}S]methionine and [^{35}S]cysteine in MEM lacking cysteine and methionine. Cell lysates were subjected to immunoprecipitation using the v-*sis* protein antibody as previously described (Hannink and Donoghue, 1986). Samples were run on a 12.5% SDS-polyacrylamide gel and processed for fluorography to visualize proteins.

Mitogenic Response Assay

Mitogenic response was assayed by incorporation of [^{3}H]thymidine into DNA. Cell lines were split into 24-well plates at 1×10^5 cells per well and grown for 3 d to confluence. Cells were then serum starved for 48 h in DME containing 0.5% calf serum. After serum starvation, cells were heat induced with prewarmed 43°C DME and placed in a 43°C incubator for 40 min after which time they were transferred to a 37°C incubator. Cells not heat induced were refed with 37°C DME or treated with various concentrations of PDGF-AB (R & D Systems, Inc., Minneapolis, MN). 17-h post-heat induction, cells were labeled for 6.5 h with 5 μCi/ml [^{3}H]thymidine in DME. Duplicate samples were lysed in 0.4 ml of 1% SDS and 5 mM EDTA. DNA was precipitated with 1 ml of 25% TCA and the precipitate collected by filtration for analysis by liquid scintillation counting.

Western Analysis of Phosphotyrosyl Proteins

Cell lines were split at 2×10^5 cells per 60-mm plate and grown for 3 d to confluence. Cells were then serum starved in DME containing 0.5% calf serum for 2 d. Cells were induced with various concentrations of PDGF-AB for 10 min at 37°C or with 43°C DME for 40 min. Heat-induced cells were allowed to recover at 37°C for various periods of time. Cells were lysed in protein gel sample buffer containing 1 mM sodium orthovanadate, 0.1 mM PMSF, and 10 μg/ml aprotinin. Samples were sonicated briefly to shear DNA and then boiled for 5 min before electrophoresis on a 7.5% SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose membrane by electroblotting at 50 V for 3 h. The blot was incubated overnight in TBS (20 mM Tris, pH 7.4, 150 mM NaCl) containing 3% BSA and 0.02% NaN_3 and then incubated for 4 h with affinity-purified anti-phosphotyrosine at a concentration of 1 μg/ml in the TBS/BSA solution. The affinity

purified anti-phosphotyrosine antibody was produced against phosphotyrosyl containing proteins made in *E. coli* expressing the v-*abl* tyrosine kinase (Maher and Pasquale, 1988; Wang, 1985). The blot was washed three times with TBS and then incubated for 30 min with 0.5 μCi/ml ^{125}I-labeled *Staphylococcus aureus* protein A in the TBS/BSA solution. The blot was washed with TBS as before, followed by two washes of TBS containing 1% NP-40, 0.1% SDS, and 0.5% deoxycholate. Washing with this solution was found to significantly reduce the background level of these blots. The blot was exposed to Kodak XAR-5 film with an intensifying screen for 3 d at −70°C.

Northern Analysis of c-fos

Cell lines were split at 5 × 10^5 cells per 100-mm plate and grown for 3 d to confluence. Cells were then serum starved in DME containing 0.5% calf serum for 48 h. Cells were induced with 10% calf serum DME at 37°C for 30 min or with 43°C DME for 40 min and allowed to recover for 1 h at 37°C. Total RNA was isolated from uninduced and induced cells by the guanidinium-cesium chloride method (Chirgwin et al., 1979) and ~10 μg of RNA from each sample was size fractionated by electrophoresis on a 1% formaldehyde agarose gel. The RNA was transferred to Nytran membrane (Amersham Corp., Arlington Heights, IL) and hybridized to a murine c-*fos* probe containing exon 1 (Kruijer et al., 1984). Hybridization was carried out overnight at 42°C in 50% formamide, 5× Denhardt's solution, 0.1% SDS, 5× SSPE, and 100 μg/ml denatured salmon sperm DNA and the membrane stringently washed with 0.1× SSPE, 0.1% SDS at 65°C. The blot was exposed to Kodak XAR-5 film with an intensifying screen for 3 d at −70°C.

Results

Expression System and Selection of Cell Lines

To characterize the interaction of membrane-anchored v-*sis* proteins with the PDGFR, we decided to express the proteins depicted in Fig. 1 in NIH 3T3 cells under control of the *Drosophila melanogaster hsp70* promoter and examine these proteins for their ability to inducibly stimulate a mitogenic response. Genes encoding the v-*sis*^{wt} and v-*sis*^{239}-G proteins were previously shown to be transforming based on their ability to efficiently induce focus formation in NIH 3T3 cells, while the gene encoding the v-*sis*^{239}-G^{338} protein was shown to be nontransforming (Hannink and Donoghue, 1986; Hannink et al., 1986). The *hsp70* promoter was chosen for its ability to be rapidly induced upon a brief increase in temperature. It should be noted that certain heat shock conditions have been reported to influence tyrosine phosphorylation and gene expression in various cultured cells (Andrews et al., 1987; Maher and Pasquale, 1989). However, as shown in subsequent control experiments, the heat induction conditions described in this report do not affect NIH 3T3 cells in this manner. This heat-inducible expression system should allow a pulse of protein to be sent through the ER, Golgi, and finally to the cell surface of NIH 3T3 cells. As the v-*sis* proteins are synthesized and travel through the secretory pathway, autocrine stimulation of the PDGFR should occur for the transforming v-*sis* proteins, and this should lead to inducible mitogenesis.

Genes encoding the three v-*sis* proteins shown in Fig. 1 were placed under transcriptional control of the *Drosophila melanogaster hsp70* promoter. Stable cell lines capable of heat-inducible expression of these proteins were selected in NIH 3T3 cells by cotransfecting the various *hsp70-sis* constructs with a plasmid containing the gene for a mutant form of dhfr which allows for selection and amplification in dhfr$^+$ cells (Simonsen and Levinson, 1983). Cells were selected in 250 nM methotrexate and individual colonies grown up as cell lines. Cell lines were screened for inducible protein expression by briefly heat shocking cells at 43°C, and after a short recovery period at 37°C, cells were examined for protein expression using indirect immunofluorescence with an antibody directed against the v-*sis* protein. Typical staining patterns for cell lines expressing these proteins are shown in Fig. 2. Strong Golgi staining is visible for the v-*sis*^{wt} (Fig. 2 *B*) and v-*sis*^{239}-G (Fig. 2 *D*) proteins. This pattern is typical for proteins which pass through the secretory pathway. The v-*sis*^{239}-G^{338} protein on the other hand shows a

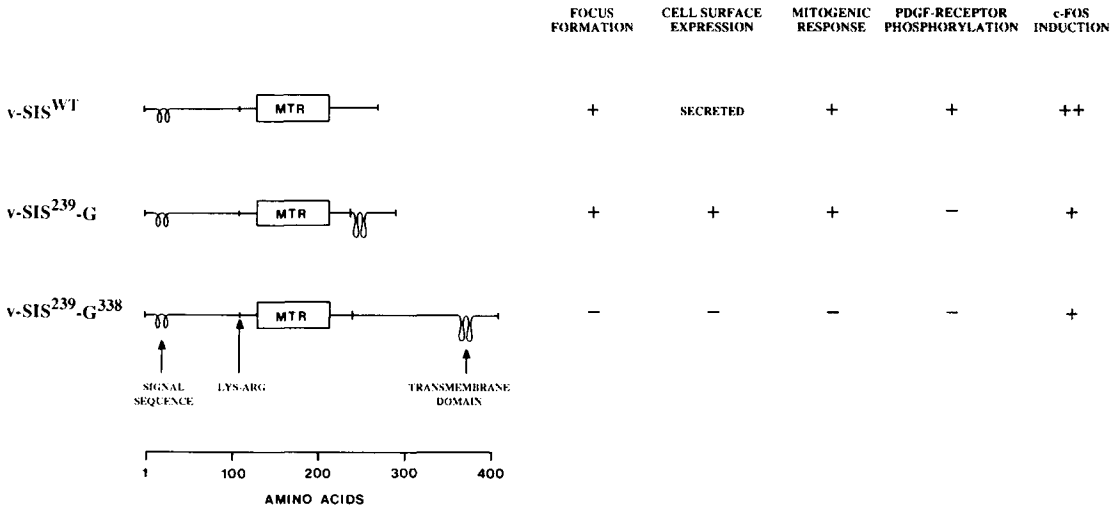

Figure 1. Properties of wild-type and membrane-anchored v-*sis* proteins. Wild-type and two membrane-anchored forms of the v-*sis* protein are depicted. The signal sequence for secretion and the dibasic proteolytic processing site within the v-*sis* protein precursor are indicated for all proteins. The transmembrane domain of the vesicular stomatitis virus G protein is indicated for the two membrane-anchored v-*sis* proteins. The minimal transforming region (Sauer et al., 1986; Hannink et al., 1986) of the v-*sis* protein is represented by the boxed MTR. The superscripted numbers indicate the amino acid codon in the respective gene at which the fusion was made (Hannink and Donoghue, 1986). Various properties of these proteins are summarized as described in the text.

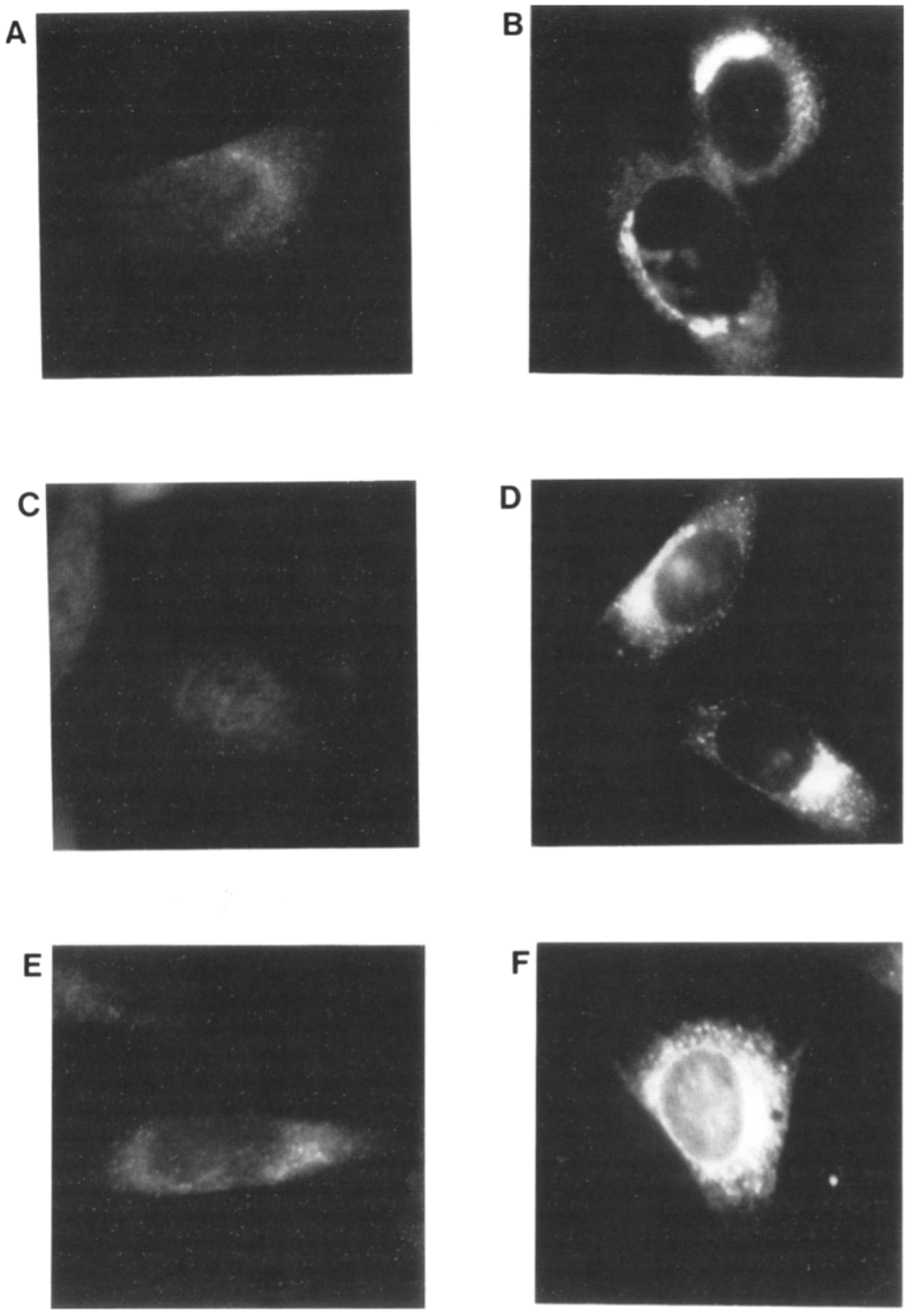

Figure 2. Indirect immunofluorescence of cell lines that inducibly express v-*sis* and membrane-anchored v-*sis* proteins. Cell lines capable of inducible expression of the proteins depicted in Fig. 1 were selected in NIH 3T3 cells by cotransfecting with a mutant form of dhfr that allows for selection in dhhfr$^+$ cells as described in Materials and Methods. Selected cell lines were screened for the ability to inducibly synthesize various v-*sis* proteins by subjecting cells to a 40-min heat shock at 43°C followed by a 2-h recovery period at 37°C. Cells were processed for immunofluorescence as described in Materials and Methods. (*A*) v-*sis*^{wt}, uninduced; (*B*) v-*sis*^{wt}, induced; (*C*) v-*sis*^{239}-G, uninduced; (*D*) v-*sis*^{239}-G, induced; (*E*) v-*sis*^{239}-G^{338}, uninduced; (*F*) v-*sis*^{239}-G^{338}, induced.

more diffuse staining pattern typical for proteins retained intracellularly in the ER (Fig. 2 *F*). It is possible that retention of v-*sis*^{239}-G^{338} in the ER may relate to its lack of transforming activity. Three cell lines, each expressing one of the three proteins depicted in Fig. 1, were expanded and further characterized.

To confirm that each cell line inducibly synthesized the v-*sis* protein of expected molecular weight, immunoprecipitations were performed. The selected cell lines described above were either not induced or heat-induced and then metabolically labeled for 2 h with [^{35}S]methionine and [^{35}S]cysteine. Cell lysates were subjected to immunoprecipitation using the antibody directed against the v-*sis* protein. As shown in Fig. 3, proteins of 31, 32, and 43 kD were inducibly synthesized from cell lines expressing v-*sis*^{wt}, v-*sis*^{239}-G, and v-*sis*^{239}-G^{338}, respectively. These proteins are of the predicted size for glycosylated v-*sis* proteins that have not as yet undergone proteolytic processing at the basic dipeptide, Lys-Arg.

Inducible Mitogenic Response

The ability of the various heat-inducible v-*sis* proteins to

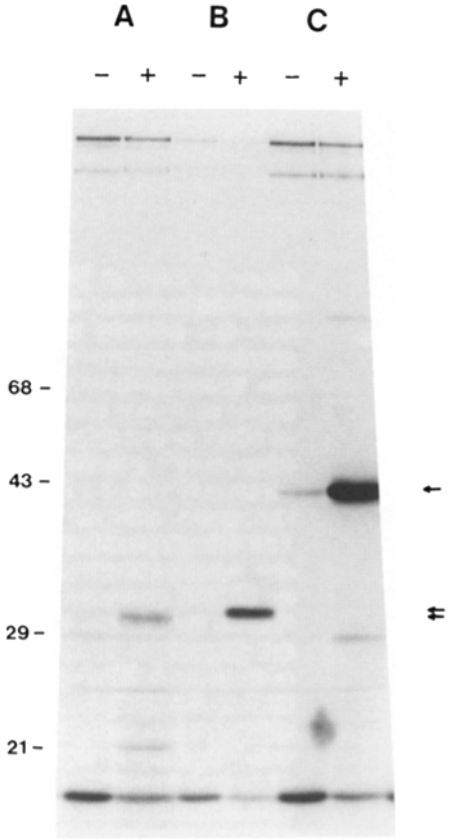

Figure 3. Immunoprecipitation of various v-*sis* proteins. Cell lines selected for inducible expression of the proteins depicted in Fig. 1 were heat induced at 43°C for 40 min and then metabolically labeled for 2 h with 100 μCi/ml each [^{35}S]methionine and [^{35}S]cysteine. Cell lysates were subjected to immunoprecipitation using an antibody directed against the v-*sis* protein. Immunoprecipitates were analyzed on a 12.5% SDS-polyacrylamide gel and radioactive proteins detected by fluorography and autoradiography. Plus and minus indicate heat induction and no heat induction, respectively. Molecular mass markers in kilodaltons are indicated. The arrows indicate the v-*sis* proteins specifically immunoprecipitated from heat-induced cell lines. (*A*) v-*sis*^{wt}; (*B*) v-*sis*^{239}-G; (*C*) v-*sis*^{239}-G^{338}.

promote mitogenesis was examined in the appropriate cell lines. Mitogenic response was assayed by incorporation of [^{3}H]thymidine into DNA following a brief heat induction. Results from a typical mitogen assay are shown in Table I. Cell lines which inducibly expressed the v-*sis*^{wt} and v-*sis*^{239}-G proteins consistently showed about a fourfold increase in thymidine incorporation compared to uninduced cells, while the cell line expressing v-*sis*^{239}-G^{338} did not show any significant increase in incorporation. This is consistent with the ability of these v-*sis* proteins to transform NIH 3T3 cells. It should be noted that heat treatment of the parental NIH 3T3 cell line does not induce mitogenesis (data not shown). It is possible that the membrane-anchored v-*sis*^{239}-G protein is transforming and mitogenic due to proteolytic release of a soluble form of the protein and subsequent interaction with the PDGFR. However, we have not been able to detect the presence of such a protein. These results indicate that inducible synthesis of wild type or a transforming membrane-anchored v-*sis* protein leads to an inducible mitogenic response, while inducible synthesis of a nontransforming membrane-anchored v-*sis* protein does not.

Table I. Mitogenic Response of Cell Lines that Inducibly Express v-sis and Membrane-anchored v-sis Proteins

Cell line	No heat shock	Heat shock
v-*sis*^{wt}	4,755	18,589
	3,322	12,415
v-*sis*^{239}-G	4,630	16,309
	4,561	14,721
v-*sis*^{239}-G^{338}	1,925	3,562
	2,269	1,890

Mitogenic response was assayed by incorporation of [^{3}H]thymidine after heat shock induction. A representative mitogen assay is shown. Cell lines were split into 24-well plates at 1 × 10^5 cells per well and grown for 3 d to confluence in DME containing 10% calf serum. Cells were then serum starved in DME containing 0.5% calf serum for 48 h. Heat-induced cells were treated with 43°C DME and placed in a 43°C incubator for 40 min then transferred to a 37°C incubator. Cells not heat shocked were refed with 37°C DME. 17 h later, cells were labeled with 5 μCi/ml [^{3}H]thymidine for 6.5 h. Duplicate samples were lysed in 0.4 ml 1% SDS, 5 mM EDTA and DNA precipitated with 1 ml of 25% TCA. Precipitated DNA was collected by filtration and counted.

Lack of Inducible Autophosphorylation

The ability of various v-*sis* proteins to induce autophosphorylation of the PDGFR was examined in the appropriate cell lines at various times after heat induction. Cells were grown to confluence, serum starved, and either heat induced, treated with PDGF, or left untreated. Tyrosine phosphorylation of the receptor was detected by Western blot analysis of cell lysates using an affinity purified anti-phosphotyrosine antibody. As shown in Fig. 4, a 40-min heat induction of the v-*sis*^{wt} protein led to tyrosine phosphorylation of a 180-kD protein that comigrated with the major tyrosine-phosphorylated protein in PDGF-treated cells. PDGFR tyrosine phosphorylation was first detected in cells expressing the v-*sis*^{wt} protein 1 h after heat induction, suggesting that this time was necessary for v-*sis*^{wt} protein synthesis and interaction with the PDGFR. HS9.1 cells also stimulated PDGFR autophosphorylation 1 h after heat induction. HS9.1 is a previously described cell line that can also express the v-*sis*^{wt} protein by heat inducement (Hannink and Donoghue, 1988). In contrast to cell lines which expressed the v-*sis*^{wt} protein, no tyrosine phosphorylation of the PDGFR was detected in cells that expressed either the v-*sis*^{239}-G or v-*sis*^{239}-G^{338} protein by inducement. All cell lines showed PDGFR autophosphorylation after a 10-min treatment with PDGF indicating the presence of functional receptors at the cell surface. These results indicate that PDGFR autophosphorylation can be detected in cells which inducibly express the v-*sis*^{wt} protein, but that under the same conditions, neither the v-*sis*^{239}-G nor the v-*sis*^{239}-G^{338} protein can stimulate detectable autophosphorylation of the PDGFR.

Why is there no detectable autophosphorylation in response to a mitogenic membrane-anchored v-*sis* protein? It is possible that the mitogenic membrane-anchored v-*sis*^{239}-G protein is limited in its mobility due to membrane association. The membrane anchor might also create a steric problem leading to inefficient binding and interaction with the PDGFR when compared to the secreted v-*sis* protein. This could increase the time necessary for v-*sis*^{239}-G to interact

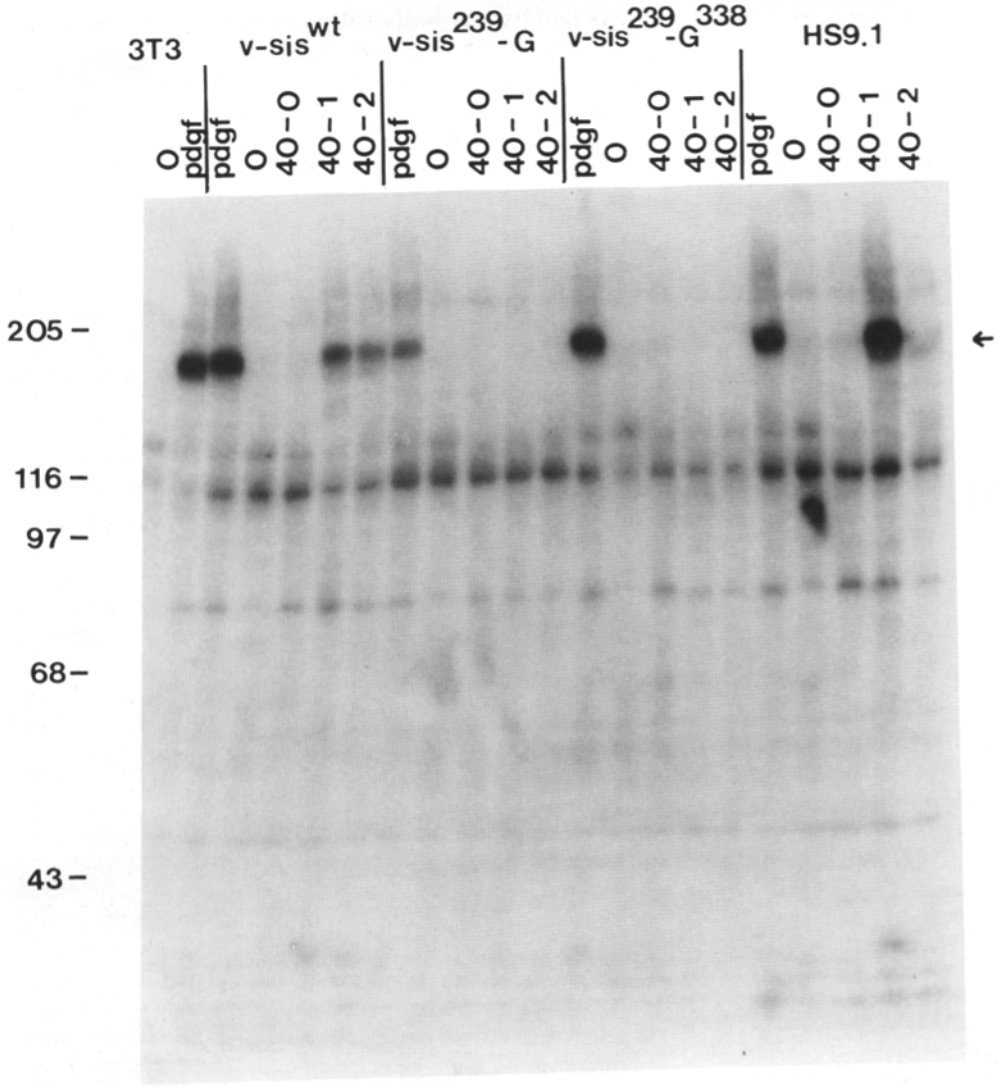

Figure 4. Lack of PDGFR autophosphorylation in cell lines that inducibly express membrane-anchored v-*sis* protein. Serum starved cells were treated for 10 min with 5 ng/ml PDGF or heat induced for 40 min at 43°C and allowed to recover at 37°C for 0, 1, or 2 h. Cell lysates were run on a 7.5% SDS-polyacrylamide gel and autophosphorylation of the PDGFR monitored using Western blot analysis and an anti-phosphotyrosine antibody as described in Materials and Methods. Phosphotyrosine containing proteins were detected with ^{125}I-protein A. Numbers above each lane represent the time of heat induction and recovery period. Molecular mass markers in kilodaltons are indicated on the left of the blot. The position of the tyrosine-phosphorylated PDGFR is indicated by the arrow. HS9.1 is another cell line similar to the cell line expressing v-*sis*^{wt} (Hannink and Donoghue, 1988).

with enough PDGFR to stimulate detectable autophosphorylation and a mitogenic response. Thus, autophosphorylation might be induced by v-*sis*^{239}-G, but the response may be delayed and/or weaker than that induced by v-*sis*^{wt}. To address this possibility, autophosphorylation of the PDGFR was examined at greater lengths of time after induction of v-*sis*^{wt} and v-*sis*^{239}-G. Although induction of v-*sis*^{wt} protein led to detectable PDGFR autophosphorylation beginning at 1 h and persisting up to 6 h after induction, no autophosphorylation was detected for up to 8 h postinduction for v-*sis*^{239}-G (data not shown). Thus, the lack of autophosphorylation in response to v-*sis*^{239}-G does not appear to be due to a time delay in PDGFR autophosphorylation.

Mitogenic Response Requires Detectable PDGFR Autophosphorylation

It is possible that the interaction of membrane-anchored v-*sis*^{239}-G with the PDGFR leads to weak but undetectable levels of PDGFR autophosphorylation. This undetectable autophosphorylation may still be above a threshold level necessary to induce mitogenesis. To show that a PDGF-induced mitogenic response actually requires detectable levels of autophosphorylation, PDGF concentrations which stimulate mitogenesis were compared to concentrations which induced PDGFR autophosphorylation (Fig. 5). Serum-starved NIH 3T3 cells were treated with various concentrations of PDGF ranging from 10 ng/ml to 62 pg/ml and receptor tyrosine phosphorylation examined after 10 min. When comparing mitogenic response and PDGFR autophosphorylation, it was consistently noted that a mitogenic response was not observed unless PDGFR autophosphorylation was also detected. A mitogenic response was not detected below 2.5 ng/ml PDGF (data not shown). In fact, at a concentration of 250 pg/ml, autophosphorylation above background levels was still observed in the absence of a detectable mitogenic response. Thus, in NIH 3T3 cells, PDGFR autophosphorylation correlates strongly with mitogenesis in response to exogenous PDGF. The possibility cannot be eliminated, however, that either mitogenesis or PDGFR autophosphorylation may require a different threshhold concentration of growth factor when it is delivered by an autocrine pathway rather than added exogenously.

Induction of c-fos

Transcription of the c-*fos* gene is one of the earliest events induced after PDGF interacts with its receptor, occurring as

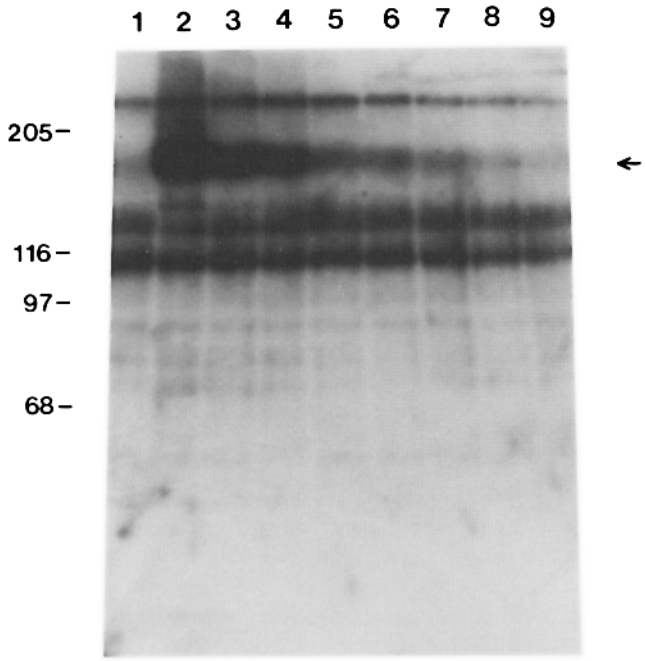

Figure 5. PDGF-induced receptor autophosphorylation. PDGF concentrations that induce receptor autophosphorylation were examined by Western blot analysis using an anti-phosphotyrosine antibody and ^{125}I-protein A. NIH 3T3 cells were treated with various concentrations of PDGF for 10 min at 37°C and then processed as described in Materials and Methods. The position of tyrosine-phosphorylated PDGFR is indicated by the arrow. Molecular mass markers in kilodaltons are indicated. Lane *1*, no PDGF; lane *2*, 10 ng/ml; lane *3*, 5 ng/ml; lane *4*, 2.5 ng/ml; lane *5*, 1 ng/ml; lane *6*, 500 pg/ml; lane *7*, 250 pg/ml; lane *8*, 125 pg/ml; lane *9*, 62 pg/ml.

soon as 10 min after growth treatment (Kruijer et al., 1984). As a measure of PDGFR activation, the ability of the membrane-anchored v-*sis* proteins to induce c-*fos* transcription was examined. Cell lines capable of inducible expression of the various v-*sis* proteins were treated with a 40-min heat shock and allowed to recover for 1 h. Total RNA was isolated from uninduced and heat-induced cells and c-*fos* message was detected by Northern blot analysis. A 1-h recovery period was chosen since this is when c-*fos* expression is maximal in NIH 3T3 cells which inducibly synthesize the v-*sis*^{wt} protein (Hannink and Donoghue, 1988). As seen in Fig. 6, the 2.2-kb c-*fos* message was detected in all cell lines which inducibly expressed the various v-*sis* proteins. However, the v-*sis*^{239}-G and v-*sis*^{239}-G^{338} proteins induced about six- to sevenfold less c-*fos* message than the v-*sis*^{wt} protein. It is interesting that the v-*sis*^{239}-G^{338} protein, which is nonmitogenic, induced c-*fos* message at a level equal to or greater than the mitogenic v-*sis*^{239}-G protein. This indicates that at least some portion of a PDGFR-mediated signal transduction pathway can be stimulated by this nonmitogenic membrane-anchored v-*sis* protein. The level of c-*fos* expression for both heat-induced HS9.1 cells and calf serum treated NIH 3T3 cells was much greater than that of the other cell lines. This is not surprising since calf serum efficiently induces c-*fos* mRNA and HS9.1 cells are known to inducibly synthesize more v-*sis*^{wt} protein than the cell line described in this paper (unpublished data). It should be noted that in contrast to HeLa cells (Andrews et al., 1987), NIH 3T3 cells apparently do not induce c-*fos* message in response to heat stress (Fig. 6). These results indicate that a low level of c-*fos* expression can be induced by both the mitogenic v-*sis*^{239}-G protein and the non-mitogenic v-*sis*^{239}-G^{338} protein, indicating that PDGFR-mediated c-*fos* induction can occur in the absence of both detectable PDGFR autophosphorylation and a mitogenic response.

Discussion

We have described membrane-anchored forms of the v-*sis* protein which can be inducibly expressed in NIH 3T3 cell lines. Expression of the v-*sis*^{239}-G^{338} protein was observed to induce c-*fos* without stimulating mitogenesis, and the v-*sis*^{239}-G protein was observed to stimulate mitogenesis in the absence of detectable PDGFR autophosphorylation. Somewhat surprisingly, naturally occurring forms of PDGF which are membrane anchored have not been reported. However, the artificial construct reported here may be viewed as a model system for the study of related growth factor receptors which are activated by membrane-anchored ligands, including TGF-α (Teixido et al., 1987; Brachmann et al., 1989; Wong et al., 1989), CSF-1 (Rettenmier et al., 1987), and the c-*kit* ligand (Huang et al., 1990; Anderson et al., 1990; Martin et al., 1990; Flanagan and Leder, 1990).

Many growth factor receptors including PDGFR possess intrinsic tyrosine kinase activity. Ligand-dependent autophosphorylation of these receptors is thought to involve receptor oligomerization or dimerization (Ullrich and Schlessinger, 1990). Some models suggest that autophosphorylation of the PDGFR may affect the interaction of several cellular proteins with the PDGFR. These cellular proteins may include PLC-gamma, PI-3 kinase, GAP, and Raf-1, all of which are potentially involved in mitogenic signal transduction pathways (Escobedo and Williams, 1988; Coughlin et al., 1989; Kazlauskas and Cooper, 1989, 1990; Morrison et al., 1989; Kaplan et al., 1990). An autophosphorylation site within the kinase insert domain appears to be required for association of PI-3 kinase with the receptor (Kazlauskas and Cooper, 1989), and mutants affecting the two major autophosphorylation sites of PDGFR exhibit reduced mitogenic responses (Kazlauskas, A., and J. A. Cooper, personal communication). Such studies suggest that PDGFR autophosphorylation sites may be required for efficient PDGF-induced mitogenesis and for association with putative receptor substrates.

In contrast, the membrane-anchored form of PDGF described here, v-*sis*^{239}-G, apparently stimulates mitogenesis and transformation without leading to detectable autophosphorylation of PDGFR. Several possible models are discussed below that may explain this phenomenon.

The first model involves the possibility that PDGFR is phosphorylated at an undetectable level in response to v-*sis*^{239}-G protein, but still above a threshold level required to simulate mitogenesis. This is rendered unlikely by control experiments that clearly demonstrate that exogenous PDGF can stimulate detectable PDGFR autophosphorylation at concentrations below that required to stimulate detectable mitogenesis (see Fig. 5). Nonetheless, the addition of exogenous PDGF may not be an adequate control for the situation where growth factor is provided by an autocrine pathway, as in the experiments described in this work.

A second model concerns the possibility of altered turn-

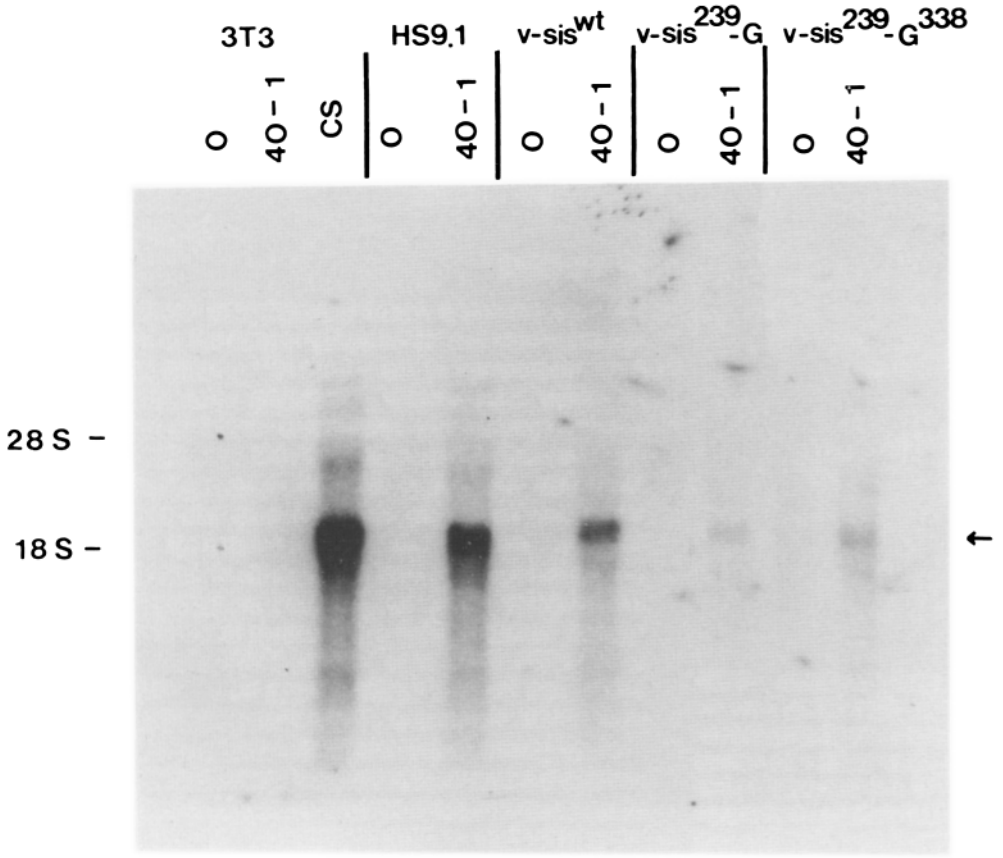

Figure 6. Northern analysis of c-*fos* induction from cell lines which inducibly express v-*sis* and membrane-anchored v-*sis* protein. Serum starved cells were heat induced for 40 min at 43°C and allowed to recover for 1 h at 37°C. Total RNA was isolated from uninduced and induced cells and ~10 μg of RNA from each sample was analyzed by Northern blotting using a murine c-*fos* probe as described in Materials and Methods. As a positive control 3T3 cells were treated with 10% calf serum for 30 min. Numbers above each lane represent the time of heat induction and recovery period. HS9.1 is a similar cell line to the one described in this paper which expresses the v-*sis*^{wt} protein (Hannink and Donoghue, 1988). The position of the 28S and 18S rRNAs are shown and the 2.2-kb c-*fos* transcript is indicated by the arrow.

over times for either the membrane-anchored growth factor or the ligand/receptor complex. Normally, addition of PDGF to cells expressing PDGFR leads to rapid internalization and degradation of the ligand–receptor complex (Bowen-Pope and Ross, 1982; Keating and Williams, 1987). The fact that v-*sis*^{239}-G is membrane-anchored may lead to altered rates of internalization and degradation of the ligand/receptor complex, resulting perhaps in a signal of longer duration. By this model, activation of quantitatively fewer receptors might still stimulate mitogenesis if v-*sis*^{239}-G activates the receptor for an extended period of time. We have determined that the v-*sis*^{wt} protein in the NIH 3T3 cell lines described here exhibits a half-life of ~15 min compared to ~50 min for v-*sis*^{239}-G; however, the half-life of the cell surface PDGFR was found to be the same after induced synthesis of either v-*sis*^{239}-G or the v-*sis*^{wt} proteins (data not shown). Further measurements of turnover rates using cell lines expressing higher levels of ligands and receptor will be required in order to fully address this model. It is relevant that internalization-defective EGF receptors exhibit a normal mitogenic response at significantly lower EGF concentrations compared with the wild-type EGF receptor (Wells et al., 1990). This demonstrates that an extended interaction of a growth factor with its receptor may lead to an increased biological response.

A third model would suggest that the membrane-anchored v-*sis*^{239}-G protein interacts with PDGFR such that mitogenic signaling is independent of receptor autophosphorylation. Precedent certainly exists for autophosphorylation-independent receptor activation, as evidenced by mutations in the cytoplasmic domain of the EGF receptor which exhibit constitutive signaling in the absence of detectable receptor autophosphorylation (Massoglia et al., 1990). A related question concerns whether the v-*sis*^{239}-G protein stimulates dimerization of PDGFR or whether it interacts only with monomeric PDGFR due to steric constraint. If receptor dimerization is prerequisite for autophosphorylation, then the constraint that the v-*sis*^{239}-G protein interacts only with monomeric PDGFR would predict the absence of receptor autophosphorylation. It is pertinent that autophosphorylation of several receptor tyrosine kinases, such as the CSF-1 receptor, EGF receptor, and PDGFR, has been shown to occur intermolecularly and likely requires receptor dimerization (Ohtsuka et al., 1990; Honegger et al., 1989, 1990; Heldin et al., 1989).

The resolution of these various models will depend upon a direct examination of the state of receptor oligomerization and/or the examination of substrates associated with PDGFR in cells expressing the v-*sis*^{239}-G protein. One approach to the study of PDGFR dimerization has relied upon antiphosphotyrosine immunopurification of "activated/dimerized" receptor in conjunction with chemical cross-linking of receptor subunits (Bishayee et al., 1989). Despite numerous attempts, we have been unable to obtain meaningful results using this approach due to the absence of detectable phosphorylation of PDGFR after induced synthesis of the v-*sis*^{239}-G protein (data not shown). Attempts to immunoprecipitate activated/dimerized PDGFR using a murine PDGFR antibody proved less sensitive than antiphosphotyrosine immunoblots (data not shown). An alternate approach (Heldin et al., 1989) has used purified PDGFR in a cell-free system to

demonstrate dimerization in response to soluble PDGF; however, the feasibility of this approach would need to be carefully evaluated since the v-*sis*^{239}-G ligand is membrane-bound.

In addition, we have examined whether the PDGFR is activated in response to v-*sis*^{239}-G protein by assaying the kinase activity of the receptor and the phosphorylation of associated substrates. Although phosphorylation of PDGFR and associated substrates of ~140, 85, and 74 kD (presumably PLC-gamma, PI-3 kinase, and Raf-1, respectively) was detected in each cell line treated externally with PDGF, neither phosphorylation of PDGFR nor any associated substrates was detected in response to induced synthesis of v-*sis*^{239}-G protein. This was in contrast to induced autocrine synthesis of v-*sis*^{wt} that resulted in a low level of detectable PDGFR autophosphorylation and phosphorylation of similar associated substrates (data not shown).

Many cellular events are stimulated by PDGF, including PDGFR autophosphorylation, tyrosine phosphorylation of associated substrates, changes in intracellular pH, phosphoinositol turnover, calcium mobilization, and induction of growth-related genes such as c-*fos* and c-*myc*. Which of these events are necessary and/or sufficient for initiating DNA synthesis and cell proliferation is not clear. For example, a PDGFR mutant with a deletion in the kinase insert domain does not induce mitogenesis in response to PDGF, despite stimulation of autophosphorylation, phosphatidylinositol hydrolysis, calcium mobilization, and c-*fos* expression (Escobedo and Williams, 1988; Severinsson et al., 1990). As another example, revertants of v-*ras* transformed cells have been reported to dissociate PDGF-induced calcium mobilization and expression of c-*myc*, c-*fos*, and *JE* from autophosphorylation of PDGFR (Quinones et al., submitted for publication). Such studies suggest that some of the classical responses induced by PDGF may be dissociated from the mitogenic response.

The data presented in this work clearly demonstrate that induced autocrine synthesis of the membrane-anchored v-*sis*^{239}-G protein leads to mitogenic signaling in the absence of detectable PDGFR autophosphorylation. This is in contrast to induced autocrine synthesis of v-*sis*^{wt} protein which results in the readily detectable incorporation of phosphotyrosine into PDGFR. Thus the v-*sis*^{239}-G ligand, by whatever biochemical mechanism, effectively dissociates PDGFR autophosphorylation from mitogenic signal transduction. These results add to the growing body of evidence that the various cellular responses to PDGF can be dissociated from one another, and that there may exist multiple pathways of signal transduction for the PDGFR. This work also suggests that for other ligand/receptor systems, membrane-anchored growth factors may be expected to elicit novel biochemical responses compared with their soluble counterparts.

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