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

Bruce A. Lee* and Daniel J. Donoghue†
Departments of *Chemistry and †Biology and Center for Molecular Genetics, University of California, San Diego, La Jolla, California 92093-0322

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<sup>239-G</sup>. The gene encoding v-sis<sup>239-G</sup> 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<sup>239-G</sup> 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<sup>239-G</sup> 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<sup>239-G</sup><sup>398</sup>, also induced c-fos without receptor autophosphorylation. These results raise interesting questions regarding the roles of autophosphorylation and c-fos induction in PDGF-mediated signal transduction and suggest the possibility of an autophosphorylation-independent signal transduction pathway.
ity to transform NIH 3T3 cells (Hannink and Donoghue, 1986). Thus, these membrane-anchored proteins can inter- 
act productively with the PDGFR. The fusion proteins were 
anchored to the cell membrane via the transmembrane do-
main 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 glyco-
sylated, dimerized, and underwent proteolytic processing 
similar to the v-sis protein. Transforming activity corre- 
lated with cell surface expression of these proteins, indicat-
ing 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-I, are synthesized as membrane-bound 
precursors (Mroczkowski, 1989; Teixido et al., 1987; Ret-
tenmeter et al., 1987). Membrane-bound TGF-alpha has 
been shown to interact productively with EGF receptors on adjac-
tent 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. Mem-
brane-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 re-
sponse 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 auto-
phosphorylation. 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 autophosphoryla-
tion-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-sis239 gene, has been previously reported (Han-
nink and Donoghue, 1988). Similar plasmids for expression of v-sis239.G 
and v-sis239.G-G38 were constructed as follows. The hsp70 promoter was 
iso-lated from pMH118 (Hannink and Donoghue, 1988) as a 640-bp HindIII(Blunt)-ClaI fragment by linearizing the plasmid with HindIII, 
setting the 5' overhang with the Klenow fragment of Escherichia coli DNA poly-
merase I in the presence of the four deoxynucleoside triphosphates, and then 
cutting with ClaI. Plasmids pMH113 and pMH85 contain the genes for 
v-sis239.G and v-sis239.G-G38, respectively, in the SV-40 late expression vec-
tor pJC119 (Sprague et al., 1983). The 5' portions of the genes encoding 
v-sis239.G and v-sis239.G-G38 were isolated as 750-bp BamHI(blunt)-BstEII 
fragments from pMH113 and pMH85 by cutting these plasmids with 
ClaI. Plasmids pMH113 and pMH85 contain the genes for 
fragments led to pAL128 and pAL129, which are heat-inducible expression 
plasmids described above with pFR400, a plasmid containing a mutant form 
of dhfr which allows for selection and amplification in dhfr- 
positive cells (Simon-
sen and Levinson, 1983). NIH 3T3 cells were grown in DME containing 
10% calf serum in a humidified 10% CO2 incubator. Subconfluent NIH 
3T3 cells (2 x 10^6 per 60-mm plate) were transfected with 1.25 g of pFR400 
and 10 µg of pMH119, pAL128, or pAL129 using a modified calcium phos-
phate precipitation technique (Chen and Okayama, 1987). 20 pL transfection, 
60-mm plates were split onto five 60-mm plates and the following 
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 immuno-
fluorescence 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% CO2 
incubator. After the heat induction period, cells were transferred to a 37°C 
humidified 10% CO2 incubator and allowed to recover for 2 h. Immediately 
after the recovery period, cells were fixed for 10 min with 3% parafo-
maldehyde 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 Laborato-
ries, 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 x 10^5 cells per 60-mm plate and grown 
for 3 d. Each plate was then re-fed with 37°C DME for 2 h, after which time 
the media was aspirated and cells heat induced with prewarming 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 [35S]methionine and [35S]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 [3H]thymidine into 
DNA. Cell lines were split into 24-well plates at 1 x 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 re-fed with 37°C DME or treated with various concen-
trations 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 [3H]thymidine 
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 immuno-
fluorescence to identify cell lines expressing v-sis proteins.

Western Analysis of Phosphotyrosyl Proteins

Cell lines were split at 2 x 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 
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 into nitrocellulose membrane 
by electroblotting at 50 V for 3 h. The blot was incubated overnight in TBS 
containing 5% nonfat dry milk and then probed with affinity-purified anti-phosphotyrosine 
at a concentration of 1 µg/ml in the TBS/BSA solution. The affinity

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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 125I-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 x 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 to confluence. Cells were then serum starved in DME containing 0.5% calf serum 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 (Krijger 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-sis239-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-sis239-G338 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. Cells 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-sis239-G (Fig. 2 D) proteins. This pattern is typical for proteins which pass through the secretory pathway. The v-sis239-G338 protein on the other hand shows a

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**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 dhfr′ 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-sis239-G, uninduced; (D) v-sis239-G, induced; (E) v-sis 239-G 33s, uninduced; (F) v-sis 239-G 33s, induced.

more diffuse staining pattern typical for proteins retained intracellularly in the ER (Fig. 2 F). It is possible that retention of v-sis239-G 33s 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 [35S]methionine and [35S]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-sis239-G, and v-sis239-G 33s, 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
promote mitogenesis was examined in the appropriate cell lines. Mitogenic response was assayed by incorporation of $[^3H]$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 protein consistently showed about a fourfold increase in incorporation, while inducible synthesis of a nontransforming membrane-anchored v-sis protein does not.

**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 treated, with PDGF, or left untreated. Tyrosine phosphorylation of the PDGFR 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$^{\text{wt}}$ 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$^{\text{wt}}$ protein 1 h after heat induction, suggesting that this time was necessary for v-sis$^{\text{wt}}$ 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$^{\text{wt}}$ protein by heat inducement (Hannink and Donoghue, 1988). In contrast to cell lines which expressed the v-sis$^{\text{wt}}$ protein, no tyrosine phosphorylation of the PDGFR was detected in cells that expressed either the v-sis$^{299}$-G or v-sis$^{299}$-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$^{\text{wt}}$ protein, but that under the same conditions, neither the v-sis$^{299}$-G nor the v-sis$^{299}$-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$^{299}$-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$^{299}$-G to interact.
with enough PDGFR to stimulate detectable autophosphorylation and a mitogenic response. Thus, autophosphorylation might be induced by v-sis239-G, but the response may be delayed and/or weaker than that induced by v-siswt. To address this possibility, autophosphorylation of the PDGFR was examined at greater lengths of time after induction of v-siswt and v-sis239-G. Although induction of v-siswt 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-sis239-G (data not shown). Thus, the lack of autophosphorylation in response to v-sis239-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-sis239-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 threshold 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 125I-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.

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-sis239-G protein was observed to induce c-fos without stimulating mitogenesis, and the v-sis239-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., 1989), 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 PDGF may affect the interaction of several cellular proteins with the PDGFR. These cellular proteins may include PLC-gamma, PI-3 kinase, 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). 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 PDGF exhibit reduced mitogenic responses (Kazlauskas, A., and J. A. Cooper, personal communication). Such studies suggest that PDGF 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-sis239-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-sis239-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 simulate 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-
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-sis239-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-sis239-G activates the receptor for an extended period of time. We have determined that the v-siswt protein in the NIH 3T3 cell lines described here exhibits a half-life of \( \sim 15 \) min compared to \( \sim 50 \) min for v-sis239-G; however, the half-life of the cell surface PDGFR was found to be the same after induced synthesis of either v-sis239-G or the v-siswt 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-sis239-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-sis239-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-sis239-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-sis239-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-sis239-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<sup>239</sup>-G ligand is membrane-bound.

In addition, we have examined whether the PDGFR is activated in response to v-sis<sup>239</sup>-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<sup>239</sup>-G protein. This was in contrast to induced autocrine synthesis of v-sis<sup>239</sup>-G 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<sup>239</sup>-G protein leads to mitogenic signaling in the absence of detectable PDGFR autophosphorylation. This is in contrast to induced autocrine synthesis of v-sis<sup>239</sup>-G protein which results in the readily detectable incorporation of phosphotyrosine into PDGFR. Thus the v-sis<sup>239</sup>-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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