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Blockade of autocrine stimulation in simian sarcoma virus-transformed cells reverses down-regulation of platelet-derived growth factor receptors

(v-*sis* oncogene/tyrosine phosphorylation/suramin)

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ABSTRACT **The viral (v)-*sis* oncogene encodes a protein ($p28^{sis}$) that is structurally homologous to platelet-derived growth factor (PDGF). We have shown that simian sarcoma virus (SSV)-transformed cells containing the v-*sis* oncogene release a M_r 20,000 substance that is recognized by antisera to synthetic peptide sequences contained in $p28^{sis}$. Medium conditioned by SSV-transformed cells competes with ^{125}I-labeled PDGF for specific PDGF receptor sites, initiates DNA synthesis, and stimulates tyrosine phosphorylation of the PDGF receptor when added to normal cells. When normal cells are cocultured with SSV-transformed cells, the PDGF receptors of the normal cells are down-regulated by factors released from the transformed cells. Thus, SSV-transformed cells release material that is functionally similar to PDGF. We have used anti-phosphotyrosine antibodies to purify PDGF receptors and to detect PDGF-stimulated receptors in normal cells. SSV-transformed cells have no PDGF receptors detectable by these antibodies or by ^{125}I-labeled PDGF binding studies. However, when SSV-transformed cells are exposed to suramin, a compound that blocks binding of PDGF to its receptors, the receptors reappear on the cell surface and within 8 hr are present at the same levels as in control cells. These "new" receptor sites can be phosphorylated in response to PDGF. Thus, the absence of PDGF receptors in SSV-transformed cells is due to down-regulation of the receptors by an autocrine mechanism that can be blocked by suramin.**

The determinants of the phenotypic differences between normal and transformed cells are poorly understood. Recent studies have suggested that transformation may be produced through mechanisms involving growth factors and their receptors (1–14). For example, fibroblast cells transformed by some RNA and DNA tumor viruses synthesize and release polypeptides that can stimulate proliferation, confer anchorage independence, and cause neoplastic morphological changes (1–4). After release, these polypeptide growth factors presumably act through specific receptor sites on the parent cell, thus altering the growth characteristics of the cell.

Cells transformed by the oncogene v-*sis* of simian sarcoma virus (SSV) are unique in that the oncogene encodes the transforming polypeptide $p28^{sis}$ (15), which shares extensive sequence homology with part of the platelet-derived growth factor (PDGF) molecule (5, 6). SSV-transformed cells process $p28^{sis}$ by glycosylation, dimerization, and proteolysis (16). Structural, immunological, and functional similarities between PDGF and v-*sis*-encoded peptides have been demonstrated (11, 13, 16, 17). However, the mechanism of transformation by SSV remains unknown. To examine this issue, we studied the manner in which PDGF-like factors released from SSV-transformed cells interact with the PDGF receptors of normal cells and investigated the role of these factors in the regulation of PDGF receptors in SSV-transformed cells.

METHODS AND MATERIALS

Cells. Cells were grown in Dulbecco's minimal essential medium supplemented with 10% calf serum, 2 mM L-glutamine, 100 units of penicillin per ml, and 100 μg of streptomycin per ml. Normal rat kidney (NRK) and SSV-transformed NRK cells were a gift from Stuart A. Aaronson and Keith C. Robbins of the Center for Cancer Research (National Institutes of Health).

Measurement of Tyrosine Kinase *in Vivo*. Labeling of cells with radioactive phosphate and immunospecific purification of phosphotyrosine-containing proteins were performed as described (18). Confluent cellular monolayers were incubated for 3 hr in buffered phosphate-free media with [^{32}P]orthophosphate. Cells were then stimulated with PDGF or conditioned medium as indicated and solubilized in cold Tris-buffered Triton X-100 containing both protease and phosphatase inhibitors. Cell extracts were then centrifuged, dialyzed, adsorbed with bovine serum albumin-conjugated Sepharose, and immunospecifically purified by using monoclonal antibody to phosphotyrosine (18). $NaDodSO_4$/polyacrylamide gel electrophoresis and subsequent autoradiographic exposure at −70°C were then used to analyze isolated ^{32}P-labeled phosphoproteins.

Immunological Detection of $p20^{sis}$. Conditioned medium from NRK or from SSV-transformed cells was concentrated 500-fold by using dialysis in 1 M acetic acid followed by lyophilization. After reduction with 10% 2-mercaptoethanol, 50 μl of concentrated medium was electrophoresed into a $NaDodSO_4$/5–17.5% polyacrylamide gel. Protein was then electrophoretically transferred to nitrocellulose. Nonspecific binding was blocked by preincubation of the transferred extract with 3% bovine serum albumin/0.1% Triton X-100 in phosphate-buffered saline (pH 7.4). Antibody (19) against synthetic peptides (20 μl) representing residues 1–17 or 73–89 of the chain of PDGF homologous to the predicted M_r 20,000 COOH-terminal sequence of $p28^{sis}$ was preincubated for 1 hr at 37°C with 100 μg of peptide prior to probing the extracts. The antibody–peptide reaction mix was diluted 1:500 with 3% bovine serum albumin/0.1% Triton X-100 in phosphate-buffered saline and incubated with the transferred extract at 4°C for 15 hr. After the nitrocellulose was

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Abbreviations: PDGF, platelet-derived growth factor; ^{125}I-PDGF, ^{125}I-labeled PDGF; SSV, simian sarcoma virus; NRK, normal rat kidney.

washed with phosphate-buffered saline, it was incubated with a 1:500 dilution of affinity-purified rabbit anti-mouse IgG_1 (Litton) for 2 hr at 25°C. Binding was visualized with 10^6 cpm of ^{125}I-labeled protein A. Autoradiography was performed at −70°C in the presence of intensifying screens (17).

PDGF and ^{125}I-Labeled PDGF (^{125}I-PDGF). Human PDGF was purified from outdated human platelets and was radioiodinated (15,000–20,000 cpm/ng) by the iodogen method as described (20).

RESULTS

Medium Conditioned by SSV-Transformed Cells Competes for PDGF Receptors and Stimulates Receptor Phosphorylation. NRK cells and SSV-transformed NRK cells were washed extensively in serum-free medium (see the legend for Fig. 1 *Upper*) and were incubated with serum-free medium for an 18-hr conditioning period. Conditioned medium from control and from SSV-transformed cells was collected and then tested for activity in stimulating DNA synthesis of BALB/3T3 cells and in inhibiting the binding of ^{125}I-PDGF to its specific receptor sites (Fig. 1 *Upper*). SSV-conditioned medium blocked specific ^{125}I-PDGF binding even at dilutions greater than 1:10 and stimulated DNA synthesis in 3T3 cells at comparable concentrations; conditioned medium from control NRK cells neither inhibited binding nor stimulated DNA synthesis at the concentrations tested (Fig. 1 *Upper*). In parallel control experiments, half-maximal concentrations of PDGF for inhibiting binding of ^{125}I-PDGF and eliciting DNA synthesis were 0.1 nM and 0.2 nM, respectively (Fig. 1 *Lower*). Thus, the undiluted conditioned medium contained the equivalent of 0.5–1.0 nM PDGF, or roughly 3 times the PDGF activity found in fetal calf serum.

When added to intact fibroblasts, PDGF rapidly stimulates the tyrosine phosphorylation of a M_r 180,000 membrane protein that has the characteristics of the PDGF receptor (18, 23–25). SSV-conditioned medium was tested for the ability to stimulate tyrosine phosphorylation of the PDGF receptor. For these experiments an anti-phosphotyrosine antibody was used to immunopurify the tyrosine-phosphorylated proteins from cells exposed to PDGF or conditioned medium (18). Previous studies showed that the predominant phosphoprotein immunopurified by this antibody has the molecular size (M_r 180,000), lectin affinity, isoelectric point, chromatographic properties, and phosphorylation pattern of the PDGF receptor (18). SSV-conditioned medium stimulated tyrosine phosphorylation of the PDGF receptor identified by this method (Fig. 2, lane 4), whereas control NRK medium lacked this ability (Fig. 2, lane 3). Other *in vivo* substrates for PDGF-activated kinase (18) also were stimulated by SSV-conditioned medium.

PDGF Receptors Are Not Detected in SSV-Transformed Cells. SSV-transformed cells were examined for the presence of ^{125}I-PDGF binding sites and PDGF-stimulated tyrosine kinase activity. SSV-transformed cells had no detectable ^{125}I-PDGF binding sites (Table 1), whereas their nontransformed counterpart (NRK cells) bound ^{125}I-PDGF in an amount comparable to that previously reported for nontransformed mesenchymal cells (20). Similarly, crude membranes prepared from SSV-transformed cells did not bind ^{125}I-PDGF, while membranes derived from NRK cells bound 8100 cpm of ^{125}I-PDGF per mg of membrane protein (Table 1). Extensive washing of the SSV-membranes in the presence of suramin, a compound known to dissociate PDGF from its receptors (22), did not restore the ^{125}I-PDGF binding sites.

To further assess the status of PDGF receptors in SSV-transformed cells, antibody to phosphotyrosine was again used to immunopurify the phosphorylated PDGF receptor and other cellular phosphoproteins. SSV-transformed cells

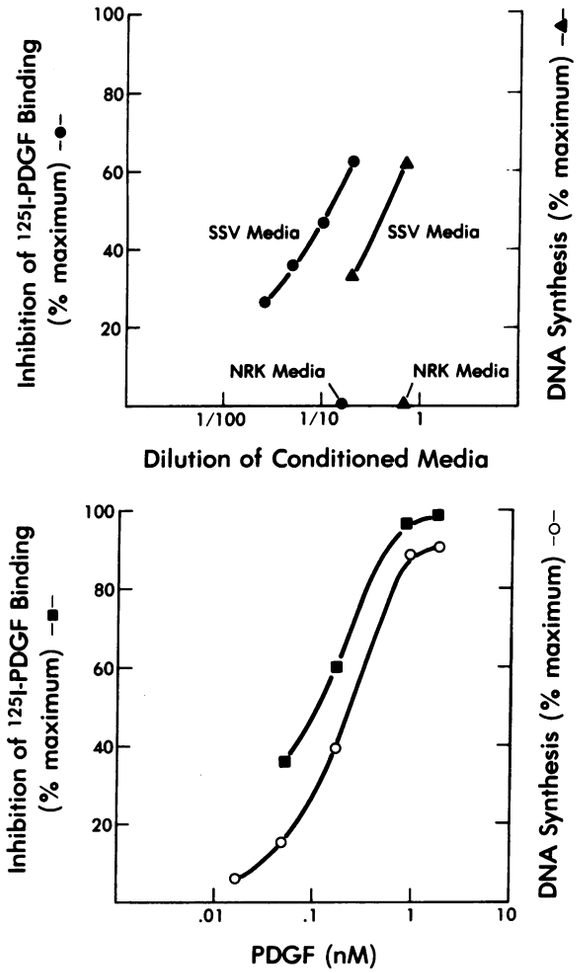

FIG. 1. (*Upper*) Effects of conditioned medium on specific ^{125}I-PDGF binding and DNA synthesis. Parallel cultures of NRK and SSV-transformed NRK cells were washed three times for 2-hr intervals and then incubated 18 hr in medium without serum (15 ml per 175-cm^2 flask). The conditioned medium was then centrifuged and stored at −70°C. At the end of medium collection, both the NRK cells and SSV-transformed cells were >95% viable by trypan blue staining. The ability of conditioned medium from normal cells and SSV-transformed cells to stimulate DNA synthesis in confluent BALB/3T3 cells (▲) was assessed by [^{3}H]thymidine uptake as described (21). The conditioned media also were tested for their ability to inhibit specific ^{125}I-PDGF binding to BALB/3T3 cell membranes (●). For these experiments 30–200 μl of conditioned medium was added to the binding assay containing 0.02–0.04 nM ^{125}I-PDGF and 105 μg of isolated 3T3 membranes in a total volume of 700 μl of buffer consisting of 10 mM Tris·HCl (pH 7.4), 154 mM NaCl, and 10% platelet-poor plasma. Specific ^{125}I-PDGF binding to membranes was determined as detailed (22). Maximum inhibition of specific ^{125}I-PDGF binding refers to complete inhibition by 10 nM unlabeled PDGF. Maximum DNA synthesis refers to the maximal stimulation of [^{3}H]thymidine uptake observed in response to PDGF within each assay. Values shown represent means of replicate determinations from two separate experiments. (*Lower*) Effects of PDGF on specific ^{125}I-PDGF binding and DNA synthesis. For each experiment in *Upper*, a parallel experiment was performed to test the effects of PDGF on ^{125}I-PDGF binding (■) and on DNA synthesis (○).

contained no detectable tyrosine-phosphorylated M_r 180,000 protein either in the presence (Fig. 3, lane 1) or absence (Fig. 3, lane 2) of added PDGF. In the control NRK cells, PDGF stimulated tyrosine phosphorylation of the M_r 180,000 PDGF receptor (Fig. 3, lanes 9 and 10). These results show that no functional PDGF receptors are detected in intact SSV-transformed cells.

SSV-Transformed NRK Cells Release Enough PDGF-Like

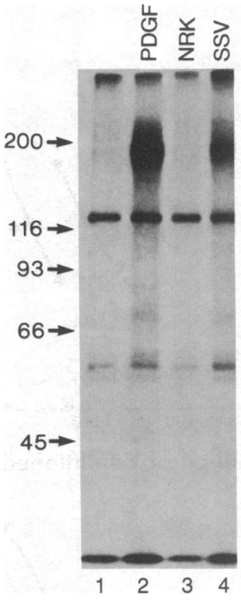

FIG. 2. Stimulation of PDGF receptor autophosphorylation by conditioned medium from SSV-transformed cells. Identical wells of BALB/3T3 cells were loaded for 3.5 hr at 37°C with $^{32}P_i$ (1 mCi per well) and were incubated in a total volume of 1 ml for 3.5 hr at 4°C with the following substances: no addition (lane 1), 2 nM PDGF (lane 2), NRK-conditioned medium concentrated 2-fold (lane 3), and SSV-conditioned medium concentrated 2-fold (lane 4). Cell extracts were immunopurified with monoclonal antibody to phosphotyrosine and analyzed by $NaDodSO_4$/polyacrylamide electrophoresis and autoradiography as described. The prominent band at M_r 180,000 has the characteristics expected of the PDGF receptor (18). Conditioned media were collected as described in Fig. 1. Molecular weight markers (shown as $M_r \times 10^{-3}$) were myosin (M_r 200,000), β-galactosidase (M_r 116,250), phosphorylase *b* (M_r 92,500), bovine serum albumin (M_r 66,250), and ovalbumin (M_r 45,000).

Material to Down-Regulate PDGF Receptor Phosphorylation in Normal Cells. Chronic exposures of cells to PDGF causes a progressive decrease in the number of PDGF receptors (26), a process termed "down-regulation." A possible explanation for the absence of PDGF receptors in SSV-transformed cells is that the receptors are down-regulated by the action of PDGF-like peptides released from these cells. To test whether SSV-transformed cells release enough PDGF-like material to down-regulate PDGF receptors, NRK cells ("target" cells) were cocultured in the presence of SSV-transformed cells ("conditioning" cells). The results showed that the SSV-transformed cells released enough material into the medium to remove all of the target-cell PDGF receptors as detected by PDGF-stimulated receptor phosphorylation (Fig. 4, lanes 3 and 4). When control cells (NRK cells) were used as the "conditioning" cells, PDGF-stimulated phosphorylation of the PDGF receptor was still observed in the "target" NRK cells (Fig. 4, lanes 1 and 2). During these experiments we noted that NRK cells cocultured with SSV-transformed cells developed a distinctive spindle-shape appearance associated with PDGF stimulation.

Table 1. ^{125}I-PDGF specific binding to SSV/NRK and NRK cells and membranes

^{125}I-PDGF binding	NRK	SSV/NRK
Whole cell binding,* cpm per 10^6 cells	2200 ± 1000	100 ± 100
Membrane binding,† cpm/mg of protein	8100 ± 300	400 ± 900

*Suspensions of confluent serum-depleted cells were used to measure specific binding as described (20). Cells (5×10^5 cells per ml) were incubated 40 min at 37°C in 700 μl of phosphate-buffered saline containing 10% platelet-poor plasma with 0.02 nM ^{125}I-PDGF.

†Determination of specific ^{125}I-PDGF binding to membranes and preparation of membranes from each cell type were performed as described (22). For each assay, cell membranes (105 μg of protein) were incubated for 40 min at 37°C in 700 μl of 10 mM Tris·HCl (pH 7.4)/154 mM NaCl/10% platelet poor plasma containing 0.02 nM ^{125}I-PDGF. Values shown represent means of duplicate or triplicate determinations from 4–6 experiments.

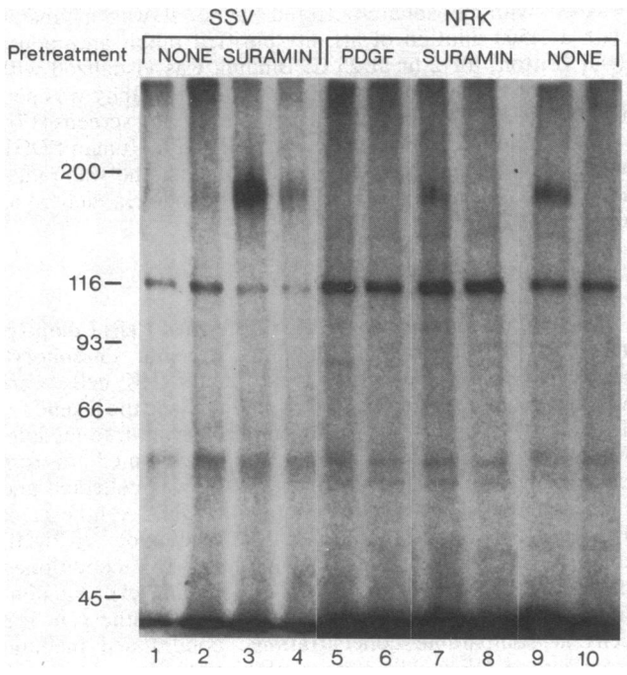

FIG. 3. Recovery of surface PDGF receptors of SSV-transformed cells by suramin treatment. SSV-transformed cells (lanes 1–4) or NRK cells (lanes 5–10) were assayed for PDGF-stimulated tyrosine kinase activity after preincubation at 37°C with suramin PDGF. Preincubation conditions were as follows: no addition (lanes 1, 2, 9, and 10), 8 hr with 1 mM suramin (lanes 3, 4, 7, and 8), and 11 hr with 10 nM PDGF (lanes 5 and 6). All cells were washed twice with 2 ml per 35-mm well of phosphate-free medium, loaded with $^{32}P_i$ (1 mCi per 10^6 cells) during the final 3 hr of preincubation at 37°C, cooled 15 min to 4°C, washed three times with 6 ml per 35-mm well of ice-cold Dulbecco's minimal essential medium, and then incubated with (odd numbered lanes) or without (even numbered lanes) 10 nM PDGF for 3.5 hr at 4°C. Cell extracts were immunopurified with monoclonal antibody to phosphotyrosine and analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis and autoradiography as described. Molecular weights are shown $\times 10^{-3}$.

PDGF Receptors Reappear when SSV-Transformed Cells Are Incubated with Suramin. If the loss of detectable PDGF receptors in SSV-transformed cells is produced through continuous down-regulation by endogenous PDGF-like material, this effect should be prevented by an agent that blocks the binding of PDGF to its receptor. Suramin (27) is a heterocyclic polyanionic compound that reversibly inhibits the binding of PDGF to its specific receptor sites (22). Incubation of SSV-transformed cells with 1 mM suramin for 8 hr restored PDGF-stimulated receptor phosphorylation (Fig. 3, lanes 1–4). Similar incubation of control NRK cells with 1 mM suramin did not alter the phosphorylation of receptors induced by PDGF (Fig. 3, lanes 7–10). Incubation with 0.1 mM suramin for 8 hr was equally effective in restoring PDGF receptor tyrosine kinase activity to SSV transformed cells. However, brief exposure (30 min) to 1 mM suramin had little effect (data not shown).

When NRK cells were exposed to 10 nM PDGF at 37°C, PDGF-stimulated receptor phosphorylation was no longer

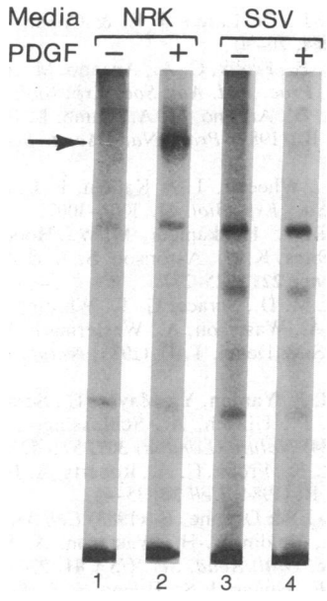

FIG. 4. Down-regulation of PDGF receptor phosphorylation in nontransformed cells by coculture with SSV-transformed cells. "Conditioning" cells were grown in the bottom of plastic wells, and "target" cells were grown on coverslips that could be added to the conditioning wells. Conditioning cells (either NRK or SSV-transformed cells) were seeded in 2 ml of 10% calf serum in Dulbecco's minimal essential medium (DME medium) at $\approx 2 \times 10^5$ cells per 35-mm well. Target NRK cells were seeded at the same density on 32-mm glass coverslips. After 24 hr, the medium was changed to 2% calf serum in DME medium. After an additional 24 hr, target cell coverslips were transferred to conditioning wells by placing the coverslips on a pedestal over the layer of conditioning cells. Cells were cocultured in this manner for 48 hr. Coverslips were then placed in wells containing 1 mCi of $^{32}P_i$ per 700 μl of phosphate-free medium for 2 hr at 37°C, then incubated 5 min with (lanes 2 and 4) or without (lanes 1 and 3) 2 nM PDGF, and assayed for phosphorylated PDGF receptor as described. For the samples in lanes 1 and 2, the conditioning cells were NRK cells; for the samples in lanes 3 and 4, the conditioning cells were SSV-transformed NRK cells. In each case NRK cells were used as target cells on the coverslips.

present. NRK cells exposed first to 10 nM PDGF for 3 hr at 37°C and then incubated 8 hr with both suramin and PDGF displayed active phosphorylation of a M_r 180,000 protein in response to PDGF. Thus, suramin restores PDGF-induced receptor phosphorylation to both SSV-transformed cells and to NRK cells whose PDGF receptors have been down-regulated by exogenous PDGF.

Medium Conditioned by SSV-Transformed Cells Contains p20^{sis}. To test for secreted forms of the *sis* gene product, conditioned medium from SSV-transformed cells and from NRK cells was collected as described in Fig. 1 and subjected to immunoblot analysis using antisera to synthetic peptides representing the amino and central portions of the predicted p20^{sis} sequence (19). Under reducing conditions, a protein of $M_r \approx 20{,}000$ was detected in the SSV-conditioned medium by antisera to the amino-terminal (Fig. 5, lanes A–D) and central (lanes E–H) portions of p20^{sis}. The specificity of this reaction was confirmed by preincubation of the antisera with the immunizing or unrelated peptide. Binding activity of the antiserum against the amino-terminal portion of p20^{sis} was blocked by preincubation with the immunizing peptide (Fig. 5, lane C) but not the unrelated peptide (Fig. 5, lane A). Similarly binding activity of the antiserum against the central portion of p20^{sis} was blocked by the immunizing peptide (Fig. 5, lane G) but not the unrelated peptide (Fig. 5, lane E). These results demonstrate that p20^{sis} is released into medium conditioned by SSV-transformed cells. Although several higher molecular weight bands reacted weakly with the anti-peptide

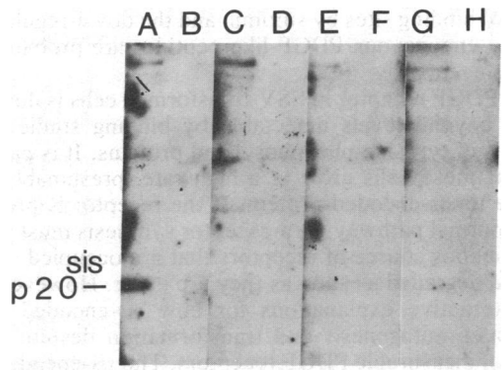

FIG. 5. Immunological detection of p20^{sis} in medium conditioned by SSV-transformed cells. The equivalent of 25 ml of medium conditioned by SSV-transformed cells (lanes A, C, E, and G) or by NRK cells (lanes B, D, F, and H) was electrophoresed into a 5–17.5% polyacrylamide gel and electrophoretically transferred to nitrocellulose. The extract was probed with mouse antisera (19) to the amino-terminal (lanes A–D) or central (lanes E–H) portion of p20^{sis}, which had been preincubated with the 100 μg of peptide representing the central portion of the molecule (lanes A, B, G, and H) or the amino-terminal portion of the molecule (lanes C, D, E, and F). Thus, the lane assignments for the SSV-conditioned medium are: A, anti-amino-terminus antiserum plus unrelated peptide; C, anti-amino-terminus antiserum plus immunizing peptide; E, anti-central-portion antiserum plus unrelated peptide; G, anti-central-portion antiserum plus immunizing peptide. The lanes for the NRK-conditioned medium (B, D, F, and H) are paired with the corresponding lanes of SSV-conditioned medium.

antisera (Fig. 5, lanes A, C, E, and G) these reactions were not blocked by the presence of immunizing peptide and were therefore nonspecific.

DISCUSSION

Our data demonstrate that SSV-transformed cells secrete a factor that binds to and activates PDGF receptors. This factor is elaborated into the SSV-transformed cells' environment at levels sufficient to stimulate DNA synthesis and down-regulate PDGF receptors. The ability of suramin to restore PDGF-induced receptor phosphorylation in SSV-transformed cells demonstrates that these cells actively synthesize and process functional PDGF receptors. Taken together, these data suggest that the PDGF receptors of SSV-transformed cells are tonically stimulated (and down-regulated) in an autocrine fashion by a secreted PDGF-like factor.

PDGF can induce some specific phenotypic properties characteristic of transformed cells (9, 14) but may not elicit the fully transformed state unless additional factors are added (28). Thus, the transformation of NRK cells by SSV may involve more than the autocrine action (29) of endogenous PDGF-like peptides on cell-surface PDGF receptors. For example, SSV-transformed cells may produce α and β transforming growth factors (2), which may potentiate the action of *sis*-encoded protein. Alternatively, structural differences between PDGF and its *sis*-encoded analogue may provide the *sis*-encoded protein with greater efficacy as a transforming agent. Finally, since p28^{sis} is formed and processed inside the cell (16), the interaction of receptor and ligand might occur in an intracellular compartment where receptors are not subject to the "down-regulation" process, which may limit the cellular response to exogenous PDGF (26).

Our data do not definitively prove that the PDGF-like peptides of SSV-transformed cells act solely at the cell surface. However, suramin is a relatively large (M_r 1429) hydrophilic molecule with structural similarity to trypan blue and, therefore, is not likely to act at intracellular sites. Thus, the resto-

ration of binding sites by suramin and the down-regulation of sites by endogenous PDGF-like peptides are probably cell-surface events.

The PDGF receptor in SSV-transformed cells is down-regulated beyond levels detectable by binding studies or by analysis of tyrosine-phosphorylated proteins. It is paradoxical that these cells grow at a high rate, presumably in response to *sis*-encoded protein. If the receptor is processed by its normal pathway, new receptor synthesis must provide a continuous source of receptors that are occupied, activated, and degraded as soon as they are made. However, there are alternative explanations for how *sis*-encoded protein stimulates mitogenesis and transformation despite the absence of measurable PDGF receptors. The *sis*-encoded product may act through intracellular pathways that do not involve the receptor. An alternative is that the unoccupied receptor exerts an inhibitory effect on cell growth in normal cells. Thus, the main function of PDGF in normal cells and its *sis*-encoded analogue in SSV-transformed cells would be to remove this inhibitory influence by stimulating internalization and degradation of the receptor. According to this theory, the growth factors and tumor promoters that could remove most efficiently the receptors from the cell surface would be the most effective growth-promoting stimuli.

It is of interest that in SSV-transformed cells there is no evidence of constituitive phosphorylation of the PDGF receptor stimulated by endogenously produced *sis*-encoded protein (Fig. 3, lane 2). In normal cells phosphorylation of the PDGF receptor is maximal within 5–10 min after exposure to PDGF and thereafter decreases rapidly (18). Thus, the tyrosine-phosphorylated form of the receptor is likely to be a transient intermediate that is formed prior to down-regulation and fails to accumulate because of a short half-life.

The *sis* oncogene is a useful tool for probing the role of growth factors in cell transformation. The biologically active form, the *sis*-encoded protein, is not known. Our data confirm the presence of a M_r 20,000 form of the *sis* protein in medium conditioned by SSV-transformed cells. Whether this protein accounts for all of the biological activity of the conditioned medium is unclear. The studies presented here suggest but do not prove that *sis*-encoded protein interacts with PDGF receptors. Recently Wang and Williams (30) have demonstrated that the v-*sis*-encoded protein expressed in *E. coli* inhibits specific binding of ^{125}I-PDGF to its receptors in 3T3 membranes. Further studies of this system should help elucidate the nature of the interaction of the *sis*-encoded protein with the PDGF receptor and the role of this interaction in cell transformation.

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