Autocrine Stimulation of Intracellular PDGF Receptors in v-*sis*–Transformed Cells

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Autocrine activation of platelet-derived growth factor (PDGF) receptors is the mechanism of transformation by the v-*sis* oncogene. Since the addition of PDGF does not transform normal cells, autocrine mechanisms may involve unique pathways of receptor activation. In this study autocrine stimulation of the PDGF receptor was observed in v-*sis*–transformed normal rat kidney (NRK) cells. In contrast to receptor activation in normal cells, autocrine activation of PDGF receptors in v-*sis*–transformed cells occurred in intracellular compartments, disrupting receptor processing and diverting receptors and their precursors to a chloroquine-sensitive degradation pathway. These findings show that intracellular activation of receptors by autocrine mechanisms may play a role in cell transformation.

THE V-*SIS* ONCOGENE ENCODES A protein that is structurally similar and functionally equivalent to platelet-derived growth factor (PDGF) (*1*). Only cells that express PDGF receptors can be transformed by v-*sis* (*2, 3*), which suggests that the receptor is essential for transformation. Whether autocrine activation of the receptor occurs in intracellular compartments or at the cell surface by secreted v-*sis*–encoded proteins has been the subject of controversy (*2–4*). To determine the site of autocrine activation of the PDGF receptor in v-*sis*–transformed cells, we studied receptor biosynthesis by metabolic labeling experiments. In normal (untransformed) cells the receptor is synthesized as a 160-kD precursor that contains core, endo-β-*N*-acetylglucosaminidase H (endo H)–sensitive, asparagine-linked sugars (*5*). The precursor is converted to a 180-kD mature form that contains complex, endo H–resistant oligosaccharide and is then expressed at the cell surface (*5*). Previous binding experiments with ^{125}I-labeled PDGF showed that few, if any, PDGF receptors are detectable is v-*sis*–transformed cells (*2, 3, 6*). The absence of detectable receptors has been attributed to down-regulation caused by the interaction of receptors with v-*sis*–encoded proteins. In metabolic labeling studies, however, receptor proteins of 140 and 160 kD were readily detected in v-*sis*–transformed cells (Fig. 1B, time 0). These receptor proteins were susceptible to digestion with endo H and therefore represented incompletely processed forms of the receptor (*7, 8*). Similar amounts of receptor precursors were expressed in normal and v-*sis*–transformed cells (Fig. 1, A and B, time 0).

Forty-five minutes after synthesis, a mature, endo H–resistant form of the PDGF receptor was expressed in both normal rat kidney (NRK) and v-*sis*–transformed NRK cells (Fig. 1). In contrast to the mature, 180-kD protein immunoprecipitated from untransformed cells, the mature form of the receptor identified in v-*sis*–transformed cells was resistant to neuraminidase treatment, was heterogeneous, and had a lower apparent molecular mass (170 kD), suggesting that this form of the receptor was partially processed. The heterogeneity of the mature form of the receptor in these cells appeared to be due to variable processing of the protein's asparagine-linked sugars, because treatment of immunoprecipitated receptors with peptide *N*-glycosidase F (*N*-glycanase) (*9*) reduced mature and precursor forms to a discrete band of 140 kD in both NRK and v-*sis*–transformed cells (*8*).

Another distinction between the mature receptor proteins seen in normal cells and those in v-*sis*–transformed cells is their half-life. Densitometric analysis of Fig. 1B and other experiments showed that the half-life of the 170-kD receptor in v-*sis*–transformed cells is only 10 to 15 minutes. In contrast, receptor half-life in untransformed cells in the absence of PDGF is approximately 3 hours (*5*).

Unexpectedly, we found that neither the mature nor the precursor form of the receptor in v-*sis*–transformed cells was susceptible to trypsin treatment of intact cells at 4°C (Fig. 1B). In v-*sis*–transformed cells, therefore, the mature form of the receptor was detected exclusively in intracellular compartments. By comparison, in NRK cells, 60% of the receptors were detected in a trypsin-sensitive pool 45 minutes after labeling, and nearly all of the receptors were sensitive to trypsin after 90 minutes (Fig. 1A). Not surprisingly, the 160-kD intracellular receptor precursor was insensitive to trypsin in NRK cells.

When the PDGF receptor is activated by PDGF, the receptor becomes phosphorylated on its own tyrosine residues (*10*). We have shown that antibodies to phosphotyrosine can be used to identify ligand-activated, tyrosine-phosphorylated PDGF receptors in normal cells (*11*). To identify the activated form of the PDGF receptor in v-*sis*–transformed cells, metabolically labeled receptors were immunoprecipitated by antibodies to phosphotyrosine (Fig. 2B) and, in parallel, by antibodies to the PDGF receptor (Fig. 2A). Both antibodies recognized the 170-kD receptor 1 hour after metabolic labeling of v-*sis*–transformed cells (Fig. 2, A and B). In contrast, the 180-kD tyrosine-phosphorylated form of the receptor in NRK cells was recognized by antibodies to phosphotyrosine only after exogenous PDGF was added to the cells (Fig. 2B). These data show that the mature form of the PDGF receptor is activated, presumably by interaction with v-*sis*–encoded proteins, in v-*sis*–transformed cells.

The activated form of the receptor recognized by antibodies to phosphotyrosine in v-*sis*–transformed cells, like the receptor proteins detected by antibody to the PDGF receptor (Fig. 1B), was found exclusively in intracellular compartments that were resistant to trypsin treatment at 4°C (Fig. 3, A and B). In untransformed cells, the ligand-activated, tyrosine-phosphorylated form of the receptor was at the cell surface and therefore was susceptible to intact cell trypsinization (Fig. 3A). These data suggest that

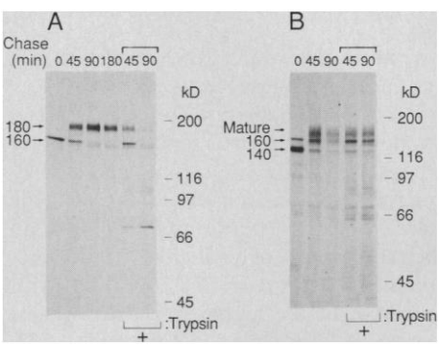

Fig. 1. Biosynthesis of the PDGF receptor in NRK and v-*sis*–transformed NRK fibroblasts. NRK (**A**) and v-*sis*–transformed NRK (**B**) cells were labeled with [^{35}S]methionine (0.25 mCi/ml) for 15 minutes and then incubated with unlabeled medium for the time periods indicated. After the chase period, intact cells were incubated with trypsin (0.1 mg/ml) (+) at 4°C for 2 hours as indicated. All other samples were also treated with trypsin, but trypsin activity was inhibited by soybean trypsin inhibitor at 0.3 mg/ml. Cell extracts were immunoprecipitated by treatment with antiserum 88, which was directed against the kinase insert region of the receptor as previously described (*5*). Immunoprecipitates were separated by SDS–polyacrylamide gel electrophoresis on 7% gels. A fluorogram is shown. Specificity of antisera to PDGF receptor proteins was demonstrated with preimmune normal rabbit sera and peptide blocking experiments as described (*5*). Antiserum 88 immunoprecipitated mature and precursor forms of the receptor equally well (*8*).

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activation of the PDGF receptor in v-*sis*–transformed cells occurs in intracellular compartments.

To exclude the possibility that PDGF receptors in v-*sis*–transformed cells are transiently expressed at the plasma membrane, activated by extracellular ligands, and then immediately internalized to a trypsin-resistant compartment before degradation, we incubated cells at 37°C with the polyanionic compound suramin. In NRK cells, suramin inhibits the interaction of PDGF with its cell surface receptor (*2, 8*). When v-*sis*–transformed cells were incubated with suramin, tyrosine phosphorylation of the receptor was unaffected (Fig. 3B) (*12*).

In v-*sis*–transformed cells, receptor precursors were degraded before they could be processed to a mature form. Densitometric analysis of Fig. 1B and other fluorograms showed that in v-*sis*–transformed cells more than 90% of ^{35}S-labeled precursors disappeared during a 45-minute chase period, but only 20 to 30% could be accounted for by processing to the mature form (Fig. 1B). Therefore, it seemed likely that in v-*sis*–transformed cells, PDGF receptor precursors were shunted from the maturation pathway to a degradative pathway. This pattern of processing contrasts with the pattern seen in normal fibroblasts, in which the 160-kD precursor is quantitatively converted to the mature 180-kD form. (*5*). To determine if the 160-kD precursor identified in v-*sis*–transformed cells was degraded by *p*H-sensitive proteases, we incubated cells with the lysosomotrophic agent chloroquine before labeling and immunoprecipitation. Chloroquine (Fig. 4A) or ammonium chloride (*8*) treatment dramatically prolonged the half-life of the 160-kD precursor in v-*sis*–transformed cells. No effect on receptor precursor metabolism was noted in control cells (*8*). These data suggest that the 160-kD precursor is degraded by chloroquine-sensitive acid hydrolases (*13*) shortly after synthesis.

To determine if an activated form of the 160-kD receptor precursor was detectable in v-*sis*–transformed cells, we treated cells with chloroquine and the phosphatase inhibitor sodium orthovanadate before metabolic labeling and immunoprecipitation with antibodies to phosphotyrosine. Under these conditions a tyrosine-phosphorylated form of the 160-kD precursor was immunoprecipitated from v-*sis*–transformed, but not untransformed, cells (Fig. 3B). The endo H sensitivity of this band was not definitively ascertained; the 160-kD phosphoprotein disappeared after endo H treatment, but glycosidase treatment reduced signal intensity and a band of lower apparent molecular weight was not detected (Fig. 3B).

In v-*sis*–transformed cells a 140-kD incompletely processed form of the receptor was seen in addition to the 160-kD precursor (Fig. 1B). Chloroquine (Fig. 4A) or ammonium chloride (*8*) treatment reversed the ratio of 160- to 140-kD forms. After endo H treatment the 140-kD band shifted to an apparent molecular weight of 120-kD, which was lower than the apparent size (135 kD) of the receptor isolated from tunicamycin-treated cells in which *N*-linked glycosylation was blocked (*8*). Thus, the lower apparent weight of the 140-kD incompletely processed form was not due to a difference in glycosylation. These data suggest that the 140-kD form was produced by degradation of the 160-kD precursor.

The half-life of the mature form of the PDGF receptor in v-*sis*–transformed cells was also prolonged by pretreatment with chloroquine (Fig. 4A) or ammonium chloride (*8*). To determine if the mature 180-kD cell surface receptor in untransformed cells is degraded by a similar mechanism, we treated NRK cells with chloroquine before metabolic labeling. Cells were then incubated with unlabeled methionine in the absence or presence of PDGF, which accelerates receptor degradation (*5*), before solubilization and immunoprecipitation. In contrast to the mature form identified in v-*sis*–transformed cells, degradation of the 180-kD cell surface PDGF receptor in NRK cells was not inhibited by chloroquine even when the cells were stimulated by PDGF (Fig. 4B). Therefore, in v-*sis*–transformed NRK cells, degradation of the mature form of the PDGF receptor occurs via a chloroquine-sensitive pathway not followed by the receptor in normal cells.

These studies provide the first direct evidence of receptor activation by an autocrine mechanism and show that in v-*sis*–transformed cells autocrine activation of the PDGF receptor occurs in intracellular compartments. By contrast, in normal cells, only cell surface receptors are activated by PDGF. We cannot be certain if, after activation, receptors in v-*sis*–transformed cells are tran-

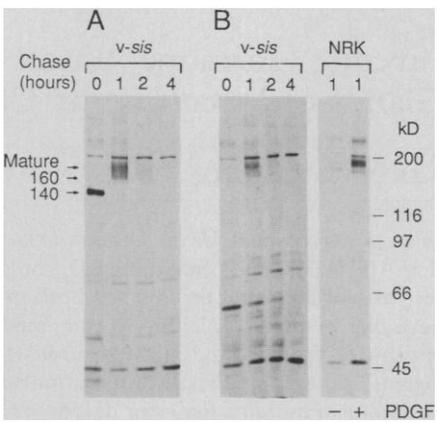

Fig. 2. Autocrine activation of PDGF receptor in v-*sis*–transformed NRK cells. Untransformed (NRK) and v-*sis*–transformed NRK (v-*sis*) cells were biosynthetically labeled with [^{35}S]methionine as described in Fig. 1. NRK cells were incubated in the absence (−) or presence (+) of a saturating concentration of PDGF. Cell extracts were immunoprecipitated by exposure to PDGF receptor antiserum 83 (**A**) or antiserum to phosphotyrosine (**B**). Receptor and tyrosine phosphoproteins are shown. The fluorogram represented here resulted from a prolonged exposure (2 weeks). This was necessary because of the low abundance of activated receptor proteins in v-*sis*–transformed cells. Nonspecific bands, therefore, are prominent. A 210-kD and other proteins of higher apparent molecular weight were nonspecifically immunoprecipitated by treatment with antisera to the PDGF receptor and to phosphotyrosine and by nonimmune serum (*8*).

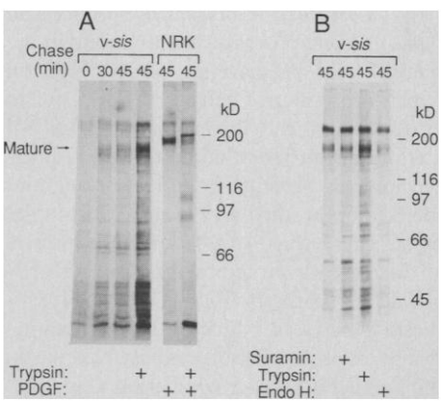

Fig. 3. Effect of trypsin, suramin, and endo H treatment on the activated form of the PDGF receptor in v-*sis*–transformed NRK cells. (**A**) Untransformed (NRK) and v-*sis*–transformed (v-*sis*) cells were labeled with [^{35}S]methionine as described in Fig. 1. NRK cells were incubated with (+) or without a saturating concentration of PDGF during the chase period. After the chase period, cells were incubated with trypsin (+) as described in Fig. 1. Cell lysates were immunoprecipitated with antibodies to phosphotyrosine. The 170-kD tyrosine-phosphorylated form of the receptor in v-*sis*–transformed cells and the 180-kD activated receptor in NRK cells are shown. In this and other experiments, lanes from trypsin-treated cells consistently had a higher background level, increasing the prominence of the trypsin-resistant bands. (**B**) Cell monolayers were incubated with 25 μ*M* chloroquine and 37.5 μ*M* sodium orthovanadate at 37°C for 1 hour. Cells were then biosynthetically labeled with [^{35}S]methionine as described in Fig. 1 in the presence of chloroquine and vanadate. Cells were then incubated with unlabeled medium for 45 minutes at 37°C in the presence of chloroquine and vanadate with or without suramin at 140 μ*M*. After the chase period, cells were incubated with trypsin as described in Fig. 1. Cell lysates were immunoprecipitated with antibodies to phosphotyrosine, released by boiling in SDS, and incubated with or without endo H as described (*5*). The 160- and 170-kD tyrosine-phosphorylated forms of the receptor are shown.

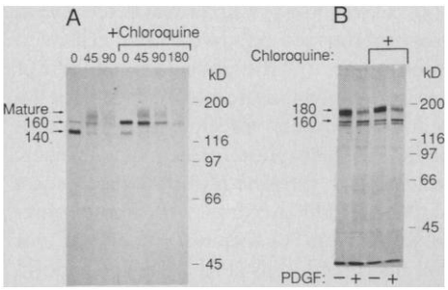

Fig. 4. Effect of chloroquine treatment on PDGF receptor metabolism in NRK and v-*sis*–transformed NRK cells. Cell monolayers were treated with Dulbecco's minimum essential medium (DMEM) with (+) or without 25 μ*M* chloroquine for 1 hour at 37°C. v-*sis*–transformed NRK cells (**A**) were biosynthetically labeled with [^{35}S]methionine as described in Fig. 1 in the absence or presence of chloroquine. NRK cells (**B**) were labeled with [^{35}S]methionine with or without chloroquine at 37°C for 2 hours in the absence (−) or presence (+) of a saturating concentration of PDGF, which causes receptor internalization and degradation. Cell extracts were immunoprecipitated with antiserum to PDGF receptor as described in Fig. 1.

siently expressed at the cell surface and then degraded or if they are shunted directly to degradation pathways. Additionally, we cannot exclude the possibility that a small percentage of receptors is expressed at the plasma membrane before activation and then immediately degraded, thus evading detection by our methodology. In v-*sis*–transformed cells, however, the majority of PDGF receptor activation clearly occurred in intracellular compartments, before receptor maturation was complete and before receptors could have reached the cell surface. Our data are consistent with reports showing that chronic exogenous PDGF treatment, by itself, will not transform cells (*14*). These findings also help explain why attempts to reverse v-*sis*–mediated transformation with antisera to PDGF have had limited success (*15*). This novel, intracellular mechanism of activation of a growth factor receptor is likely to be responsible for v-*sis*–induced cell transformation. Attempts to reverse transformation by v-*sis*, therefore, should be directed to intracellular receptors.

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12. Previous ^{125}I-labeled PDGF binding studies (*2*) have shown that suramin "up-regulates" cell surface PDGF receptors in v-*sis*–transformed cells. Although suramin treatment did not inhibit intracellular receptor activation in v-*sis*–transformed cells, in separate experiments (*8*) we found that suramin prolonged the half-life of the mature form of the receptor in these cells. In the presence of suramin, mature receptor proteins were detectable at the cell surface as shown by their sensitivity to trypsin (data not shown). As noted previously, suramin inhibits binding of PDGF to cell surface receptors (*2*). However, suramin also has access to intracellular compartments (*13*). It is not clear, therefore, if an intracellular or an extracellular mechanism accounts for suramin's capacity to up-regulate cell surface PDGF receptors in v-*sis*–transformed cells.
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Juvenile Hormone Action Mediated in Male Accessory Glands of *Drosophila* by Calcium and Kinase C

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In an in vitro system for the *Drosophila melanogaster* male accessory gland, it was found that $10^{-9}M$ juvenile hormone III could accurately mimic the copulation-induced response of increased protein synthesis in glands from virgin flies. Stimulation by this hormone required calcium in the medium. Experiments with tumor-promoting phorbol esters indicated that activation of protein kinase C can also cause the glands to increase protein synthesis. Stimulation of protein synthesis by juvenile hormone did not occur in mutants deficient in kinase C activity. These results suggest a membrane-protein–mediated effect of juvenile hormone that involves calcium and kinase C.

THE MALE ACCESSORY GLANDS OF *Drosophila melanogaster* produce and secrete proteins that are found in the seminal fluid and have important effects on female reproductive behavior (*1*). In our in vitro system, the incorporation of [^{35}S]-methionine into trichloroacetic acid (TCA)–precipitable proteins increases throughout the 3 hours studied and is linear within the first 2 hours (Fig. 1A). Copulation induces an increase in protein synthesis (Fig. 1B) and RNA synthesis (Fig. 1C). Protein synthesis is increased in glands dissected immediately after copulation and is maximal in the flies 2 hours after copulation (Fig. 1B). Our in vitro measurements of the timing and magnitude of the stimulation of overall protein and RNA synthesis following copulation are in agreement with previous in vivo studies of the copulation-induced stimulation of protein and RNA synthesis in these glands (*2, 3*).

Since it is known that juvenile hormone (JH) effects the correct development, maturation, and functioning of the male accessory glands in other insects (*1, 4, 5*), and is important in the regulation of *D. melanogaster* female reproductive physiology (*6–9*), we examined the effects of JH on the male accessory glands in vitro. At concentrations of $10^{-9}M$ juvenile hormone III (JH III), the amount of incorporation of [^{35}S]methionine into TCA-precipitable proteins is maximally increased to nearly 300% of that of control values after a 1-hour incubation (Fig. 2A). There is a partial effect even at concentrations of $10^{-11}M$. The hormone 20-hydroxyecdysone at $10^{-7}M$ causes little or no increase in protein synthesis (Table 1). Each reagent listed in Table 1 was tested at

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