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Dominant-Negative Mutants of Platelet-Derived Growth Factor Revert the Transformed Phenotype of Human Astrocytoma Cells

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Malignant astrocytoma is the most common primary human brain tumor. Most astrocytomas express a combination of platelet-derived growth factor (PDGF) and PDGF receptor which could close an autocrine loop. It is not known whether these autocrine loops contribute to the transformed phenotype of astrocytoma cells or are incidental to that phenotype. Here we show that dominant-negative mutants of the PDGF ligand break the autocrine loop and revert the phenotype of BALB/c 3T3 cells transformed by the PDGF-A or PDGF-B (c-*sis*) gene. Then, we show that these mutants are selective in that they do not alter the phenotype of 3T3 cells transformed by an activated Ha-*ras* or v-*src* gene or by simian virus 40. Finally, we show that these mutants revert the transformed phenotype of two independent human astrocytoma cell lines. They have no effect on the growth of human medulloblastoma, bladder carcinoma, or colon carcinoma cell lines. These observations are consistent with the view that PDGF autocrine loops contribute to the transformed phenotype of at least some human astrocytomas.

The platelet-derived growth factors (PDGFs) are composed of two subunits, PDGF-A and PDGF-B (c-Sis), which associate through disulfide bonds to form PDGF-AA, PDGF-AB, and PDGF-BB (8, 15, 18, 35). Two PDGF receptor subunits, α and β, associate noncovalently to form functional homodimers or heterodimers which differ in the ability to interact with the three isoforms of PDGF (19, 20, 26, 42). PDGF-α receptor subunits can affiliate with either an A or B chain of the PDGF ligand. PDGF-β receptor subunits recognize PDGF-B chains preferentially. Thus, α:α homodimers function as the universal PDGF receptor, responsive to all three isoforms of PDGF. The β:β receptor homodimers respond preferentially to PDGF-BB, and α:β receptor heterodimers have an intermediate response profile (19, 21, 26, 42).

PDGF autocrine loops are established when PDGF ligands and responsive PDGF receptor subunits are expressed in the same cell. In tissue culture, these autocrine loops can both initiate and sustain a transformed phenotype (4, 11, 23, 28). In human neoplastic disease, PDGF autocrine loops have been linked to sarcoma, lung carcinoma, and malignant astrocytoma (1, 22, 45). The link is especially pervasive in malignant astrocytoma. Although the PDGF ligand and receptor genes are autonomous and independently regulated, the majority of human astrocytomas and astrocytoma cell lines express a combination of these genes that could, in principle, close a PDGF autocrine loop and drive the unregulated mitotic process in these tumors (14, 22, 39). Still, the role of PDGF autocrine loops in human astrocytoma has remained unclear. At one extreme, these loops may be completely incidental to the disease. As an intermediate view, the closure of PDGF autocrine loops may be an early initiating event which becomes irrelevant to the malignant phenotype as the tumor progresses and acquires additional genetic lesions. Finally, it is possible that PDGF autocrine loops contribute to the transformed phenotype of astrocytoma cells.

To document the contribution of such loops to malignant growth, it is necessary to disrupt them and demonstrate that normal growth regulation is restored. A bulk of evidence suggests that although v-*sis* can activate intracellular forms of PDGF-β receptor (10, 28), these receptors must be localized to the cell surface for v-*sis* to induce mitogenic signalling and autocrine transformation (10, 16, 32). Accordingly, reagents that antagonize the interaction between PDGF and its receptor at the cell surface, such as suramin and PDGF-neutralizing antibodies, have been found to reduce the mitogenicity and revert the phenotype of v-*sis*-transformed cells (5, 11, 32). However, other groups have demonstrated mitogenically active intracellular forms of v-*sis* and maintain that intracellular PDGF autocrine loops involving PDGF-β receptors can initiate cellular transformation (3, 4, 33). Thus, PDGF autocrine loops might be more comprehensively targeted for disruption by interfering with PDGF ligand formation within the cell interior.

In previous studies, we constructed two mutations within a PDGF-A cDNA clone which exhibit dominant-negative properties (36). In COS cell cotransfection studies, one mutant, 1308, suppresses biologic activity of PDGF-A and -B subunits by forming unstable heterodimers. A second mutant, 1317, forms stable but inactive heterodimers with PDGF-A subunits. Heterodimers between 1317 and PDGF-B can also form. However, these heterodimers are functional, thus restricting the dominant-negative action of 1317 to PDGF-A. It occurred to us that these mutants might be capable of disrupting autocrine loops involving the PDGFs and their receptors. In principle, these mutants could act at the very onset of autocrine loop closure by suppressing the formation of PDGF ligand within the Golgi apparatus. In this study, we tested the mutants initially in a defined context, using PDGF-transformed BALB/c 3T3 cells, and then in

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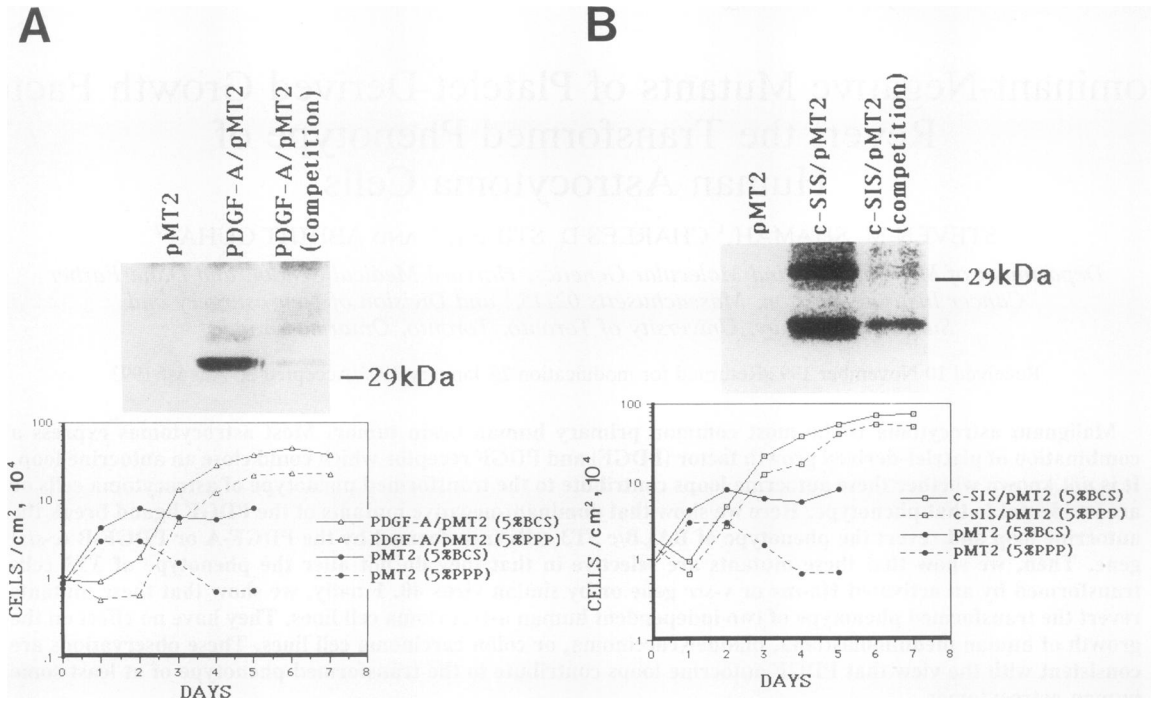

FIG. 1. Growth of PDGF-transformed cells in the presence or absence of serum. (A) Immunoprecipitations of metabolically [^{35}S]cysteine-labeled PDGF-A-transformed CM with a PDGF-AA-specific antibody demonstrated PDGF-AA homodimers (~32 kDa) which could be competed for with 1 μg of unlabeled, recombinant PDGF-AA. The growth of PDGF-A-transformed cells in 5% BCS versus 5% PPP was measured at the indicated days following plating at 2,000 cells per cm^2. (B) The CL of metabolically labeled c-*sis*-transformed cells was immunoprecipitated with a PDGF-BB-specific antibody, revealing two bands (~30 and ~24 kDa) that were specifically competed for with 1 μg of unlabeled, recombinant PDGF-BB. The growth of c-*sis*-transformed cells in BCS versus PPP was determined as in panel A.

several human astrocytoma cell lines. We show that PDGF dominant-negative mutants revert the phenotype of PDGF-transformed cells and at least some human astrocytomas. These data are consistent with the notion that PDGF autocrine loops are an active factor in the transformed phenotype of these tumors.

MATERIALS AND METHODS

cDNA plasmid constructs. Full-length mouse PDGF-A (37) and human c-*sis* (a kind gift from the laboratory of D. Bowen-Pope, University of Washington, Seattle) cDNAs were cloned into the *Eco*RI site of the pMT2 expression vector (27) and expressed under the control of the adenovirus major late promoter. The 1308 and 1317 PDGF dominant-negative mutants were cloned into the *Hin*dIII site of the pLNCX vector (38) and expressed under the control of the cytomegalovirus immediate-early promoter. The neomycin resistance gene is expressed by pLNCX; resistance to hygromycin B was conferred to cells by cotransfection with pY3 (7).

Cell transfections. Transfection of BALB/c 3T3 cells was performed by a calcium phosphate precipitation technique. Approximately 18 h prior to transfection, 10^6 cells per dish were plated on 100-mm-diameter tissue culture dishes in Dulbecco's modified Eagle medium (DMEM; GIBCO) supplemented with 10% bovine calf serum (BCS; HyClone). A mixture of DNA (20 μg of pMT2 constructs with 1 μg of pY3, or 20 μg of pLNCX constructs) in 0.5 ml of 0.125 M $CaCl_2$ was added dropwise to 0.5 ml of 2× HBS buffer (280 mM NaCl, 1.5 mM $Na_2HPO_4 \cdot 7H_2O$, 50 mM *N*-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid [HEPES]) with constant aeration and incubated for 30 min at room temperature (RT) to facilitate the formation of a calcium phosphate precipitate. The precipitate was added to cells without medium for 20 min at RT, and then the cells were incubated for 4 h with 4 ml of 10% BCS at 37°C. After the precipitate was removed, the cells were shocked with 15% glycerol–1× HBS for 3 min, rinsed twice with phosphate-buffered saline (PBS), and grown in 10% BCS. Clonal lines were isolated in the third to fourth weeks of drug selection with 200 μg of hygromycin B (Calbiochem) per ml and/or 500 μg of neomycin sulfate (GIBCO) per ml. Four-millimeter squares of trypsin-soaked Whatman 3MM filter paper were applied directly to PBS-rinsed cell colonies, incubated for 3 min at 37°C, and transferred to six-well tissue culture dishes (Falcon) for subsequent clonal expansion.

For the astrocytoma cell lines, transfections were performed with Lipofectin reagent (GIBCO/BRL) as follows. Eighteen hours prior to transfection, 10^6 cells were plated on fibronectin-coated (1 μg/cm^2 for 1 h; Collaborative Biomedical Products) 100-mm-diameter tissue culture dishes. Lipofectin was added to DMEM at a concentration of 16 μl/ml and then mixed with an equal volume of DMEM containing DNA (pLNCX constructs) for a final concentration of 8 μl/ml. Cells were rinsed twice with PBS and incubated with the Lipofectin-DNA mixture (4 ml per dish) at 37°C for 5 h, at which time an equal volume of 10% BCS was added. Drug-resistant cultures were selected in 500 μg of neomycin sulfate per ml.

Metabolic labeling and immunoprecipitation. Primary clones were first screened for expression of PDGF-A or c-*sis* mRNA by Northern (RNA) analysis (data not shown). One

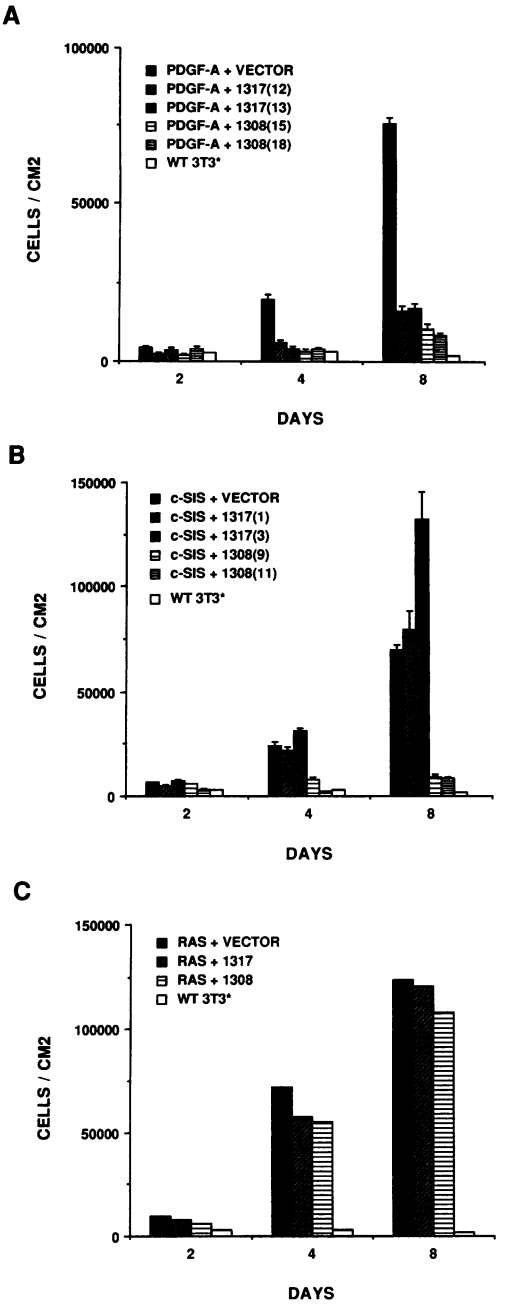

FIG. 2. Growth suppression of PDGF-A and PDGF-B (c-*sis*)-transformed cells by dominant-negative mutants. The indicated cell lines were plated in duplicate wells at 2,000 cells per cm^2 and grown in 5% PPP. Cells were harvested and counted at the indicated days. (A) PDGF-A-transformed cells with or without 1317 or 1308; (B) c-*sis*-transformed cells with or without 1317 or 1308; (C) Ha-*ras*-transformed cells with or without 1317 or 1308. Numbers in parentheses represent different clonal cell lines. Each value (with the exception of Ha-*ras* values) represents the average ± standard error of the mean of two independent growth curve experiments. WT, wild type.

subline expressing abundant PDGF-A mRNA and another expressing abundant c-*sis* mRNA were metabolically labeled to verify PDGF protein expression. Cells were grown to confluence on five 100-mm-diameter tissue culture dishes (Falcon), rinsed twice with cysteine-free DMEM, and incubated with 250 μCi of [^{35}S]cysteine (NEN) in 1 ml of cysteine-free DMEM for 4 h at 37°C with intermittent rocking. After the conditioned medium (CM) was harvested, the cells were rinsed twice with PBS and lysed with 1 ml of radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 50 mM Tris [pH 8], 1% Nonidet P-40, 10 mM deoxycholic acid, 0.1% sodium dodecyl sulfate [SDS]) containing 1 mM phenylmethylsulfonyl fluoride (PMSF). The CM and cell lysates (CL) were concentrated in Amicon Centriprep-10 concentrators (10,000-molecular-weight cutoff) and then clarified by centrifugation before immunoprecipitation.

Immunoprecipitations were performed with a polyclonal mouse PDGF-AA-specific antibody (1:100; kindly provided by Chiayeng Wang) and a monoclonal human PDGF-BB-specific antibody (1:400; Upstate Biotechnology Inc. [UBI]). Antibodies were added directly to CL, or with RIPA-PMSF to CM, and incubated for 3 h at 4°C with constant rocking. For competition experiments, 1 μg of unlabeled, recombinant PDGF-AA (UBI) or PDGF-BB (UBI) was added to the CL or CM prior to addition of antibodies. Protein A-Sepharose CL-4B resin (Pharmacia) was added for 1 h at 4°C and subsequently rinsed twice with RIPA-PMSF. The samples were reconstituted in nonreducing Laemmli sample buffer, boiled for 5 min, and analyzed by SDS-polyacrylamide gel (14% acrylamide) electrophoresis (PAGE).

Growth in serum and PPP. The PDGF-A-transformed, PDGF-B (c-*sis*)-transformed, and pMT2-transfected primary cell lines were plated in 10% BCS at a density of 2,000 cells/cm^2 into 24-well plates. After 24 h, the medium was changed to either 5% BCS or 5% platelet-poor plasma (PPP), and new medium added every third day. Duplicate wells were trypsinized and counted with a Coulter Counter (Coulter Electronics, Inc.), and the average number of cells per square centimeter was recorded daily for 8 days. Cells transfected with the dominant-negative mutants were similarly plated into six-well dishes also at a starting density of 2,000 cells per cm^2.

Colony formation assay. Cells were transfected with the dominant-negative mutants as described above. Following the transfection, cells were grown in 10% BCS for 24 h and then split 1:2 into either 5% BCS or 5% PPP with neomycin sulfate (500 μg/ml) and hygromycin B (200 μg/ml). For transfection of astrocytoma cells, the medium was changed to 5% PPP and 500 μg of neomycin sulfate per ml 48 h after transfection for simultaneous selection of drug resistance and growth in PDGF-deprived medium. After approximately 3 weeks, drug-resistant colonies were photographed at 10× magnification or fixed with 10% formalin and stained with 0.625% crystal violet for analysis.

Anchorage-independent growth. Sterilized 1.8% agar in water was mixed with equal volumes of 2× DMEM–20% BCS, layered on the bottom of 60-mm-diameter culture dishes (Falcon), and allowed to gel at RT. A methocellulose suspension with 1× DMEM–10% BCS was prepared as previously described (40). A suspension of 10^5 cells in 4 ml of methocellulose-BCS was layered on top of the agarose bed. Fresh methocellulose-BCS (3 ml) was added weekly.

Western immunoblotting. Cells were grown to confluence in 150-mm-diameter dishes and incubated in 5% PPP for 48 h. As controls, BALB/c 3T3 cells were incubated for 15 min in the absence or presence of 30 ng of PDGF-AA or PDGF-BB (UBI) per ml. Cellular protein was isolated at 4°C with 0.5 ml of lysis buffer (10 mM NaP buffer [pH 7.2], 150 mM NaCl, 1% Nonidet P-40, 10% glycerol, 0.2% NaF, 0.44% $NaPP_i$) containing 1 mM sodium orthovanadate, 5 μg

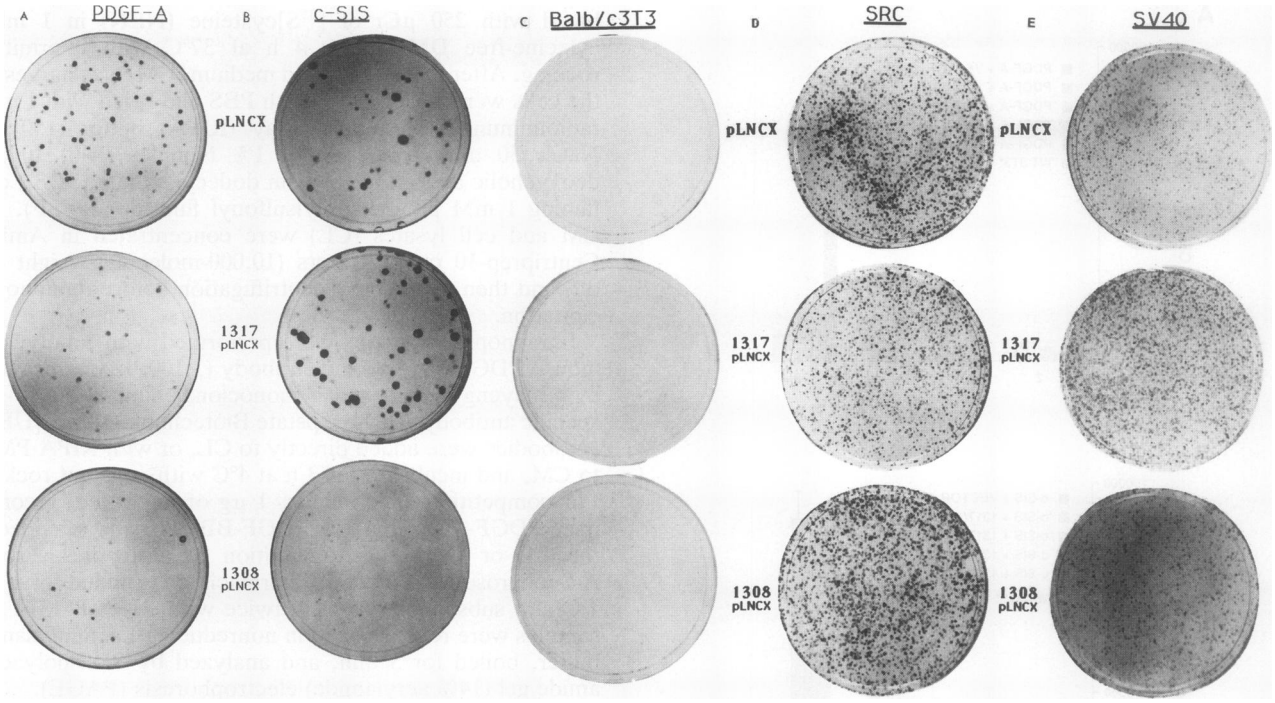

FIG. 3. Inhibition of colony formation by dominant-negative mutants. PDGF-A-transformed (A), PDGF-B (c-*sis*)-transformed (B), normal (neomycin resistant) (C), *src*-transformed (D), or SV40-transformed (E) BALB/c 3T3 cells were transfected with the dominant-negative mutant constructs as described in Materials and Methods and grown in 5% PPP with hygromycin B and neomycin drug selection. After approximately 3 weeks, cells were fixed and stained as described in the text and evaluated for the number and size of cell colonies.

of aprotinin per ml, and 1 mM PMSF. The CL were microcentrifuged to remove cellular debris. The protein content was quantified (Bio-Rad protein analysis), and the CL were stored at −70°C.

For anti-PDGF-α receptor blots, PDGF-A-transformed (~300 μg) or wild-type (~150 μg) 3T3 CL were resuspended in (1×) reducing Laemmli buffer, boiled for 5 min, and separated on SDS–7.5% polyacrylamide gels. The gels were transblotted to nitrocellulose and blocked at 37°C with 2% gelatin in Tris-buffered saline (TBS; 10 mM Tris [pH 8], 0.9% NaCl) for 1 h. A polyclonal anti-PDGF-α receptor antibody (diluted 1:100 in TBS containing 0.1% Tween 20 [TBST]; kindly provided by Chiayeng Wang) or a monoclonal antiphosphotyrosine antibody (diluted 1:5,000; kindly provided by Thomas Roberts) was applied overnight at RT. For anti-PDGF-β receptor blots, c-*sis*-transformed (~3,000 μg) or wild-type (~30 μg) 3T3 CL were first enriched for PDGF-β receptors by immunoprecipitation (as outlined above) with a polyclonal anti-PDGF-β receptor antibody (1:100; UBI) prior to Western blotting. The immunoprecipitates were resuspended in reducing Laemmli buffer, boiled for 5 min, and separated on SDS–7.5% polyacrylamide gels. The gels were transblotted to Immobilon-P (Millipore) and blocked at RT with 5% bovine serum albumin in PBS for 2 h. Anti-PDGF-β receptor antibody or antiphosphotyrosine antibody (both diluted 1:5,000 in blocking buffer) was applied for 1.5 h at RT. After a rinse with TBST, the secondary anti-rabbit or anti-mouse alkaline phosphatase-conjugated antibody (1:7,000; Promega) was applied for 1 h at RT in TBST. Alkaline phosphatase reactions were carried out with the addition of 66 μl of nitroblue tetrazolium and 33 μl of 5-bromo-4-chloro-3-indolyl phosphate to 10 ml of developing buffer (100 mM Tris [pH 9.5], 100 mM NaCl, 5 mM $MgCl_2$). CL used in all immunoprecipitations and immunoblots were normalized on the basis of total protein content.

Nude mice. Pooled colonies of U343 astrocytoma cells transfected with the 1308 dominant-negative mutant or the control expression vector (pLNCX) were trypsinized and resuspended in DMEM. By using a tuberculin syringe and a 27-gauge hypodermic needle, 10^7 cells (0.3 ml) were injected into the subcutaneous interscapular tissue of 4- to 6-week-old male Swiss nude mice (Taconic). The tumor diameter was recorded for four animals per experimental set on a weekly basis starting at 3 weeks postinjection. The mean tumor diameter ± standard error of the mean was calculated, and the statistical significance at 10 weeks was determined by Student's *t* test (STAT-SAK; G. E. Dallal).

RESULTS

Transformation of BALB/c 3T3 cells with PDGF-A or PDGF-B (c-*sis*) enhances cell growth in PDGF-deficient medium. PDGF-A or PDGF-B (c-*sis*) cDNAs were subcloned into the pMT2 expression vector and cotransfected with the hygromycin B resistance plasmid, pY3, into BALB/c 3T3 fibroblasts. Two hygromycin B-resistant sublines expressing high levels of PDGF-A or -B mRNA (data not shown) were selected for further characterization. One cell line produces PDGF-AA homodimer and releases it into the cell culture medium (Fig. 1A). Another produces PDGF-BB homodimer, the bulk of which remains cell associated, as noted by others (31) (Fig. 1B). Both of the PDGF-producing sublines were released from their growth dependence on exogenous PDGF (Fig. 1). This release was demonstrated by comparing cell growth in medium supplemented with PPP with growth in medium supplemented with BCS. As the product of unclot-

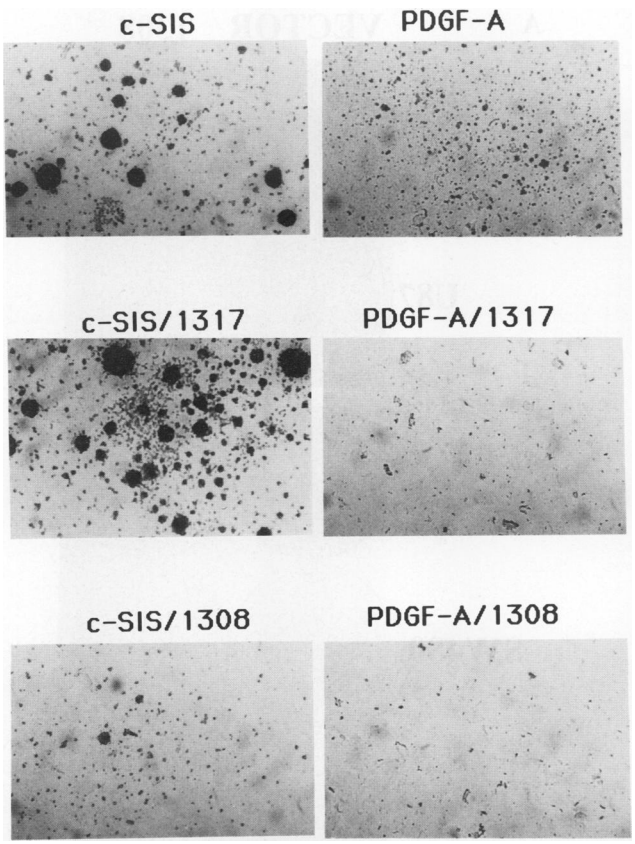

FIG. 4. Inhibition of anchorage-independent growth by dominant-negative mutants. PDGF-A and c-*sis*-transformed cells with or without 1317 or 1308 expression were analyzed for anchorage-independent growth. Cells (10^5) were plated in a methocellulose-BCS suspension and allowed to grow for 3 to 4 weeks, with fresh methocellulose-BCS added weekly. The suppressive effect of the dominant-negative mutants was measured as the ability to prevent the growth of colonies in methocellulose.

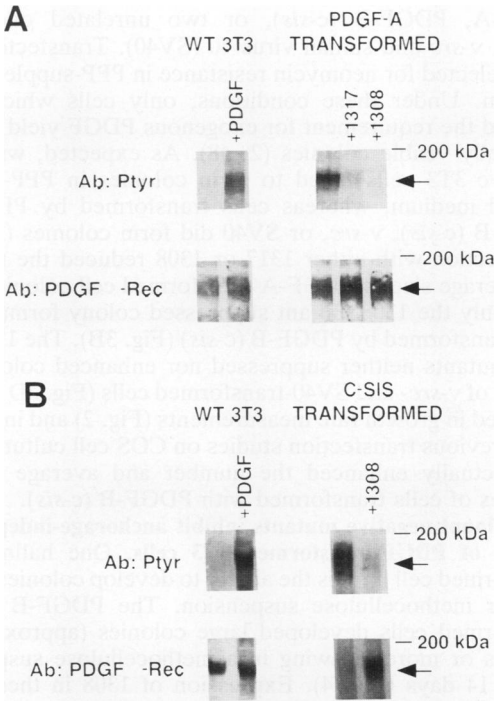

FIG. 5. Dominant-negative mutants disrupt PDGF autocrine loops. PDGF autocrine loops were analyzed by measuring the level of constitutive receptor tyrosine phosphorylation in cells preincubated with 5% PPP for 48 h at 37°C. (A) Cell lysates of wild-type (WT) 3T3 cells (untreated or PDGF treated) or PDGF-A-transformed cells (untreated or transfected with 1317 or 1308) were resolved by SDS-PAGE, transferred to nitrocellulose, and analyzed by Western immunoblotting with antiphosphotyrosine (Ptyr; top) and anti-PDGF-α receptor (α-Rec; bottom) antibodies (Ab). The PDGF treatment for the wild-type 3T3 cells was with 30 ng of PDGF-AA per ml for 15 min at 37°C. (B) CL of wild-type 3T3 cells (untreated or PDGF treated) or PDGF-B (c-*sis*)-transformed cells (untreated or transfected with 1308) was immunoprecipitated with an anti-PDGF-β receptor (β-Rec) antibody to enrich for receptors prior to Western immunoblotting with antiphosphotyrosine (top) and anti-PDGF-β receptor (bottom) antibodies. For the experiments in panel B, PDGF treatment for the wild-type 3T3 cells was with 30 ng of PDGF-BB per ml for 15 min at 37°C. To compensate for receptor downregulation, we had to load unequal quantities of CL in the various lanes. For the experiments in panel A, we used ~150 μg of CL from BALB/c 3T3 cells and ~300 μg of CL from PDGF-A-transformed cells. For the experiments in panel B, the differential was even greater: ~30 μg of CL from BALB/c 3T3 cells and ~3,000 μg of CL from PDGF-B (c-*sis*)-transformed cells.

ted blood, PPP contains little, if any, PDGF (8, 44). Cells transformed with either PDGF-A or PDGF-B (c-*sis*) grew nearly as well in PPP- or BCS-supplemented medium. By contrast, a control culture of BALB/c 3T3 cells, transfected with vector alone, showed superior growth in the BCS-supplemented medium. In addition to reducing the growth differential between PPP- and BCS-supplemented media, transformation with either PDGF-A or PDGF-B (c-*sis*) increased the final cell density at confluence (Fig. 1).

PDGF dominant-negative mutants restore serum dependence of 3T3 cells transformed by PDGFs. The PDGF-A and PDGF-B (c-*sis*)-transformed 3T3 cell sublines were subjected to secondary transfections with expression vectors encoding either of two PDGF dominant-negative mutants, 1317 and 1308. Two subclones from each transfection group which expressed high levels of 1317 or 1308 mRNA (data not shown) were selected for further study. First, we tested the secondary transfectants for growth in PPP-supplemented medium. Expression of either mutant suppressed the growth of PDGF-A-transformed sublines (by approximately 70%) compared with a control PDGF-A-transformed subline which received only the pLNCX vector during secondary transfection (Fig. 2A). The PDGF-B (c-*sis*)-transformed sublines expressing 1308 were likewise growth inhibited (by approximately 90%) compared with the control PDGF-B (c-*sis*)-transformed subline (Fig. 2B). The 1317 mutant failed to suppress the growth of c-*sis*-transformed cells and, in fact, facilitated growth, consistent with our previous findings which suggest that the 1317:PDGF-B heterodimer retains functional activity (36). The inhibition of growth by 1308 and 1317 is specific to PDGF-transformed cells, as they were unable to inhibit the growth of Ha-*ras*-transformed BALB/c 3T3 cells (Fig. 2C).

A colony formation assay for the action of PDGF dominant-negative mutants. To eliminate the possibility of clonal selection bias and to rapidly screen other cell lines, we used a colony formation assay for growth in PDGF-free medium. The dominant-negative mutant expression vectors were transfected into 3T3 cells which had been transformed with

PDGF-A, PDGF-B (c-*sis*), or two unrelated oncogenic agents, v-*src* and simian virus 40 (SV40). Transfected cells were selected for neomycin resistance in PPP-supplemented medium. Under these conditions, only cells which have escaped the requirement for exogenous PDGF yield macroscopically visible colonies (2, 48). As expected, wild-type BALB/c 3T3 cells failed to form colonies in PPP-supplemented medium, whereas cells transformed by PDGF-A, PDGF-B (c-*sis*), v-*src*, or SV40 did form colonies (Fig. 3). Transfection with either 1317 or 1308 reduced the number and average size of PDGF-A-transformed cell colonies (Fig. 3A). Only the 1308 mutant suppressed colony formation of cells transformed by PDGF-B (c-*sis*) (Fig. 3B). The 1308 and 1317 mutants neither suppressed nor enhanced colony formation of v-*src*- and SV40-transformed cells (Fig. 3D and E). As noted in growth rate measurements (Fig. 2) and in accord with previous transfection studies on COS cell cultures (36), 1317 actually enhanced the number and average size of colonies of cells transformed with PDGF-B (c-*sis*).

Dominant-negative mutants inhibit anchorage-independent growth of PDGF-transformed 3T3 cells. One hallmark of transformed cell lines is the ability to develop colonies in soft agar or methocellulose suspension. The PDGF-B (c-*sis*)-transformed cells developed large colonies (approximately 50 cells or more) growing in a methocellulose suspension within 14 days (Fig. 4). Expression of 1308 in these cells inhibited growth in methocellulose. In agreement with results in two different growth assays (Fig. 2 and 3), expression of 1317 actually enhanced anchorage-independent growth of the PDGF-B (c-*sis*)-transformed cells. The PDGF-A-transformed 3T3 cells also developed colonies in methocellulose suspension, albeit smaller than the ones generated by PDGF-B (c-*sis*)-transformed cells (Fig. 4). Expression of either 1308 or 1317 in PDGF-A-transformed cells inhibited anchorage-independent growth, in accord with our growth rate measurements and colony formation assays (Fig. 2 and 3).

Disruption of PDGF autocrine loops by the dominant-negative mutants. The tyrosine phosphorylation status of PDGF receptors was examined by Western immunoblotting with an antiphosphotyrosine antibody. When normal 3T3 cells were cultured in PPP-supplemented medium, the PDGF receptors were not phosphorylated unless challenged with exogenous PDGF. In the same medium, receptors were constitutively tyrosine phosphorylated in cells transformed with PDGF-A or PDGF-B (c-*sis*) (Fig. 5). Expression of the 1308 mutant eliminated this constitutive tyrosine phosphorylation in either PDGF-A- or PDGF-B (c-*sis*)-transformed cells (Fig. 5). In keeping with its biologic and biochemical action spectrum, expression of 1317 eliminated the constitutive phosphotyrosine signal in cells transformed by PDGF-A (Fig. 5A). Western immunoblotting with anti-PDGF-α and anti-PDGF-β receptor antibodies showed that loss of the constitutive tyrosine phosphate signal on PDGF receptors signal did not reflect a loss of PDGF receptor protein. Indeed, total receptor protein was more abundant following transfection with dominant-negative mutants. This apparent upregulation was especially evident in the PDGF-B (c-*sis*)-transformed cells following transfection with 1308 (Fig. 5B).

Taken together, these data indicate that PDGF autocrine loops are closed in 3T3 cells that express either PDGF-A or PDGF-B and that our dominant-negative mutants disrupt these loops. It should be noted, however, that acquisition and reversion of the transformed phenotype do not correlate in a linear way with the abundance of tyrosine-phosphorylated PDGF receptor. The total abundance of PDGF receptor protein in our PDGF-B (c-*sis*)-transformed 3T3 cells is less than 1% of that of control 3T3 cells (see the legend to Fig. 5). Although receptors are upregulated when PDGF autocrine loops are broken by our dominant-negative mutants, they do not return to the levels seen in wild-type 3T3 cells (see Discussion).

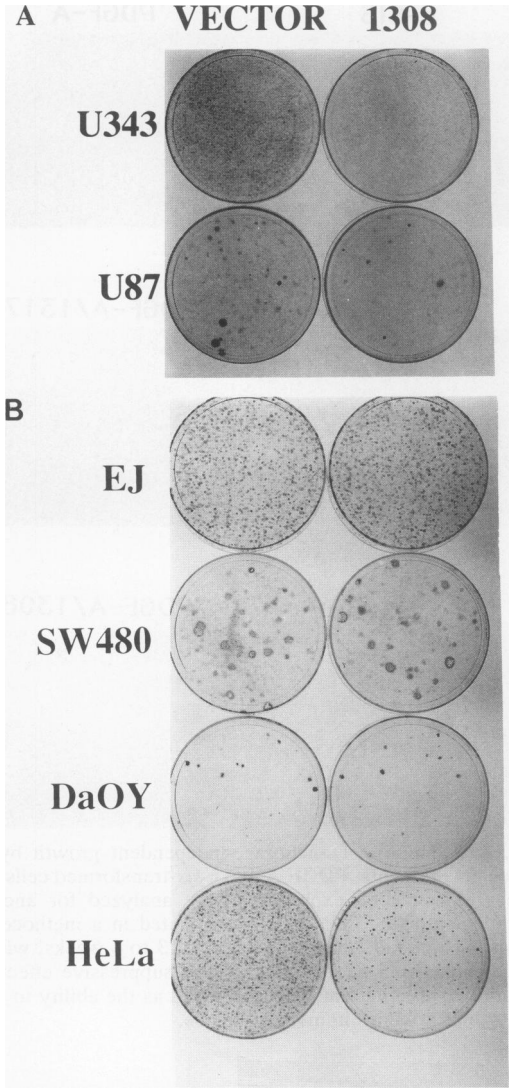

FIG. 6. PDGF dominant-negative mutant inhibits astrocytoma cell growth. Astrocytoma cell lines U87 and U343 were transfected with 1308 and analyzed for colony formation in 5% PPP. Cells (10^6 per dish) were transfected with 20 μg of 1308/pLNCX (1308) or 20 μg of pLNCX (vector) and grown in 5% PPP supplemented with 500 μg of neomycin sulfate per ml. Drug-resistant colonies were stained in 0.05% cresyl violet approximately 3 weeks after transfection.

Inhibition of astrocytoma cell growth by the 1308 dominant-negative mutant. Of the two mutants that we characterized, only 1308 was active on both PDGF-A- and PDGF-B (c-*sis*)-transformed 3T3 cells. For this reason, we expressed 1308 in two independent astrocytoma cell lines, U87 MG (U87) and U343 MGa Cl 2:6 (U343). These cell lines were previously characterized and found to express both PDGF-A and PDGF-B together with PDGF-β receptors; U343 expresses higher levels of the ligands, whereas U87 expresses more receptors (39). As judged from these expression patterns,

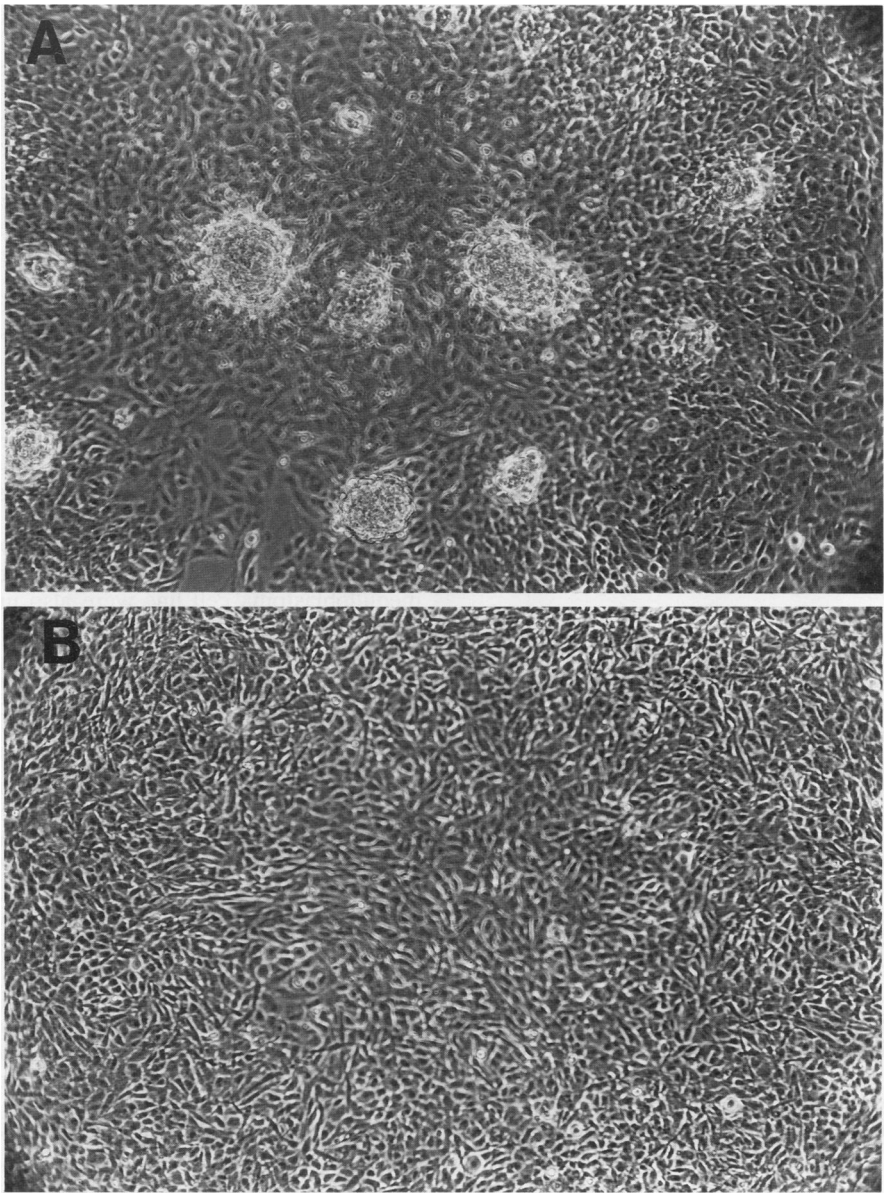

FIG. 7. Morphological changes in astrocytoma cells induced by a PDGF dominant-negative mutant. U343 astrocytoma cells transfected with the control expression vector (A) or with 1308 (B) were pooled and plated at equal density (5×10^5 cells per dish) in 5% PPP and photographed on the same day at 10× magnification.

both cell lines are candidates for possessing PDGF autocrine loops. Using the colony formation assay described above (Fig. 3), we found that both U87 and U343 cells formed colonies in PPP-supplemented medium. Thus, as predicted, these cells do not require exogenous PDGF for growth (Fig. 6A). When either astrocytoma line was transfected with 1308, colony formation in PPP-supplemented medium was markedly inhibited (Fig. 6A).

As controls for specificity, we studied the response of one human cell line derived from a different type of brain tumor (the medulloblastoma cell line DaOY) and two human tumor cell lines that express an activated *ras* gene (the EJ bladder carcinoma line and the SW480 colon carcinoma). None of these cell lines were affected by 1308 (Fig. 6B), although they expressed equivalent amounts of 1308 mRNA (data not shown). We were somewhat surprised to find that 1308 suppresses colony formation in HeLa cells, a human cervical carcinoma cell line. However, Western blot analysis (data not shown) indicates that our HeLa cell stocks express PDGF-β receptors. For this reason and others (see Discussion), it is possible that the response of HeLa cells to 1308 reflects the presence of a PDGF autocrine loop in a hitherto unexpected context.

Morphological changes in astrocytoma cells. The U343 astrocytoma cell line forms multilayered, refractile foci when grown in culture (Fig. 7A). Expression of the 1308 mutant in these cells resulted in distinct morphological changes. Both the number and size of foci that developed when grown in PPP-supplemented medium were reduced relative to control cells transfected with vector alone (Fig.

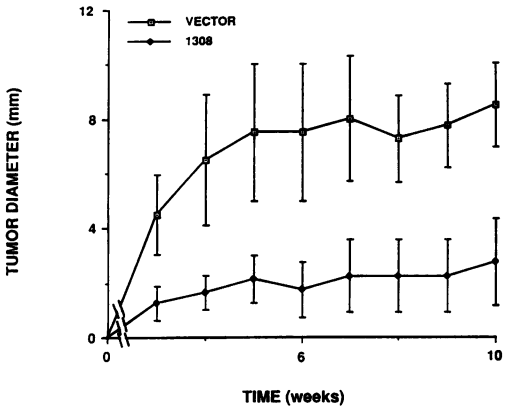

FIG. 8. A PDGF dominant-negative mutant reduces tumorigenicity of astrocytomas in nude mice. U343 astrocytoma cells transfected with 1308 or the control expression vector were pooled and injected subcutaneously into four nude mice per experimental set at 10^7 cells per animal. The data represent mean tumor diameters ± standard errors of the means. The tumor diameter of cells expressing 1308 is 70% smaller than that of control cells at 10 weeks ($t = 2.6$, $P < 0.05$).

7B). Microscopic analysis also supports the view that 1308 does not function as a toxin. We never observed cell debris, cytoplasmic vacuoles, or other visual indicators of a toxic response to 1308 expression (Fig. 7B). Cells expressing the dominant-negative mutant remained capable of sustained growth in serum and PPP-supplemented medium, albeit at rates slower than those of control cells. Similar morphological changes were observed for U87 cells expressing the 1308 dominant-negative mutant (data not shown).

Decreased tumorigenicity in nude mice of astrocytoma cells expressing 1308. U343 cells expressing 1308 were injected into nude mice as a measure of the tumorigenicity of these cells. Each of four mice injected in the interscapular region with the control U343 cells developed moderate-size tumors that persisted through 10 weeks (Fig. 8). In contrast, U343 cells expressing the 1308 dominant-negative mutant induced small tumor formation in only two of four injected mice. At 10 weeks, the mean diameter of tumors formed by U343 cells was reduced by 70% in cells expressing 1308.

DISCUSSION

Malignant astrocytomas are the most common primary intracranial tumors. In the United States, these tumors account for approximately 15,000 deaths per year and 4 to 5% of all cancer-related deaths (34). Several lines of evidence suggest that PDGF may contribute to the growth of these tumors. The majority of astrocytoma cell lines tested express PDGFs and PDGF receptor subunits which could potentially close autocrine loops and lead to stimulation of the tumor cells (39). Operative specimens from high-grade astrocytomas express greater amounts of both PDGF-A and PDGF-B than do lower-grade astrocytomas or nonneoplastic glial tissue (14, 22). These high-grade astrocytomas also express PDGF-α receptors, implicating autocrine-mediated growth (12, 14, 22, 30). It is not clear, however, that PDGF autocrine loops contribute to the malignant growth of these human brain tumors. High levels of expression of other growth factors and/or growth factor receptors have been demonstrated in astrocytomas (9, 17, 46, 47). Moreover, these tumors almost certainly accumulate other kinds of genetic lesions in the process of becoming a clinically detectable tumor mass (6, 13, 25, 43).

In this study, we used dominant-negative mutants of the PDGF ligand to address the role of PDGF autocrine loops in human astrocytoma. Starting with well-characterized 3T3 cell lines, we found that our mutants break PDGF autocrine loops, as indicated by Western blot analysis of receptor phosphorylation status. The mutants revert the transformed phenotype of 3T3 cells that display these loops. The selective action of the mutants on 3T3 cells transformed by PDGF-A or PDGF-B (c-*sis*) is in accord with the biochemical properties of the mutant proteins (36). One of the mutants, 1308, reverts the transformed phenotype of astrocytoma cell lines which express both PDGF ligands and β receptor subunits.

To interpret these results, it is important to show that growth responses to PDGF dominant-negative mutants are confined to cells that exhibit PDGF autocrine loops. Neither mutant affects the phenotype of 3T3 cells transformed by activated *ras*, v-*src*, or SV40, agents that function downstream of PDGF receptor activation. Two human carcinoma cell lines possessing activated *ras* oncogenes and a human medulloblastoma cell line were also unaffected by expression of the 1308 mutant. We would not have expected 1308 to inhibit growth of the two carcinoma lines because *ras*-transformed 3T3 cells were not affected. Thus, these unresponsive human lines represent an important positive control for species specificity. Growth inhibition of the two astrocytoma lines does not reflect a generalized nonspecific, toxic response to expression of a mutated mouse protein within human cells. We were surprised to see that expression of 1308 inhibits growth of the HeLa cell line, which was derived decades ago from a cervical carcinoma (29). We are unaware of any data linking HeLa cells or cervical carcinoma to PDGF autocrine loops. However, Western blot analysis shows that the HeLa cell stocks in our laboratory express PDGF-β receptors (data not shown). Others have shown that the endometrial cells which line the porcine uterus express PDGF-β receptors in vivo (41). Moreover, PDGF autocrine loops have been implicated as a growth control mechanism for placental cytotrophoblasts that have invaded the maternal endometrium (24). Further studies are needed to determine whether the HeLa cell response to 1308 is a nonspecific artifact or, alternatively, whether the mutant has revealed a PDGF autocrine loop in an interesting and hitherto unexpected context.

Our Western blot analysis of PDGF-transformed 3T3 cells raises other interesting questions for future study. As shown in Fig. 5, the total abundance of constitutively phosphorylated (and presumably active) PDGF-β receptor in PDGF-B (c-*sis*)-transformed 3T3 cells is less than 1% of that detected in normal 3T3 controls exposed to moderate concentrations of exogenous PDGF. The low amplitude of the phosphotyrosine signal reflects the fact that the total abundance of PDGF-β receptor protein in the transformed 3T3 cells is less than 1% of wild-type levels. Even when the autocrine loop is broken by expression of 1308, the upregulated receptor abundance is no more than 10% of that of wild-type 3T3 cells. These quantitative findings raise several interesting questions. The mitogenic signals delivered by autocrine-activated PDGF-β receptors appear to be disproportionately effective, and it would be interesting to know why. It would also be interesting to know whether the loss of PDGF receptors in PDGF-transformed cells is adaptive. Since signals from autocrine-activated receptors appear to be extraordinarily potent, one might imagine that, in excess,

they could be toxic. Further studies are required to address these issues.

In summary, our studies on 3T3 cells show that dominant-negative mutants of a PDGF ligand disrupt autocrine loops which can form in the cell interior. These mutants revert the phenotype of PDGF-transformed 3T3 cells and do not perturb the growth of cells transformed by other agents. By extension, our studies on human tumor-derived cell lines are consistent with the view that PDGF autocrine loops contribute to the unregulated growth of some astrocytomas. A broader survey of human astrocytoma cell lines is needed to determine whether the responses to PDGF dominant-negative mutants exhibited by the U343 and U87 lines are typical or atypical. Additional studies could also determine whether growth responses to PDGF dominant-negative mutants have diagnostic, prognostic, or therapeutic utility.

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