Growth Control Variant Cell Line Having Increased Serum Requirement and Decreased Response to Platelet-derived Growth Factor: Reversion by 5-Azacytidine

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ABSTRACT Variants of the mouse embryo fibroblast x melanoma hybrid clone 100A have been isolated by a procedure that selects against cells that are able to grow in medium containing low concentrations of serum plus insulin. Three variant clones derived from this selection were found to have a much higher serum requirement than the parental clone 100A cells, as evidenced by a very low rate of DNA synthesis and growth in medium containing low concentrations of serum. Two of the variants had approximately double the number of chromosomes as the parental cell line, while one had approximately the same number of chromosomes as the parental cells. One of the variants was very strongly reverted by 5-azacytidine but not by ethyl methanesulfonate, suggesting that it reverted by a nonmutational mechanism such as a stable change in DNA methylation. Analysis of the growth requirements in hormone-supplemented serum-free media of the 100A parent, the INS 471 variant, and revertants of the variant indicated that the variant had a specific deficiency in its growth response to platelet-derived growth factor (PDGF). PDGF dose-response curves obtained with the variant cells were shifted approximately an order of magnitude toward higher PDGF concentrations relative to PDGF dose-response curves obtained with the parental 100A cells. This quantitative increase in PDGF requirement of the INS 471 variant appears to explain the increased serum requirement of this variant. Equilibrium binding experiments performed with 125I-PDGF suggest that the variant does not have a decreased number of PDGF receptors.

Serum contains a complex mixture of nutrients and hormonal growth factors that stimulates the proliferation of mammalian cells. Included among the constituents of this mixture are platelet-derived growth factor (PDGF) (1–5), insulin-like growth factors (6–9), steroids (10, 11), transforming growth factor-β (12), the ion transport protein transferrin (13, 14), fibronectin (15), cell-spreadin factor (16), lipids (17), and low molecular weight nutrients (18, 19). An early observation regarding the oncogenic transformation of normal fibroblasts (20, 21) and fibroblastic cell lines (21–23) by tumor viruses was that malignantly transformed cells required lower concentrations of serum for growth than their untransformed counterparts. More recent results suggest that this quantitative decrease in serum requirement is a distinct event along a multistep pathway for conversion of normal somatic cells to tumor cells (see for example references 21 and 24). The reduction in serum requirement of transformed fibroblasts has recently been traced to a loss or reduction in requirement for specific growth factors such as PDGF, epidermal growth factor (EGF), fibroblast growth factor, or insulin (25–31). The identity of genes involved in the control of cell proliferation by serum growth factors is a subject of considerable current interest. We have recently undertaken a detailed study of the growth characteristics of the mouse embryo fibroblast x melanoma hybrid clone 100A, which exhibits a strong growth response to insulin and the insulin-like growth factor MSA (32–36). We report here the isolation of variants of this hybrid clone that have a markedly increased serum requirement. One of the variants appears to have a specific deficiency in its...
growth response to PDGF. This variant is reverted at a high frequency by 5-azacytidine (5-azaC).

MATERIALS AND METHODS

Cells, Culture Procedures, and Materials: Cells were grown at 37°C in Eagle's minimal essential medium (MEM) with 10% fetal bovine serum (FBS) (from Reheis Chemical Co. [Kankakee, IL], Gibco Laboratories [Grand Island, NY], or Centaurus), penicillin (71 U/ml), and streptomycin (100 µg/ml) under a humidified atmosphere containing 95% air plus 5% CO2. Cell sizes were determined with a Coulter counter (Coulter Electronics Inc., Hialeah, FL) equipped with a channelizer. Hybrid clone 100A is a biparental hybrid that was derived from a fusion between an HPRT- clone of a spontaneous mouse melanoma and secondary mouse embryo cells. The original, karyotypically uniform, and growth-restricted clone 100A have been described in detail (32-37). PDGF, purified through the CM Sephadex step of Antoniades et al. (38), was obtained from Collaborative Research Inc. (Lexington, MA). The specific activity of the PDGF was 6.6-17 ng/ml in the lots used in these experiments. (For the definition of PDGF units, see reference 38). This preparation of PDGF was used for growth assays. More highly purified preparations of PDGF were used for receptor binding assays (see below). Porcine insulin (25.5 U/mg) was obtained from Elanco Products Co. (Indianapolis, IN). EGF (receptor grade) was from Collaborative Research Inc. Human fibronectin was purchased from Meloy Laboratories Inc. (Springfield, VA) or Collaborative Research Inc. Soybean trypsin inhibitor (type I-S, chromatographically prepared), transferrin (substantially iron free), and BSA (crystalline) were from Sigma Chemical Co. (St. Louis, MO).

Isolation of Variants: An exponential culture of 100A cells was exposed to 0.5 µg/ml ethyl methanesulfonate (EMS) for 18 h and grown for 8 d (three passages at a 1:4 dilution) to permit the expression of mutations. This concentration of EMS gave 80% killing as determined by relative cloning efficiencies and more than a 50-fold induction of ouabain-resistant variants. (In a test for resistance to 1 mM ouabain, the frequency of resistant colonies was 1.1 x 10^-7 for unmutagenized cultures and 6 x 10^-5 for the second passage of a mutagenized culture.)

The mutagenized cultures of 100A were subjected to four to seven rounds of a negative selection procedure modified from that used by Pruss and Herschman (39) to select for EGF-unresistant variants of the 3T3 cell line. For the first round, 10^6 cells were plated per 25 cm^2 tissue culture flask in MEM with 2% FBS. After 24 h, the medium was changed to MEM with 0.3% FBS. After a further incubation of 24 h, when there were few mitotic cells, the medium was aspirated and MEM with 0.3% FBS, colchicine (3 µg/ml), and insulin (10 µg/ml) was added to permit growth at 37°C. Individual clones were picked and named systematically with three letters followed by three digits, e.g., "INS 456" means the sixth clone from the fifth bottle that underwent four rounds of the selection.

Measurement of DNA Synthesis by Autoradiography: Cells (60,000 per well) were plated in Linbro trays (Flow Laboratories, McLean, VA) with 24 wells, each containing one 1.5-cm coverslip in 1 ml of medium. DNA synthesis was measured by adding \[^{3}H\]thymidine to the medium at a final concentration of 5 µCi/ml (6.25 x 10^8 M). Coverslips were fixed as described (40), mounted on glass slides, dipped in NTB-2 (Kodak) emulsion, exposed for 24 h, and developed according to manufacturer's instructions. Nuclei were stained for 50 min with 4% Giemsa stain (Fish Scientific Co., Pittsburgh, PA) in 0.07 M sodium phosphate buffer, pH 7.0. At least 400 nuclei were counted in a minimum of five fields for each coverslip.

Determination of Chromosome Number: An exponentially growing culture was exposed to Colcemid (0.1-0.2 µg/ml) for 25-30 min to colcemidize the cells. Cells were allowed to swell in 0.56% KCl for 10 rain, fixed twice with methanol/acetic acid (3:1), and spread on cleaned slides. After staining with 4% Giemsa in 0.07 M sodium phosphate, pH 7.0, 50 metaphase chromosome spreads were counted under ×1,250 magnification.

Reversion Analysis: Exponential cultures were exposed to EMS or 5-azaC in MEM plus 10% FBS for 24 h at 37°C. Parallel unreated control cultures were included in each experiment. The medium was then removed and the cultures were washed once with PBS. Survival was determined by trypanblueing some cultures and plating cells at densities of 100 and 1,000 cells per 10-cm dish in MEM plus 10% FBS. Colonies on these dishes were scored at 1 wk by fixing cells and staining with 0.1% crystal violet. Fresh medium was added to other mutagen-treated and control cultures, and the cells were allowed to grow to confluence (48 h at 37°C). The cultures were then subcultured at a 1:4 dilution, to allow recovery from the mutagen. After the cells had grown to confluence (3-6 d), cells were removed from the plates by trypsinization and plated at 5 x 10^5 per 10-cm dish in MEM plus 0.3% FBS, or 10^6 per 10-cm dish in MEM containing 10% FBS and 10 mM ouabain. In some experiments, forward mutation rate to ouabain resistance was measured by plating cells at 10^5 cells per 10-cm dish in MEM containing 10% FBS and 1 mM ouabain. Mutation frequencies obtained by this method agreed closely with those obtained by the colony assay (data not shown). At the higher plating density, the higher ouabain concentration was required to suppress growth of the background. Revertant clones capable of growing in medium with 0.3% FBS were fixed, stained, and counted after 3 wk. Ouabain-resistant colonies that had diameters >3 mm were scored after 2 wk.

Growth of Cells in Hormone-supplemented Serum-free Media: The basal serum-free medium consisted of a 1:1 mixture of Ham's F12 medium and Dulbecco's modified Eagle's medium, 1.2 g/liter sodium bicarbonate (Gibco Laboratories, tissue culture grade). 15 µl HEPES (Calbiochem-Behring Corp., La Jolla, CA), 15 µl 2-aminoethanol (Sigma Chemical Co.). 100 µg/ml streptomycin (Calbiochem), and 71 U/ml penicillin (E. R. Squibb & Sons, Princeton, NJ), adjusted to pH 7.3. coli medium and all solutions used in the serum-free growth experiments were made in high-performance liquid chromatography-grade water (Fisher Scientific Co.). Basal medium was stored at 4°C for periods up to 1 mo before use. Immediately before use, the medium was supplemented with 3 x 10^-4 M insulin, and, as indicated, fibroblast growth factor (5 µg/ml), PDGF (0.5 U/ml), or EGF (200 ng/ml). Some batches of PDGF were found to contain cytotoxic contaminants. To eliminate these, we routinely heated the PDGF to 100°C for 10 min before use.

For serum-free growth experiments, a log-phase culture in a 75 cm^2 flask was washed two times with 15-20 ml ice-cold (4°C) PBS. At t = 0, 10 ml of ice-cold trypsin (0.5%/PBS in PBS) was added. At t = 1 min, the trypsin was aspirated and incubation was continued at room temperature. At t = 2.5 min, the flask was shaken to dislodge the cells, and 10 ml of ice-cold soybean trypsin inhibitor (1 mg/ml in PBS) was added. Cells were washed once with unsupplemented serum-free medium, resuspended in the same medium, counted, and plated at a density of 10,000 cells per 3.5-cm dish. After 3-4 d at 37°C, cells were removed by trypsinization and counted with a Coulter counter.

PDGF Binding Assays: Cells were grown to confluence in 3.5-cm dishes in MEM plus 10% FBS. Washed twice with 2 ml of serum-free MEM, and incubated at 37°C for 1 h with 2 ml of serum-free MEM. The MEM was aspirated and replaced with 1 ml binding mix which consisted of PBS plus 10 µg/ml MgSO4, 7 H2O, 10 µg/ml CaCl2, 2 H2O, 1 mg/ml BSA, 100,000 cpm of homologous [3H]-PDGF (specific activity 20,000 cpm/ng, generously provided to us by Dr. Harry Antoniades), and with or without 500 µg/ml of partially purified PDGF (purified through the CM Sephadex step [41] and provided by Dr. Antoniades). The binding assays were incubated at 4°C for 3 h, and the monolayers were then rapidly washed six times with 2 ml of ice-cold PBS containing 1 mg/ml BSA. The cells were solubilized with 2 ml of 10% glycerol and 1% Triton X-100, and radioactivity was determined in a Beckman Bio- gamma counter (efficiency 78%; Beckman Instruments Inc., Irvine, CA). All results represent the average of triplicate determinations. Cell counts were performed on triplicate parallel cultures using a Coulter counter.

RESULTS

Variant Isolation

Clone 100A is a mouse embryonic fibroblast × melanoma hybrid cell line that exhibits a very strong growth response to insulin and the insulin-like growth factor MSA (33-36). Our aim was to select for variants of this cell line that had a reduced response to the mitogenic action of insulin by stimulating growth-arrested cells with insulin in the presence of colchicine and removing cells that entered mitosis in response to the hormone (see Materials and Methods). A total of 37 clones were isolated from cells that survived between four and seven rounds of selection. These clones were screened for ability to respond to the stimulatory effect of insulin in the [3H]thymidine incorporation assay (performed as in ref
Although none of the clones were completely resistant to the growth-stimulatory effects of insulin, several clones differed dramatically from the parental clone 100A cells in that they arrested growth much more completely in medium with low serum than did the parental cells. These clones represented 16% of clones that survived the selection. Three variant clones of this class were chosen for further study.

Chromosomal analysis (Fig. 1) revealed that two of the variants had approximately double the number of chromosomes observed in the parental clone 100A cells (INS 471 and INS 794), while one variant (INS 643) had a median chromosome number very close to that of clone 100A. None of the three variant clones were resistant to colchicine (Table I).

**Serum and Growth Factor Response of Variants**

As shown in Table II, the three variant clones were strongly serum dependent in comparison with the parental clone 100A, which was capable of slow growth in medium with 0.3% FBS. The generation times observed for the variants in medium with 10% FBS were approximately equal to the generation time of the parental cells. This indicated that the variants were not simply slow growers, but rather had an increased serum requirement. The parental clone 100A cells had a relatively high saturation density, while that of the variants was much lower (Table II). This may also reflect an increased serum requirement of the variants (e.g., see reference 42).

The response of the variant clones to insulin and serum in nuclear labeling experiments is shown in Fig. 2. The basal level of nuclear labeling in the variant clones in medium with low serum was much lower than that of the parental cells, reflecting the more complete growth arrest of the variants under these conditions. Addition of 1% FBS to resting cultures of the variant clones recruited only a very small fraction of cells to enter S phase, while the same amount of serum strongly stimulated the parental cells (Fig. 2, bar F). Addition of 10% FBS to resting cultures of the variant clones caused most cells to enter S phase. These results again demonstrate the increased serum requirement of the variants relative to that of the parental clone 100A cells. All of the variants exhibited a response to insulin in the nuclear labeling assay (Fig. 2, bars B and C). However, in each case the total fraction of cells that entered S phase in medium with low serum plus insulin was considerably lower than for the parental cells.
This explains the selective advantage of the variants under the conditions used for their isolation.

Since higher concentrations of serum were required to elicit a growth response in the variants than in the parental clone 100A cells, we examined the response of the variant clones to PDGF, which is one of the principal mitogenic factors in serum (1, 2). Dose-response curves for stimulation of the parental 100A and variant cells with PDGF are shown in Fig. 3. The parental clone 100A cells exhibited a strong growth response to PDGF, while the response of the variant clones to PDGF was very weak. As with insulin, the total fraction of cells that entered S phase in response to PDGF was high for the clone 100A cells and low for the variants. In experiments in which high concentrations of PDGF and insulin were added together to the variants, we observed a stimulation that was more than the sum of the individual effects of the two hormones (Fig. 2, bars C, D, and E). This suggests synergism in the effects exerted by the two hormones, similar to that observed with other cells (43, 44). However, even the combination of a high concentration of insulin and PDGF induced <50% of the variant cells to enter S phase during the 24-h labeling period. In contrast to the results obtained with the variant cells, a less than additive effect of insulin and PDGF was observed with the parental clone 100A cells, since either hormone alone was strongly stimulatory to the parental cells.

Reversion Analysis

The increased serum/growth factor requirement of the variants was also manifested in an inability to form colonies capable of sustained growth in MEM with 1% FBS. The parental clone 100A cells formed large, rapidly growing clones in this medium (13–22% cloning efficiency). INS 643 and INS 794 formed minute clones in this medium, while INS 471 did not form clones at all (Fig. 4). This difference between the parental cells and variants was specific for medium with low serum; in MEM containing 10% FBS, the variants and parental cells both formed clones with high efficiency (40–70% cloning efficiency). The variant that gave the lowest background growth in medium with low serum (INS 471) was selected for further analysis.

The background of growth of the INS 471 variant in medium with low serum was sufficiently low that it was possible to select for revertants of this variant that were able to grow in medium with low serum (LS+ revertants) by plating the variant at a low density (104 cells per 10-cm dish) in medium with 0.3–1% FBS (Table III). Interestingly, the base analogue 5-azaC caused the variant to revert to LS+ at a very high frequency (~1%). In contrast, the alkylating agent EMS, which is a strong mutagen, was relatively ineffective in reverting the variant. The mutagenic activity of EMS and 5-azaC in the same cells has been examined in parallel experiments in which forward mutation to ouabain resistance (45–47) has been measured. EMS, but not 5-azaC, induced ouabain-resistant mutants of the INS 471 cell line. This is consistent with the known relative mutagenic potency of the two compounds as measured in other systems: EMS is a very strong mutagen (48, 49) and 5-azaC is a very weak mutagen (50). 5-azaC is known to cause undermethylation of DNA and reexpression of genes that have been inactivated by DNA methylation (51–55). The very high reversion frequency of INS 471 induced by 5-azaC suggested that 5-azaC reverted INS 471 via a nonmutational mechanism such as a change in DNA methylation.

To determine whether the LS+ phenotype of the revertants was stable, four 5-azaC-induced LS+ revertants of INS 471, were picked, cultured in MEM plus 10% FBS, and replated at clonal density in MEM and 1% FBS. All four revertants were able to form large clones in MEM plus 1% FBS, indicating that the LS+ phenotype was stable. The cloning efficiency of the revertants in this medium ranged from 3–14%, as compared with a cloning efficiency of 13–22% for the parental hybrid clone 100A cells in the same medium.

Growth of 100A and INS471 in Hormone-supplemented Serum-free Media

The analysis of the growth response of the variants to insulin and PDGF shown in Figs. 2 and 3 involved short term stimulation (24 h) in medium that contained a low concentration of serum. These experiments were complicated by the presence of PDGF and insulin-like growth factors in the serum. The growth response of the parental 100A cells, the INS 471 variant, and two independent LS+ revertants of INS 471 to PDGF and insulin was studied further under completely serum-free conditions. The parental 100A cells grew extremely well in a serum-free medium supplemented with fibronectin, transferrin, insulin, and PDGF (Fig. 5, bar D). In this medium, a 74-fold increase in cell numbers over the inoculum was observed with the 100A cells: this corresponds to a generation time of ≈14 h. This is one of the most rapid growth rates ever observed for any cell line in any hormone-supplemented serum-free medium (56), and it is as fast as the growth rate of the same cells in medium with 10% serum (Table II). In the same medium in which PDGF was replaced by EGF; good growth was also obtained, although less growth...
was obtained in the medium with EGF than in that with PDGF. The growth response of the INS 471 variant cells to PDGF and EGF was also examined under serum-free conditions (Fig. 5). Interestingly, the growth response of the variant cells to PDGF was drastically reduced relative to that of the 100A cells: there was an approximately 15-fold reduction in cell yield in response to PDGF with the INS 471 variant as compared with the 100A parent. In contrast, the INS 471 variant grew about as well as the 100A parent in the serum-free medium supplemented with EGF (Fig. 5, bar E). Thus the variant exhibited a specific deficiency in its response to PDGF. No significant difference was observed in the growth response of the parental 100A and INS 471 variant cells to insulin. For example, in the experiment illustrated in Fig. 5, addition of insulin to the basal medium containing fibronectin and transferrin allowed both cell lines to maintain their inoculum density, but no net growth was observed in either case (Fig. 5, bars B and C). The results of these growth experiments performed in serum-free medium thus indicated a specific deficiency of the variant cells in their response to PDGF.

To determine whether the revertants of INS 471 that were selected for ability to grow in medium with low serum had also reverted for their response to PDGF, we grew two independent 5-azaC-induced LS⁺ revertants of INS 471 in hormone-supplemented serum-free medium under conditions identical to those used for the 100A and INS 471 cells in the experiment shown in Fig. 5. The results (Fig. 6) indicated that the two LS⁺ revertants had a significantly increased growth response to PDGF compared with that of the INS 471 variant. In addition, both LS⁺ revertants resembled the parental 100A cells in that the growth obtained in medium with 0.5 U/ml PDGF was greater than that obtained in medium with EGF. These results indicate that reversion to a high PDGF response occurs concomitantly with reversion to LS⁺.

To further analyze the deficiency of the INS 471 variant in its response to PDGF, we performed dose-response experiments in which growth was measured as a function of PDGF concentration (Fig. 7). The yield of cells obtained with the INS 471 variant was lower than that obtained with the parental 100A cells at every PDGF concentration tested, whereas the response of the INS 471 cells to EGF (200 ng/ml) was
TABLE III
Reversion of INS 471 to Growth in Medium with Low Serum

| Treatment* | Survival* | LS+ Clones* | Oua+ Clones* |
|------------|-----------|-------------|--------------|
| µg/ml      | % per 10^4 cells | per 10^6 cells |               |
| None (control) | (100)      | 0            | 0             |
| EMS        | 200       | 42           | 0             | 42            |
|            | 400       | 22           | 2             | 78            |
|            | 650       | 0.6          | 0             | 29            |
| 5-azaC     | 0.03      | 110          | 0             | 0             |
|            | 0.3       | 54           | 0.33          | 0             |
|            | 1.0       | 54           | 16            | 0             |
|            | 3.0       | 25           | 86            | 0             |
|            | 10.0      | 22           | 12            | 0             |

* Cells were treated for 24 h as described in Materials and Methods.
* Numbers represent the ratio of cloning efficiency of cells from mutagen-treated versus control cultures. The control cloning efficiency was 67% (average of six dishes). Each number for the mutagen-treated cultures represents the average of three dishes.

J Cells were subcultured once after treatment with mutagen and then plated at a density of 10^4 cells per 10-cm dish in MEM plus 0.3% FBS. The control represents the average of six dishes; the numbers for mutagen-treated cultures represent the average of three dishes.

J Cells were subcultured once following treatment with mutagen and then plated at a density of 10^6 cells per 10-cm dish in MEM plus 10% FBS + 10 mM ouabain. Results represent the average of duplicate dishes.

![Figure 5](image)

**Figure 5** Growth of 100A and INS 471 in serum-free medium. Cells were plated at a density of 10,000 cells per 3.5-cm dish, and were harvested and counted 4 d later. Each bar represents the average of three determinations. (---) Represents the inoculum density. (A) Basal serum-free medium + 10 µg/ml fibronectin. (B) Basal serum-free medium + 10 µg/ml fibronectin + 5 µg/ml transferrin. (C) Basal serum-free medium + 10 µg/ml fibronectin + 5 µg/ml transferrin + 5 µg/ml insulin. (D) Basal serum-free medium + 10 µg/ml fibronectin + 5 µg/ml transferrin + 5 µg/ml insulin + 0.5 U/ml PDGF. (E) Basal serum-free medium + 10 µg/ml fibronectin + 5 µg/ml transferrin + 5 µg/ml insulin + 200 ng/ml EGF.

![Figure 6](image)

**Figure 6** Growth of INS 471 LS^+^11 and INS 471 LS^+^102 in serum-free medium. Cells were plated at a density of 10,000 cells per 3.5-cm dish, and were harvested and counted 4 d later. Each bar represents the average of three determinations. (---) Represents the inoculum density. (A) Basal serum-free medium + 10 µg/ml fibronectin. (B) Basal serum-free medium + 10 µg/ml fibronectin + 5 µg/ml transferrin. (C) Basal serum-free medium + 10 µg/ml fibronectin + 5 µg/ml transferrin + 5 µg/ml insulin. (D) Basal serum-free medium + 10 µg/ml fibronectin + 5 µg/ml transferrin + 5 µg/ml insulin + 0.5 U/ml PDGF. (E) Basal serum-free medium + 10 µg/ml fibronectin + 5 µg/ml transferrin + 5 µg/ml insulin + 200 ng/ml EGF.

actually slightly better than that of the 100A cells (Fig. 7, broken line). This again illustrates the specificity of the defect in the PDGF response of the variant. The dose-response curve of the variant was clearly shifted to the right relative to that obtained with the 100A cells. For any given growth response, approximately one order of magnitude higher PDGF concentration was required by the INS 471 variant than by the 100A parent. For example, a yield of 200,000 cells (corresponding to a generation time of ~17 h) was obtained with the 100A cells at a PDGF concentration of 0.1 U/ml versus 1.0 U/ml for the variant. This shift in the dose-response curve for PDGF very likely explains the increased serum requirement of the variant.

**PDGF-receptor Binding Experiments**

A possible explanation that we have considered for the poor growth response of the INS 471 variant to PDGF is that this variant might have reduced numbers of PDGF receptors, and that the revertants might have reverted for receptor expression. To test this possibility, we performed equilibrium binding experiments in which we examined the binding of ^125^I-labeled PDGF to the parental 100A cells, the INS 471 variant cells, and one LS^+^ revertant of the INS 471 variant. The results (Table IV) indicated that all three cell lines exhibited...
specific binding of PDGF. Normalizing the data to cell numbers, the INS 471 variant actually bound more PDGF than the parental 100A cells. The variant cells have approximately 1.5-fold more surface area than the 100A parent cells (Table II); however, this difference in surface area does not account for the greater than threefold increase in specific PDGF binding observed with the variant. In any case, these results suggest that the diminished PDGF response of the INS 471 variant is not attributable to decreased number of PDGF receptors having an unaltered affinity for PDGF.

DISCUSSION

The selection procedure used to isolate the variants was based on removal of cells that underwent mitosis when stimulated with insulin in medium containing low concentrations of serum. The range of possible phenotypes that could arise from this selection is quite broad. The use of inhibitors of microtubule polymerization has previously yielded density revertants of simian virus 40 (SV40)-transformed 3T3 cells (57), variants of 3T3 cells that do not respond to the tumor promoter 12-O-tetradecanoylphorbol-13-acetate (59). The presence of agents that destabilize microtubules has actually been shown to enhance the effect of growth factors in dense cultures of 3T3 cells (60, 61). None of the variants described here were resistant to colchicine.

One class of variants that survived the present selection had a drastically reduced ability to grow in medium with low concentrations of serum. The lesion in these variants appeared to relate specifically to serum requirement, since all clones in this class grew as rapidly as the parental cells in medium containing high serum concentrations. In medium containing limiting serum concentrations the variants exhibited a weak growth response to insulin and PDGF, in that a relatively low proportion of cells were stimulated to traverse S phase by addition of insulin or PDGF. The fact that only a small fraction of the variant cells entered the cell cycle in medium containing low concentrations of serum plus insulin explains the selective advantage of the variants under the conditions.

TABLE IV

Binding of PDGF to Clone 100A, INS 471, and INS 471 LS+1

| Cells             | Total binding cpm/dish | Nonspecific binding cpm/dish | Specific binding cpm/dish | Specific binding cpm/10^5 cells |
|-------------------|------------------------|------------------------------|---------------------------|---------------------------------|
| 100A (parent)     | 4,226 ± 870            | 1,450 ± 149                  | 2,776                      | 145                             |
| INS 471 (variant) | 4,617 ± 1,076          | 1,534 ± 144                  | 3,083                      | 548                             |
| INS 471 LS+1 (revertant) | 4,302 ± 402          | 1,863 ± 438                  | 2,439                      | 439                             |

Figure 7 PDGF dose response curves for the parental 100A cells (A), and INS 471 variant cells (B). Cells were plated at a density of 10,000 cells per 3.5-cm dish in basal serum-free medium supplemented with 10 μg/ml fibronectin, 5 μg/ml transferrin, 5 μg/ml insulin, and PDGF as indicated. Each point represents the average of duplicate determinations. (--) Represents growth obtained in basal serum-free medium supplemented with 10 μg/ml fibronectin, 5 μg/ml transferrin, 5 μg/ml insulin, and 200 ng/ml EGF (average of duplicate determinations).
used for their isolation. Addition of insulin and PDGF to medium containing limiting serum concentrations resulted in a more than additive effect in the variants; however, even with high concentrations of insulin and PDGF, <50% of the variant cells entered S phase.

The nuclear labeling experiments were complicated by the presence of serum, which contains both PDGF (1) and insulin-like growth factors (6–9). Thus, it was not possible to say with certainty which growth factor was actually limiting in a given experiment. To circumvent this problem, we studied in detail the growth of the parental 100A cells and the INS 471 variant, which had the “tightest” LS" phenotype (Fig. 4), in hormone-supplemented serum-free media. The parental 100A cells grew extremely well in a medium containing fibronectin, transferrin, insulin, and PDGF. The parental cells also grew well in a similar medium in which PDGF was replaced with EGF, although less growth was obtained in this medium than in the PDGF-containing medium. In the hormone-supplemented serum-free medium, no obvious difference was detected between the growth response to insulin of the parental 100A cells and the INS 471 variant cells. The INS 471 variant cells did, however, exhibit a dramatically reduced growth response to PDGF. This defect in the growth response to PDGF of the variant cells was specific, as the variant cells did not exhibit a decreased growth response to EGF. The PDGF dose-response curve obtained with the INS 471 variant was shifted to the right by approximately an order of magnitude in PDGF concentration relative to the PDGF dose-response curve obtained with the 100A parent. This very likely explains the requirement of the variant for higher serum concentrations. Also, the decreased saturation density of the variant in serum-containing medium (Table II) is consistent with the variant having a defective growth response to PDGF, since PDGF appears to be the major serum determinant of saturation density (1).

The genetic basis for the phenotype of the variants is a subject of considerable interest. Two of the three variants had chromosome numbers that were approximately double that of the parental 100A cells. This hyperploidy very likely resulted from the use of colchicine in the selection procedure. Interestingly, when Pollack, Vogel, and co-workers (57, 62–64) selected flat revertants of SV40-transformed 3T3 cells, hyperploidy was associated with suppression of the ability to grow in low amounts of serum and low saturation density. Revertants picked on the basis of increased serum requirement were always density revertants, but the opposite was not true (62, 63). In the present study, hyperploidy was not a necessary condition for increased serum dependence. For example, INS 643 had an increased serum dependence but had a modal chromosome number similar to that of the parental 100A cells. A comparison of the hormonal growth factor requirements of SV40-transformed 3T3 cells and serum-dependent revertants of these cells under totally serum-free conditions has not been reported. However, in experiments in which cells were plated in medium containing serum or on serum-coated dishes and then transferred to serum-free medium, serum-dependent revertants of SV40-transformed 3T3 cells appeared to have an increased insulin requirement (31). In contrast, the principal defect in the INS 471 variant appears to be a poor growth response to PDGF.

In the experiment shown in Table IV, the variant cells exhibited, if anything, increased specific binding of the 125I-PDGF tracer relative to the parental 100A cells. This observation would not be consistent with a lesion involving a decrease in receptor number, with no alteration in receptor binding affinity. The binding data shown in Table IV would be consistent with other types of defects in the PDGF receptor, or a postreceptor defect in the intracellular pathway of PDGF action.

The base analogue 5-azaC, which reverts INS 471 at a high frequency, is known to cause hypomethylation of DNA (51) and reexpression of the genes that have been inactivated by DNA methylation. Genes that have previously been found to be activated by 5-azaC, possibly by a mechanism involving hypomethylation, include a cryptic metallothionein-I gene in W7 mouse thymoma cells (53), genes on an inactive X chromosome (52), endogenous retroviral genes (54), an inactive thymidine kinase gene in a thymidine kinase-deficient variant hamster cell line (55), and developmentally inactivated fetal globin genes (65). 5-azaC also induces malignant transformation of cultured C3H 10T½ cells (66) and CHEF/18 cells (67), and 5-azaC is a carcinogen that induces leukemias and lymphomas in mice when assayed by the standard National Cancer Institute bioassay protocol (68). This suggests that 5-azaC is capable of activating cellular oncogenes in some cell types.

The very high frequency of reversion of INS 471 induced by 5-azaC, compared with the low reversion frequency induced by EMS, suggested that 5-azaC reverted INS 471 by a nonmutational mechanism, possibly involving a change in DNA methylation. In parallel control experiments in which forward mutation to ouabain resistance was measured with the same doses of 5-azaC and EMS, a strong induction of ouabain-resistant mutants was observed with EMS but not with 5-azaC. This is consistent with the known mutagenic potency of the two chemicals: EMS is a very strong mutagen (48, 49), whereas 5-azaC is a very weak one (50). The extraordinarily high frequency of reversion of INS 471 induced by 5-azaC (nearly 1% at a dose of 3 μg/ml) is also suggestive of reversion by a nonmutational mechanism.

The INS 471 variant arose in a culture that had been mutagenized by EMS. EMS is primarily a base pair substitution mutagen (69), and no effect of EMS on DNA methylation has been described to date. Thus, reversion of an EMS-induced mutant with 5-azaC is a surprising observation. One similar example of EMS-induced variants that are reverted at a high frequency by 5-azaC has been reported recently: these are prolactin-deficient variants of the rat GH3 cell line (70). There are a number of possible explanations for the reversion characteristics of the INS 471 cell line. The principal lesion in this variant could involve methylation of a regulatory site of a gene involved in the PDGF response, which is reverted by treatment of the variant by 5-azaC. Alternatively, the lesion in INS 471 could be a true mutation that is reverted by 5-azaC-induced demethylation at a second site. The identity of genes involved in regulating the cellular response to PDGF and other growth factors is a subject of considerable current interest. Recently, PDGF has been shown to regulate several specific messenger RNAs, including transcripts of the c-myc gene (71–73). It will be of considerable interest to determine whether there is a defect in the induction by PDGF of any of these mRNAs in the INS 471 variant.

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