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Analysis of the Reduced Growth Factor Dependency of Simian Virus 40-Transformed 3T3 Cells

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We have measured in a defined serum-free medium the platelet-derived growth factor (PDGF) and insulin requirements of normal Swiss 3T3 cells, simian virus 40-transformed 3T3 cells, and partial revertants of simian virus 40-transformed 3T3 cells. Swiss 3T3 cells displayed strong requirements for both PDGF and insulin. Both of these requirements were significantly diminished in simian virus 40-transformed 3T3 cells. Analysis of the PDGF and insulin requirements of the revertants indicated that the loss of either of these two growth factor requirements was not necessarily linked to the other; rather, the growth factor requirements were specifically associated with other parameters of transformation. The reacquisition of a PDGF requirement cosegregated with reversion to density-dependent growth inhibition, whereas reacquisition of a normal insulin requirement cosegregated with reversion to a normal growth dependence on calf serum. Anchorage dependence was dissociable from both growth factor requirements. The relationship between the PDGF requirement and density-dependent growth inhibition was further analyzed in normal 3T3 cells by measuring the PDGF requirement at different cell densities. At high cell densities, the requirement for PDGF became significantly greater. We suggest that at least in part the ability of transformed cells to grow to high saturation densities results from their loss of a requirement for PDGF.

Transformation by simian virus 40 (SV40) and other oncogenic agents often reduces the high serum growth requirement of normal fibroblasts (11, 15, 22, 27, 30). Several reports have led to the conclusion that a reduced serum requirement results from the loss of a requirement for specific growth factors. For example, Rous sarcoma virus transformation of chicken embryo fibroblasts reduced their requirement for the insulin-replaceable activity in serum (30), and abortive SV40 infection of normal mouse 3T3 cells stimulated DNA synthesis in the absence of platelet-derived growth factor (PDGF) and the insulin-like growth factor somatomedin C, both of which were necessary for the stimulation of uninfected cells (24, 28). Additionally, Cherington et al. discovered that a reduced epidermal growth factor (EGF) requirement was associated with tumorigenicity in a set of hamster fibroblast cell lines (5).

Not all of these reported changes need be directly related to a reduced serum dependence. Transformed cells often exhibit other alterations in growth control, such as the loss of density-dependent growth inhibition and the loss of a normal anchorage requirement (22). If the loss of a specific growth factor requirement is directly linked to a reduced serum requirement, then the two phenotypes should cosegregate upon reversion. Revertants of SV40-transformed cells that have regained a normal serum dependence have been isolated (33, 34). Since wild-type SV40 can be rescued from these revertants and since they continue to express SV40 T-antigen, it is thought that these revertants harbor a cellular mutation that reverses the phenotype of a reduced serum dependence or, alternatively, that due to the increased chromosomal content of these revertants extra copies of some normal cellular gene can suppress the transformed phenotype (33, 34). For some of these revertants, the presumptive cellular alteration affects the serum require-

ment without affecting the ability to grow without anchorage to a solid substrate (34).

Previously, evidence was provided that intracellular cyclic AMP concentrations in mouse fibroblasts responded specifically to growth regulation by serum and not to density-dependent growth regulation (19). Serum-sensitive revertants regained the property of responding to serum deprivation by a sharp rise in intracellular cyclic AMP levels. In contrast to this linkage of cyclic AMP response to serum dependence, serum-sensitive revertants did not regain the normal response to serum deprivation or to a reduction in the transport rates of either glucose or amino acids (9). This finding is in agreement with other reports that cell growth is dissociable from increased nutrient uptake (32, 37). In this paper, we report that the reversion to calf serum (CS) dependence for mouse fibroblasts is linked to the reacquisition of an insulin requirement but is dissociable from the requirement for PDGF. The requirement for PDGF, in contrast, appears to be tightly associated with density-dependent growth inhibition.

MATERIALS AND METHODS

Cell lines. Swiss 3T3 cells, SV40-transformed Swiss 3T3 cells (clone SV101), and the revertants derived from SV101 were maintained in 10% fetal calf serum-Dulbecco modified Eagle medium (FCS/DME; vol/vol) with 100 U of penicillin per ml and 100 µg of streptomycin per ml. SV101 was cloned from SV40-infected Swiss 3T3 cells (21). Revertant FLSV101 (FLSV) was derived from SV101 by 5-fluorodeoxyuridine-negative selection at high density (21). Serum revertants AvSV101-4 (Av4) and LSSV101-1 (LS1) were derived from clone SV101 by bromodeoxyuridine-negative selection at low density in a gamma globulin-depleted serum and in low (1%) serum, respectively (34). Kirsten murine sarcoma virus (Ki-MuSV)-transformed Av4 cells (Ki-Av4) were isolated from a morphologically altered, densely growing colony after Ki-MuSV infection of Av4

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(35). NIH-3T3 cells were obtained from M. Wigler, and NIH-DT cells, a Ki-MuSV-transformed derivative of NIH-3T3 cells isolated in the laboratory of E. Scolnick, were obtained from V. Vogt. All cell lines were found to be free of mycoplasma contamination by the fluorescence method of Chen (4).

The published properties of these cell lines (anchorage requirement, serum requirement, and saturation density) were confirmed recently in this laboratory. However, the use of FCS in place of CS proved unsatisfactory for the measurement of the serum requirement. All cell lines grew significantly faster in 1% FCS than they did in 1% CS. The ratios of doubling times in 1% CS to 10% CS were as reported, except that previously Ki-Av4, like Av4, did not grow at all in 1% CS (35), and in recent experiments, Ki-Av4 retained a normal serum requirement, but like 3T3, grew slowly in 1% CS. Saturation densities in 10% FCS were within a 0.4-fold range of previously published values (35). Plating efficiency (PE) in Methocel (Dow Chemical Co.) with 10% FCS yielded values that were within a twofold range of published values, except that clone SV101 had a 7% PE instead of a 20% PE, Ki-Av4 had a 6% PE instead of a 20% PE, and serum revertant LS1 had a 0.01% PE instead of a 0.001% PE (35).

Growth factors and basal medium. Insulin, EGF, and fibroblast growth factor (FGF) were purchased from Collaborative Research, Inc. Human transferrin was purchased from Sigma Chemical Co. Fibronectin was purified from human plasma by gelatin-affinity chromatography essentially as described previously (12), with the modification that bound fibronectin was eluted with 1 M NaBr–10 mM sodium phosphate (pH 5.5). Fibronectin was dialyzed against phosphate-buffered saline and diluted to 0.1 µg/ml or less before filter sterilization. Filtration resulted in a considerable loss of protein; hence, the fibronectin concentration was determined after filtration. Partially purified PDGF was prepared from 100 U of outdated human platelets (New York Blood Center) by the method of Heldin et al. (14). Platelet lysate was purified sequentially by carboxymethyl cellulose-Sephadex chromatography, Blue-Sepharose chromatography, and molecular sizing on a Bio-Gel P150 column. The final steps were carried out aseptically to avoid filter sterilization. The assay for PDGF mitogenic activity consisted of measuring the additional growth of 3T3 cells in totally defined serum-free medium in the presence of insulin, EGF, and transferrin as described below. Of the partially purified PDGF, 60 ng/ml displayed mitogenic activity, corresponding to 20 ng of purified PDGF per ml (kindly provided by C. H. Heldin).

MCDB 402 medium was prepared as described previously (26) from double glass-distilled water (2) with the following modifications for use with 10% CO₂ incubators: 4.6 g of NaCl per liter, 3.0 g of NaHCO₃ per liter, and 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid). The final concentrations of three components were changed to the following: biotin, 100 ng/ml; vitamin B12, 3×10^{-7} M; and sodium silicate, 10^{-6} M. We also added either 100 µg of streptomycin per ml and 100 U of penicillin per ml or 50 µg of gentamicin per ml. We found it beneficial to make fresh stock solutions for MCDB 402 medium every 6 months. All glassware, including pipettes, were acid washed before use.

Serum-free cell culture. Our protocol utilized culture dishes that were sequentially coated with sterile 0.1% poly-D-lysine (Sigma Chemical Co.) for 10 min, washed once with phosphate-buffered saline, and treated with 1 µg of fibronectin per cm² in DME for 30 min at 37°C, followed by one wash with serum-free DME. Cells were harvested and plated in

MCDB 402 medium as follows: subconfluent dishes of cells grown in 10% serum were washed once with 0.5 mM EDTA (ethylenediaminetetraacetic acid)–phosphate-buffered saline and allowed to detach in a small volume of phosphate-buffered saline. The detached cells were diluted into MCDB 402 medium, centrifuged, resuspended in MCDB 402 medium, and counted with the aid of a Coulter Counter. The cells were plated with sterile polypropylene pipette tips. Plates were fed twice weekly. The growth factors used were insulin, 10 µg/ml; transferrin, 5 µg/ml; EGF, 40 ng/ml; and partially purified PDGF, 60 ng/ml. Experiments with FGF utilized 10 ng/ml in place of PDGF.

To determine the relative growth rates, cells were plated on 35- or 60-mm Nunc dishes as described in the text. Replicate plates for each condition were counted on the indicated days. The average number of doublings in the restrictive condition was divided by the average number of doublings in the nonrestrictive condition to determine the relative growth rate. In some cases, the growth factor whose mitogenic response was being measured was not added until day 1.

The standard errors of relative measurements were determined by the relationship $S_{rm}^2/X_{rm}^2 = S_r^2/X_r^2 + S_{nr}^2/X_{nr}^2$ (1), where X_{rm} , X_r , and X_{nr} are the average values of the relative measurement, growth in the restrictive condition, and growth in the nonrestrictive condition, respectively, and S_{rm} , S_r , and S_{nr} are the corresponding standard errors. Analysis of variance was used to determine the significance of the difference in relative measurements between two cell lines.

RESULTS

Growth of Swiss 3T3 cells in defined medium. We have modified the serum-free formula for Swiss 3T3 cells developed by Serrero and co-workers (25). The protocol we employed involves the treatment of culture dishes with poly-D-lysine and fibronectin, the replacement of DME with an enriched modification of DME termed MCDB 402 medium (26), and the addition of the following growth factors: PDGF, insulin, EGF, and transferrin. When Swiss 3T3 cells were plated in this serum-free medium, the growth rate was comparable to that obtained with 10% serum (doubling time, 22 h). The requirement for PDGF and insulin will be discussed in detail below. However, EGF and transferrin could be deleted from the medium without any significant change in growth rate (data not shown). It is likely that the addition of freshly dissolved ferrous sulfate to the basal medium obviates the requirement for transferrin, as has been shown in several other systems (26). The lack of requirement for EGF was somewhat surprising in view of the work of Wharton et al. (38), who showed that EGF, in addition to PDGF and insulin, was required for reentry into the cell cycle of confluent 3T3 cells. One possible explanation for our results is that our cells were not in stationary phase when placed into the serum-free medium and that EGF is only required during the transition from stationary phase to active proliferation.

Reduced PDGF requirement in SV40-transformed 3T3 cells. Swiss 3T3 cells displayed a strong requirement for PDGF. In its absence, the growth rate was about one half that with PDGF (Table 1). In sharp contrast, SV40-transformed 3T3 cells (clone SV101) did not require PDGF at all (Table 1). This finding is in agreement with earlier work with platelet-poor plasma that indicated that SV40-transformed 3T3 cells did not require PDGF (24). Eight other independent SV40-transformed 3T3 cell lines also displayed a significantly reduced requirement for PDGF (data not shown).

TABLE 1. PDGF requirement of mouse fibroblasts^a

Cell line	Growth control phenotypes			Relative growth rate (-PDGF/+PDGF)
	Serum	Density	Anchorage	
Swiss 3T3	N	N	N	0.52 ± 0.03
SV101	T	T	T	1.04 ± 0.06
FLSV	T	N	N	0.22 ± 0.05
LS1	N	N	N	0.62 ± 0.03
Av4	N	N	T	0.48 ± 0.07
Ki-Av4	N	T	T	1.01 ± 0.05

^a The PDGF requirement was determined by plating 2×10^4 cells per 35-mm dish on poly-D-lysine-fibronectin-coated dishes containing 2 ml of MCDB 402 medium plus (per ml): 10 μ g of insulin, 5 μ g of transferrin, and 40 ng of EGF. Twenty-four hours later, duplicate plates were counted, and 60 ng of partially purified PDGF per ml was added to half of the remaining dishes. Cell counts were determined 4 days later. N, Normal; T, transformed.

We examined the PDGF requirements of three partial revertants of clone SV101. All three of these revertants were previously found to express T-antigens (both 96,000 and 17,000 molecular weight) and to yield wild-type SV40 upon fusion with monkey cells (3, 35). They have regained some but not necessarily all growth properties of Swiss 3T3 cells (Table 1). All three revertants had reacquired a stronger PDGF requirement (Table 1). It is of interest that FLSV, a flat revertant selected for survival at high densities in the presence of the killing agent 5-fluorodeoxyuridine, had reacquired a strong PDGF requirement but retained the low serum requirement of clone SV101 (Table 1). Thus, it does not appear that the requirement for high serum concentrations, as measured by comparison of growth rates in 10 versus 1% CS, correlates with the requirement for PDGF. Besides PDGF dependence, the phenotype that is shared by all three of the revertants, but not by clone SV101, is density-dependent growth inhibition (Table 1). Retransformation of the revertant Av4 with Ki-MuSV reduced both PDGF dependence and density-dependent growth inhibition (Table 1). This tentative relationship between density-dependent growth inhibition and a requirement for PDGF may be fortuitous, but the following additional lines of evidence suggest that it is substantial: (i) Vogel et al. (36) found that PDGF, and not plasma, which contains insulin-like growth factors, was the major determinant of the final saturation density achieved by 3T3 cells; (ii) there was a significant correlation between the degree of PDGF independence and the ability to grow to high saturation densities in the eight independent SV40 transformants (data not shown); and (iii) we have found that the requirement for PDGF by 3T3 cells is more stringent at higher cell densities (Table 2).

PDGF affects the insulin requirement of 3T3 cells. The final step in our development of the serum-free medium for 3T3 cells was the replacement of PDGF for FGF. PDGF and FGF are interchangeable in many short-term assays (24, 28). However, 10 ng of PDGF per ml was more potent than 10 ng

TABLE 2. Effect of cell density on PDGF requirement

Inoculum cell density ^a	Doublings		Relative growth rate
	+PDGF	-PDGF	
1×10^5	3.4	0.4	0.11
3×10^4	3.6	1.4	0.39
1×10^4	3.3	1.7	0.52
3×10^3	2.0	1.1	0.55

^a Cells per 60-mm dish. Growth was monitored in a 4-day assay as described in Table 1, footnote a.

of FGF per ml for the growth of Swiss 3T3 cells in a 3-day growth assay (Table 3). However, PDGF severely depresses the strong requirement for insulin that we previously observed with FGF in the medium (Table 3). PDGF is known to be a potent stimulator of the secretion of somatomedin C (insulin-like growth factor I [IGF-I]) by fibroblasts (6). Thus, a more sensitive assay of the insulin requirement necessitated the use of FGF instead of PDGF.

Insulin requirement of SV40-transformed 3T3 cells and revertants. Previously, other investigators have reported that SV40-transformed cells require insulin for growth in serum-free medium (23). However, no comparison was drawn with normal cells. The strong insulin requirement of normal 3T3 cells is severely diminished in clone SV101 (Table 4). Since clone SV101 mitogenically responds to insulin, we agree that SV40-transformed 3T3 cells require insulin for optimal growth. However, when the response or requirement is compared with that of normal 3T3 cells, it is apparent that clone SV101 does not slow down its growth in response to insulin deprivation to nearly the same extent as do normal 3T3 cells (Table 4). This differential effect is also observed when PDGF is employed; however, the difference is not as dramatic (Table 4). Other SV40-transformed 3T3 cells also displayed a significant reduction in their requirement for insulin (data not shown).

The serum-sensitive revertant LS1 regained a stronger insulin requirement, paralleling its reacquisition of a normal serum requirement (Table 4). The serum-sensitive revertant Av4 also grew very inefficiently without insulin. Thus, both serum-sensitive revertants of clone SV101^a were found to have regained a 3T3-like insulin requirement.

FLSV, a flat anchorage-dependent revertant of clone SV101 that retained a low serum requirement (Table 1), displayed a minimal requirement for insulin, similar to that of clone SV101 (Table 4). This shows that the loss of the insulin requirement cosegregates with the loss of serum dependence and not with the loss of the anchorage requirement or the loss of density-dependent growth inhibition. Retransformation of revertant Av4 with Ki-MuSV affected neither its serum dependence nor its insulin requirement (Table 4).

DISCUSSION

Our results indicate that SV40 transformation can drastically alter the growth factor requirements of normal fibroblasts. Both the PDGF and insulin requirements found in normal 3T3 cells were diminished or abolished after SV40 transformation. It will be important to determine whether either or both of these diminished growth factor requirements are necessary for tumor formation. Neoplastic transformation can alter many cellular properties, but only some of them have been shown to be directly related to tumorigenicity. For example, the loss of cell surface fibronectin is not

TABLE 3. PDGF affects the insulin requirement of 3T3 cells

Condition ^a	Doublings	Relative growth rate
Insulin, EGF, FGF	1.5	0.33
EGF, FGF	0.5	
Insulin, EGF, PDGF	2.6	0.73
EGF, PDGF	1.9	

^a 3T3 cells were plated onto poly-D-lysine-fibronectin-coated 60-mm dishes containing 4 ml of MCDB 402 medium supplemented with or without (per ml): 10 μ g of insulin, 10 ng of FGF, 40 ng of EGF, or 10 ng of pure PDGF.

TABLE 4. Insulin requirement of mouse fibroblasts^a

Cell line	Inclusion of:		Relative growth rate (-Ins/+Ins)
	PDGF	FGF	
Swiss 3T3	-	+	0.30 ± 0.06
SV101	-	+	0.73 ± 0.05
FLSV	-	+	0.79 ± 0.07
LS1	-	+	0.53 ± 0.02
Av4	-	+	0.10 ± 0.04
Ki-Av4	-	+	0.19 ± 0.05
Swiss 3T3	+	-	0.71 ± 0.03
SV101	+	-	0.85 ± 0.02

^a Cells were plated at a density of 2×10^4 per 35-mm dish. The medium was supplemented with either 10 ng FGF per ml or 60 ng of partially purified PDGF per ml as indicated, plus 40 ng of EGF per ml and 5 μ g of transferrin per ml. Some plates received insulin (Ins). Duplicate plates for each condition were counted on days 1 and 5. The numbers were converted to doublings, and the values represent the relative growth rates \pm standard errors.

necessary for tumor formation (16). However, it has been well established that anchorage independence is a necessary prerequisite for tumor formation of both fibroblastic and epithelial cells (16). Recently, Perez-Rodriguez (20) reported that a reduced serum requirement, in addition to anchorage independence, was a necessary prerequisite for tumorigenicity in hamster fibroblasts.

Many transformed cells and human tumor cell lines would appear to be able to escape the requirement for specific growth factors by virtue of their secretion of growth factors homologous to either EGF or the insulin-like growth factor multiplication-stimulating activity (MSA) (8, 13). Murine sarcoma virus- and adenovirus-transformed rodent cells secrete an EGF-like growth factor that induces certain normal rat cell lines to grow in soft agar and also prevents the binding of exogenous, labeled EGF (8, 13). Secretion of homologous growth factors by SV40-transformed cells may underlie their ability to grow well in the absence of PDGF or insulin. SV40 T-antigen could conceivably stimulate the synthesis of PDGF or insulin-like growth factors at the level of RNA transcription. It is of interest that clone SV101 has been found to have a reduced level of PDGF receptors and also appears to secrete a PDGF-like peptide into culture medium (A. Vogel, personal communication). We are currently measuring PDGF receptors in the set of revertants examined in this report.

Two significant findings emerged from the selective reacquisition of growth factor requirements by revertants: (i) the loss of the PDGF requirement was directly associated with the loss of density-dependent growth inhibition, and (ii) the loss of the strong insulin requirement was directly associated with a reduced serum dependence. In light of the first finding, it has been known for some time that the final saturation density of normal fibroblasts is directly proportional to the serum concentration (15). Recently, Vogel et al. have provided evidence that PDGF is the serum component responsible for this determination of final saturation density (36). Our results are in concert with this finding and suggest that the loss of the PDGF requirement enables cells to grow to high saturation densities. Selective reacquisition of only the PDGF requirement appears to be a sufficient alteration to restore density-dependent growth inhibition to the flat revertant FLSV.

McClure (18) found that SV40-transformed 3T3 cells displayed a requirement for PDGF only when suspended in agar or when deprived of fibronectin-coated substrate. This seems to suggest that cell shape is involved in the require-

ment for PDGF. We have shown that 3T3 cells display a much stronger requirement for PDGF when plated at high cell densities, which may result from changes in cell shape as cells approach confluence.

Our finding that the loss of a stringent insulin requirement is directly related to a reduced serum dependence is in agreement with the work of Temin, who found that Rous sarcoma virus-transformed chicken fibroblasts had a reduced serum dependence (30) and that this reduced serum dependence resulted from a diminished requirement for the insulin-replaceable activity in serum (31). Partial purification of the major mitogenic activity in CS for the growth of chicken fibroblasts revealed that this activity was closely related to the insulin-like growth factor MSA (10). Other laboratories have reported a reduced insulin requirement after neoplastic transformation. Moloney sarcoma virus-transformed dog kidney epithelial cells have been reported to have a reduced requirement for insulin when compared with their normal counterparts (29). The insulin growth requirement for fibroblasts is likely to represent a requirement for somatomedin or insulin-like growth factors (7, 17). The somatomedin IGF-I totally substitutes for insulin in our growth assay (S. Powers, unpublished data). Somatomedins are thought to mediate many if not all of the growth-promoting effects of growth hormone (17). It would not be surprising if escape from this major humoral growth regulatory system by a single cell influenced its growth potential in vivo.

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