Multiple Pathways Lead to Activation of the Survival Mechanism in Quiescent BALB/c-3T3 Cells (43524)

IGOR TAMM AND TOYOKO KIKUCHI
Cell Physiology and Virology Laboratory, The Rockefeller University, New York, New York 10021

Abstract. **The survival of density-arrested quiescent murine BALB/c-3T3 cells in serum-free Dulbecco's medium requires the presence of cell growth factors or second messenger agonists. The protein synthesis inhibitor anisomycin blocks the survival-mediating action of the basic fibroblast growth factor (bFGF) and of 12-*O*-tetradecanoylphorbol 13-acetate (TPA), but has little or no effect on the protective action of platelet-derived growth factor or 8-bromoadenosine 3′:5′-cyclic monophosphate (Br-cAMP). The effects of anisomycin are concentration dependent in the range from 2.5 to 25 μM and show that the survival-enhancing abilities of bFGF and TPA critically require protein synthesis, whereas those of platelet-derived growth factor and Br-cAMP do not. The survival-mediating action of bFGF and TPA can also be blocked with the RNA synthesis inhibitors actinomycin D and 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB), whereas the action of platelet-derived growth factor and Br-cAMP is largely resistant. Results on the time course of action of DRB, a selective inhibitor of the synthesis of mRNA precursor molecules, suggest that the RNA required for the survival-enhancing action of bFGF and TPA is present in cells at the time of serum withdrawal and addition of the survival factor and has a half-life greater than 3 h. The new evidence provides further support for the hypothesis that protection of serum-deprived, density-arrested BALB/c-3T3 fibroblasts against death can be achieved either via pathways that entail the synthesis of protein and RNA (e.g., via diacylglycerol-protein kinase C) or via pathways that do not involve *de novo* biosynthesis (e.g., via cAMP-protein kinase A).**
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The survival of density-inhibited quiescent BALB/c-3T3 fibroblasts in the absence of serum requires the presence of growth factors (1–3). In their absence, most of the cells die with approximately first-order kinetics over the course of 5 hr from serum withdrawal (2, 3). The death process is characterized by contraction of the cells and cytoplasmic blebbing at the plasma membrane. This mode of death is unusual in that it is not associated with the commonly recognized signs of either necrosis (swelling before rupture) or apoptosis (nuclear condensation and lobulation before cleavage of nuclear DNA and cell fragmentation) (3).

Of special note are the observations that second messenger agonists can replace growth factors in protecting density-inhibited cells against death (2, 3). We have reported that whereas the survival-enhancing activity of some growth factors and second messenger agonists is dependent on protein and RNA synthesis, that of others is not or is less so (1–3). Our present hypothesis is that the cellular death process in serum-deprived, density-arrested BALB/c-3T3 cells is initiated by an as yet unidentified event whose occurrence is prevented by the presence of appropriate growth factors and/or cytokines acting as survival factors (4). Different factors may use distinct and sometimes multiple pathways that presumably converge on a mechanism critical for the maintenance of cellular integrity (4).

In the present studies, we have investigated the effects of the duration of treatment of cells with 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB), an inhibitor of messenger precursor RNA synthesis, on the survival of serum-deprived quiescent BALB/c-3T3 cells mediated by platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), 8-bromoadenosine 3′:5′-cyclic monophosphate (Br-cAMP), or 12-*O*-tetradecanoylphorbol 13-acetate (TPA). The results of

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short-term treatment with actinomycin D are also presented. In addition, we report on the survival-blocking effects of anisomycin, an inhibitor of protein synthesis. Our results provide evidence for several distinctive pathways, the activation of which averts the cell death process.

Materials and Methods

Materials. PDGF was obtained from Collaborative Research, Inc. (Bedford, MA), bFGF was from Biosource International (Westlake Village, CA), and TPA, Br-cAMP, and anisomycin were purchased from Sigma Chemical Co. (St. Louis, MO). Anisomycin was dissolved and stored as described by Tiwari *et al.* (5). Actinomycin D and DRB were obtained from Merck, Sharp & Dohme (Rahway, NJ).

Cell Culture and Survival Assays. BALB/c-3T3 cells were obtained from Dr. W. J. Pledger, Vanderbilt University, TN, and stock cultures were passaged as described previously (1). Cultures for experiments were seeded at a density of 3500 cells/cm^2 in 96-well plates. The growth medium was Eagle's medium modified by Dulbecco and supplemented with 10% bovine serum (BS) (Hyclone, Logan, UT). After planting, cultures were incubated for 3 days at 37°C. The serum-containing medium was changed and the cultures were used after an additional 4 days of incubation, at which time they were quiescent. Cultures were washed once with serum-free medium prior to the addition of survival factors with or without inhibitors. After varying periods, cell survival was assayed by neutral red uptake as described previously (1, 2, 6) or by Coulter count of trypsinized samples.

We have shown previously that determination of the survival-enhancing activity of cell growth factors by neutral red uptake measurement and cell enumeration in a Coulter counter gives equivalent results (2). In the present study, this was confirmed with the second messenger agonists Br-cAMP and TPA. Cells that are morphologically recognizable as intact (2, 3) exclude erythrocin B (2). The cell count after 20 hr of incubation in 10% BS is unchanged from the 0-hr cell count. A full discussion of the rationale behind the use of neutral red uptake measurements in cell survival studies on quiescent BALB/c-3T3 cells has been presented previously (2).

Results

Effects of Inhibition of RNA Synthesis on Cell Survival. Table I shows that in 3-hr experiments, actinomycin D (5 μg/ml) treatment had little if any effect on cell survival mediated by PDGF or Br-cAMP, but it markedly decreased survival in medium supplemented with bFGF or TPA. These results suggest that rapidly turning over RNA species synthesized either constitutively or in response to PDGF or Br-cAMP are not involved in the pathway(s) by which these two agents prevent death of quiescent cells. In sharp contrast, RNA synthesis is required for the survival-promoting action of bFGF and TPA. It should be noted that actinomycin D cannot be used to prove unambiguously that an mRNA has a short half-life (7).

In unsupplemented Dulbecco's medium, actinomycin D decreased survival further, indicating that not only was RNA synthesis not required for the death process to occur, but that suppression of RNA synthesis increased the probability of the occurrence of cell death.

The results obtained with DRB (40 or 60 *μM*), a selective inhibitor of messenger-precursor RNA synthesis, are generally consistent with those obtained with actinomycin D used at high concentration (5 μg/ml), at which it inhibits messenger precursor RNA synthesis in addition to ribosomal RNA synthesis. Table II shows that in 5-hr experiments, DRB had little effect on the survival-enhancing actions of PDGF and Br-cAMP, whereas it substantially decreased the protective effects of bFGF and TPA (see also Ref. 2).

It is noteworthy, however, that in 3-hr experiments, 40 or 60 *μM* DRB had little effect on the survival mediated by any of the four agents examined (see also Ref. 2). Thus, whereas the bFGF- and TPA-mediated pathways that are operating in cell survival can be targeted with actinomycin D within 3 hr of serum deprivation, they cannot be so targeted with DRB. Our findings suggest that the mRNA species involved in the bFGF- or TPA-mediated survival may be present in the cells at the time of bFGF or TPA addition to serum-free medium, but that they become depleted in DRB-treated cells over the course of several hours, and in actinomycin D treated cells much more rapidly so.

The results of 20-hr treatment with DRB reveal a number of new aspects of the cell survival-promoting actions of PDGF, bFGF, Br-cAMP, and TPA. It is important to evaluate the effects of the survival factors in the context of the fate of DRB-treated cells incubated either in 10% BS-containing medium or in the absence of serum. Table III shows that DRB in serum-free medium increases cell deaths in a concentration-dependent manner. In sharp contrast, presence of serum essentially completely preserves cells regardless of whether DRB is present or absent. Serum not only provides factors necessary for cell survival when DRB is not present, but it also provides factors that block the cell-killing action of DRB.

Although PDGF affords marked protection in the presence of DRB in short-term experiments, the protection decreases with time as DRB treatment is prolonged to 20 hr (see also Ref. 3). With bFGF and TPA, the time-dependent loss of protective activity is faster than with PDGF.

The results with Br-cAMP are remarkable in that this agonist, like serum, is capable of completely block-

Table I. Effects of Actinomycin D on Growth Factor- and Second Messenger Agonist-Stimulated Short-Term (3-hr) Cell Survival

Agents and concentrations	Cell survival[a]		
	No actinomycin D	Actinomycin D (5 μg/ml)	$\frac{\text{Actinomycin D}}{\text{No actinomycin D}} \times 100$[b]
BS (10%)	100	74	74 ± 4
None	24	12	50 ± 6
PDGF (5 ng/ml)	65	59	91 ± 11
bFGF (20 ng/ml)	77	24	31 ± 13
Br-cAMP (1.45 m*M*)	59	57	97 ± 7
TPA (100 n*M*)	55	30	55 ± 21

[a] Cell survival is expressed as the percentage of 10% BS control. Neutral red uptake in 10% BS controls was 0.810 O.D. unit. Cultures were pretreated with actinomycin for 30 min prior to wash with serum-free medium and treatment with growth factors or agonists with or without actinomycin D. Shown are mean results of four experiments in each of which all variables were examined.
[b] Actinomycin D is expressed as mean percentage of no actinomycin D ± SD. Standard deviations were derived using survival values calculated as ratios of survival in actinomycin D/survival in the absence of actinomycin D, each expressed as percentage of 10% BS control, from the individual experiments.

Table II. Effects of DRB on Growth Factor- and Second Messenger Agonist-Stimulated Short-Term (3-hr or 5-hr) Cell Survival

Agents and concentrations	Cell survival[a]		
	No DRB	DRB as percentage of no DRB[b]	
		40 μ*M*	60 μ*M*
3 Hr			
BS (10%)	100	87 ± 10	92 ± 7
None	19 ± 14	78 ± 31	83 ± 23
PDGF (5 ng/ml)	63 ± 10	101 ± 18	94 ± 10
bFGF (20 ng/ml)	68 ± 17	86 ± 16	84 ± 22
Br-cAMP (1.45 m*M*)	60 ± 13	111 ± 11	86 ± 13
TPA (100 n*M*)	62 ± 15	108 ± 15	99 ± 19
5 Hr			
BS (10%)	100	81 ± 7	79 ± 7
None	14 ± 7	69 ± 11	53 ± 22
PDGF (5 ng/ml)	60 ± 10	81 ± 14	80 ± 11
bFGF (20 ng/ml)	71 ± 16	64 ± 16	54 ± 17
Br-cAMP (1.45 m*M*)	62 ± 12	90 ± 13	90 ± 17
TPA (100 n*M*)	65 ± 9	74 ± 26	60 ± 29

[a] Data are expressed as percentage of 10% BS control. Neutral red uptake in 10% BS controls for 3-hr and 5-hr incubation was 0.899 and 0.937 O.D. unit, respectively. Shown are mean results ± SD of 13 experiments; not all variables were examined in all 13 experiments.
[b] Based on values normalized with respect to 10% BS controls in the different experiments. *t* Tests were performed to evaluate the significance of the differences between the survival-enhancing effects of the agents at 5 hr with the following results, expressed as *P*, %: (a) for 40 μ*M* DRB: PDGF versus bFGF, < 0.1; Br-cAMP versus TPA, 0.1–1.0; and b) for 60 μ*M* DRB: PDGF versus bFGF, 1–2; Br-cAMP versus TPA, 2. Differences between PDGF and Br-cAMP and between bFGF and TPA were not statistically significant (*P* > 10%) either at 40 or 60 μ*M* DRB.

Table III. Effects of DRB on Growth Factor- and Second Messenger Agonist-Stimulated Long-Term (20-hr) Cell Survival

Agents and concentrations	Cell survival[a]		
	No DRB	DRB as percentage of no DRB[b]	
		40 μ*M*	60 μ*M*
BS (10%)	100	99 ± 4	93 ± 7
None	12 ± 3	62 ± 10	38 ± 7
PDGF (5 ng/ml)	64 ± 5	72 ± 8	49 ± 4
bFGF (20 ng/ml)	88 ± 8	70 ± 4	43 ± 4
Br-cAMP (1.45 m*M*)	54 ± 9	124 ± 19	138 ± 23
TPA (100 n*M*)	48 ± 5	74 ± 13	51 ± 14

[a] Data are expressed as percentage of 10% BS control. Neutral red uptake in 10% BS controls was 0.832 O.D. unit. Shown are mean results ± SD of four experiments.
[b] Based on values normalized with respect to 10% BS controls in the different experiments.

ing the killing action of DRB employed for 20 hr. However, Br-cAMP, even at maximally effective concentrations (2), falls short of serum in its ability to mediate survival of quiescent cells not treated with DRB.

Effects of Inhibition of Protein Synthesis on Cell Survival. We extended our earlier studies with cycloheximide (1, 3) to a detailed examination of the effects of anisomycin on the survival of quiescent BALB/c-3T3 cells. Figure 1 shows that anisomycin had little if any effect on the survival-promoting activity of PDGF, whereas it markedly decreased the activity of bFGF. It decreased the protective activity of bFGF in a concentration-dependent manner, with 25 μ*M* anisomycin causing a 90% reduction in cell survival. There was a similar striking difference between anisomycin effects on cell survival mediated via Br-cAMP versus TPA. Anisomycin slightly decreased Br-cAMP-mediated cell survival, but it markedly decreased survival mediated by TPA.

Figure 2 documents the fact that anisomycin had little effect on serum-mediated cell survival. It also shows that in the concentration range from 2.5 to 25

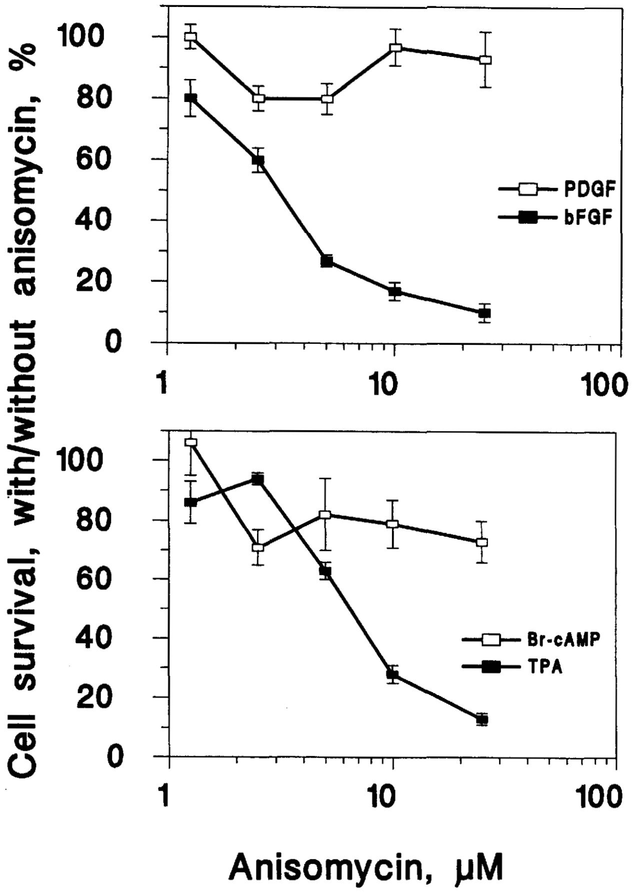

Figure 1. Relationship between anisomycin concentration and effects on PDGF-, bFGF-, Br-cAMP-, or TPA-mediated survival of BALB/c-3T3 cells. Three-hour survival assays with density-arrested quiescent cells were carried out as described in Materials and Methods. Mean results of six experiments. Not all variables were examined in all experiments. Cell survival in the absence of anisomycin, expressed as a mean percentage ± SE of that in the 10% BS controls, was as follows: PDGF (5 ng/ml), 62 ± 5.0%; bFGF (20 ng/ml), 71 ± 10%; Br-cAMP (1.45 m*M*), 56 ± 7.0%; TPA (100 n*M*), 58 ± 7.0%.

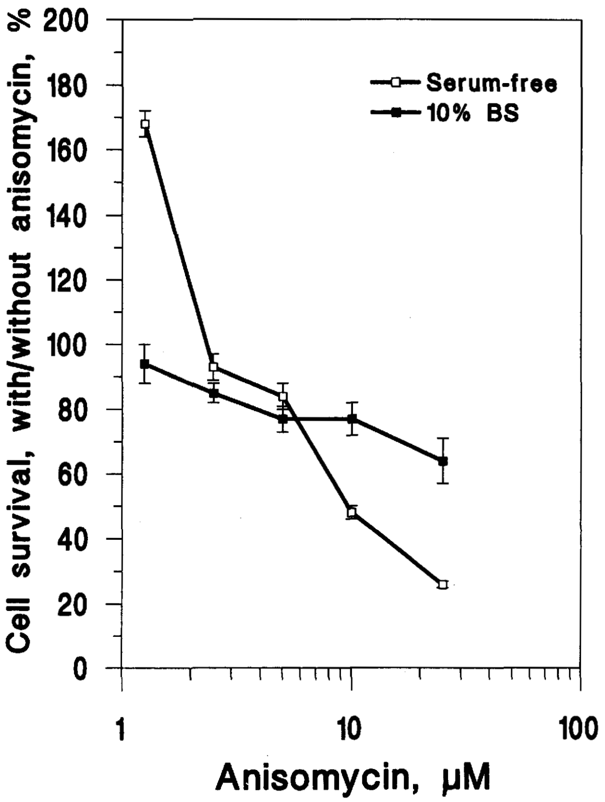

Figure 2. Effects of anisomycin on cell survival in serum-free or 10% BS-containing medium. The data shown are derived from the same experiments as those summarized in Figure 1. The mean (± SE) neutral red uptake in 10% BS controls was 0.755 ± 0.062 O.D. unit and in serum-free controls it was 0.140 ± 0.047 O.D. unit or 19% of that in 10% BS controls.

μM, anisomycin decreased further the already low survival of serum-deprived cells. Surprisingly, 1.25 *μM* anisomycin appeared to increase the survival of serum-deprived cells. The basis of this paradoxical result is not clear. It suggests that a slight reduction in the level of protein synthesis below that in serum-deprived cells favors survival or that proteins exist in these cells that play a role in cell death and whose synthesis is highly anisomycin sensitive.

Overall, the results of experiments with inhibitors of RNA and protein synthesis are consistent and establish a fundamental difference in the pathways activated by PDGF and Br-cAMP versus bFGF and TPA that mediate survival of serum-deprived cells.

Discussion

The present results and those obtained previously (1–3) establish that quiescent density-inhibited BALB/c-3T3 cells can be protected against death due to serum deprivation by activation of pathways that either involve or do not involve *de novo* protein synthesis. Experiments with inhibitors indicate that the protective effects of TPA and bFGF are closely tied to the ability of the cell to synthesize protein and RNA. The requirement for protein synthesis is demonstrable as early as 1 hr after deprivation of cells of growth factors and becomes fully expressed within 2–3 hr (3). Growth factors and tumor promoters induce a large number of proteins which include transcription factors, structural protein, enzymes, cytokines, and proteins of as yet unknown function (reviewed in Ref. 8). Among candidate proteins that may play a role in TPA- or bFGF-mediated cell survival, the product of the *N10/nur77/NGFI-B/TIS1* gene should be noted as this gene is induced in 3T3 cells by TPA and FGF (9). The protein product is nuclear and is a member of the superfamily of ligand-binding transcription factors that includes the steroid and thyroid receptors (10). In sharp contrast, the cell survival-enhancing action of Br-cAMP and PDGF is not dependent on protein synthesis. Br-cAMP or PDGF may more directly affect one or more metabolic pathways of critical importance for cell survival.

Although Br-cAMP and PDGF can protect cells against death in a protein synthesis-independent manner, the present evidence still leaves open the possibility that under conditions when RNA and protein synthesis are not blocked, these factors may exert their protective action, at least in part, through a protein and RNA synthesis-dependent pathway.

The results that we have obtained with two inhibitors of RNA synthesis, i.e., DRB and actinomycin D, are consistent. However, it is apparent that actinomycin D blocked the cell survival-enhancing action of bFGF and TPA much faster than did DRB, in spite of the fact that DRB inhibits messenger precursor RNA synthesis very rapidly after addition to the culture medium (11). Some inhibition of synthesis can be detected within 1 min and becomes maximal between 5 and 15 min. Actinomycin D effects on cell metabolism are, however, broader than those of DRB, which may account for the difference in the time required to show an effect.

The effectiveness of DRB and actinomycin D as transcription inhibitors in murine fibroblasts has been demonstrated (12). Northern blot analysis of cytoplasmic RNA from quiescent Swiss 3T3 cells has shown that 78 μM DRB and 5 μg/ml of actinomycin D both block by approximately 90% the induction of two growth-factor-inducible genes by serum. The expression of these genes peaks 5 hr after serum induction and is also suppressed by 10 μg/ml of cycloheximide and 100 μg/ml of anisomycin (12). The dose-dependence curve for DRB is steep in the 5–15 μM range and reaches a plateau beyond the 60 μM concentration (13, 14). As is well known, actinomycin D is effective in inhibiting rRNA synthesis at 0.5 μg/ml and messenger RNA synthesis at 5 μg/ml.

Based on our results with DRB, it appears that mRNA required for growth factor- or second messenger-mediated survival of cells may be present in quiescent cells at the time of the initial medium change and the addition of a factor or agonist, but that such mRNA decay with time.

The results obtained with anisomycin strikingly illustrate the critical requirement for protein synthesis in the bFGF- or TPA-mediated cell survival and lack of such a requirement in PDGF or Br-cAMP action. The dose-response curves (cf., Fig. 1) indicate that bFGF action is approximately twice as sensitive to inhibition of protein synthesis as is TPA action. Results of experiments with actinomycin D (Table I) and DRB (Tables II and III) are consistent with the anisomycin findings.

Inhibitors of RNA and protein synthesis have a lethal effect on quiescent serum-deprived BALB/c-3T3 cells, which increases with time of treatment as shown by present and earlier results (1–3). The remarkable finding is that the lethal effect of DRB can be blocked not only by 10% BS, but also by 1.45 mM Br-cAMP. This conclusion is suggested by the findings summarized in Table III. Overall, in the 20-hr experiments, Br-cAMP is ~50% as effective as 10% BS in mediating the survival of the quiescent cells. However, when the results are expressed as the survival ratio, with DRB to without DRB, Br-cAMP is ≥100% effective. Thus, Br-cAMP does not permit any deaths to occur in the presence of DRB that would not occur in its absence.

This is not the case with TPA. TPA (100 nM) is also ~50% effective in keeping quiescent cells alive for 20 hr in serum-free medium, but in contrast to the findings with Br-cAMP, DRB decreases the survival of TPA-treated cells. It is likely that DRB acts by suppressing the synthesis of mRNA species that are required for the expression of the survival-enhancing effect of TPA; however, other mechanisms for the lethal effect of DRB cannot be excluded (3).

The DNA enhancer elements responsive to cAMP-protein kinase A and diacylglycerol/phorbol ester-protein kinase C signaling pathways consist of closely related core motifs. The octamer sequence TGACGTCA is the cAMP-responsive enhancer sequence, whereas the heptamer TGAGTCA is the TPA (phorbol ester)-responsive enhancer (TRE) (15–18). The cAMP-responsive enhancer site is recognized by a family of cAMP-responsive enhancer-binding proteins, which are implicated in cAMP-induced alterations in transcription (reviewed in Ref. 19). The TRE site is also recognized by a group of proteins, including the products of the c-*fos* and c-*jun* families of proto-oncogenes (reviewed in Ref. 20). The JUN proteins bind to the TRE predominantly as heterodimers with the FOS and FOS-related proteins (20). These proteins are induced by mitogenic and differentiation-inducing stimuli (21). The gene(s) whose expression is required for the cell survival-enhancing action of TPA and bFGF may be activated via TRE, but not necessarily so. The identification of the protein(s) required for bFGF- or TPA-mediated cell survival would be of great interest.

In many cells, there appears to be extensive cross-talk between the protein kinase C and the protein kinase A pathways (22). However, there is at present no evidence of cross-talk between these two major signal transduction pathways in BALB/c-3T3 cells. In these cells, the TPA- and the cAMP-stimulated pathways mediate interleukin 1 receptor expression independent of each other (23). It remains to be demonstrated whether induction of synthesis of interleukin 1 receptor RNA by TPA is dependent on *de novo* protein synthesis in BALB/c-3T3 cells and whether induction by Br-cAMP may not be.

Recent studies in other systems have produced new evidence that a given factor can act via multiple signal transduction pathways. For example, nerve growth factor (NGF) appears to employ at least three distinct pathways in PC12 cells to induce the primary response genes c-*fos*, c-*jun*, TIS1, TIS8, and TIS11, as shown by inhibitor studies with 2-aminopurine and 6-thioguanine (24). These early genes have different properties and are activated by NGF with different kinetics. It has been suggested that rapid activation of the tyrosine kinase gp140^{prototrk} response to NGF binding (25, 26) could be the single initial step in the induction of these early genes; however, the NGF mechanism appears thereafter to diverge into at least three separable pathways, each of which regulates early gene transcripts (24). Conversely, evidence has been obtained that different agents can utilize independent signaling pathways to activate the same gene (24, 27).

A possible interpretation of these complex findings may be that maintenance of homeostasis requires very fine tuning of a wide range of chemical processes in the cell under a wide variety of changing conditions and that the multiplicity of signal transduction pathways has provided the evolutionary answer to this requirement.

It has been demonstrated for certain cell types, such as neurons (28–33), that growth factors play a role in regulating cell survival in the living organism during embryologic development and after lesion. There is not always a complete correspondence between *in vivo* and *in vitro* findings, as illustrated by the fact that the ciliary neurotrophic factor promotes the survival of ciliary sensory and sympathetic neurons *in vitro* but not *in vivo*, although ciliary neurotrophic factor does promote the survival of spinal motoneurons both *in vitro* and *in vivo* (33). The regulation of the number of fibroblasts through limitation of cell viability remains to be investigated in the organism.

Our evidence shows that there is a redundancy in signal transduction pathways whose activation enables the quiescent density-arrested BALB/c-3T3 mouse fibroblast to survive, and that different growth factors may use different pathways. The fundamental biological fact is that such quiescent cells require exogenous growth factors for survival. The autonomy of transformed cells is illustrated by the fact that neither simian virus 40 (34) nor spontaneously transformed BALB/c-3T3 cells require exogenous growth factors for survival (1). Whether the survival of the transformed cells is mediated via autocrine factors or by some other mechanism is not clear.

The view that emerges from studies of growth factors and cytokines as survival factors for cells in culture is that such factors provide a regulatory environment that plays a key role in cell survival, and that, as would be expected, signal transduction is required for the operation of the system (1–4, 34–44).

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