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Platelet-derived growth factor-modulated proteins: Constitutive synthesis by a transformed cell line

(competence/fibroblast growth factor/polypeptide hormones/BALB/c-3T3 mouse cells)

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ABSTRACT **Platelet-derived growth factor (PDGF) initiates replication of density-arrested BALB/c-3T3 mouse cells by rendering them "competent" to respond to factors contained in plasma. Treatment of quiescent cells with PDGF rapidly stimulates the preferential synthesis of several cytoplasmic proteins (molecular weights 29,000 to 70,000). Four of these proteins were noted within 1.5 hr of PDGF addition and one (pI) within 40 min. Inhibitors of RNA synthesis prevented the synthesis of these proteins. Both the synthesis of pI and the stimulation of DNA synthesis displayed a similar dose response to PDGF concentration. Pituitary fibroblast growth factor, which also induces competence, stimulated pI and pII synthesis. Plasma, epidermal growth factor, or insulin, which do not induce competence, did not stimulate selective synthesis of these proteins. A transformed variant of BALB/c-3T3 cells, which has retained the growth requirement for plasma factors but lost the requirement for PDGF, synthesizes these PDGF-modulated proteins constitutively.**

The platelet-derived growth factor (PDGF) (1, 2) is a heat-stable cationic polypeptide which has been purified to homogeneity (3, 4). It induces the first event in the growth response of density-arrested BALB/c-3T3 mouse cells by rendering them "competent" to respond to a second set of growth factors contained in platelet-poor plasma (PPP) (5–6). Competence persists for many hours after PDGF removal (5–6); subsequent addition of plasma causes the cells to progress through G_0/G_1 and into S phase of growth. Plasma can be replaced by two growth factors: epidermal growth factor (EGF) and somatomedin (or insulin) (7, 8). Although PDGF, EGF, or somatomedin alone induces a weak growth response in our BALB/c-3T3 cells, in combination they function synergistically (at 1 nM) to stimulate the majority of cells to synthesize DNA.

Some information is available regarding the action of EGF and somatomedins. Although binding and internalization occur rapidly (9–11), continuous treatment is required for DNA synthesis (9, 12). The growth-stimulatory action of these factors on PDGF-treated BALB/c-3T3 cells or a human glial cell line may be related to nutrient supply (13, 14).

Little is known regarding the action of PDGF. This hormone induces a pleotypic response but nutrients are not required for its action (13). It appears to regulate cellular receptors for EGF and somatomedin (11, 15). PDGF may have a different mechanism of action than other growth factors because a brief treatment is sufficient to induce a growth response (5, 16). Furthermore, unlike EGF or insulin, it causes transient centriole deciliation in density-arrested BALB/c-3T3 cells (17).

Because little is known about PDGF function, our laboratories have examined the early molecular events in the growth response. In a companion article (18), Smith and Stiles demonstrate that competence can be transferred from PDGF-treated donor BALB/c-3T3 cells (or their cytoplasts) to untreated recipients by using fusion techniques. Treatment of donors with inhibitors of RNA synthesis prevents acquisition of this cytoplasmic signal. We now show that PDGF selectively promotes rapid synthesis of a family of cytoplasmic proteins within quiescent BALB/c-3T3 cells; this response is blocked by inhibitors of RNA synthesis. A functional association between these proteins and the PDGF-induced competent state is further documented by several observations, including the constitutive synthesis of the proteins by a variant BALB/c-3T3 cell line that does not require PDGF for growth.

MATERIALS AND METHODS

BALB/c-3T3 (clone A31) or ST3T3 (clone 2) cells were grown in Dulbecco's modified Eagle's medium (DME medium) (5). Experiments were done on confluent cells 3–4 days after a medium change. Confluent cells were transferred to incorporation medium (DME medium containing 2.5% of the usual methionine concentration) with or without purified growth factors or plasma; at various times, 30–50 μCi of [^{35}S]methionine (Amersham) per ml was added for 10–20 min (1 Ci = 3.7×10^{10} becquerels). The cells were washed, removed with a rubber policeman, collected by centrifugation, and suspended in 100 μl of reticulocyte saline buffer (0.01 M NaCl/0.01 M Tris·HCl/1.5 mM $MgCl_2$ pH 7.4) containing 1% Nonidet P-40 (Sigma); nuclei were removed by centrifugation. PDGF-modulated proteins (termed cytoplasmic proteins) were found in the nonnuclear fraction containing cytoplasm and membranes. $NaDodSO_4$ and 2-mercaptoethanol were each added to 1% and samples were heated to 100°C. The quantity of [^{35}S]methionine incorporated into trichloroacetic acid-precipitable material was determined and equal quantities of incorporated [^{35}S]methionine (usually 20,000 cpm) from these fractions were applied to polyacrylamide gels (19). After electrophoresis, gels were processed for fluorography (20). Autoradiograms of gels were scanned with a densitometer and the density of protein bands was determined. Two-dimensional gel electrophoresis of cytoplasmic fractions was performed by a modification of the described technique (21).

Most experiments were done with "partially purified PDGF," purified 500-fold by heat treatment and CM-Sephadex chromatography (3). "Highly purified PDGF" was obtained by further chromatography on Bio-Gel P150 (Bio-Rad) (3). Electrophoretically homogeneous PDGF was prepared by a modification

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Abbreviations: DRB, 5,6-dichloro-β-D-ribofuranosylbenzimidazole; DME medium, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; FGF, pituitary fibroblast growth factor; PDGF, platelet-derived growth factor; PPP, platelet-poor plasma.

of published procedures (3). Pituitary fibroblast growth factor (FGF) (22), EGF (23) (Collaborative Research, Waltham, MA), insulin (Sigma), and PPP (American Red Cross) (5) were also used. Actinomycin D, 5,6-dichloro-β-D-ribofuranosylbenzimidazole (DRB) (Sigma), and camptothecin (a gift of P. Vilk) were used to inhibit RNA synthesis.

RESULTS

PDGF Stimulates Rapid Selective Synthesis of Proteins. Density-inhibited BALB/c-3T3 cells were transferred to incorporation medium prewarmed to 37°C. Some cultures were treated with partially purified PDGF, and others with plasma for 1.5–4.5 hr; [^{35}S]methionine was then added. Twenty minutes later the cytoplasmic fractions were harvested, analyzed on a 6–18% exponential gradient of polyacrylamide, and processed for fluorography. Treatment with plasma for 1.5 hr did not stimulate selective protein synthesis compared to medium alone. PDGF induced preferential accumulation of four proteins within this time (Fig. 1); a fifth was detectable within 3.0–4.5 hr. Molecular weights of these proteins were approximated as: pI, 29,000; pII, 35,000; pIII, 45,000; PIV, 60,000, and pV, 70,000.

Accumulation of pI was studied in detail. Density-inhibited cells were treated with partially purified PDGF for 20–60 min and the newly synthesized proteins were analyzed on a 15% polyacrylamide gel. Preferential accumulation of pI was detected within 40 min (Fig. 2).

To learn if the PDGF-modulated proteins appear as a result of degradation, [^{35}S]methionine was added to quiescent cells in medium containing a low concentration of plasma (0.25%). The cells were washed and transferred to medium containing no [^{35}S]methionine and a 40-fold excess of unlabeled methionine. Treatment of these cultures with partially purified PDGF (25 μg/ml) for 2 hr after transfer to [^{35}S]methionine-free medium did not increase the amounts of labeled pI or pII; treatment with an optimal concentration of plasma (5%) did not decrease them (data not shown). These proteins do not accumulate as a result of modification of proteins synthesized in the absence of PDGF.

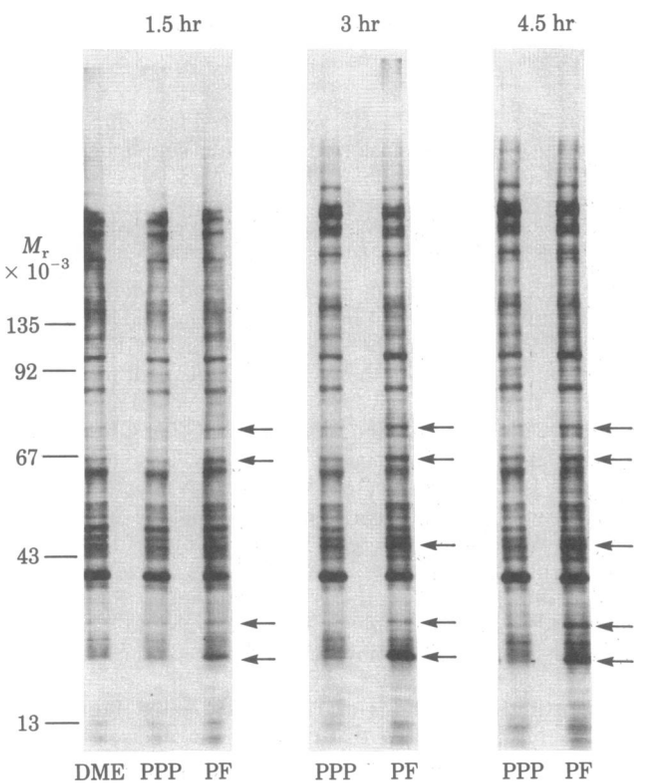

FIG. 1. PDGF stimulates selective synthesis of five cytoplasmic proteins. Density-inhibited BALB/c-3T3 cells were transferred to incorporation medium alone (DME) or medium supplemented with either dialyzed PPP at 5% or partially purified PDGF (PF) at 25 μg/ml. At the indicated times, [^{35}S]methionine was added, and 20 min later the cultures were harvested for gel electrophoresis. The arrows indicate proteins that were preferentially synthesized in response to PDGF. The positions of molecular weight markers are shown.

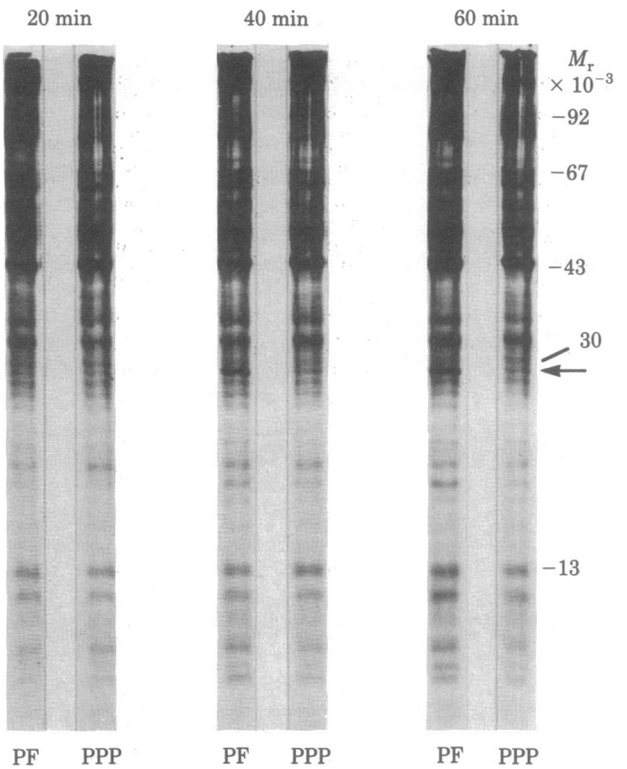

FIG. 2. PDGF stimulates preferential synthesis of pI within 40 min. Density-arrested cells were treated with partially purified PDGF (PF) at 25 μg/ml or 5% PPP for the indicated times prior to the addition of [^{35}S]methionine. Ten minutes later the cultures were harvested. The arrow indicates the position of pI.

Two-dimensional gel electrophoresis of cytoplasmic proteins indicated that pI is a single protein (Fig. 3). The density of the pI spot from the PDGF-treated cultures appeared to be at least 10-fold greater than that of plasma-treated cultures. Although pI was predominant, several other proteins were also preferentially synthesized after PDGF treatment.

RNA Synthesis Requirement. Some cultures of density-inhibited cells were treated with actinomycin D (5 μg/ml) for 30 min to inhibit >95% of RNA synthesis; they were then transferred to incorporation medium supplemented with partially purified PDGF. Other cultures received no actinomycin D; they were simply transferred to incorporation medium containing PDGF or plasma. [^{35}S]Methionine was added to the cultures 1.5–4.5 hr after addition of PDGF or plasma and the cellular proteins were separated on a 15% acrylamide gel. In the absence of actinomycin D, partially purified PDGF stimulated the preferential synthesis of pI and pII. In its presence, there was no detectable increase in pI or pII synthesis compared to plasma-supplemented cultures (Fig. 4). Inhibition of pI, pII, and pV synthesis by actinomycin D was also demonstrated by using two-dimensional gel electrophoresis to resolve cytoplasmic proteins (data not shown). Other inhibitors of RNA syn-

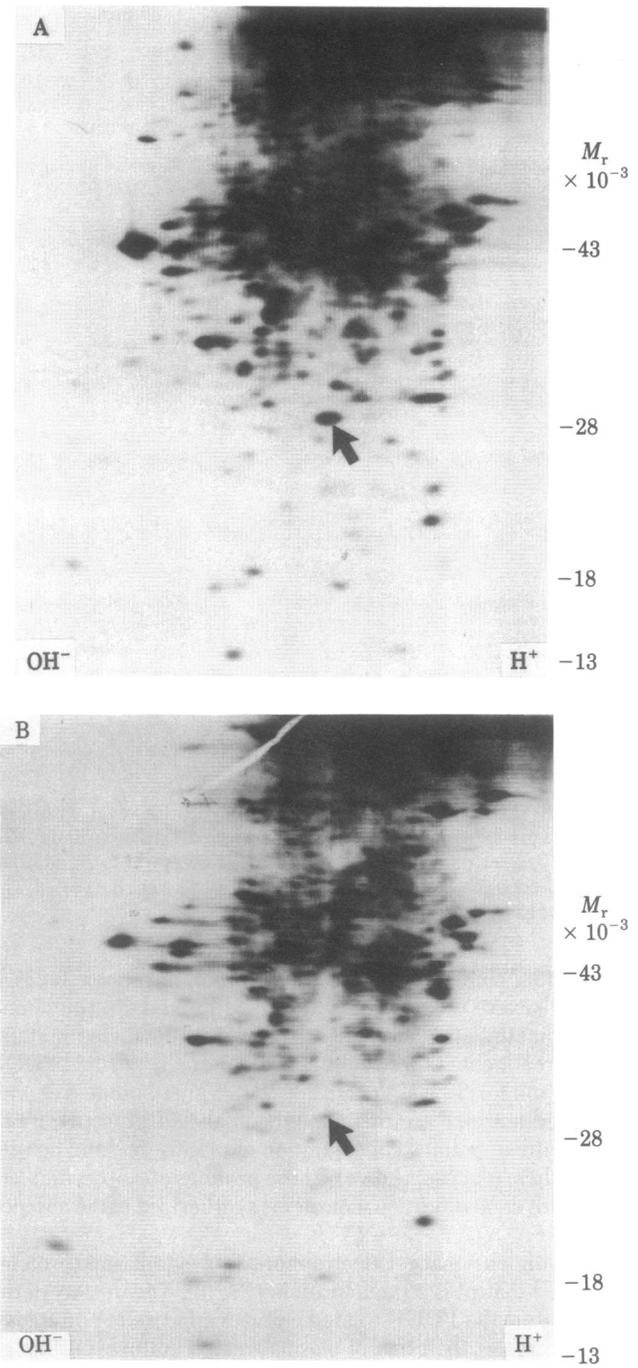

FIG. 3. Two-dimensional gel electrophoresis of PDGF- or plasma-treated cells. Density-inhibited cells were treated with either highly purified PDGF at 12 μg/ml (*A*) or 5% PPP (*B*) for 2 hr prior to [^{35}S]methionine addition. Cultures were harvested 20 min later and cytoplasmic contents (200,000 cpm per sample) were applied to a non-equilibrium pH gradient two-dimensional gel electrophoresis system. The position of pI is indicated.

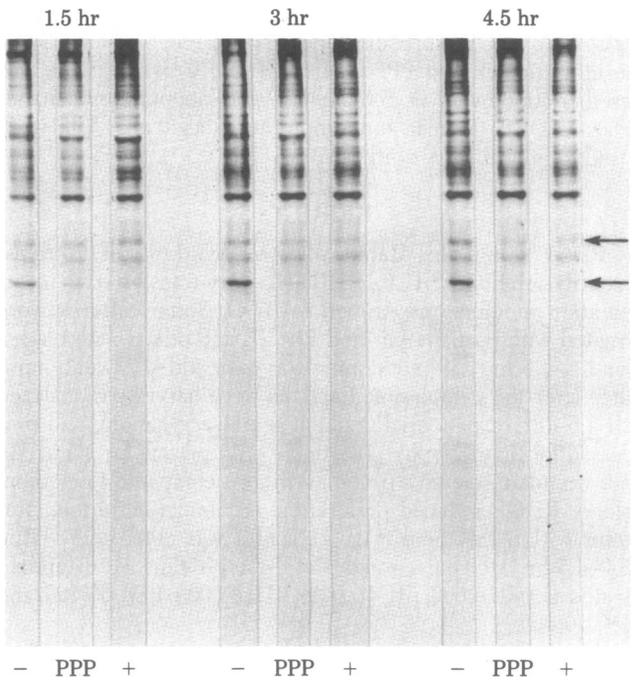

FIG. 4. Actinomycin D inhibits PDGF-stimulated pI and pII synthesis. Density-inhibited cells were treated with 25 μg/ml of partially purified PDGF (+ and −) or 5% PPP for the indicated times prior to addition of [^{35}S]methionine. The cultures were harvested 20 min later. Some PDGF-treated cultures (+) were incubated with actinomycin D at 5 μg/ml for 30 min prior to growth factor addition, whereas other PDGF-treated cultures (−) received no actinomycin D. Plasma-treated cultures received no actinomycin D. The arrows indicate pI (lower) and pII (upper).

thesis, including camptothecin (30 μg/ml) or DRB (50 μM), also prevented selective synthesis of these proteins (data not shown). Synthesis of these proteins in response to PDGF appears to require RNA synthesis.

pI Synthesis and Competence. Quiescent cells were treated with various concentrations of partially purified PDGF or with 5% PPP for 3 hr. Some cultures were labeled with [^{35}S]methionine for one-dimensional gel analysis of pI. Synthesis of pI was estimated by scanning the fluorograms with a densitometer. The amount of pI synthesized was regulated by the PDGF concentration (Fig. 5). After PDGF was removed from duplicate cultures, the cells were transferred to medium supplemented with 5% PPP and [^{3}H]thymidine. The percentage of cells that synthesized DNA (competent cells) was also a function of the PDGF concentration (Fig. 5). The dose response for DNA synthesis was similar to that for pI synthesis.

Complementation analysis has been used to define the hormonal growth factors that induce competence. PDGF or pituitary FGF induces competence but insulin, EGF, or plasma does not (7). To learn if those growth factors that induce competence stimulate selective synthesis of pI, confluent cells were treated with electrophoretically pure PDGF, FGF, EGF, insulin, plasma, or medium alone for 3 hr. PDGF and FGF stimulated a 4- to 7-fold increase in the synthesis of both pI and pII as compared to plasma-treated cultures (Table 1). EGF (50 ng/ml) stimulated a small increase in pII (1.5-fold) and no increase in pI synthesis. Higher concentration (>1 μg/ml) stimulated a small increase in pI synthesis (data not shown). Similarly, insulin had little effect. Only growth factors that induce BALB/c-3T3 cells to become competent cause a large increase in the synthesis of these proteins.

Constitutive Synthesis of PDGF-Modulated Proteins. To provide further evidence that PDGF-modulated selective protein synthesis regulates competence, we studied a variant of BALB/c-3T3 cells (termed ST3T3), which spontaneously became transformed and lost the growth requirement for PDGF. A full characterization of this cell line will be reported elsewhere. Briefly, like BALB/c-3T3 cells, the ST3T3 cells become arrested at confluence with a G_1 DNA content when grown in medium supplemented with 10% serum. However, ST3T3 cells become arrested at a 6-fold higher density (30×10^4 cells per cm^2) than BALB/c-3T3 cells. Density-arrested ST3T3 cells can

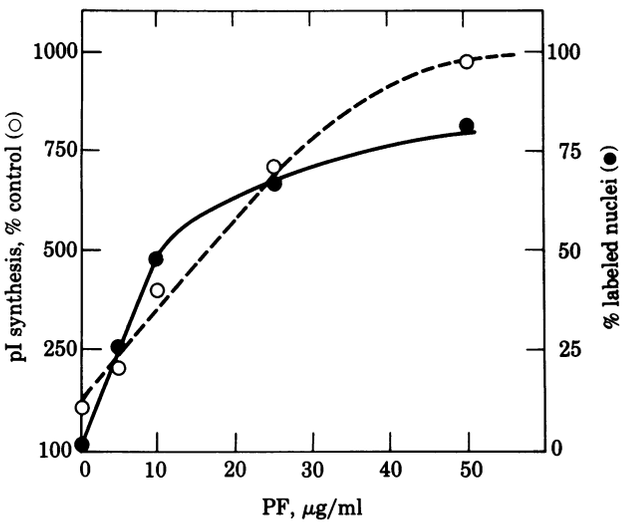

FIG. 5. PDGF concentration regulates both the synthesis of pI and the percentage of cells that synthesize DNA. Density-inhibited cells were treated with various concentrations of partially purified PDGF (PF) for 3 hr or with 5% PPP. One set of cultures was labeled with [^{35}S]methionine for 20 min; after electrophoresis, the synthesis of pI was estimated with a densitometer. Synthesis of pI in the PDGF-treated cultures (○) is plotted as percentage of control PPP-treated cultures). After treatment with PDGF or PPP, a duplicate group of cultures was transferred to medium containing the usual methionine concentration, supplemented with 5% PPP and [^{3}H]thymidine to determine the percentage of cells entering the S phase. The plates were fixed 20 hr later and processed for autoradiography (●).

be stimulated to replicate by the addition of plasma-supplemented medium and enter the S phase after a 10-hr lag. The percentage of cells that synthesize DNA is governed by the plasma concentration. Addition of PDGF or FGF, with or without plasma, has little or no effect on the percentage of cells that synthesize DNA. Density-arrested BALB/c-3T3 or ST3T3 cells were treated for 3 hr with highly purified PDGF or with PPP. PDGF-induced the increased synthesis of two proteins (pI, pII) by BALB/c-3T3 cells; pII was particularly prominent. The ST3T3 cells synthesized these same proteins in the plasma-supplemented medium; treatment with PDGF did not increase the amount synthesized (Fig. 6). The relative amount of these proteins synthesized by plasma-treated ST3T3 cells was equal to or greater than that synthesized by PDGF-treated BALB/c-3T3 cells. It appears that ST3T3 cells synthesize these PDGF-modulated proteins constitutively.

Table 1. Effect of various growth factors on selective protein synthesis

Growth factor	Synthesis, % plasma control	
	pI	pII
PDGF	700	550
FGF	600	400
EGF	95	150
Insulin	120	100
None	110	100

Density-inhibited BALB/c-3T3 cells were treated with pure PDGF (16 ng/ml), FGF (50 ng/ml), EGF (50 ng/ml), insulin (6 μg/ml), no growth factor, or PPP (5%) for 3 hr and then labeled with [^{35}S]methionine for 20 min for electrophoresis. The density of pI and pII was determined with a densitometer and is given as the percent of the plasma controls. This table is a composite of several experiments, each of which had a PPP control that was normalized to 100%.

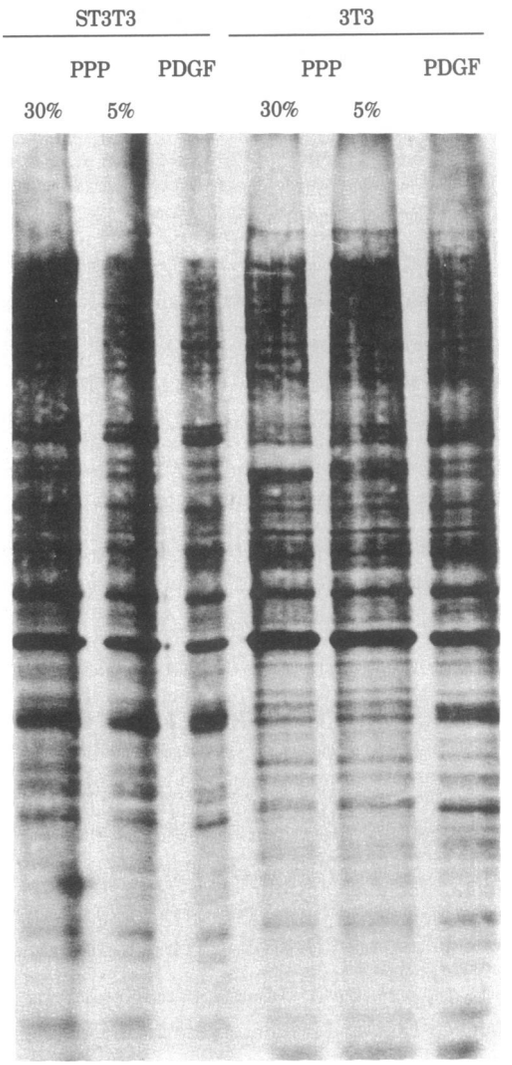

FIG. 6. Constitutive synthesis of PDGF-modulated proteins by ST3T3 cells. BALB/c-3T3 cells and ST3T3 cells were grown to confluence in medium supplemented with 10% serum. Three days after the last medium change, the density-arrested cultures were transferred to incorporation medium supplemented with either 30% or 5% PPP or highly purified PDGF at 14 μg/ml for 3 hr. [^{35}S]Methionine was added and 20 min later the cultures were harvested. Equal amounts of trichloroacetic acid-insoluble contents (20,000 cpm per sample) were applied to a gel. The arrows (from top to bottom) indicate the positions of pII (M_r 35,000) and pI (M_r 29,000).

DISCUSSION

Treatment with PDGF stimulates selective synthesis of several cytoplasmic proteins in BALB/c-3T3 cells. This response is very rapid and appears to be contingent upon cellular RNA synthesis. Four independent lines of evidence point to a tight association between the synthesis of these proteins and PDGF-induced competence. First, the PDGF dose response for pI induction is similar to that for DNA synthesis. Second, the time course for pI synthesis is similar to that for acquisition of competence. A brief treatment with PDGF induces both competence (5) and pI synthesis. Third, a functional analogue of PDGF (pituitary FGF) (7) induces pI and pII; plasma growth factors, EGF, or insulin, which do not induce competence (7), do not induce the synthesis of these proteins. Finally, a BALB/c-3T3 cell variant that synthesizes pI and pII constitutively has lost the growth requirement for PDGF although it retains a requirement for plasma factors.

Previous studies have noted that serum-stimulated cultures synthesize specific proteins prior to S phase (24–30); however, our experiments differ in a fundamental way. In previous studies, synthesis of specific proteins was detected during growth as cells progressed through G_0/G_1 towards S phase. Because the cells were growing, it could not be determined if the proteins were regulatory in nature or appeared as a result of growth. Growth does not appear to be required for the synthesis of pI and pII, because PDGF-treated cells display only a weak growth response in the absence of plasma.

Like other growth factors, PDGF and FGF (31) trigger a rapid pleotypic response; components of this response include increased potassium uptake (32) and increased protein synthesis. However, unlike other growth factors, PDGF and FGF stimulate the rapid selective synthesis of several cytoplasmic proteins. Stimulation of pI is detected within 40 min of PDGF addition. In mouse fibroblasts, the delay between transcription and appearance of new mRNA in the cytoplasm is about 20 min (33). Thus, the delay between addition of PDGF and modulation of genes controlling pI expression is no greater than 20 min, and it may be less; time intervals between 20 and 40 min were not examined in these studies. To our knowledge, a comparable rapid response at the level of gene transcription has not been described for other polypeptide hormones (34–37). Induction of pI by PDGF is comparable to induction of the heat shock proteins in *Drosophila* (38) in terms of kinetics.

A variant cell line appears to synthesize two of the PDGF-modulated proteins in a constitutive fashion. These proteins were identified by using one criterion, molecular weight as determined by gel electrophoresis. Additional criteria will have to be utilized to unequivocally demonstrate identity of the PDGF-modulated and constitutively synthesized proteins.

The saturation density of BALB/c-3T3 is regulated, in part, by the concentration of PDGF (5), which in turn modulates the selective synthesis of several proteins. These proteins might have a role in mediating the response to PDGF; other growth factors, such as EGF and insulin, would act in a different manner. In support of this model, competence can be transferred from PDGF-treated donor cells to untreated recipient cells by using cell fusion (18). Furthermore, a variant cell line that synthesizes these proteins in a constitutive fashion grows in the absence of PDGF. This variant, which was initially recognized because it is morphologically transformed, grows to a 6-fold higher density than BALB/c-3T3 cells. It is tempting to speculate, as Sachs (39) has done, that constitutive synthesis of PDGF-modulated proteins provides one mechanism for altering cellular growth.

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1. Ross, R. & Vogel, A. (1978) *Cell* **14**, 203–210.
3. Scher, D. C., Shepard, R. C., Antoniades, H. N. & Stiles, C. D. (1979) *Biochim. Biophys. Acta* **560**, 217–241.
3. Antoniades, H. N., Scher, C. D. & Stiles, C. D. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 1809–1813.
4. Heldin, C. H., Westermark, B. & Wasteson, A. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 3722–3726.
5. Pledger, W. J., Stiles, C. D., Antoniades, H. N. & Scher, C. D. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 4481–4484.
6. Pledger, W. J., Stiles, C. D., Antoniades, H. N. & Scher, C. D. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 2839–2843.
7. Stiles, C. D., Capone, G. T., Scher, C. D., Antoniades, H. N., Van Wyk, J. J. & Pledger, W. J. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 1279–1283.
8. Leof, E. B., Wharton, W., Van Wyck, J. J. & Pledger, W. J. (1980) *J. Cell Biol.* **87**, 5 (abstr.).
9. Carpenter, G. & Cohen, S. (1976) *J. Cell Biol.* **71**, 159–171.
10. Schlessinger, J., Schechter, Y., Willingham, M. C. & Pastan, I. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 2659–2663.
11. Clemmons, D. R., Van Wyk, J. J. & Pledger, W. J. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 6644–6648.
12. Schechter, Y., Hernaez, L. & Cuatrecasas, P. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 5788–5791.
13. Stiles, C. D., Isberg, R. R., Pledger, W. J., Antoniades, H. N. & Scher, C. D. (1979) *J. Cell. Physiol.* **99**, 395–406.
14. Heldin, C. H., Wasteson, A. & Westermark, B. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 6611–6615.
15. Wrann, M., Fox, C. F. & Ross, R. (1980) *Science* **210**, 1363–1365.
16. Rutherford, R. B. & Ross, R. (1976) *J. Cell Biol.* **69**, 196–203.
17. Tucker, R. W., Scher, C. D. & Stiles, C. D. (1979) *Cell* **18**, 1065–1072.
18. Smith, J. C. & Stiles, C. D. (1980) *Proc. Natl. Acad. Sci. USA* **78**, 4363–4367.
19. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
20. Bonner, W. J. & Laskey, R. A. (1974) *Eur. J. Biochem.* **46**, 1279–1283.
21. O'Farrell, P. Z., Goodman, H. M. & O'Farrell, P. H. (1977) *Cell* **12**, 1133–1142.
22. Gospodarowicz, D. (1974) *Nature (London)* **249**, 123–127.
23. Cohen, S. (1962) *J. Biol. Chem* **237**, 1555–1562.
24. Gates, B. J. & Friedkin, M. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 4959–4961.
25. Melero, J. A. & Finchman, V. (1978) *J. Cell. Physiol.* **95**, 295–306.
26. Benecke, B. J., Ben-Ze'ev, A. & Penman, S. (1978) *Cell* **14**, 931–939.
27. Riddle, V. G. H., Dubrow, R. & Pardee, A. B. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 1298–1302.
28. Jimenez de Asua, L., Richmond, K. M. V., Otto, A. M., Dubler, A. M., O'Farrell, M. K. & Rudland, P. S. (1979) in *Hormones and Cell Culture*, Cold Spring Harbor Conferences on Cell Proliferation, eds. Sato, G. & Ross, R. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), Vol. 6, pp. 403–424.
29. Riddle, V. G. H. & Pardee, A. B. (1980) *J. Cell. Physiol.* **103**, 11–15.
30. Rudert, W. A., Graves, M. W., Chadwick, D. E. & Lieberman, I. (1980) *J. Cell. Physiol.* **104**, 73–81.
31. Rudland, P. S., Seifert, W. & Gospodarowicz, D. (1974) *Proc. Natl. Acad. Sci. USA* **71**, 2600–2604.
32. Mendoza, S. A., Wigglesworth, N. M., Pohjanpelto, P. & Rosengurt, E. (1980) *J. Cell. Physiol.* **103**, 17–27.
33. Perry, R. P., Kelley, D. E. & Latorre, J. (1974) *J. Mol. Biol.* **82**, 315–332.
34. Marks, P. A. & Rifkind, R. A. (1972) *Science* **175**, 955–961.
35. Dannies, P. S. & Tashjian, A. H. (1973) in *Tissue Culture: Methods and Application*, eds. Kruse, P. & Patterson, M. K. (Academic, New York), pp. 561–569.
36. Johnson, L. K., Baxter, J. D., Vlodavsky, I. & Gospodarowicz, D. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 394–398.
37. Nilsen-Hamilton, M., Shapiro, J. M., Massoglia, S. L. & Hamilton, R. T. (1980) *Cell* **20**, 19–28.
38. Asburn, M. & Bonner, J. J. (1979) *Cell* **17**, 241–254.
39. Sachs, L. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 6152–6156.