Platelet-derived Growth factor–induced Alterations in Vinculin and Actin Distribution in BALB/c-3T3 Cells

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ABSTRACT Exposure of BALB/c-3T3 cells (clone A31) to platelet-derived growth factor (PDGF) results in a rapid time- and dose-dependent alteration in the distribution of vinculin and actin. PDGF treatment (6–50 ng/ml) causes vinculin to disappear from adhesion plaques (within 2.5 min after PDGF exposure) and is followed by an accumulation of vinculin in punctate spots in the perinuclear region of the cell. This alteration in vinculin distribution is followed by a disruption of actin-containing stress fibers (within 5 to 10 min after PDGF exposure). Vinculin reappears in adhesion plaques by 60 min after PDGF addition while stress fiber staining is nondetectable at this time. PDGF treatment had no effect on talin, vimentin, or microtubule distribution in BALB/c-3T3 cells; in addition, exposure of cells to 5% platelet-poor plasma (PPP), 0.1% PPP, 30 ng/ml epidermal growth factor (EGF), 30 ng/ml somatomedin C, or 10 μM insulin also had no effect on vinculin or actin distribution. Other competence-inducing factors (fibroblast growth factor, calcium phosphate, and choleragen) and tumor growth factor produced similar alterations in vinculin and actin distribution as did PDGF, though not to the same extent. PDGF treatment of cells for 60 min followed by exposure to EGF (0.1–30 ng/ml for as long as 8 h after PDGF removal), or 5% PPP resulted in the nontransient disappearance of vinculin staining within 10 min after EGF or PPP additions; PDGF followed by 0.1% PPP or 10 μM insulin had no effect. Treatment of cells with low doses of PDGF (3.25 ng/ml), which did not affect vinculin or actin organization in cells, followed by EGF (10 ng/ml), resulted in the disappearance of vinculin staining in adhesion plaques, thus demonstrating the synergistic nature of PDGF and EGF. These data suggest that PDGF-induced competence and stimulation of cell growth in quiescent fibroblasts are associated with specific rapid alterations in the cellular organization of vinculin and actin.

Platelet-derived growth factor (PDGF)[1] is the major mitogenic growth factor in serum for cells of mesenchymal origin (2, 44), in addition to being a major chemoattractant for human neutrophils and monocytes (13), fibroblasts (45, 49), and smooth muscle cells (50), thereby potentially playing an important role in wound healing, atherogenesis, and cell growth. The biochemical mechanisms by which PDGF regulates the growth of cells is not known. Recent work has shown that PDGF and growth factors in platelet-poor plasma (PPP) function in a synergistic manner to control the growth of BALB/c-3T3 fibroblasts in tissue culture (42). PDGF alone cannot initiate growth of quiescent cells; however, transient treatment of cells with PDGF followed by plasma results in DNA synthesis and cell growth (41). Further work revealed that the plasma requirement for the stimulation of DNA synthesis in BALB/c-3T3 cells can be replaced by epidermal growth factor (EGF) and either insulin or Somatomedin C (SmC) (36). An interesting finding of these studies was that only brief exposure to PDGF was required to prime cells for responsiveness to plasma, and that this sensitized state (competence) lasted long after removal of PDGF from cells (57). This PDGF-induced competent state was found to decay with a half life of 18 h, and could also be induced by treatment of cells with fibroblast

[1] *Abbreviations used in this paper:* C.T. Cholera toxin; DME, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; FGF, fibroblast growth factor; PDGF, platelet-derived growth factor; PPP, platelet-poor plasma; SmC, somatomedin C; TGF, tumor growth factor.

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growth factor (FGF), macrophage-derived growth factor, or calcium phosphate (53, 57). Lastly, choleragen treatment of cells was found to potentiate the action of PDGF in competence induction as well as abrogate the requirement for EGF in cell cycle progression (32). To date, no cellular response, other than induction of DNA synthesis, has been demonstrated in cells following exposure to EGF which required prior exposure of cells to PDGF.

The finding that only brief exposure to PDGF is required to induce competence suggests that rapid biochemical events which follow PDGF binding are important in the induction of competence. Shortly after binding of growth factors, a plethora of metabolic changes in cells has been shown to occur. These rapid changes include alterations in the activity of plasma membrane Na^+/H^+ antiport systems (31), alterations in ion fluxes (5), mobilization of intracellular calcium (34), alkalinization of intracellular pH (4), and phosphorylation of plasma membrane phospholipids (18) and growth factor receptors (8). The latter two observations have caused an intense amount of interest based on the findings that tumor promotors have been shown to phosphorylate specific plasma membrane phospholipids (22), and that growth factor receptors for PDGF and EGF share with some oncogene proteins a tyrosine protein kinase activity (8). Exposure of cells to PDGF has been found to result in the phosphorylation of 42-, 43-, and 45-kD proteins which seem to be localized at the plasma membrane (10). To date, the identity of the proteins which are phosphorylated upon PDGF addition have yet to be identified.

In addition to the rapid events which occur at the plasma membrane of cells following PDGF addition, subsequent effects on gene expression have been documented (30). A number of specific proteins have been identified in BALB/c-3T3 cells induced by PDGF addition (40); one of these proteins which occurs early after PDGF exposure, a 29-kD protein, has been localized to the nucleus (38), but its function is unknown. Furthermore, PDGF rapidly stimulates the expression of *myc* and *fos* in quiescent BALB/c-3T3 cells (30). If tyrosine phosphorylation of growth factor receptors and cellular substrates is important for growth factor action, and for growth factor activity that can be modulated at the transcriptional level in the nucleus, it is important to identify the intracellular proteins that may mediate the passage of information from the plasma membrane to the nucleus. The findings that certain growth factor receptors and oncogene protein products possess tyrosine kinase activity (23, 26), in conjunction with the localization of oncogene products to the plasma membrane (and in one specific case, to adhesion plaques [43]), prompted us to examine whether PDGF addition to cells would affect adhesion plaque structure. PDGF addition to BALB/c-3T3 cells results in a rapid and reversible alteration in the distribution of vinculin and actin that is very reminiscent of that observed in virally transformed cells (26, 48, 50), which is thought to be mediated through tyrosine phosphorylation. We demonstrate that this alteration is specific for PDGF and other competence-inducing factors and not for other growth factors required for progression (e.g., EGF and SmC), and that PDGF and EGF can exert a synergistic effect with respect to the distribution of vinculin from adhesion plaques, which can be related to mitogenesis in these cells. We also report that the loss of vinculin from adhesion plaque in response to EGF first requires pretreatment with PDGF. This finding constitutes the first description of a specific cellular response to EGF treatment which occurs only in PDGF-induced competent BALB/c-3T3 cells, but not in quiescent cells. Previous portions of this work have appeared in abstract form (25).

MATERIALS AND METHODS

Cell Culture: BALB/c-3T3 cells (clone A31) were grown in Dulbecco's modified Eagle's medium (DME)/10% heat-inactivated calf serum/4 mM glutamine, supplemented with penicillin at 50 U/ml and streptomycin at 50 μg/ml in humidified 5% CO_2/95% air at 37°C. Cells were seeded at 4–5 × 10^5 cells/1.76 cm^2 well on glass coverslips, and cultures were used for experiments 4–5 d after plating.

Preparation of Growth Factors: Highly purified PDGF was prepared from boiled extracts of outdated human platelets by a combination of reported procedures (24, 41, 42). The PDGF was greater than 25% pure and was highly mitogenic at 2–10 ng/ml in microtiter assays with density-dependent inhibited BALB/c-3T3 cells as described by Pledger et al. (40). The PDGF preparation did not contain measurable tumor growth factor (TGF) activity as demonstrated by radio receptor assay. PPP was purified as previously described from peripheral venous blood drawn from healthy volunteers (41). EGF was purified from mouse submaxillary glands by the procedure of Savage and Cohen (46). FGF was a gift of Dr. Dennis Gospodarowicz (University of California at San Francisco), while platelet-derived β-TGF was a gift from Dr. Hal Moses (Mayo Clinic, Rochester, MN). Bovine insulin and cholera toxin (C.T.) were purchased from Sigma Chemical Co., St. Louis, MO. SmC was a gift from Dr. Judson Van Wyk.

[^{3}H]Thymidine (DNA Synthesis) Assay: BALB/c-3T3 cells were grown in Falcon 24-well tissue culture plates for 4–5 d containing 2.0 ml of serum-supplemented DME. Confluent quiescent cultures were transferred to fresh medium containing PDGF, PPP, and [^{3}H]thymidine. After incubation for 36 h, the cultures were processed for autoradiography as previously described (41, 42).

Immunofluorescence: After exposure of BALB/c-3T3 cells to varying concentration of growth factors, serum, or hormones in 0.5 ml of medium for appropriate times, cells were processed for the demonstration of vinculin, talin, or actin as follows.

Cells were fixed in 3.7% formaldehyde in phosphate-buffered saline (PBS) (pH 7.4), 1 mM magnesium chloride ($MgCl_2$), and 0.1 mM calcium chloride ($CaCl_2$), for 5 min at room temperature. The coverslips containing the cells were rinsed three times in PBS and extracted for 3 min at room temperature in 0.1% Triton X-100 containing 1 mM $MgCl_2$ and 0.1 mM $CaCl_2$. After rinsing in PBS, the coverslips were incubated in either a 1:25 dilution of rabbit anti-vinculin antibody, a 1:25 dilution of rabbit anti-talin antibody, or a combination (final dilution of 1:25) of rabbit anti-talin and guinea pig anti-vinculin. The antibodies directed against vinculin and talin were kindly supplied to us by Keith Burridge (University of North Carolina at Chapel Hill) and have been previously characterized with regard to their specificity (19). Coverslips were incubated for 30 min at 37°C, rinsed three times in PBS and incubated in the following second antibodies. The coverslips were incubated in fluorescein goat anti-rabbit IgG or fluorescein goat anti-guinea pig IgG (Flow Laboratories, McLean, VA). The second antibodies also contained Rhodamine-Phalloidin (which binds to F-actin), obtained from Molecular Probes (Junction City, OR), at a concentration of 5 μl of Rhodamine-Phalloidin/100 μl second antibody; this allowed us to visualize the distribution of vinculin or talin and actin in the same cell. When cells were stained with a mixture of rabbit anti-talin and guinea pig anti-vinculin, the second antibody incubation was performed using a mixture of fluorescein goat anti-rabbit IgG and Rhodamine-sheep anti guinea pig IgG which did not contain any Rhodamine-Phalloidin. The coverslips were incubated in second antibody for 30 min at 37°C, rinsed three times in PBS, and mounted onto slides in a mixture of 60% glycerol/40% PBS containing 5% *n*-propyl gallate. Tubulin and vimentin were visualized after the fixation of cells in −20°C methanol for 10 min with a 1:50 dilution of rabbit antitubulin antibody which had been raised against sea urchin vinblastine crystals (kindly supplied by Keigi Fujiwara at the Harvard Medical School) and a 1:5 dilution of mouse monoclonal antivimentin antibody purchased from LAB systems of Chicago, IL. Incubation conditions were as previously described except that cells were incubated in a 1:20 dilution of fluorescein goat anti-rabbit IgG (tubulin) and Rhodamine guinea pig anti-mouse IgG (vimentin). This fixation procedure did not allow the visualization of actin with Rhodamine-Phalloidin. After mounting onto slides, photographs of the cells were taken on a Zeiss IM-35 inverted microscope using epi-illumination and filter sets which discriminate between fluorescein and Rhodamine fluorescence. Photographs were taken on Tri-X film and developed at an effective ASA of 1600. For each experiment, 150–200 cells per treatment were counted and scored for the presence or absence of vinculin and actin staining, which were distributed in adhesion plaques (focal contacts) and stress fibers, respectively.

RESULTS

PDGF Effects on the Cytoskeleton

Exposure of quiescent BALB/c-3T3 cells to PDGF results in a rapid time- and dose-dependent alteration in the distribution of vinculin and actin. In untreated cells, vinculin staining is concentrated in adhesion plaques near the cell edge or at areas of cell-cell contact (Fig. 1 *A*), as well as being diffusely distributed throughout the cytoplasm. The higher intensity observed in the perinuclear region may be due to a higher concentration of vinculin or a larger accessible volume in this region of the cell. Actin is found disposed in stress fibers (Fig. 1 *D*) which course throughout the cell or are concentrated around the cell periphery. These stress fibers can often be seen terminating in vinculin-containing adhesion plaques. Within 2.5 min after addition of 50 ng/ml PDGF to BALB/c-3T3 cells, vinculin staining is lost from adhesion plaques (Fig. 1 *B*), and assumes a more punctate distribution in the cytoplasm, primarily concentrated at the cell center. Actin organization is unaffected at this time (Fig. 1 *E*). By 60 min of exposure to PDGF, the vinculin staining has reappeared in adhesion plaques (Fig. 1 *C*), while there is little staining of actin in cells at this time (Fig. 1 *F*). This latter finding is in agreement with the data of Bochus and Stiles (3) and Mellström et al. (33), who have reported that PDGF addition to BALB/c-3T3 cells resulted in the rapid and reversible loss of microfilaments and that treatment of human diploid glial cells with PDGF resulted in early reorganization of actin microfilaments and membrane ruffling.

To determine whether exposure of cells to PDGF specifically caused disruption of vinculin and actin or also caused alterations in other cytoskeletal structures as well, we exposed cells to PDGF (12 ng/ml for 20 min at 37°C), fixed them, and processed them for the immunofluorescent detection of talin (adhesion plaque protein [6]), vimentin (intermediate filaments), or tubulin (microtubules). As can be seen in Fig. 2, although PDGF treatment caused the disappearance of vinculin staining in cells (Fig. 2, *A* and *B*), talin distribution was unaffected (cf. Fig. 2, *C* and *D*). While the images in this figure were obtained using rabbit anti-talin antibody, identical experiments using guinea pig anti-vinculin antibodies and rabbit anti-talin antibodies demonstrated disruption of vinculin staining with the preservation of talin adhesion plaque staining. These results suggest that PDGF exposure results in a specific disruption or removal of vinculin from adhesion plaques while still maintaining the adhesion plaque structure intact (as evidenced by the lack of effect of PDGF treatment on talin). Whether the reappearance of vinculin in adhesion plaques by 60 min after PDGF exposure results from vinculin accumulation to previously existing adhesion plaques is not

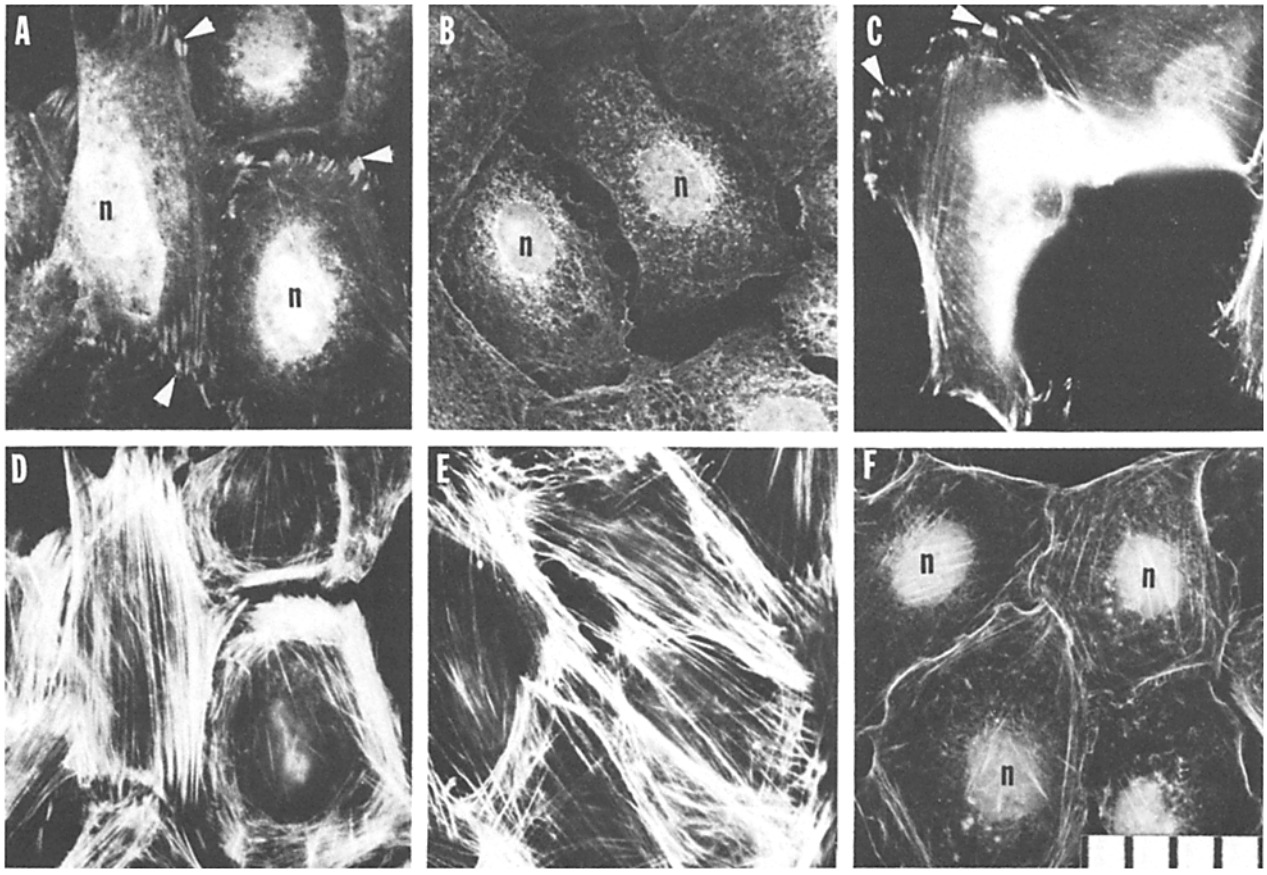

FIGURE 1 Effect of PDGF treatment on vinculin (*A–C*) and actin (*D–F*) distribution in BALB/c-3T3 cells. In untreated cells, vinculin staining is found predominantly in adhesion plaques (arrowheads in *A*) near sites of cell-cell contact or cell-substrate interaction and in a diffuse distribution throughout the cytoplasm. Actin is disposed as stress fibers which course throughout the cell or are concentrated around the cell periphery (*D*). Within 2.5–5 min after exposure of cells to 50 ng/ml of PDGF, vinculin staining has disappeared from adhesion plaques and assumes a punctate perinuclear distribution (*B*). The distribution of actin is unchanged at this time (*E*). By 60 min after continual exposure of cells to 50 ng/ml of PDGF, vinculin has reappeared in adhesion plaques (arrowheads in *C*), while stress fiber staining is essentially absent (*F*). *n*, Nucleus. Bars, 10 μM.

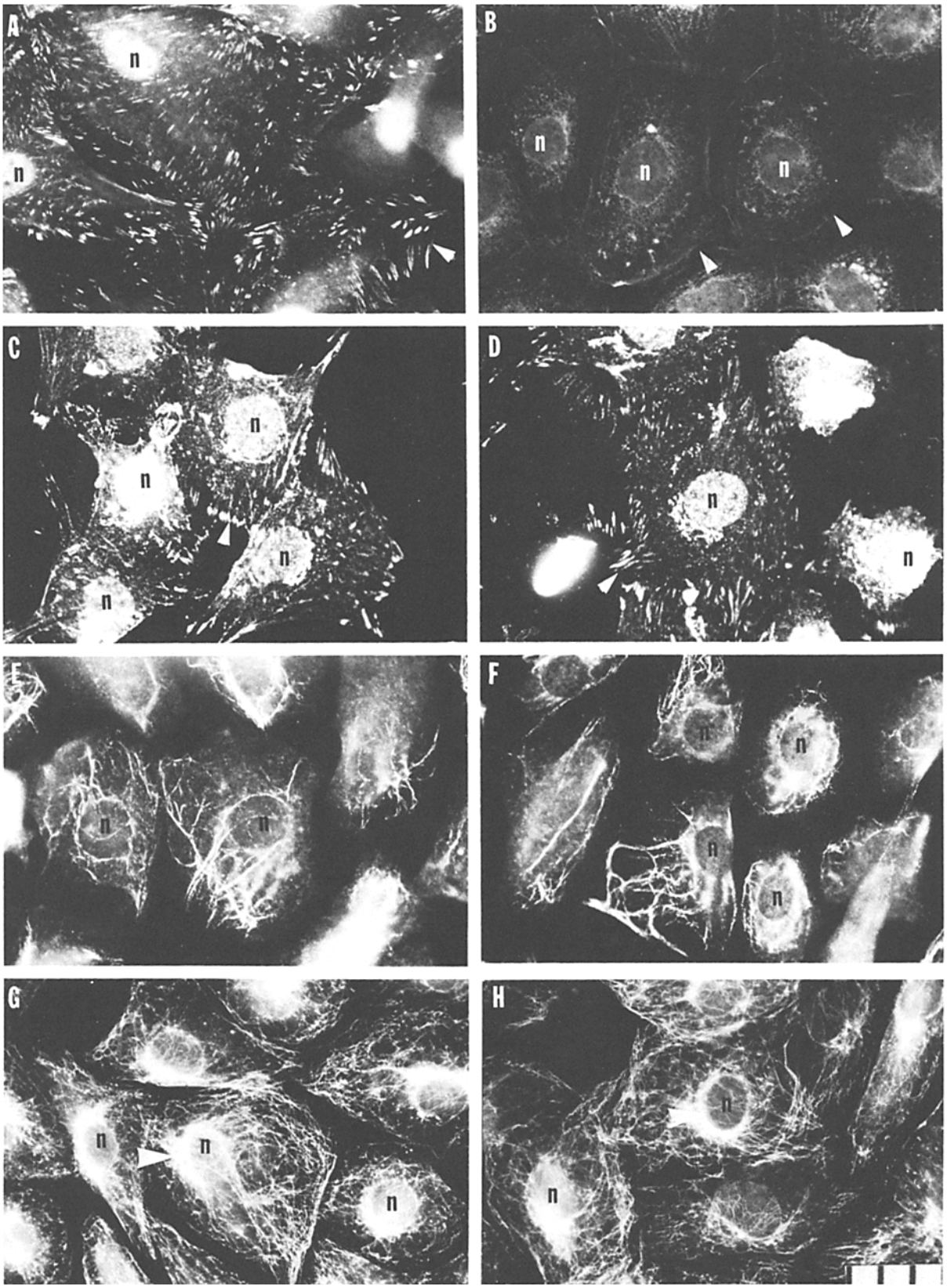

FIGURE 2 Effect of PDGF treatment (12 ng/ml for 20 min at 37°C) on vinculin (*A* and *B*), talin (C and *D*), vimentin (*E* and *F*), and microtubule (*G* and *H*) distribution in BALB/c-3T3 cells. Vinculin is concentrated in adhesion plaques at sites of cell-cell or cell-substratum contact in untreated cells (arrowheads in *A*). After exposure to PDGF, vinculin staining in adhesion plaques is lost (arrowheads in *B*) and assumes a punctate distribution within the cytoplasm. Talin, another adhesion plaque protein (C), has an identical distribution to that of vinculin, being localized in adhesion plaques. Unlike vinculin, PDGF treatment has no effect on talin distribution or staining (*D*; cell edge delineated by arrowhead). Intermediate filaments (vimentin) display an interlacing network of fibers (*E*) which are also unaffected by PDGF treatment (*F*). Microtubules are disposed as a radial network of fibers originating from a perinuclear organizing center (arrowhead in G) and this distribution is unaltered by PDGF treatment (*H*). *n*, Nucleus. Bars, 10 μM.

known. PDGF addition did not alter the organization of intermediate filaments (vimentin [Fig. 2, *E* and *F*]) or microtubules (tubulin [Fig. 2, *G* and *H*]). No change in cell shape was noted during these experiments, suggesting that the cells remained attached to the substrate for the duration of PDGF exposure. Exposure of BALB/c-3T3 cells to EGF (10 ng/ml), insulin (10 μM), SmC (30 ng/ml), 5% PPP, or 0.1% PPP had no effect on vinculin or actin distribution in BALB/c-3T3 cells.

Fig. 3 illustrates the time- and dose-dependent disappearance of vinculin staining from adhesion plaque structures or actin stress fiber staining in cells after exposure of cells to varying doses of PDGF. At very high doses (50 ng/ml), adhesion plaque staining disappears (up to 95%) within 2.5 min after PDGF addition (Fig. 3*A*), while stress fiber staining does not disappear until 5–10 min after exposure to PDGF (Fig. 3*B*). Progressively lower doses of PDGF (25 or 12 ng/ml) bring about a somewhat slower but quantitatively similar disappearance in vinculin staining; however, the kinetics of the disruption of actin stress fibers appears independent of dose at these concentrations. Cells exposed to 6.25 ng/ml PDGF display only a partial disappearance of vinculin staining from adhesion plaques (~50% at 15 min after treatment), whereas no effect was found on stress fibers at this concentration. This figure also illustrates that between 30 and 60 min after exposure of cells to PDGF, vinculin staining reappears in adhesion plaques (Fig. 3*A*), while stress fibers have not recovered even by 90 min after PDGF addition (Fig. 3*B*). The findings that PDGF treatment results in the rapid disappearance of vinculin from the adhesion plaques before the disappearance of actin-containing stress fibers suggests that the primary effect of PDGF treatment is to cause vinculin disruption from adhesion plaques. The subsequent disruption of actin-containing stress fibers may be secondary to the disruption of vinculin in the adhesion plaques since many morphological and biochemical studies have indicated that actin-containing stress fibers are tethered at adhesion plaques or focal contacts through an interaction with vinculin (21).

Specificity of Growth Factor Modulation of Vinculin Distribution

When BALB/c-3T3 cells are rendered competent by brief exposure to PDGF, the subsequent addition of EGF and insulin-like growth factors (or PPP) induces progression through the G0/G1 phases of the cell cycle and entry into S phase. In addition to PDGF, other agents, including FGF, calcium phosphate ($Ca_3[PO_4]$), and a macrophage-derived growth factor have been shown to be able to induce this competent state in BALB/c-3T3 cells. Table I illustrates the effect some of these compounds and various other growth factors or hormones on the distribution of vinculin and actin staining shortly after addition to density-arrested BALB/c-3T3 cells. Both FGF and $CaCl_2$ disrupted adhesion plaque and stress fiber staining, though to a lesser extent than did PDGF (Table I). TGF at higher concentrations (1–10 ng/ml) also caused a disappearance of vinculin and actin staining in cells. At the concentrations of PDGF used in our studies, the

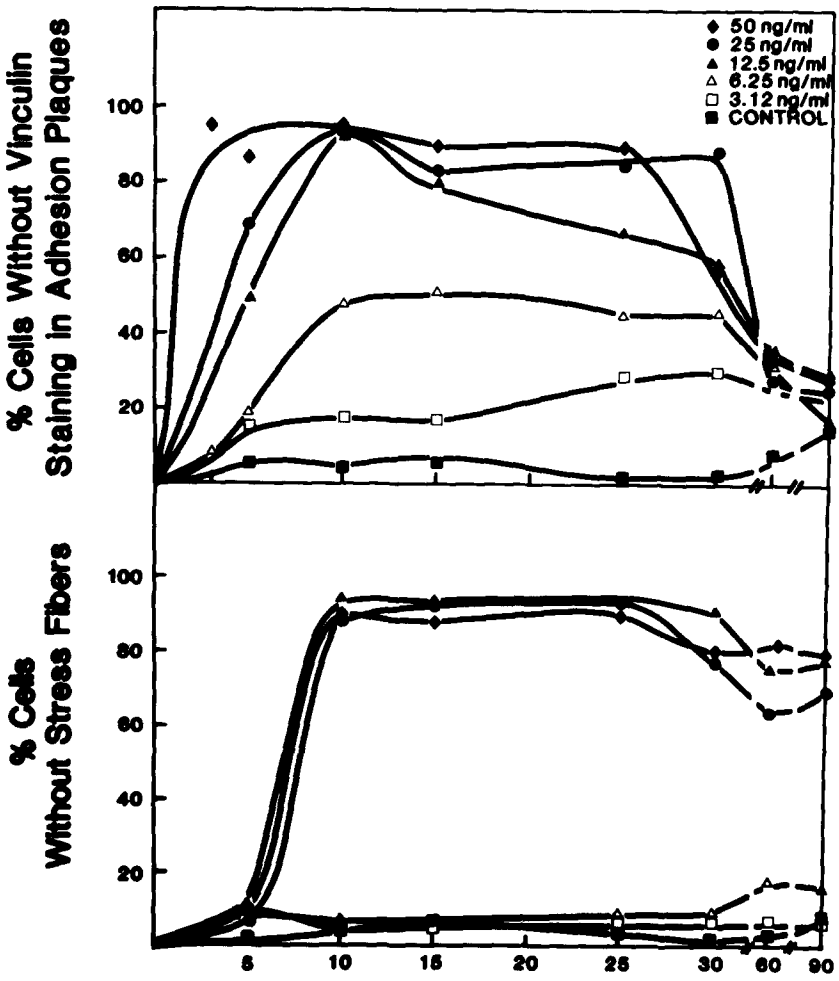

FIGURE 3 Time- and dose-dependent effect of PDGF treatment on vinculin adhesion plaque and actin stress fiber staining in BALB/c-3T3 cells exposed to PDGF. At high PDGF concentrations, vinculin staining disappears within 2.5 min after PDGF addition (*top*), while actin-containing stress fibers do not undergo disruption until 10 min after exposure of cells to PDGF (*bottom*). Whereas the disappearance of vinculin staining is dependent on the dose of PDGF (*top*), stress fiber break-down appears relatively dose-independent (at the higher PDGF concentrations) (*bottom*). By 60 min after PDGF addition, vinculin staining has reappeared in adhesion plaques (*top*), while most cells are still devoid of actin-containing stress fibers (*bottom*).

level of contaminating TGF was shown to be negligible by radio receptor assay (personal communication, Dr. Hal Moses), and therefore could not be responsible for PDGF's effect on vinculin and actin distribution. Choleragen (cholera toxin [C.T.]), which has been shown to enhance DNA synthesis by potentiating the action of PDGF (32), caused a very efficient and rapid disruption of vinculin staining in adhesion plaques and stress fiber staining in BALB/c-3T3 cells. In contrast to PDGF or other BALB/c-3T3 competence factors, exposure of cells to EGF (0.1–30 ng/ml), PPP (5% or 0.1%), SmC (30 ng/ml), or insulin (10 μM) had no effect on vinculin adhesion plaque or actin stress fiber staining in the BALB/c-3T3 cells. None of the agents listed in Table I altered the staining patterns of any other cytoskeletal structures during these brief incubation periods.

Effect of Addition of EGF, PPP, or Insulin on Vinculin and Actin Distribution after Brief Exposure to PDGF

Previous work has shown that subsequent to transient exposure to PDGF, cells could be induced to enter the S phase of the cell cycle by plasma (39). Further investigations have demonstrated that the requirement for plasma could be replaced by EGF, and either insulin or SmC (35). In addition, it was found that the competent state induced by PDGF decayed with a half life of 18 h; addition of plasma for up to 13–18 h after removal of PDGF still resulted in the entry of cells into S phase (50). We investigated whether addition of EGF, insulin, or PPP after removal of PDGF led to any rearrangement in vinculin or actin distributions in BALB/c-3T3 cells. The results of this study are presented in Fig. 4. As demonstrated earlier, vinculin staining within adhesion plaques reappears within 60 min after PDGF exposure, while actin stress fibers have not reformed by this time (Fig. 1, *b* and *c*, and Fig. 4*a*). When PDGF was removed from the cells at this time and EGF was added, vinculin staining within adhesion plaques disappeared within 15 min (Fig. 4*b*). This response was found to occur up to 8 h after removal of PDGF (Fig. 4*c*), and could also be brought about by the addition of 5% PPP after the removal of PDGF. These treatments had no effect on talin staining or the distribution (staining) of other cytoskeletal structures. Addition of 10 μM insulin (Fig. 4*f*) or 0.1% PPP (Fig. 4*e*) after the removal of PDGF had no effect on the distribution of vinculin staining in these cells. These results suggest that the alterations observed in the vinculin component of adhesion plaques, and stress fiber structure, are related to the induction of competence by PDGF and the further signaling of cells to enter the S phase by PPP or EGF.

TABLE I

Percent Disruption of Vinculin and Actin Staining at 15 Min after Ligand Addition

Ligand	Dose	% cells without vinculin staining in adhesion plaques	% cells without stress fiber staining
None (control)	—	<3	<5
PDGF	6.5 ng/ml	18	7
	12 ng/ml	83	93
	25 ng/ml	93	88
TGF	0.1 ng/ml	65	50
	1 ng/ml	67	54
	10 ng/ml	30	50
FGF	25 ng/ml	15	12
	50 ng/ml	27	23
	100 ng/ml	29	30
C.T.	1 ng/ml	68	88
	100 ng/ml	90	92
	1,000 ng/ml	88	90
$CaCl_2$	15 mM	35	50
EGF	0.1 ng/ml	<5	<5
	1 ng/ml	<5	<5
	30 ng/ml	<5	<5
PPP	5%	<5	<5
	0.1%	<5	<5
SmC	30 ng/ml	<5	<5
Insulin	10 μM	<5	<5

5-d cultures of BALB/c-3T3 cells grown on coverslips and maintained in DME containing 10% calf serum were treated with DME containing PDGF, TGF, FGF, EGF, C.T., $CaCl_2$, PPP, SmC, or insulin at the concentrations indicated for 15 min at 37°C, and fixed and processed for the immunofluorescent demonstration of vinculin and actin as described in the Materials and Methods. 150–200 cells per coverslip were counted and scored for the presence or absence of adhesion plaque or stress fiber staining. The percentage of cells without staining for adhesion plaques or stress fibers is indicated.

To further test the hypothesis that vinculin disappearance from adhesion plaques is correlated with the initiation of growth of quiescent cells by PDGF, we measured the amount of DNA synthesis ([^{3}H]thymidine uptake) as a function of PDGF concentration (after further treatment of cells with 5% PPP). The percentage of cells which took up [^{3}H]thymidine was correlated with the percentage of cells lacking adhesion plaque vinculin staining as a function of PDGF concentration. These data are plotted and shown in Fig. 5. As can be seen, the actual percentage of cells without adhesion plaque vinculin staining is almost identical to the percentage of the cells which were labeled with [^{3}H]thymidine. The close correlation between the disappearance of vinculin from adhesion plaques and cell growth (see *inset*, Fig. 5) suggests that an early event in the initiation of cell growth by PDGF in quiescent BALB/c-3T3 cells may involve the reversible disruption of vinculin from adhesion plaques or focal contacts.

Synergy between PDGF and EGF or PPP

PDGF has been shown to sensitize BALB/c-3T3 cells to EGF and reduce the concentration of EGF required for mitogenic stimulation of 10T½ cells (39). This effect of PDGF may occur through reactions that affect the EGF receptor, since PDGF treatment alters EGF receptor number (56). We wondered if this synergy between PDGF and EGF (the increased sensitivity of cells to EGF after exposure to PDGF) was also reflected in changes in the distribution of vinculin and actin staining in cells. We approached this question by first treating cells with a high (12 ng/ml) or low dose (3.25 ng/ml) of PDGF for 60 min followed by treatment with EGF (10 ng/ml), insulin (10 μM), or PPP (5% or 0.1%). The results of these experiments are shown in Table II. Treatment of cells with low doses of PDGF (3.25 ng/ml) did not result in loss of vinculin staining from adhesion plaques; exposure of cells to high doses of PDGF (12 ng/ml) results in the transient disappearance of vinculin staining in adhesion plaques (see Fig.

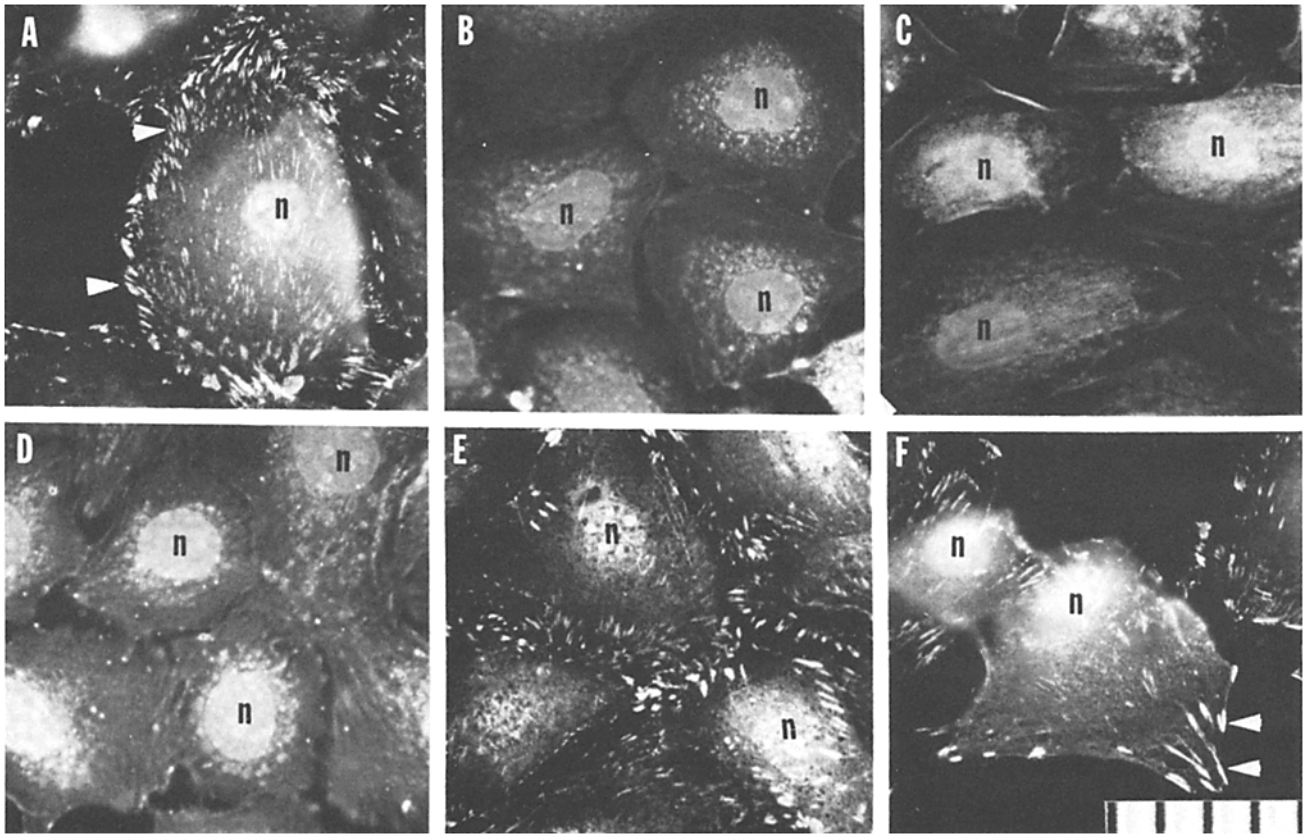

FIGURE 4 Vinculin staining in cells treated with 12 ng/ml PDGF for 60 min (*A*) followed by 10 ng/ml EGF (*B* and *C*), 5% PPP (*D*), 0.1% PPP (*E*), or 10 μM insulin for 15 min at 37°C. Vinculin staining reappears in adhesion plaques within 60 min after exposure to PDGF (*A*); subsequent removal of PDGF followed by addition of 10 ng/ml EGF results in the disappearance of vinculin staining within 15 min (*B*). Addition of EGF up to 8 h after removal of PDGF continues to result in the abolishment of vinculin staining in adhesion plaques within 15 min of EGF addition (*C*). PDGF treatment followed by addition of 5% PPP also abolished vinculin adhesion plaque staining (*D*), while 0.1% PPP (*E*) or 10 μM insulin (*F*) following PDGF treatment had no effect on vinculin staining (compare arrowheads in *A* and *F*). *n*, Nucleus. Bars, 10 μM.

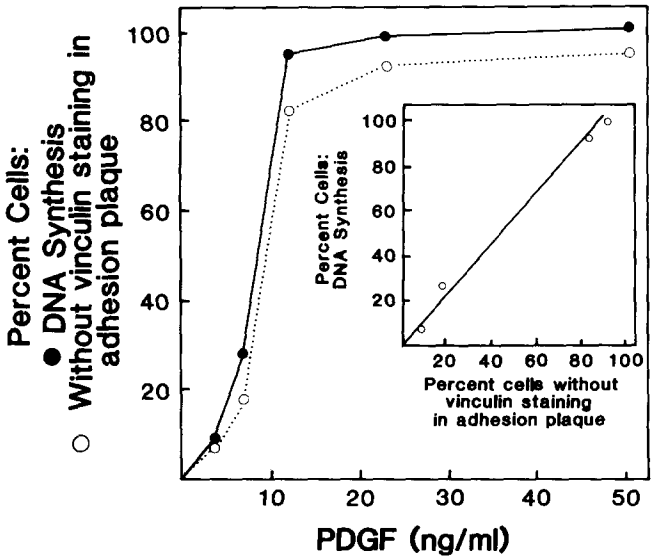

FIGURE 5 DNA synthesis (●) and disappearance of vinculin staining from adhesion plaques (○) as a function of PDGF concentration. The percentage of cells which contain labeled nuclei closely parallels the percentage of cells without vinculin adhesion plaque staining at all doses of PDGF (see *inset*).

1), which had returned by 60 min after PDGF addition (Table II). Addition of EGF to cells after a 60-min exposure to low doses of PDGF results in the rapid disappearance of vinculin adhesion plaque staining and actin stress fiber staining. Similar treatment with 5% PPP yielded the same results, while treatment of cells with EGF alone or with 0.1% PPP or 10 μM insulin following PDGF had no effect. Thus, PDGF and EGF seem to act in a synergistic manner with respect to alterations in the cellular distribution of vinculin and actin.

DISCUSSION

The biochemical mechanisms which regulate cell growth are unknown. The use of serum-derived growth factors in conjunction with tissue culture cells has allowed investigators to study biochemical changes associated with growth. These approaches have led to the discovery that different phases of the cell cycle can be regulated by different serum-derived growth factors (35, 36, 39). Specifically, PDGF stimulates arrested (quiescent) BALB/c-3T3 cells, in a concentration- and temperature-dependent manner, to leave G_0 and enter the cell cycle (41). The traverse of G_0/G_1 and entry into S phase can be facilitated by EGF and SmC (40, 42). The induction of competence and growth initiation by PDGF apparently involves increases in gene expression as illustrated by the induction of unique mRNA and proteins (52). The mechanism whereby PDGF alters gene expression by binding specific receptors is unknown and remains an important focus of investigation.

The findings that only brief exposure of cells to PDGF is necessary to render cells sensitive to EGF and SmC (51)

TABLE II

Vinculin and Actin Staining 75 Min after Sequential Additions of Ligands

Primary ligand	Secondary ligand	% cells without vinculin staining in adhesion plaques	% cells without stress fiber staining
Dose	*Dose*		
PDGF (3.25 ng/ml)	—	11	10
PDGF (12 ng/ml)		23	85
EGF (10 ng/ml)	—	<5	<5
PDGF (12 ng/ml)	EGF (10 ng/ml)	68	74
PDGF (3.25 ng/ml)	EGF (10 ng/ml)	72	71
PDGF (3.25 ng/ml)	5.0% PPP	68	35
PDGF (3.25 ng/ml)	0.1% PPP	13	11
PDGF (3.25 ng/ml)	Insulin (10 μM)	21	18

5-d old BALB/c-3T3 cells grown in DME were exposed to the same medium containing the primary ligand at the concentrations indicated in the table for 60 min at 37°C. The medium containing the primary ligand was removed and replaced by DME containing the secondary ligand at the concentrations indicated for 15 min at 37°C. Cells were then fixed and processed for the immunofluorescent demonstration of vinculin and actin as described in the Materials and Methods section. 150–200 cells were scored for the presence or absence of adhesion plaque or stress fiber staining. The percentage of cells without adhesion plaque or stress fiber staining is indicated.

suggest that rapid alterations in cellular structure or function which occur early after PDGF addition to cells may be responsible for PDGF-induced competence. We have found that exposure of BALB/c-3T3 cells to PDGF leads to rapid and reversible, time- and dose-dependent alterations in vinculin and actin distribution. Within 2.5 min after PDGF exposure, vinculin staining disappears from adhesion plaques (Fig. 1) while actin staining, in the form of stress fibers, becomes disrupted between 5 and 10 min after PDGF addition. Adhesion plaques (focal contacts) are regions where actin microfilaments terminate in cells such as cultured fibroblasts and where the plasma membrane comes close to the underlying substrate. Various actin-binding proteins have been localized to this region of the plasma membrane (1, 7, 20, 21), and include α-actinin, fimbrin, talin, and vinculin. Previous findings using immunocytochemistry indicate that actin microfilaments terminate in vinculin contained in adhesion plaques or focal contacts (21). The PDGF-induced disappearance of vinculin from adhesion plaques is reversible in that, by 60 min after treatment of cells with PDGF, vinculin staining has reappeared in adhesion plaques, whereas stress fibers have not reformed by this time. Stress fiber staining does eventually reappear within 120–180 min after PDGF addition (3) (also Herman, B., and W. J. Pledger, unpublished data). The slower kinetics of actin stress fiber disruption, coupled with the findings that stabilization of actin microfilaments with phalloidin (3) does not prevent PDGF-induced competence, suggests that microfilament breakdown is not required for induction of competence but is probably secondary to the disruption of vinculin from adhesion plaques. The findings that no microfilament disruption occurs at concentrations of PDGF which result in disappearance of ~50% of the detectable vinculin staining (see Fig. 3), and that a synergistic effect of PDGF and EGF with respect to vinculin disappearance from adhesion plaques and cell growth can be demonstrated at these low doses of PDGF, strengthens the argument that microfilament breakdown is not important in the induction of competence.

That the effect of PDGF is specific for vinculin is shown by the findings that neither talin (another adhesion plaque protein), vimentin (intermediate filament), nor tubulin (microtubule) staining is affected by PDGF treatment (see Fig. 2). This suggests that focal contacts or adhesion plaques remain intact in cells after exposure to PDGF, and that vinculin is specifically removed from these structures after PDGF addition. These results are in agreement with our observations that no change in cell shape occurs during the alterations in vinculin distribution after exposure of cells to PDGF. Previous work from other laboratories (33) has shown that PDGF treatment (1.3 nM) of serum-starved glial cells results in a large increase in membrane ruffling activity and extension of lamellapodia. These changes, which occur within 1 to 60 min of PDGF addition, are also associated with translocation of membrane from the cell periphery to the cell center and an increased shuttling of intracellular particles. These alterations in membrane activity were associated with a rearrangement of actin microfilaments yielding a substrate set of stress fibers as well as a high concentration of actin filaments in the membrane ruffles. PDGF treatment did not cause the disappearance of actin staining in these cells as it does in BALB/c-3T3 (see Fig. 1); it is not known whether similar changes in membrane ruffling activity occur shortly after PDGF treatment of BALB/c-3T3 cells and if so whether the alterations we observe in vinculin distribution may be related to this phenomenon. It is interesting to note that similar changes in ruffling activity, increases in micropinocytic activity, and microfilament organization have been reported in A431 cells shortly after treatment with EGF (9). Thus an early effect of growth factors in general may be to enhance membrane turnover activity and cause alterations in intracellular actin distribution. It is not known, however, whether these alterations in membrane activities and microfilament distributions are related to cell growth and whether concomitant disruption of vinculin from adhesion plaques occurs during these processes.

The hypothesis that alterations in vinculin may be related to competence induction in BALB/c-3T3 cells is strengthened by the findings that other agents which have been shown to induce competence in BALB/c-3T3 cells (FGF, choleragen, and Ca^{2+}), also cause the disappearance of vinculin from adhesion plaques. Other growth factors (e.g., EGF or SmC) and insulin had no effect on vinculin staining in these cells, suggesting that the disruption of vinculin staining in adhesion plaques is not a general biological effect of growth factor or hormone binding. How PDGF might induce competence in BALB/c-3T3 cells is not known. The findings that (*a*) choleragen potentiates the effects of suboptimal PDGF concentrations (56), (*b*) IBMX enhances the action of choleragen (32), and (*c*) the differential sensitivity of various cell lines to PDGF can be related to their intracellular cAMP concentration (37), suggest that PDGF can sensitize cells to the action of EGF possibly through a cAMP-mediated mechanism. It is interesting to note that PDGF has also been shown to decrease EGF binding to BALB/c-3T3 cells (56) which is augmented by cholera toxin treatment. Whether the decrease in the amount of EGF bound is due to EGF receptor internalization or due to masking of plasma membrane receptors is not known; this decrease suggests, however, that PDGF may make cells competent by causing down regulation of the EGF receptor which is necessary for mitogenesis. Alternatively, the action of

PDGF and EGF may be via two different pathways necessary for the entry of cells into S phase.

Further support for a role of vinculin in the interaction of PDGF and EGF in cell cycle progression comes from our findings that vinculin staining disappears from adhesion plaques in cells treated with EGF after exposure to low doses of PDGF, which by itself does not disrupt vinculin adhesion plaque staining (see Table II). This strengthens the interpretation that PDGF can sensitize cells to the action of other specific growth factors; the observation that this vinculin disruption from adhesion plaques can still occur even up to 8 h after PDGF removal (Fig. 4) is consistent with the properties of PDGF-induced competence. Lastly, the nearly equivalent percentage of cells which demonstrate absence of vinculin adhesion plaque staining and [^{3}H]thymidine-labeled nuclei at various concentrations of PDGF suggests that vinculin disruption is important for the induction of competence and entry of cells into the cell cycle.

Binding of PDGF to purified plasma membranes results in the rapid stimulation of a membrane-associated protein kinase, which phosphorylates two major polypeptides (molecular weights of 185,000 and 130,000) and several minor components of the membrane fraction (17). The 185,000-mol-wt protein is thought to be the PDGF receptor, whereas the identity of the 130,000-mol-wt protein is not known (although this is the molecular weight of vinculin as determined by gel electrophoresis). Part of this phosphorylation is on the tyrosine residues in these proteins (10, 17, 26, 27, 48). In vivo, PDGF addition to Swiss 3T3 cells has been shown to phosphorylate two 45-kD, two 43-kD, and one 42-kD proteins at tyrosine residues (10). Similar changes were noted in these cells when they were exposed to EGF, which suggests that phosphorylation of cellular substrates at tyrosine residues may be a common mechanism of action of the two growth factors. The lack of detectability of higher molecular weight phosphorylated proteins (as was found in isolated membrane fractions) may be due to the biochemical fractionation procedures which involve the use of fluoride and EDTA, which stimulate phospho-tyrosine protein phosphatase in whole cells and may rapidly dephosphorylate certain proteins (16).

The fact that the PDGF and EGF receptors contain a tyrosine kinase activity (8) which is similar to the tyrosine kinase activity of oncogene protein products (23, 26, 52) suggests that the action of the receptor and oncogene proteins may be similar with respect to growth-promoting activity. It has recently been found that the *src* oncogene protein product has been localized to adhesion plaques (43, 50), and the alterations in actin distribution observed after oncogene transformation (11) are very similar to the early changes in actin distribution we observe after exposure of BALB/c-3T3 cells. Additional work demonstrates that the tyrosine kinase *src* oncogene protein phosphorylates vinculin (28, 48). The recent findings which demonstrate that vinculin is a substrate for protein kinase c (55), that phorbol esters have been shown to cause vinculin phosphorylation (54) and the specific redistribution of vinculin and actin (47), and that PDGF addition results in rapid increases in intracellular Ca^{2+} (34), suggest that phosphorylation of vinculin may regulate its activity with respect to cell growth. The very recent findings that the simian sarcoma virus oncogene (v-sis) codes for the β chain of PDGF (12, 14, 15, 29) suggests that studies of the biochemical regulation of growth by PDGF may be important in helping elucidate the mechanisms of oncogenesis.

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