Platelet-derived Growth Factor-stimulated Secretion of Basement Membrane Proteins by Skeletal Muscle Occurs by Tyrosine Kinase-dependent and -independent Pathways

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The basement membrane of skeletal muscle is produced by the muscle cells it ensheathes and by non-muscle cells located in the surrounding extracellular matrix. In this study, we have shown that platelet-derived growth factor (PDGF) stimulates secretion of three basement membrane components of skeletal muscle: laminin (70% increase), fibronectin (30%), and type IV collagen (70%). Furthermore, we have found using the signal transduction inhibitors, genistein (tyrosine kinase C (PKC) inhibitor), phorbol 12-myristate 13-acetate (protein kinase C (PKC) inhibitor), thapsigargin (depletes intracellular Ca\(^{2+}\) stores), and H89 (protein kinase A inhibitor), that PDGF-stimulated secretion of these proteins occurs through distinct signaling pathways. Densitometry of Western blots of L6 myoblast supernatant indicates that the PDGF-induced increase in secretion of laminin and type IV collagen is tyrosine kinase-dependent. The increase in type IV collagen secretion also shows dependence on PKC, as well as the release of intracellular Ca\(^{2+}\). Inhibition of either of these pathways reduces the increase in type IV collagen secretion to 20%. In contrast, the PDGF-induced increase in laminin secretion is unaffected by inhibition of either PKC or intracellular Ca\(^{2+}\) release. The increase in fibronectin secretion by PDGF uses yet a third set of signals. PDGF-induced fibronectin secretion is not dependent on tyrosine kinase activity but is dependent on protein kinase A as well as the release of intracellular Ca\(^{2+}\). These divergent signaling pathways provide for independent regulation of basement membrane protein secretion, allowing a muscle cell to modify both the quantity and composition of its basement membrane in response to its environment.

The basement membrane of skeletal muscle forms a continuous layer of connective tissue that completely ensheathes each individual muscle fiber. Thus, all signals that reach the muscle fiber must traverse the basement membrane, all forces generated by muscle must be transmitted across the basement membrane before acting upon other extracellular structures, and any change in muscle fiber size or shape that occurs during development, growth, or adaptation must involve remodeling of the basement membrane. Previous investigations have also shown that the composition of the basement membrane of muscle shows regional variability in composition and thickness. For example, the thickness and composition of the basement membrane at myotendinous junctions and neuromuscular junctions differs from the basement membrane at other sites on the fiber (1, 2). Furthermore, the assembly of the basement membrane during development and regeneration occurs by the sequential, rather than concurrent, release of basement membrane proteins onto the surface of the muscle fiber (3, 4). Together, these observations support the hypothesis that the synthesis and secretion of individual basement membrane proteins can be regulated independently.

Basement membrane proteins can also influence proliferation and differentiation of muscle cells. Surprisingly, these influences of different basement membrane proteins on muscle development can be antagonistic. For example, laminin stimulates differentiation of skeletal myoblasts in culture while fibronectin inhibits differentiation, but stimulates proliferation (5, 6). Thus, one would expect that muscle cells would independently regulate the synthesis and secretion of basement membrane proteins during development, if these proteins play a significant role in regulating developmental events. One means by which independent regulation could occur would be if the synthesis and secretion of each individual basement membrane component were regulated by a unique set of intracellular signals. This would enable muscle cells that were stimulated by growth factors or other substances that regulate development, to independently regulate the synthesis and secretion of basement membrane molecules in response to the stimulation.

In the present investigation, we have tested the hypotheses that platelet-derived growth factor (PDGF) stimulates secretion of basement membrane proteins in skeletal muscle cells and that the intracellular signaling mechanisms that regulate secretion of distinct basement membrane proteins differ downstream from receptor-ligand binding. PDGF was selected for study because previous studies have shown that PDGF stimulates the synthesis and secretion of many basement membrane proteins in non-muscle cells, including laminin, fibronectin, and type IV collagen (7). Furthermore, it is feasible that PDGF stimulation of muscle cells can cause local changes in basement membrane synthesis because previous investigations have shown that PDGF receptors become heterogeneously distributed on the cell surface during muscle differentiation (8, 9). We have tested the above hypotheses by measuring changes in basement membrane protein secretion by PDGF-stimulated cells in the presence of inhibitors to specific components of selected signaling pathways. By quan...

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† The abbreviations used are: PDGF, platelet-derived growth factor; DMEM, Dulbecco’s modified Eagle’s medium; PAGE, polyacrylamide gel electrophoresis; PLC, phospholipase C; PKA, CAM-dependent protein kinase; FuDR, 5-fluor-2-deoxyuridine; IP3, inositol trisphosphate; PMA, phorbol 12-myristate 13-acetate.
cultures were then transferred to 20 ng/ml PDGF, 6\textmu g/ml uridine (FuDR; Sigma) or DMEM only for 8 h. FuDR-treated cultures were then incubated with either 20 ng/ml PDGF or DMEM only for 10 min and then washed with DMEM to remove all residual PDGF. Supernatant was collected and prepared for SDS-PAGE analysis.

**Experimental Procedures**

**Cell Culture**—Rat L6 myoblasts were grown on plastic tissue culture dishes in Dulbecco’s minimal essential medium (DMEM) with 10% fetal bovine serum supplemented with 1% penicillin and streptomycin at 37 °C and 5% CO2. The culture medium was changed every other day until the cells were confluent. The cells were then incubated in DMEM alone for 24 h. Cultures were subsequently washed with DMEM, and then the control cultures transferred to DMEM only and experimental cultures transferred to DMEM supplemented with PDGF-BB at selected concentrations (Upstate Biotechnology, Inc. (UBI), Lake Placid, NY). At the end of PDGF stimulation, supernatant was collected from experimental and control cultures and centrifuged for 10 min at 3000 \times g. Supernatants were then prepared for gel samples by adding 15 \mu l of 1% bromophenol blue, 15 \mu l of 2% sodium dodecyl sulfate, 15 \mu l of \beta-mercaptoethanol, and 15 \mu l of 50% glycerol to 500 \mu l of supernatant. The samples were then boiled and electrophoresed on 8% acrylamide gels (SDS-PAGE; Ref. 10).

**Time Course**—PDGF-BB was added at 20 ng/ml to confluent L6 cells that were then incubated for 1, 4, 8, or 24 h. Control cultures for each time point were treated identically to PDGF-stimulated cells, except that their media were PDGF-free. Duplicate cultures were prepared for each experimental and control time point, and supernatants and cells were collected separately for analysis by immunoblots. Supernatant was also collected from one unstimulated culture at the onset of the experiment (\textit{t} = 0 h).

**Dose Response**—PDGF-BB was added to the culture medium at 0, 2, 10, 20, 50, and 100 ng/ml. Two plates of each concentration were incubated for 24 h, and then the supernatant was collected and prepared for SDS-PAGE.

**Pulse Stimulation**— Cultures were stimulated with 20 ng/ml PDGF-BB for 10 min and then washed with DMEM to remove all residual PDGF from the cultures. The cells were then incubated for 24 h in DMEM only. Other treatments consisted of three plates of cells that were stimulated with DMEM only and incubated for 24 h in DMEM only (controls), and three plates that experienced continuous 20 ng/ml PDGF stimulation for the entire 24-h incubation period (chronic stimulation).

In an additional stimulation experiment, cultures were incubated in DMEM containing 6 \mu g/ml uridine (Sigma) and 2.4 \mu g/ml 5-fluor-2’-deoxyuridine (FuDR, Sigma) or DMEM only for 8 h. FuDR-treated cultures were then transferred to 20 ng/ml PDGF, 6 \mu g/ml uridine, and 2.4 \mu g/ml FuDR in DMEM for 24 h. DMEM-treated cultures were then treated with either 20 ng/ml PDGF or DMEM only for 10 min and then rinsed three times in DMEM. After 24 h, supernatant was collected from PDGF/uridine/FuDR cultures, PDGF cultures, and DMEM-only cultures and prepared for SDS-PAGE and immunoblots.

**Signal Transduction Inhibitor Experiments**—The signaling pathways involved in PDGF stimulation of basement membrane protein secretion were analyzed by stimulating L6 myoblasts in the presence of a variety of signal transduction inhibitors. These included: genistein (122 nM), which inhibits tyrosine kinase activity; thapsigargin (50 nM), which prevents the release of intracellular Ca^{2+}; PMA (600 nM), which inhibits protein kinase C; and H89 (30 \mu M), which inhibits protein kinase A. Cells were preincubated in inhibitor for 14 h prior to stimulation with 20 ng/ml PDGF, 6 \mu g/ml uridine, 2.4 \mu g/ml FuDR, and the desired inhibitor.

In some experiments, cells were incubated with brefeldin A (10 \mu g/ml), which inhibits protein secretion by disrupting the Golgi apparatus, during PDGF stimulation. The brefeldin A treatments enabled distinction between PDGF stimulation of protein secretion and stimulation of protein release from the cell surface that did not involve secretion. Triplicate cultures were prepared for each experimental condition. Supernatant was collected 24 h after PDGF stimulation and prepared for SDS-PAGE analysis.

**Fig. 1.** Immunoblots of supernatants from L6 cells stimulated with 20 ng/ml PDGF-BB (PDGF) or unstimulated (CONT). Lanes are labeled to indicate hours of stimulation (0, 2, 4, 8, and 24). Upper blot shows immunoblot labeled with anti-laminin; middle blot, labeled with anti-collagen type IV; lower blot, labeled with anti-fibronectin. Arrowheads indicate position of each protein predicted by known mass in reducing SDS-PAGE. Lower bands are proteolytic fragments that accumulate during longer experiments.

**Fig. 2.** Densitometric measurements of alkaline phosphatase reaction products of immunoblots of L6 cell supernatants following stimulation with 20 ng/ml PDGF-BB for 0, 2, 4, 8, 24 h, or non-stimulated cell supernatants collected at the same time points. Immunoblots were incubated with anti-laminin (upper), anti-collagen type IV (middle), or anti-fibronectin (lower).
L6 myoblasts stimulated with 20 ng/ml PDGF-BB showed loss of PDGF receptors that were recognizable by antibodies to PDGF-β receptor oligopeptide. Therefore, we used PDGF receptor blots of cell extracts to confirm that stimulation and receptor internalization were taking place in each experiment.

**Immunoblots**—Gel samples prepared in each experiment were electrophoresed by SDS-PAGE as described above, and then electrophoretically transferred to nitrocellulose paper for 4 h at 1 A. The blots were blocked overnight at 4 °C in 50 mM Tris, pH 7.6, containing 150 mM NaCl and 0.1% NaN₃ (buffer A). The blots were then overlaid with one of the following primary antibodies diluted in buffer A containing 0.05% Tween 20 and 0.3% bovine serum albumin: 1) goat anti-type IV collagen (Southern Biotech, Birmingham, AL), diluted 1:300; 2) rabbit anti-laminin (Life Technologies, Inc.), diluted 1:40; or 3) rabbit anti-fibronectin (Calbiochem, San Diego, CA), diluted 1:200 (Fig. 1). The primary antibodies recognized proteins under reducing conditions which had apparent molecular masses of 220 kDa (laminin), 200 kDa (fibronectin), and 190 kDa (type IV collagen). The collagen IV antibody only recognized a single band at the molecular mass of 220 kDa, whereas the laminin and fibronectin antibodies recognized a doublet at 6.5 and 5.3 kilobases, the laminin 1 probe recognized a single band at 6.5 kilobases, and the fibronectin 1 probe recognized a 5.3-kilobase message, and the fibronectin probe recognized an 8-kilobase message.

**Densitometry of antibody reaction product on the blots** was performed using an imaging system/densitometer (Alpha Innotech). A standard curve containing known amounts of purified laminin, fibronectin, or collagen type IV was done to determine the linear range of detection for each antibody. The standard curves were done on blots that also contained a known volume of control supernatant so that we were able to determine the amount of laminin, fibronectin or collagen type IV in the samples. Densitometric values were compared by t tests with confidence level set at p < 0.05.

The total amounts of laminin, collagen type IV, and fibronectin in the supernatant of 24-h control samples were determined by comparing densitometric measurements of the supernatant to a loading curve composed of known amounts of purified extracellular matrix protein. The densitometric values measured from the supernatant could then be directly compared to those of known amounts of purified protein. Thus an estimate of the amount of laminin, collagen IV, and fibronectin in the supernatant was determined.

**Northern Blots**—Total RNA was isolated from L6 myoblasts stimulated with PDGF-BB for 0, 2, 4, 6, 8, 10, 12, 16, 20, and 24 h. The total RNA was then transferred to a nylon membrane and probed with a 32P-labeled cDNA probe to collagen type IV α1 chain (COL4A1, ATCC; Ref. 12), laminin β1 chain (LAMB1, ATCC; Ref. 13), and fibronectin (FN1, ATCC; Ref. 14). The collagen IV probe recognized a single band at 6.5 and 5.3 kilobases, the laminin β1 probe recognized a 6-kilobase message, and the fibronectin probe recognized an 8-kilobase message.

**Cell Counting**—The possibility that changes in secreted protein concentration could represent changes in cell number resulting from PDGF stimulation, rather than from changes in rate of protein secretion, was tested by assaying for changes in cell concentration in PDGF-stimulated cultures. Two plates of the cultured cells were stimulated with 20 ng/ml PDGF for 24 h and two plates served as controls. After 24 h, the supernatant was discarded and the cells were rinsed with buffer A and then fixed with 2% paraformaldehyde for 10 min. The cells were rinsed again with buffer A and stained with hematoxylin for 1 min. Cells in each of five randomly selected fields observed by light microscopy using a 20× objective were counted. At this magnification, each sampled field was 0.1225 mm². In an additional experiment, cells were pulse-stimulated for 10 min with PDGF, PDGF in the presence of uridine, and FuDR or DMEM alone. Cultures were then rinsed with DMEM and stained, and cell counts were performed 24 h following stimulation.

**RESULTS**

**PDGF Stimulates Secretion of Collagen Type IV, Fibronectin, and Laminin**—L6 myoblasts grown in DMEM only secreted ap-
approximately 0.14 pg/cell collagen type IV, 1.4 pg/cell laminin, and 88 pg/cell fibronectin in 24 h. Stimulation of L6 myoblasts for 24 h with 20 ng/ml PDGF-BB resulted in a significant increase in collagen type IV, laminin, and fibronectin in the culture media relative to control cultures (Figs. 1 and 2). Densitometric measurement of alkaline phosphatase reaction products in immunoblots showed that laminin concentration in the culture media after 24 h of PDGF stimulation was 115% greater than the concentration in control culture media, collagen type IV concentration in stimulated cell media was 188% greater than control values, and the fibronectin concentration in stimulated cell culture media was 36% greater than in control. Although a portion of this increase in laminin and collagen type IV concentration is attributable to a greater rate of cell proliferation in PDGF-stimulated cultures, stimulated cultures contained only 35% more cells than unstimulated cultures at the end of 24 h. Thus, the majority of laminin and collagen IV accumulation in the media of stimulated cells is attributable to an increase in secretion of these proteins by the stimulated cells.

Using inhibitors of cell proliferation, we were able to determine that the increase in laminin, type IV collagen, and fibronectin secretion resulting from PDGF stimulation did not result from an increase in cell density induced by PDGF stimulation. Cells stimulated with PDGF or PDGF in the presence of anti-mitotic agents (FuDR/uridine) did not differ significantly in laminin or fibronectin secretion. Although type IV collagen secretion was decreased with anti-mitotic treatments, fibronectin, collagen type IV, and laminin concentrations exceeded that in unstimulated cell supernatants (Figs. 3 and 4).

Matrix protein secretion was examined as a function of PDGF-BB concentration following 24 h of stimulation (Figs. 5 and 6). Maximum collagen type IV secretion occurred at 20 ng/ml PDGF-BB. Maximum laminin concentration was measured following stimulation with 50 ng/ml PDGF-BB, although the value did not differ significantly from that obtained following stimulation with 10 or 20 ng/ml. Fibronectin secretion that occurred at 100 ng/ml PDGF-BB was significantly greater than that measured at 20 ng/ml, although the 5-fold increase in PDGF concentration produced only a 10% increase in fibronectin concentration.

No decreases were observed in the concentration of laminin, fibronectin, or collagen type IV in extracts of myoblasts stimulated with 20 ng/ml PDGF for 24 h (Fig. 7). Thus, the increase in collagen type IV, fibronectin, and laminin in the culture media of stimulated cells represents a net increase in the quantities of these proteins in the preparations, rather than simply a shift in distribution of laminin, fibronectin, and collagen type IV from myoblasts to the supernatant.

Further evidence, which demonstrates that laminin, collagen IV, and fibronectin are secreted by the muscle cells, comes from experiments where the muscle cells were stimulated with PDGF in the presence of brefeldin A. In the presence of brefeldin A, laminin and collagen type IV were undetectable in the supernatant and fibronectin levels were greatly reduced and showed no increase with PDGF stimulation (Fig. 8). These data further demonstrate that the increases in laminin, collagen IV, and fibronectin in the supernatant result from PDGF stimulation of secretion rather than simply a PDGF-stimulated release of proteins from the cell surface.

Finally, Northern blots done on cells over a time course following PDGF stimulation demonstrate that the myoblasts are actively up-regulating the message levels for these three proteins (Fig. 9). The message levels for laminin, collagen IV, and fibronectin all showed dramatic increases by 6 h following PDGF stimulation with peak levels occurring at 16 h for lami-
nin (300% increase), 8 h for collagen IV (180% increase), and 16 h for fibronectin (130% increase). Thus, the increase in mRNA for each of these three proteins and the increase in concentration of the proteins in the cell supernatant confirm that PDGF stimulation is causing an increase in the production and secretion of these proteins by regulating their level of transcription.

**Brief Periods of PDGF Stimulation and Chronic Stimulation Produce Similar Changes in Matrix Secretion by Myoblasts**—The concentration of laminin in culture media of cells stimulated for 24 h with 20 ng/ml PDGF-BB (chronic stimulation) produced an increase in laminin in the culture media (190% increase relative to controls) that was significantly different from that observed following 10 min of incubation with PDGF-BB (pulse stimulation; 133% increase). Pulse stimulation also produced a significant increase in secreted collagen type IV (68% increase) that was significantly less than that produced by chronic stimulation (131% increase). Chronic (28% increase) and pulse stimulation (25% increase) produced identical increases in fibronectin secretion (Figs. 10 and 11).

**PDGF Stimulates Secretion of Laminin, Fibronectin, and Type IV Collagen via Tyrosine Kinase-dependent and -independent Pathways**—Stimulating L6 myoblasts with PDGF in the presence of various signal transduction inhibitors demonstrated that the PDGF receptor uses distinct intracellular signaling pathways to increase basement membrane protein secretion. The increase in fibronectin secretion stimulated by PDGF was independent of tyrosine kinase activity, with genistein having no effect on the PDGF-induced secretion of this protein (Figs. 12 and 13). However, incubation of the cells in H-89 prior to PDGF stimulation, or depletion of intracellular Ca\[^{2+}\] with thapsigargin, completely abolished the PDGF-induced increase in fibronectin secretion (Figs. 12 and 13). Thus, the release of intracellular Ca\[^{2+}\] that is essential for PDGF stimulation of fibronectin secretion occurs through a tyrosine kinase-independent pathway.

The PDGF-induced increase in type IV collagen secretion was completely inhibited by genistein, thereby indicating dependence on tyrosine kinase activity (Figs. 14 and 15). The PDGF-induced secretion of type IV collagen also showed de-
pendence on the release of intracellular Ca\textsuperscript{2+} stores, as well as on protein kinase C activity, although inhibition by thapsigargin or PMA was not complete in either case (Figs. 14 and 15). The similar levels of inhibition by thapsigargin and PMA suggest that they both function in the same signaling pathway rather than through independent mechanisms. We could not confirm this because incubation with both of the inhibitors resulted in substantial cell death.

PDGF-stimulated laminin secretion was not inhibited by any of the signal transduction inhibitors except the tyrosine kinase inhibitor, genistein (Figs. 16 and 17). Thus, the PDGF-induced increase in laminin secretion is dependent on tyrosine phosphorylation, but it does not involve phospholipase C activity or the generation of inositol trisphosphate (IP\textsubscript{3}) and diacylglycerol, which in turn cause release of intracellular Ca\textsuperscript{2+} and activation of PKC.

**DISCUSSION**

**PDGF Stimulation of Laminin, Type IV Collagen, and Fibronectin Secretion Is Mediated by Distinct Sets of Second Messengers—**Unidentified, soluble factors have been shown previously to stimulate secretion of extracellular matrix molecules into the supernatant of myoblasts grown in vitro (15, 16). Those factors could feasibly play a regulatory role in production of the connective tissues surrounding muscle cells during normal development and growth, or during adaptation or response to injury. It is also feasible that pathologically high concentrations of those matrix stimulatory factors could lead to fibrotic changes observed in muscle that are known to occur in several myopathies, such as Duchenne muscular dystrophy (17). We have begun to examine these possibilities by investigating the influence of PDGF on myoblast secretion of basement membrane proteins.

We have shown that PDGF-induced secretion of basement membrane proteins is mediated by distinct, intracellular signaling pathways, including pathways that are dependent and independent of tyrosine kinase activity. PDGF-stimulated increases in fibronectin secretion occur via a tyrosine kinase-independent pathway that is dependent on PKA activity. A tyrosine kinase-independent, PDGF-stimulated signaling pathway has been demonstrated previously in Balb/c 3T3 cells in which PDGF stimulation induces expression of egr-1 without detectable tyrosine phosphorylation of the PDGF receptor (18). Furthermore, in our experiments, PDGF receptor down-regulation in response to stimulation was still observed in the presence of genistein. This observation agrees with previous studies showing that in a kinase-inactivated PDGF receptor mutant, PDGF receptors are internalized and degraded in response to ligand binding (19). Therefore, tyrosine phosphorylation is not necessary for at least some of the biological responses that occur due to the PDGF receptor binding its ligand. In other studies, PDGF stimulation of 3T3 cells has been shown to release PKA from the cell membrane, resulting in an increase in activated cytosolic PKA (20). Although this latter study did not specifically test whether PKA release occurred independently of PDGF receptor phosphorylation, it is possible that the reported release of PKA from the cell membrane in PDGF-stimulated 3T3 cells is a component of the tyrosine kinase-independent pathway identified here. Results of the present investigation also show that PDGF stimulation of fibronectin secretion is sensitive to depletion of intracellular Ca\textsuperscript{2+} by thapsigargin. This is interesting because the major pathway for the release of intracellular Ca\textsuperscript{2+} by PDGF stimulation is thought to be through the activation of phospholipase

**FIG. 11.** Densitometric measurements of alkaline phosphatase reaction products in immunoblots of L6 cell supernatants stimulated with 20 ng/ml PDGF-BB. Supernatants were collected 24 h after start of the experiment. Cells were stimulated continuously (chronic), for 10 min at the beginning of the experiment (pulse), or not stimulated (control). *, p < 0.05 versus control; †, designates p < 0.05 versus chronic.

**FIG. 12.** Anti-fibronectin immunoblots of L6 supernatants stimulated for 24 h with 20 ng/ml PDGF BB, in the presence of the signal transduction inhibitors: genistein (Gen), PMA, thapsigargin (Thaps), and H89.
Cγ (PLC-γ), which then cleaves phosphoinositol bisphosphate into the second messengers IP₃ and diacylglycerol (21). IP₃ is then capable of releasing intracellular Ca²⁺ by activating the IP₃ receptor in the sarcoplasmic reticulum (reviewed in Ref. 21). Thus, the release of intracellular Ca²⁺ by this mechanism should be inhibited by using the tyrosine kinase inhibitor genistein, since it will prevent the activation of PLC by preventing the autophosphorylation of the PDGF receptor. Treatment with genistein has no effect on the PDGF-induced increase in fibronectin secretion, suggesting that there must be an alternate signaling pathway that is dependent upon the release of intracellular Ca²⁺ without the generation of IP₃ by PLC.

The finding reported here, that PDGF-induced secretion of type IV collagen is inhibited by tyrosine kinase inhibitors, indicates that this signaling pathway relies on the well characterized generation of the second messengers IP₃ and diacylglycerol, following PLC phosphorylation and activation. Furthermore, the PDGF-induced secretion of type IV collagen is sensitive to inhibition of protein kinase C, as well as the release of intracellular Ca²⁺. Signaling through both of these second messengers can be attributed to PLC activation and therefore would also be inhibited by the tyrosine kinase inhibitor genistein. However, inhibition of either protein kinase C or the release of intracellular Ca²⁺ did not completely inhibit the PDGF-induced increase in type IV collagen secretion. Since these experiments were done in the presence of the anti-mitotic agents FuDR and uridine, the remaining increase in type IV collagen secretion cannot be explained by an increase in cell number. This suggests that the mechanism of increased type IV collagen secretion may include more than one signaling pathway that is tyrosine kinase-dependent.

The PDGF-induced increase in laminin secretion is also dependent on tyrosine phosphorylation, but it is not sensitive to protein kinase C inhibition or intracellular Ca²⁺ depletion. In fact, inhibiting tyrosine phosphorylation was the only way in which we were able to prevent the induction of laminin secretion by PDGF. This eliminates any involvement of PLC and therefore implicates other signaling pathways that may be initiated by receptor autophosphorylation on tyrosine. These include activation of Ras through GRB2 as well as the activation of the mitogen-activated protein kinases (21). This arrangement of distinct signaling pathways gives the cell the potential to regulate the amount of secretion of a given protein independently of the others, making it possible for the cell to change the quantity, as well as the composition, of its basement membrane (see Diagram 1).

**Biological Implications of Distinct Signaling Mechanisms Regulating Basement Membrane Protein Secretion**—Although these findings would indicate that PDGF stimulation increases the production of basement membrane by myoblasts, electron microscopic evidence shows that basement membrane is not discernible on the surface of muscle cells *in vivo* until they have fused to form myotubes (22). Furthermore, the present study shows an increase in laminin, collagen type IV, and fibronectin in the culture media of PDGF-stimulated cells, but no increase in cell-associated laminin, collagen type IV, or fibronectin. These observations suggest that the ECM molecules released

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**FIG. 13.** Densitometric measurements of reaction products in immunoblots of L6 cell supernatants stimulated with 20 ng/ml PDGF-BB in the presence of the signal transduction inhibitors: genistein (Gen), PMA, thapsigargin (Thaps), and H89. *, p < 0.05 versus control without inhibitor; †, designates p < 0.05 versus control with inhibitor.

**FIG. 14.** Anti-type IV collagen immunoblots of L6 supernatants stimulated for 24 h with 20 ng/ml PDGF-BB, in the presence of the signal transduction inhibitors: genistein (Gen), PMA, thapsigargin (Thaps), and H89.

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*PDGF Stimulates Basement Membrane Protein Secretion*
by myoblasts following PDGF stimulation may serve some function other than basement membrane formation.

Previous investigations (5, 23, 24) have shown that laminin can stimulate myoblast locomotion, proliferation, and differentiation. These functions are antagonistic, at least to some degree, to those associated with fibronectin, which is present at high concentrations in muscle in vivo at the time that morphogenetic migrations of myogenic cells cease (25, 26). Fibronectin is also capable of stimulating myoblast proliferation in vitro (26), delaying myoblast fusion (27) and delaying expression of muscle myosin and desmin (5). The results of the present study that show a large increase in laminin secretion and only a slight change in fibronectin secretion following PDGF stimulation could suggest that the end effect of PDGF stimulation of myogenic cells would be promoting cell differentiation via laminin-mediated effects. The duration of stimulation in the present study was too brief to observe fusion of myogenic cells, and no other indicators of differentiation were assayed. However, previous studies have shown that PDGF stimulation of L6 myoblasts used at concentrations employed in the present study will inhibit myotube formation (8, 28), reduce the proportion of muscle cells expressing myosin heavy chain (29), and reduce the expression of creatine phosphokinase and sarcomeric actin (28). Furthermore, mRNA for muscle myosin heavy chain does not appear in L6 cells until after mRNA for PDGF receptor is no longer detectable (28). Thus, PDGF treatment of muscle cells causes an inhibition of differentiation, even though the treatment also elevates laminin secretion, which can stimulate muscle cell differentiation and muscle myosin expression when administered alone (5).

The influence of laminin on promoting differentiation may become apparent only after the influence of PDGF has been removed. This would be consistent with the sequence of events observed in vivo (30). An additional function for PDGF stimulation of laminin and collagen type IV secretion may be to provide a positive chemotactic signal for myoblasts and other cells present in developing muscle. Both laminin and type IV collagen have been shown to stimulate directed migration of myogenic, neuronal, and other cell types (24, 31), while PDGF is chemotactic for many mesenchymal and myogenic cells (32). The mechanisms for chemotactic signaling by collagen type IV and PDGF may share some characteristics. Type IV collagen-stimulated chemotaxis is mediated by elevating intracellular concentrations of free calcium in target cells (33), while PDGF stimulation also causes an elevation in intracellular free calcium (34, 35), although it has not yet been tested whether that elevation in calcium following PDGF stimulation is necessary for myoblast migrations. The positive chemotactic effects of PDGF, laminin, and type IV collagen, together with observations that PDGF stimulates laminin and collagen type IV secretion, suggest that the elevation in laminin and collagen type IV secretion following PDGF stimulation can provide a mechanism for amplifying the effects of PDGF on chemotaxis. Since PDGF has a brief half-life following its secretion (36), it can function only transiently as a chemoattractant. However, the effects of PDGF can be amplified over longer periods if...
PDGF also stimulates continued, elevated synthesis of other chemoattractants, such as laminin. The results of the present study that show laminin secretion remains elevated for periods of at least 24 h following 10 min of PDGF stimulation support this hypothesis. It may be significant that laminin and type IV collagen increase the frequency of neurite sprouting (reviewed in Ref. 37) and that muscle cells bearing PDGF receptors are most prevalent in muscle during the latter third of fetal development (38), at the stage when neurite invasion into muscle occurs most extensively.

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FIG. 17. Densitometric measurements of reaction products in immunoblots of L6 cell supernatants stimulated with 20 ng/ml PDGF-BB in the presence of the signal transduction inhibitors: genistein (Gen), PMA, thapsigargin (Thaps), and H89. * p < 0.05 versus control without inhibitor; † p < 0.05 versus control with inhibitor.