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PDGF Stimulation Induces Phosphorylation of Talin and Cytoskeletal Reorganization in Skeletal Muscle

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Abstract. Modifications in the interactions of the muscle cytoskeleton with the cell membrane occur during cell growth and adaptation, although the mechanisms regulating these interactions are unknown. We have observed that myotendinous junctions (MTJs), which are the primary sites of turnover of the thin filament-membrane associations in skeletal muscle, are greatly enriched in receptors for PDGF. The high concentration of PDGF receptors at MTJs suggested to us that receptor binding may initiate cytoskeletal remodeling in skeletal muscle. We tested this possibility by examining the organization and phosphorylation of cytoskeletal components of L6 myocytes after PDGF stimulation. We have found that 10 min after PDGF stimulation, L6 myoblasts exhibit no stress fibers discernible by phalloidin binding, and that vinculin relocates from focal contacts into a diffuse cytoplasmic distribution. After 60 min of incubation, these changes are largely reversed. Indirect immunofluorescence shows that at 10-min PDGF stimulation, there are no changes in the distribution of talin, the β_1 subunit of integrin, pp125^{FAK} or desmin. Phosphotyrosine distribution changes upon stimulation from focal contacts to being located both in focal contacts and granules concentrated in perinuclear regions. These

granules also immunolabel with anti-PDGF receptor.

Immunoprecipitations with anti-phosphotyrosine show that polypeptides at 180 and 230 kD show the greatest increase in tyrosine phosphorylation after PDGF stimulation. Immunoblots of anti-phosphotyrosine precipitates show that these polypeptides are the PDGF receptor and talin. We also examined the possibility that the cytoskeletal reorganization observed may result from calpain activation caused by elevated intracellular calcium induced by PDGF stimulation. However, immunoblots of control and stimulated cells show no decrease in the inactive calpain proenzyme or increase in the proteolytic, autolyzed forms of calpain pursuant to stimulation. Furthermore, stimulation produces no increase in the proportion of the 190-kD talin fragment characteristic of calpain-mediated cleavage. The retention of talin and integrin at focal contacts after talin phosphorylation, while vinculin is redistributed, indicate that phosphorylation of talin in PDGF-stimulated cells leads to separation of talin-vinculin associations but not talin-integrin associations. We propose that PDGF binding to PDGF receptors at MTJs may provide one means of regulating myofibril associations with the muscle cell membrane.

INTERACTIONS between the actin cytoskeleton and the cell membrane are mediated by a population of proteins that play both structural and regulatory roles. Modifications in these interactions occur when cells change shape, grow, divide, or locomote. Thus, regulation of cytoskeletal-membrane interactions is a basic cellular function occurring throughout the life of a cell. Although knowledge concerning the mechanisms regulating these interactions is scant, two general regulatory mechanisms are expected to occur broadly: controlled proteolytic cleavage of specific links in the cytoskeletal-membrane association to permit

remodeling, or, phosphorylation-dephosphorylation of specific links to enable modifications in their interactions.

Proteolysis as a mechanism for regulating cytoskeletal-membrane associations has been well characterized in platelets. During platelet activation and ensuing cell shape changes (Adelstein and Conti, 1975; Kajiwara et al., 1987; Tsujinaka et al., 1988), intracellular calcium is elevated (Feinstein, 1980), thereby activating calcium-dependent proteases, or calpains, that cleave proteins, such as talin (O'Halloran et al., 1985; Fox et al., 1985, 1990; Beckerle et al., 1986), that mediate thin filament membrane associations. Evidence also has been obtained that implicates calpain mediated modifications in the cytoskeleton of several cell types *in vitro*, such as smooth muscle (Evans et al., 1984) and osteoblasts (Tram et al., 1993).

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Protein phosphorylation may also play a role in regulating these interactions. Much of the evidence supporting this view relies upon studies of cells treated with tumor promoters or transformed cells *in vitro* in which there is a reduction in thin filament-membrane interactions and an increase in phosphorylation of several proteins implicated as structural links in these assemblies, such as talin (Pasquale et al., 1986; DeClue and Martin, 1987), vinculin (Sefton et al., 1981; Ito et al., 1983) and the β_1 subunit of integrin (Hirst et al., 1986; Tapley et al., 1989). A causal relationship between elevated phosphorylation of these cytoskeletal proteins in transformed cells and cytoskeletal reorganization characteristic of the transformed phenotype has not yet been proved. Studies of *in vivo* systems also support the hypothesis that protein phosphorylation modulates cytoskeletal organization. For example, paxillin (Turner, 1991) is a focal contact protein expected to play a regulatory role in cytoskeletal-membrane interactions and is the most highly phosphorylated at the developmental stages when turnover of thin filament-membrane interactions is most rapid.

Under some conditions, exogenous factors can regulate cytoskeletal-membrane associations by activating kinase activity in the receptor-bearing cell. For example, PDGF applied to 3T3 cells can result in dramatic remodeling of the actin cytoskeleton (Herman and Pledger, 1985). PDGF effects are mediated by the transmembrane PDGF receptors, which are autophosphorylating tyrosine kinases (Pike et al., 1983; Heldin et al., 1983; Frackelton et al., 1984) that have not been reported to phosphorylate structural proteins. Although the substrate for PDGF receptor phosphorylation that results in fibroblast cytoskeletal reorganization is unknown, candidate proteins include talin, vinculin and the β_1 subunit of integrin.

PDGF receptors are also present on skeletal muscle cells *in vitro* and *in vivo*. PDGF stimulation of skeletal myoblasts have been shown to stimulate proliferation, delay differentiation (Yablonka-Reuveni et al., 1990; Jin et al., 1990) and play a role in chemotaxis (Venkatasubramanian and Solursh, 1984). PDGF receptor concentration is also elevated in regenerative dystrophic muscle (Tidball et al., 1992). These observations, together with Northern blot data showing no measurable mRNA for PDGF receptor in adult muscle (Jin et al., 1990), indicate that PDGF plays a role in skeletal muscle function only during embryonic growth or during muscle regeneration, while many genes expressed during myogenesis undergo renewed expression. There are no published findings that implicate PDGF stimulation with remodeling of the cytoskeleton of skeletal muscle.

In this study, we find that PDGF receptors are prominent components of myotendinous junctions (MTJs)¹ in fully differentiated skeletal muscle fibers. This observation indicates that PDGF receptors may play regulatory roles in fully differentiated muscle, and suggests the hypothesis that PDGF receptors may be particularly important in regulating functions occurring at MTJs. Because MTJs are sites specialized for thin filament interactions with the cell membrane, we have examined whether PDGF can stimulate remodeling of the cytoskeleton of skeletal muscle, and whether talin, vinculin or the β_1 subunits of integrin are substrates for phosphorylation following PDGF stimulation.

1. *Abbreviations used in this paper:* MTJ, myotendinous junctions; PY, phosphotyrosine.

Materials and Methods

Immunohistochemistry

L6 myoblasts (American Tissue Culture Collection, Rockville, MD) were grown in 10% FBS in DME containing penicillin and streptomycin at 37°C, 5% CO₂, on glass coverslips. The coverslips had been coated with 0.05% fibronectin (human serum fibronectin; Upstate Biotechnology Inc., Lake Placid, NY) for 90 min at 37°C and then rinsed briefly with DME immediately before applying the L6 cells. Cells were grown until 60–70% confluent and then rinsed three times for 10 min each in DME before transferring to 0.5% FBS in DME for 12 h at 37°C.

The coverslips were then transferred to DME, prewarmed to 37°C, and containing 20 ng/ml PDGF-BB (Upstate Biotechnology) or DME only for 0, 2, 10, 30, or 60 min before fixation in 2% paraformaldehyde, if to be used for anti-vinculin, anti-talin, anti-PDGF receptor, phalloidin, anti-pp125^{FAK}, anti-phosphotyrosine or anti-desmin immunolabeling, or fixation in acetone, if to be used for anti-integrin β_1 subunit immunolabeling. After 10 min of fixation, the coverslips were rinsed in 50 mM Tris, pH 7.6, containing 150 mM NaCl and 0.02% NaN₃ (buffer A), and then immersed in buffer A containing 0.2% gelatin, 0.5% Tween-20 and 0.3% BSA (blocking buffer). The coverslips were then incubated in one of the following antisera: rabbit anti-recombinant PDGF β receptor (0.2 μ g/ml in buffer A; UBI), mouse anti-pp125^{FAK} (clone 2A7; 10 μ g/ml in buffer A), mouse anti-chicken vinculin (clone VIN-11-5; diluted 1:50; Sigma Chemical Co., St. Louis, MO), mouse anti-chicken talin (clone 8d4; diluted 1:40; Sigma Chemical Co.), mouse anti-phosphotyrosine (PY20; 1 μ g/ml; ICN, Cleveland, OH) rabbit anti-chicken desmin (1 mg/ml total protein in buffer A; Sigma Chemical Co.) or rabbit anti-human integrin β_1 subunit (5 μ g/ml in buffer A; Chemicon International Inc., Temecula, CA). Other coverslips were incubated in TRITC-conjugated phalloidin (10 μ g/ml in buffer A; Molecular Probes, Inc., Eugene, OR) to label thin filaments or in normal rabbit serum as control samples. After 2 h in primary antibody, samples were rinsed in buffer A for 5 min, immersed in blocking buffer for 30 min and then buffer A for an additional 5 min. The coverslips were incubated in TRITC or FITC conjugated goat anti-mouse IgG or in FITC-conjugated goat anti-rabbit IgG for 2 h, as appropriate for primary antibody used. The coverslips were then rinsed in several changes of buffer A, in water and then mounted on glass slides for observation by indirect immunofluorescence. Coverslips incubated in TRITC-phalloidin only were rinsed and mounted on slides immediately after phalloidin treatment.

Some coverslips were also prepared for double immunofluorescence labeling. Reagents used together for double labeling experiments were: anti-talin/anti-PDGF β receptor, anti-vinculin/anti-PDGF β receptor, phalloidin/anti-PDGF β receptor, phalloidin/anti-vinculin, phalloidin/anti-talin, and anti-phosphotyrosine/anti-PDGF β receptor. Second antibodies used in double immunolabeling experiments were selected so that each antigen labeled in a single preparation would fluoresce at a characteristic wavelength.

All immunolabeling preparations between which comparisons were made in this investigation were processed in parallel using identical reagents, labeling conditions, photographic exposure, developing, and printing conditions. Immunofluorescent images used for comparison were photographed on the same roll of film so no differences in processing conditions would influence comparisons.

In other immunohistochemical preparations, hindlimb muscles were removed from adult mice or rats and rapidly frozen in 10% polyvinyl alcohol, 4.3% polyethylene glycol. 10- μ m-thick sections were cut longitudinally at -20°C through muscle fibers at MTJs. Sections were mounted on gelatin/chrome alum-coated glass slides and immunolabeled with anti-PDGF receptor using the procedures described above.

Immunoprecipitations

L6 cells were cultured in fibronectin-coated tissue culture plates under the conditions described above. At ~70% confluency, the cells were rinsed three times for 10 min in DME and then cultured for 12 h at 37°C in 0.5% FBS in DME. Cultures were then incubated for 20 min at 37°C DME containing 200 μ M o-vanadate prewarmed to 37°C. Cultures were then incubated for 2, 5, 10, 30, or 60 min in 20 ng/ml PDGF-B in DME containing 200 μ M o-vanadate or DME/vanadate only. Cells were then harvested in 3 ml buffer A containing 5 mM EDTA, 0.1% Triton X-100, 0.1% deoxycholate, 50 μ M o-vanadate and 10 μ M leupeptin (lysis buffer), using a rubber policeman to scrape the cells from the plates. The samples were then triturated 5 times through a Pasteur pipette and centrifuged at 100,000 g for 30 min at 4°C. The supernatants were then aliquoted into 1.5 ml samples. 50 μ l of anti-phosphotyrosine coupled to agarose beads (PY-20/agarose; ICN) were added to each sample and the aliquots incubated at 7°C with constant

mixing by inversion. After a 3-h incubation, the samples were centrifuged at 10,000 g for 10 min, after which the supernatant was discarded. The pellets were washed three times in 1.5 ml lysis buffer followed by centrifugation at 10,000 g. After the final wash and centrifugation, the supernatant was discarded and 10 μ l of 80 mM Tris, pH 6.8, containing 0.1 M DTT, 70 mM SDS and 1.0 mM bromophenol blue (sample buffer) were added to the pellet. The pellet was heated to 100°C for 1 min, centrifuged for 10 min at 10,000 g and the supernatant analyzed by SDS-PAGE using 8% acrylamide gels.

After protein separation by SDS-PAGE, proteins in the gels were transferred electrophoretically to nitrocellulose for immunoblotting using previously described procedures (Tidball et al., 1986). Immunoblots were incubated with primary antisera to talin, vinculin, PDGF β receptor, paxillin, integrin β 1, and phosphotyrosine (PY), followed by alkaline phosphatase coupled second antibodies.

Immunoblots of PDGF-stimulated Cells

The possibility that PDGF stimulation could stimulate endogenous protease activity, resulting in cleavage of the structural proteins studied here was tested by assaying for proteolytic fragments of those proteins in PDGF-stimulated cells. L6 cells were cultured, serum starved, and stimulated for 0, 2, 5, 10, 30, or 60 min using 20 μ g/ml PDGF-B in DME, as described above. At the end of the stimulation period, DME was replaced by sample buffer, the cells were scraped from the plates with a rubber policeman and the samples were heated for 1 min at 100°C. The samples were then centrifuged for 3 min at 10,000 g and the supernatants analyzed by SDS-PAGE (Laemmli, 1970) and immunoblotting (Burnette, 1981).

Assays for Calpain Activation

The possibility that PDGF stimulation caused activation of calpain was tested by analyzing PDGF-stimulated and control L6 cell extracts prepared for immunoblots. Immunoblots were incubated with anti-pro- μ -calpain and anti-pro-m-calpain, the inactive precursors of calpain, to determine whether PDGF stimulation resulted in a decrease in inactive procalpains. The antibodies used do not recognize the active forms of calpain, so a reduction in antibody binding to the proenzyme in immunoblots is an indicator of enzyme activation (Croall et al., 1992). An additional assay used an antibody generated against the proteolytic domain of calpains, that recognizes active and inactive forms of both μ -calpain and m-calpain (Spencer and Tidball, 1992). Calpain undergoes autolysis to a lower mass activated form (Croall et al., 1992), so calpain activation results in an increase in the quantity of the lower molecular weight form of calpain in immunoblots. This was tested in immunoblots of control and PDGF-stimulated cells using anti-calpain proteolytic domain.

Results

PDGF Receptors Are Concentrated at MTJs

Indirect immunofluorescence shows that MTJs of fully differentiated rodent muscles are enriched with PDGF-receptors (Fig. 1). The distribution resembles that described previously for proteins involved in force transmission from myofibrils across the cell membrane, to extracellular structural proteins or proteins expected to regulate interactions between those structural proteins. No PDGF receptors were found distributed in a periodic pattern, i.e., in costameres, along the lateral surface of the muscle fibers, although costameres are also sites of force transmission (Danowski et al., 1992) and contain a similar population of structural proteins (Tidball, 1991).

PDGF Stimulates Reorganization of the Cytoskeleton in Skeletal Muscle Cells

Observations by indirect immunofluorescence show that the cytoskeleton of L6 myoblasts consists of distinct stress fibers terminating at focal contacts at the cell membrane. The focal contacts contain β 1 integrin, talin and vinculin (Figs. 2 and 4), each of which is a structural protein enriched at MTJs

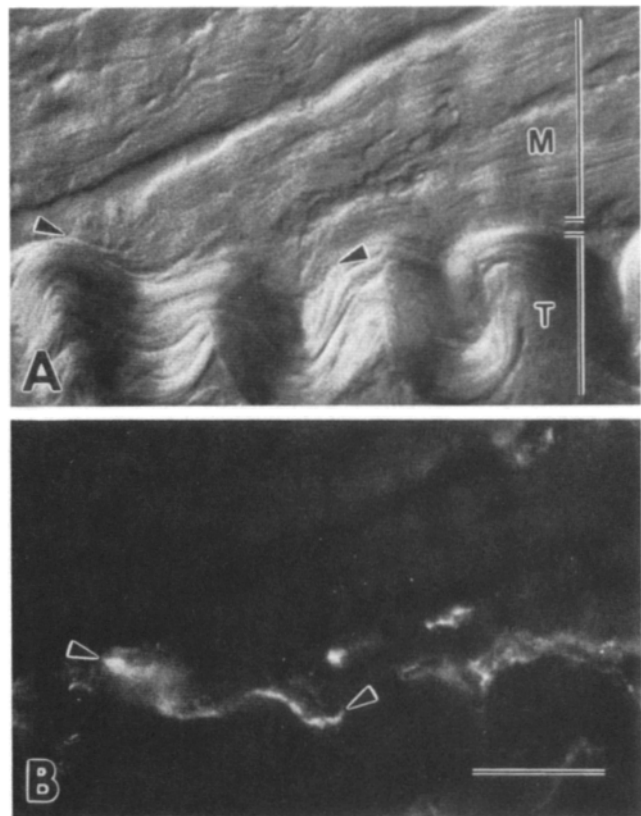


Figure 1. Longitudinal section through muscle fibers at myotendinous junction. (A) Nomarski optics image showing MTJ (between arrowheads) lying between muscle (M) and tendon (T). (B) Indirect immunofluorescence of same section incubated with antibodies to PDGF receptor. Bar, 40 μ m.

in vivo (Shear and Bloch, 1985; Tidball et al., 1986; Swadison and Mayne, 1989; Bozyczko et al., 1989). The kinase pp125^{FAK} that is expected to regulate interactions of proteins at focal contacts (Burridge et al., 1992) was found at the edge of extended, cell processes in the present study (Fig. 2). We found no evidence for pp125^{FAK} distribution in numerous, elliptical focal contacts similar to those observed in anti-vinculin stained cells (Fig. 4). PDGF stimulation caused no change in pp125^{FAK} distribution.

PDGF-stimulation of L6 myoblasts resulted in a rapid and reversible disassembly of the stress fibers in most cells (Fig. 3). TRITC-phalloidin labeling of unstimulated cells showed that throughout well-spread cells there were bundles of thin filaments arranged in stress fibers terminating preferentially at the cell margins. After a 10-min stimulation with PDGF-B, few stress fibers were demonstrable in most cells. However, after 30 min of exposure to PDGF, bundles of thin filaments increased in prominence, so that at 60 min of stimulation stress fibers were again prominent features in all cells. The stress fiber organization after 60 min of stimulation was not as extensive as unstimulated cells and the thin filaments appeared most prominent in perinuclear areas. At all time points, diffusely distributed thin filaments that were not arranged in stress fibers remained unperturbed by PDGF stimulation.

Vinculin distribution was also modified by PDGF stimulation (Fig. 4). Unstimulated cells showed vinculin concen-

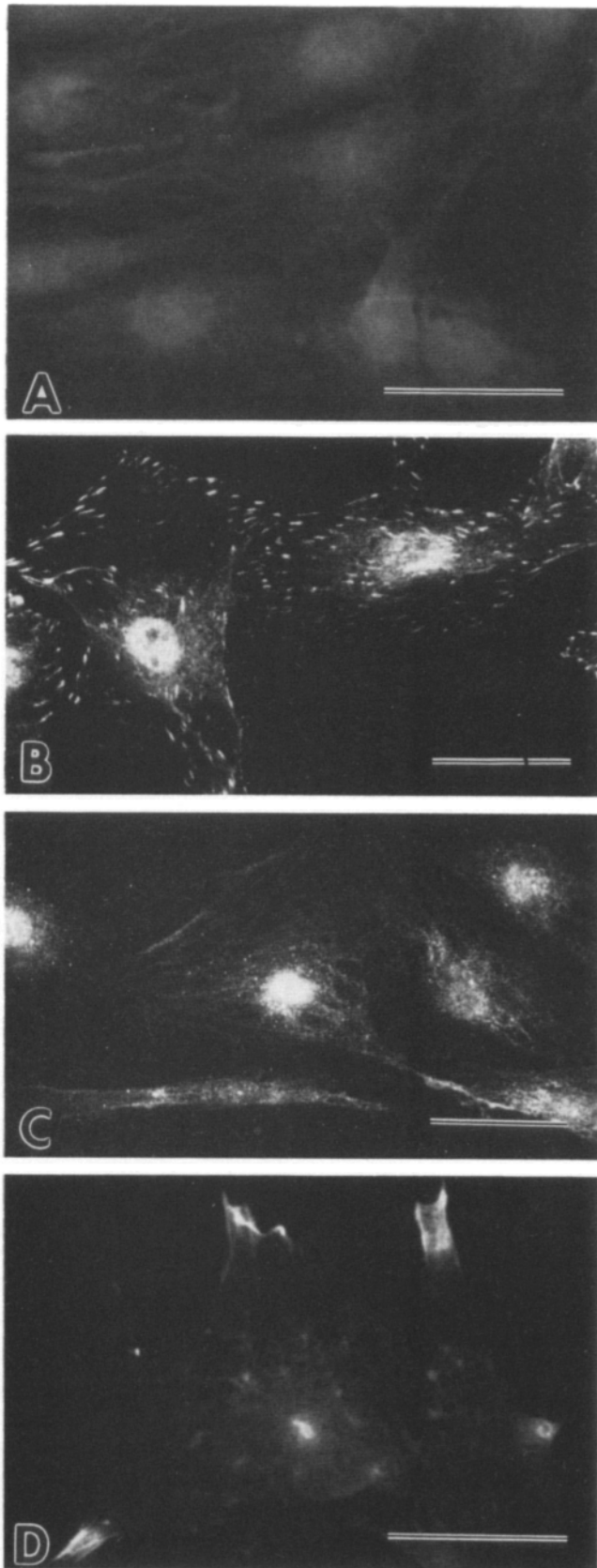


Figure 2. Indirect immunofluorescence of L6 myoblasts that were not stimulated with PDGF. (A) Cells labeled with anti-PDGF receptor. (B) Labeled with anti-talin. (C) Labeled with anti-desmin. (D) Labeled with anti-pp125^{FAK}. Bar, 15 μ m.

trated in focal contacts, primarily at the most peripheral areas of the cells. 10 min of PDGF stimulation resulted in redistribution of vinculin from most focal contacts to perinuclear regions. At 60 min of stimulation, vinculin appeared once again in scattered focal contacts, although most of the protein remained in perinuclear sites.

Talin distribution in unstimulated cells resembled vinculin's distribution with the most intense anti-talin binding at peripheral focal contacts and at the nucleus (Fig. 2). PDGF stimulation for 10, 30, or 60 min resulted in little change in talin distribution. Most talin labeling in stimulated cells was located at focal contacts (Fig. 5), although its appearance was more punctate rather than in elliptically shaped focal contacts as seen in controls. Immunolabeling for the β 1 subunit of integrin showed no change in integrin distribution following PDGF stimulation (data not shown).

Changes in PDGF receptor distribution after PDGF stimulation that were observed in the present study were identical to those reported previously (Tidball et al., 1992). PDGF receptors were diffusely distributed on the cell surface in unstimulated cells but became concentrated in intensely labeled granules within the cell soon after stimulation (Fig. 5). These granules then accumulated at the cell nucleus, where they remained for periods exceeding 1 h. Some cells did not stain for PDGF receptors in control or PDGF-stimulated preparations. Double immunolabeling with anti-PDGF receptor/anti-vinculin or anti-PDGF receptor/phalloidin showed that those cells that did not contain internalized receptor after stimulation were cells that did not show disassembly of the actin cytoskeleton or vinculin redistribution. Thus, the unresponsive cells observed in PDGF-stimulation experiments may be at an undetermined stage of the cell cycle at which PDGF receptors are not expressed at the cell surface.

Comparisons of phosphotyrosine distribution with the distribution of the proteins studied in this investigation show overlapping PDGF receptor distribution and PY location in PDGF-stimulated cells. PY is found concentrated at focal contacts in unstimulated cells and after PDGF stimulation, some PY is relocated to granules that become located in perinuclear regions while other PY remains concentrated in focal contacts. Double immunofluorescence shows that PY and PDGF receptors are colocalized in the same perinuclear granules (Fig. 5).

The dramatic disassembly of stress fibers after PDGF stimulation that is unaccompanied by dramatic cell shape changes indicates the continued existence of an organized cytoskeleton in stimulated cells. One mechanism by which these cells could retain their shape in the absence of stress fibers would be through the persistent existence of focal contacts, as demonstrated in anti-talin labeling shown here. In addition, anti-desmin labeling showed that PDGF stimulation produced no modification in this intermediate filament cytoskeletal organization (data not shown), which may be adequate to maintain cell shape.

PDGF Stimulation Results in Phosphorylation of Tyrosine in PDGF Receptors and Talin

Anti-PY immunoblots of cells stimulated with PDGF for 0, 10, or 30 min shows polypeptides at \sim 180, \sim 230, and \sim 280 kD that contain PY as a result of PDGF stimulation (Fig. 6). Of these polypeptides, the 180-kD band is the strongest sig-

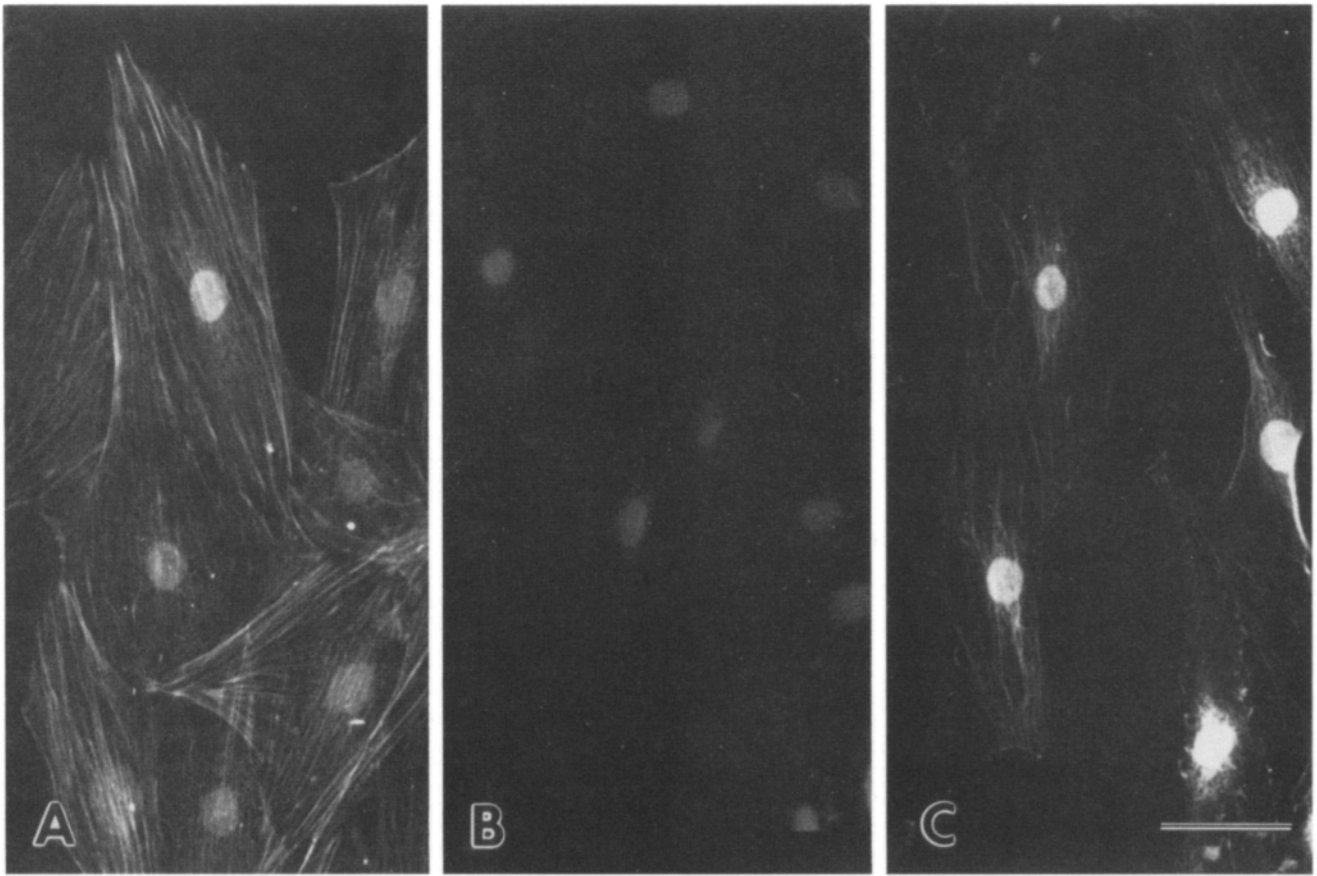


Figure 3. TRITC-phalloidin labeled L6 myoblasts. (A) Nonstimulated cells. (B) Cells after 10 min of stimulation with 20 ng/ml of PDGF-B. (C) Cells after 60 min of stimulation. Bar, 15 μ m.

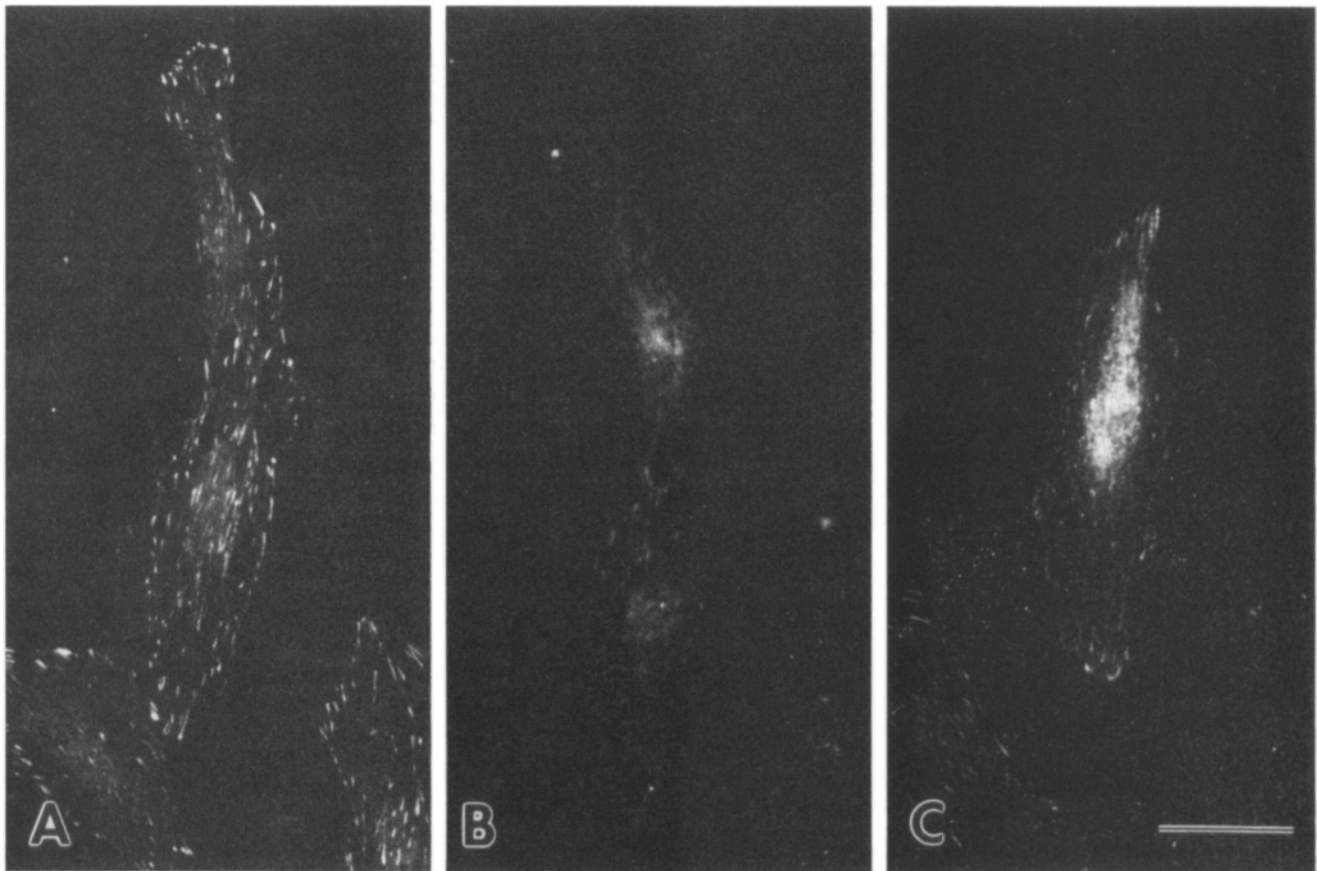


Figure 4. Indirect immunofluorescence of L6 cells labeled with anti-vinculin. (A) Nonstimulated cells. (B) Cells after 10 min of stimulation with 20 ng/ml of PDGF-B. (C) Cells after 60 min of stimulation. Bar, 15 μ m.

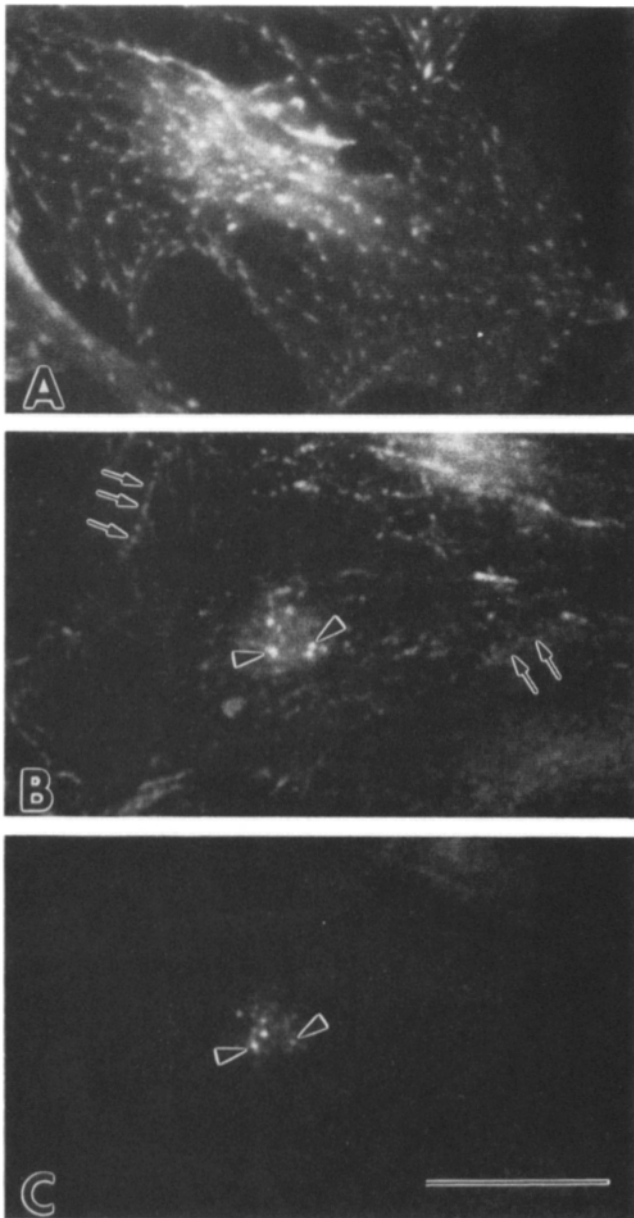


Figure 5. (A) Single L6 myocyte stimulated with 20 ng/ml of PDGF-B for 10 min and then labeled with anti-talin. Talin is located in focal contacts and in perinuclear regions. (B and C) Double immunolabeling of single L6 myocyte after PDGF stimulation and then labeled with anti-phosphotyrosine (B) and anti-PDGF-receptor (C). Some granules lying at the cell nucleus label with both anti-phosphotyrosine and anti-PDGF receptor (arrowheads). Anti-phosphotyrosine is also located in focal contacts, especially at the cell's periphery (arrows). Bar, 6 μ m.

nal. Anti-PY immunoprecipitates of 10 min PDGF-stimulated cultures followed by immunoblots of the precipitate and incubation with anti-PDGF receptor show that PDGF receptor is a 180 kD, tyrosine phosphorylated protein in these samples (Fig. 7). Identical anti-PY immunoprecipitations and immunoblots of cells stimulated for 2 or 5 min with PDGF show that the PDGF receptor phosphorylation is imperceptible at 2 min of stimulation, but apparent after 5 min of stimulation (data not shown).

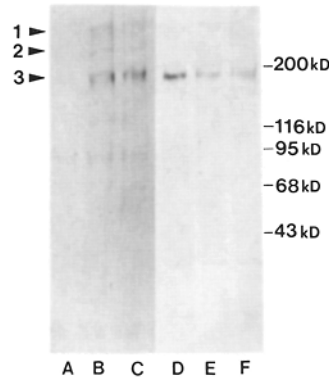


Figure 6. Immunoblots of extracts from nonstimulated cells (lanes A and D), cells after 10 min of stimulation with PDGF-B (lanes B and E) or 30 min of stimulation with PDGF-B (lanes C and F). (Lanes A-C) blot was incubated with anti-phosphotyrosine. (Lanes D-F) blot was incubated with anti-PDGF receptor. Band 1, 280 kD; band 2, 230 kD; band 3, 180 kD.

Immunoblots of anti-PY precipitates of cells stimulated for 10 min with PDGF show no perceptible tyrosine phosphorylation of vinculin, paxillin, integrin β 1, or p125^{PAK}. However, anti-talin immunoblots of anti-PY precipitates show that PDGF stimulates talin phosphorylation (Fig. 7). Anti-talin binds to a 230-kD polypeptide in the immunoprecipitates, which corresponds to the approximate mass of talin in SDS-PAGE. Furthermore, anti-talin immunoblots of the supernatant remaining after anti-PY immunoprecipitation show a decrease in talin remaining in the supernatants obtained from stimulated cells, and show that PDGF stimulation causes no talin proteolysis to its 190-kD fragment (Fig. 7).

Cytoskeletal Remodeling After PDGF Stimulation Does Not Involve Calpain Activation

In view of the observations that the association of talin and vinculin is disrupted in the PDGF-stimulated L6 cells and previous findings showing that: (a) PDGF stimulation can cause elevation in intracellular free calcium concentrations (Moolenaar et al., 1984) that may feasibly activate calpain; (b) calpain cleaves talin into 190- and 46-kD fragments (O'Halloran et al., 1985); (c) calpain cleaves vinculin to a 98-kD fragment (Evans et al., 1984); and (d) calpain is located at focal contacts of some cells (Beckerle et al., 1987), we investigated whether PDGF stimulation activated calpain in muscle cells in our preparations. Immunoblots of PDGF-

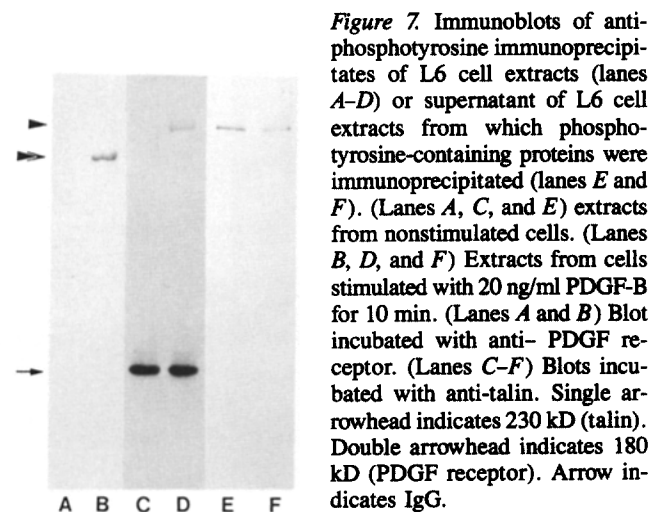


Figure 7. Immunoblots of anti-phosphotyrosine immunoprecipitates of L6 cell extracts (lanes A-D) or supernatant of L6 cell extracts from which phosphotyrosine-containing proteins were immunoprecipitated (lanes E and F). (Lanes A, C, and E) extracts from nonstimulated cells. (Lanes B, D, and F) Extracts from cells stimulated with 20 ng/ml PDGF-B for 10 min. (Lanes A and B) Blot incubated with anti-PDGF receptor. (Lanes C-F) Blots incubated with anti-talin. Single arrowhead indicates 230 kD (talin). Double arrowhead indicates 180 kD (PDGF receptor). Arrow indicates IgG.

stimulated cells using anti-pro- μ -calpain and anti-pro-m-calpain show no decrease in concentration of the inactive proenzyme following PDGF stimulation (Fig. 8). Immunoblots using antisera recognizing the proteolytic domain of μ -calpain showed that the only detectable μ -calpain appears at 80 kD, the mass of the inactive form, while no 78-kD μ -calpain, the mass of the active enzyme, appeared following PDGF stimulation (Fig. 8). This indicates that there was no measurable calpain activation resulting from the stimulation. Furthermore, immunoblots of control and PDGF-stimulated preparations show that PDGF-stimulated cells do not contain the 190-kD talin fragment (Fig. 7) or 98-kD vinculin fragment (Fig. 9) produced by calpain cleavage, thereby indicating that PDGF stimulation did not cause talin or vinculin proteolysis. Immunoblots of control and stimulated preparations also show no difference in concentration or mass of integrin β 1, or α -actinin, supporting the conclusion that proteolysis of the structural proteins located at focal contacts is not the basis of PDGF-stimulated cytoskeletal remodeling (Fig. 9).

Discussion

A persistent question in muscle biology concerns the mechanism by which sarcomere addition and deletion occurs (Epstein and Fischman, 1991). Previous studies have shown that these processes occur primarily at the ends of myofibrils, where myotendinous junctions are located (Goldspink, 1980). Thus, there must be present at MTJs a mechanism for detaching and attaching thin filaments of the terminal sarcomeres from the MTJ membrane during muscle growth and adaptation. At the onset of this study, several observations supported our hypothesis that thin filament association with the muscle membrane may be regulated by PDGF: (a) we observed high concentrations of PDGF-receptors at the MTJs of fully differentiated muscle fibers; (b) PDGF receptors are tyrosine kinases (Heldin et al., 1983; Frackelton et

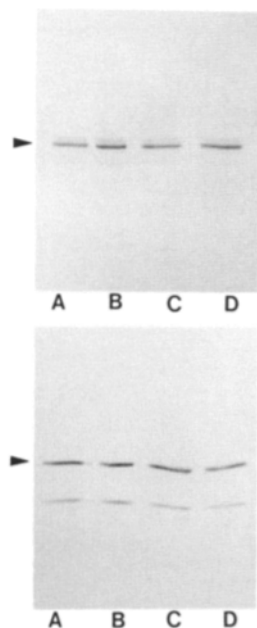


Figure 8. Immunoblots of extracts from L6 cells that were either not stimulated (lanes A and B) or stimulated (lanes C and D) with 20 ng/ml of PDGF-B for 10 min. Upper blot was incubated with anti-calpain; arrow indicates position of 80-kD calpain. Lower blot was incubated with anti-pro-m-calpain; arrow indicates position of 80-kD pro-calpain. Immunoblot using anti-pro- μ -calpain (not shown) resembled anti-pro-m-calpain blot.

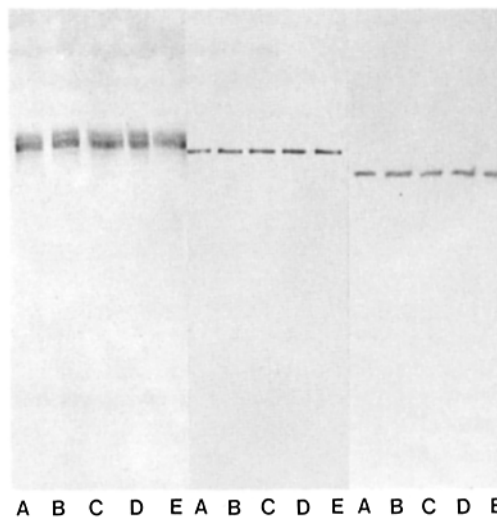


Figure 9. Immunoblots of L6 cell extracts of nonstimulated cells (lanes A and E), 10-min stimulation with 20 ng/ml PDGF-B (lanes B), 30-min stimulation (lanes C), or 60-min stimulation (lanes D). Left blot, anti-integrin β 1; center blot, anti-vinculin; right blot, anti- α -actinin.

al., 1984); (c) MTJs are highly enriched in talin (Tidball et al., 1986), vinculin (Shear and Bloch, 1985) and the β 1 subunit of integrin (Swadlow and Mayne, 1989; Bozyczko et al., 1989), all of which can be phosphorylated on tyrosine (Glenney, 1992); and (d) phosphorylation of talin, vinculin and the β 1 subunit of integrin has been associated with cytoskeletal remodeling in transformed cells and cells treated with tumor promoters (Sefton et al., 1981; Ito et al., 1983; Pasquale et al., 1986; Hirst et al., 1986; DeClue and Martin, 1987; Tapley et al., 1989).

The findings reported here show that PDGF stimulation of muscle cells results in phosphorylation of PDGF receptors and talin, accompanied by receptor internalization and cytoskeletal reorganization. The transient cytoskeletal reorganization we observe is similar to that reported for 3T3 cells in that thin filaments are depolymerized and vinculin is redistributed from focal contacts (Herman et al., 1985). Our observations indicate that talin phosphorylation may be an important control in thin filament-membrane interactions, at least in skeletal muscle.

The immunofluorescence data showing that talin remains at focal contacts while vinculin is relocated after PDGF stimulation indicate that talin phosphorylation may disrupt the talin-vinculin associations, but not talin-integrin associations. The 190-kD carboxy region of talin contains binding sites for integrin β 1 (Horwitz et al., 1986) and vinculin (Burrige and Mangeat, 1984), although the precise location of those binding sites relative to the location of phosphorylation sites is unknown. Results presented here indicate that separate phosphorylation sites may regulate interactions with integrin β 1 and vinculin. Phosphorylation sites regulating talin-integrin binding may be either tyrosines that are not substrates for PDGF receptor or perhaps serine or threonine in talin that previous studies have shown to be substrates for protein kinase C in *in vitro* assays.

Immunocytochemical observations of PY distribution are consistent with the conclusions that PDGF stimulation

results in: (a) receptor phosphorylation and internalization, as shown in other cell types (Rosenfeld et al., 1984); and (b) talin phosphorylation and dissociation from vinculin, with no change in talin distribution. PY is found concentrated at focal contacts before stimulation and in focal contacts and perinuclear granules after stimulation. Thus, the phosphotyrosine remaining in focal contacts after stimulation is co-distributed with the talin, while that located in perinuclear granules corresponds to the location of internalized PDGF receptors. The identity of the PY-containing protein enriched in focal contacts of nonstimulated muscle cells remains unknown. Although pp125^{FAK} is present in nonstimulated muscle cells, it is located primarily at the periphery of these L6 cells and not observed in the elliptically shaped patches characteristic of focal contacts. Thus, another tyrosine phosphorylated, focal contact protein that does not contain the epitope recognized by anti-pp125^{FAK} is present in these cells.

Our finding that PDGF stimulation disrupted talin–vinculin codistribution suggested the possibility that talin or vinculin cleavage rather than talin phosphorylation alone may be important in regulating cytoskeletal–membrane associations in muscle cells. A prominent possibility would be calpain-mediated cleavage of talin or vinculin because PDGF stimulation can elevate intracellular calcium concentrations (Ives and Daniel, 1987; Nishimura et al., 1992), and thereby activate calpain, which is capable of cleaving talin and vinculin. A role for calpain cleavage of focal contact proteins in 3T3 cells has been indicated by previous findings (Herman et al., 1986) in which a calcium-dependent mechanism involving proteolysis was implicated in vinculin disruption from focal contacts. Furthermore, protein phosphorylation can render some proteins more susceptible to proteolysis (Hemmings, 1980; Reichsteiner, 1990), so that the phosphorylation of talin by PDGF receptors could feasibly result in facilitated cleavage and subsequent cytoskeletal rearrangements. However, we have shown that calpain cleavage of talin or vinculin is not a PDGF-responsive regulatory event by demonstrating that PDGF stimulation causes no increase in calpain activation. Observations that support this claim are: (a) there is no decrease in the concentration of inactive procalpain after stimulation; (b) there is no increase in the mass of the activated form of calpain in immunoblots of stimulated cells; (c) there is no change in the relative proportion of the 190-kD talin fragment relative to the non-cleaved protein after stimulation; and (d) there is no appearance of a vinculin fragment or reduction in the concentration of uncleaved vinculin in PDGF-stimulated cells.

These findings therefore indicate that the phosphorylation of talin on tyrosine after PDGF binding by its receptor may regulate cytoskeletal interactions with the membrane by disrupting talin's association with vinculin. We expect that talin phosphorylation on serine and threonine, which has been induced experimentally by stimulating protein kinase C activity with tumor promoters, will have biological functions distinct from those associated with tyrosine phosphorylation. Tumor-promoter-mediated talin phosphorylation on serine and threonine can result in loss of talin from focal contacts and disruption of the actin cytoskeleton in BS-C-1 cells (Turner et al., 1989), or movement of cytoplasmic talin to the cell membrane where talin colocalizes with capped integrin in lymphocytes (Burn et al., 1988). It may be noteworthy

that these examples of tumor-promoter mediated phosphorylation of serine and threonine on intact talin appear to influence talin binding to integrin, while PDGF receptor-mediated phosphorylation of tyrosine on talin appears to influence talin interaction with vinculin.

The data presented here suggest that regulation of myofibril interactions with the MTJ membrane may be controlled to some degree by activation of kinase activity of the PDGF receptor. This speculation is supported by several observations of MTJ structure during episodes of rapid turnover of myofibril–membrane associations. Electron microscopic observations have shown that during MTJ growth and remodeling, there are high concentrations of mononucleated cells closely opposed to the remodeling MTJ (Zamora and Marini, 1988; Tidball and Lin, 1989; Tidball and Quan, 1992). Mononucleated cells, such as macrophages and fibroblasts, are PDGF-secreting cells (Shimokado et al., 1985); thus, they may be the effectors in regulating myofibril–membrane associations. In addition, experimentally induced MTJ remodeling shows that vinculin is more broadly distributed at the junctional regions of fibers in which thin filament associations with the membrane are undergoing modification (Dix and Eisenberg, 1990). This resembles the events we observe in vitro after PDGF stimulation. Thus far, our attempts to induce MTJ remodeling by application of PDGF to whole muscle have not yielded any electron microscopically discernible evidence of junction remodeling. This may reflect the presence of other, parallel systems at MTJs that are involved in myofibril–membrane associations that are not regulated by PDGF receptor-mediated phosphorylation. These parallel systems include dystrophin binding to the transmembrane, dystrophin associated glycoproteins present at MTJs (Shimizu et al., 1989; Samitt and Bonilla, 1989; Ibraghimov-Beskrovnaya et al., 1992), and desmin intermediate filaments that also appear to attach myofibrils to the junctional membrane (Tidball, 1992). Thus, remodeling of myofibril interactions with the MTJ membrane may require coincident activation of more than one process regulating interactions between these protein assemblies.

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