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The adhesion plaque protein, talin, is phosphorylated in vivo in chicken embryo fibroblasts exposed to a tumor-promoting phorbol ester

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Talin is a high molecular weight phosphoprotein that is localized at adhesion plaques. We have found that talin phosphorylation increases 3.0-fold upon exposure of chicken embryo fibroblasts to the tumor-promoting phorbol ester, phorbol 12-myristate 13-acetate. Talin isolated from tumor promoter-treated cells is phosphorylated on serine and threonine residues. Vinculin, a 130 kDa talin-binding protein, also exhibits increased phosphorylation in vivo in response to tumor promoter, but to a lesser degree than does talin. Because tumor-promoting phorbol esters augment protein kinase C activity, we have compared the ability of purified protein kinase C to phosphorylate talin and vinculin in vitro. Both talin and vinculin were found to be substrates for protein kinase C; however, talin was phosphorylated to a greater extent than was vinculin. Cleavage of protein kinase C-phosphorylated talin by the calcium-dependent protease (Type II) revealed that while both the resulting 190–200 and 46 kDa proteolytic peptides were phosphorylated, the majority of label was contained within the 46-kDa fragment. Although incubation of chicken embryo fibroblasts with tumor-promoting phorbol ester induces a dramatic increase in talin phosphorylation, we detected no change in the organization of stress fibers and focal contacts in these cells. Exposure of the cells to tumor promoter did, however, result in a loss of actin and talin-rich cell surface elaborations that resemble focal contact precursor structures.

Introduction

Cultured cells adhere to extracellular substrata via specialized regions of the plasma membrane called focal contacts or adhesion plaques. Receptors for extracellular matrix molecules such as fibronectin accumulate at adhesion plaques (Chen *et al.*, 1985; Damsky *et al.*, 1985) and actin filament bundles terminate and associate with these adhesion-competent membrane domains (Singer, 1979). A number of cytoplasmic proteins that are specifically localized at adhesion plaques have been identified (for review, see Burridge, 1986). Two of these, talin and vinculin, have been shown to interact with each other (Otto, 1983; Burridge and Mangeat, 1984), and both have been proposed to be involved in generating the transmembrane connection between the extracellular matrix and the cytoskeleton that occurs at adhesion plaques.

Adhesion plaques are dynamic structures that are responsive to the physiological state of the cell, as well as the biochemical composition of the extracellular environment. For example, adhesion plaques are assembled and disassembled in a predictable manner as cells progress through the cell cycle. In chicken embryo fibroblasts, infection with Rous sarcoma virus induces a dramatic change in cell morphology that includes the loss of adhesion plaques (David-Pfeuty and Singer, 1980). Moreover, in certain cell types, exposure of cells to platelet-derived growth factor (Mellström *et al.*, 1983; Herman and Pledger, 1985) or to tumor-promoting phorbol esters (Schliwa *et al.*, 1984; Meigs and Wang, 1986) results in loss of adhesion plaques and increased cell surface ruffling reminiscent of the transformed phenotype. The molecular mechanism by which the assembly state of the adhesion plaque is regulated is not known, although it has been widely postulated that a post-translational modification such as protein phosphorylation of an adhesion plaque component could be involved.

Since talin and vinculin have both been shown to be phosphoproteins in vivo (Sefton *et al.*, 1981; Burridge and Connell, 1983), and since the tumor-promoting phorbol esters that cause loss of adhesion plaques in many cells activate the phospholipid-dependent kinase, protein kinase C (Nishizuka, 1984), it is an intriguing possibility that phosphorylation of talin or vinculin by protein kinase C could be involved in regulating the assembly state of the adhesion plaque. Vinculin (Werth *et al.*, 1983) and talin (Litchfield and Ball, 1986; Beckerle *et al.*, 1985) isolated from avian smooth muscle have both

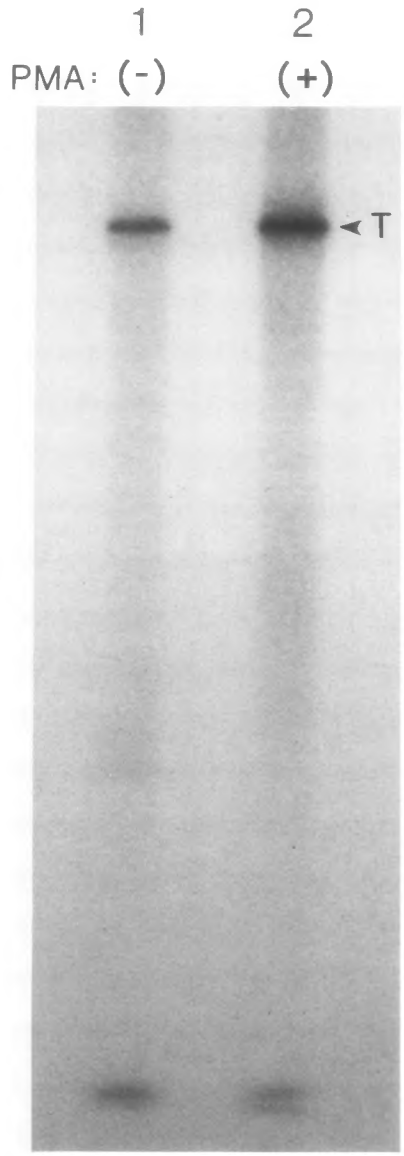


Figure 1. **Talin is phosphorylated in vivo in response to the tumor-promoting phorbol ester, PMA.** (Lane 1) Control. Talin was immunoprecipitated from chicken embryo fibroblasts labeled with ^{32}P-orthophosphate and exposed to 0.1% DMSO for 60 min. (Lane 2) Talin was immunoprecipitated from chicken embryo fibroblasts labeled with ^{32}P-orthophosphate and exposed to 50 ng/ml PMA, 0.1% DMSO for 60 min. In this particular experiment, the level of talin phosphorylation increased 2.7-fold over control levels in response to PMA.

been reported to be substrates for purified protein kinase C in vitro. Moreover, vinculin's phosphorylation increases in vivo in avian fibroblasts exposed to a tumor-promoting phorbol ester (Werth and Pastan, 1984).

In this paper we have examined whether talin is phosphorylated in vivo in avian fibroblasts exposed to phorbol 12-myristate 13-acetate (PMA), and we have compared talin phosphorylation with that of vinculin. We have also compared talin and vinculin phosphorylation by protein kinase C in vitro. Both in vitro and in vivo, talin phosphorylation increases more dramatically than does vinculin phosphorylation. The implications of our observations for the regulation of the assembly state of adhesion plaques are discussed.

Results

Phosphorylation of talin and vinculin in cells exposed to tumor-promoting phorbol ester

We have investigated whether talin phosphorylation is stimulated in chicken embryo fibroblasts exposed to the tumor promoting phorbol ester, PMA, an agent known to increase protein kinase C activity in a variety of living cells (Nishizuka, 1984). Chicken embryo fibroblasts were labeled with ^{32}P-orthophosphate and exposed to 50 ng/ml PMA, 0.1% DMSO for 10 or 60 min. Control cells were treated for the same amount of time with solvent (DMSO) alone. A

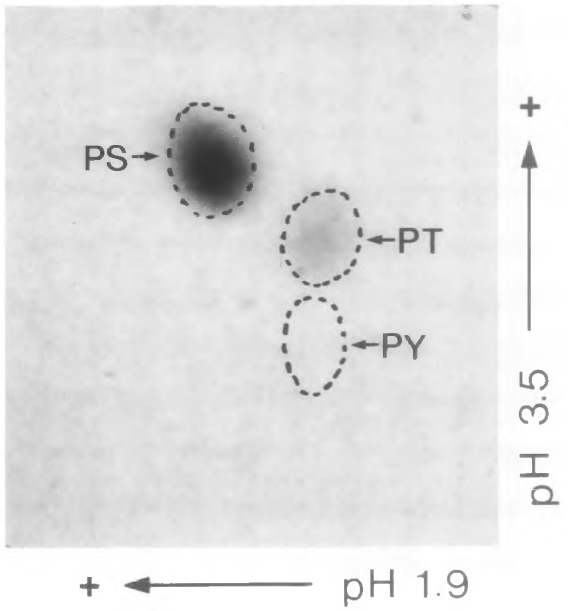


Figure 2. **Phosphoamino acid analysis of talin isolated from PMA-treated chicken embryo fibroblasts.** Two-dimensional high-voltage electrophoresis reveals that radiolabeled talin from PMA-treated cells is phosphorylated on serine and threonine residues. No phosphotyrosine was detected in this experiment. The marker amino acids, phosphoserine (PS), phosphothreonine (PT), and phosphotyrosine (PY), were included in the sample and their positions were determined by ninhydrin staining and are marked by the dashed line.

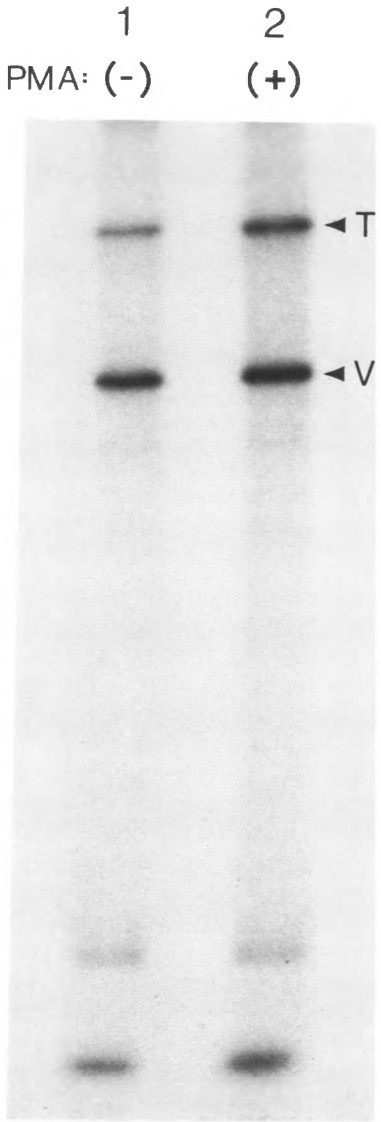


Figure 3. **Comparison of the relative increases in talin and vinculin phosphorylation in PMA-treated chicken embryo fibroblasts.** (Lane 1) Talin (T) and vinculin (V) immunoprecipitated from the same sample of untreated, ^{32}P-labeled chicken embryo fibroblasts. (Lane 2) Talin and vinculin immunoprecipitated from the same sample of ^{32}P-labeled, PMA-treated chicken embryo fibroblasts. Talin phosphorylation increases more dramatically than does vinculin phosphorylation in response to PMA. In the experiment shown here, it is not possible to compare the extent of vinculin phosphorylation with that of talin since only a small proportion of the total cellular talin was immunoprecipitated in this case.

polyclonal antibody was used to immunoprecipitate talin from control and experimental cell lysates. The immunoprecipitates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by autoradiography. Anti-talin antibody specifically immunoprecipitated a single labeled protein that migrated at an apparent molecular mass of 225 kDa (Figure 1). Based on our previous characterization of the polyclonal antiserum used in these experiments (Beckerle *et al.*, 1986), we have identified the immunoprecipitated protein as talin. As can be seen in Figure 1, talin is phosphorylated in control cells (lane 1) as well as PMA-treated cells (lane 2); however, talin phosphorylation is enhanced in cells exposed to PMA. Quantitative analysis of results from a number of experiments by scintillation counting or scanning densitometry revealed that talin phosphorylation increased an average of 3.0-fold above control levels during a 10 min exposure to PMA (n = 3 experiments) and 2.9-fold above control levels during a 60 min exposure to PMA (n = 4 experiments).

Phosphorylated talin isolated by immunoprecipitation from either control or PMA-treated cells exhibited alkali sensitivity, suggesting that the phosphoamino acids are P-ser and/or P-thr (Cooper *et al.*, 1983). To analyze directly the phosphoamino acid content of immunoprecipitated talin from PMA-treated cells, the protein was subjected to trypsinization and acid hydrolysis followed by two-dimensional high-voltage electrophoresis (Figure 2). This analysis revealed that talin is phosphorylated on serine and threonine residues. No phosphotyrosine was detected in talin isolated from either control (not shown) or PMA-treated cells; however, it should be noted that the hydrolysis procedure we utilized does not favor recovery of phosphotyrosine (Cooper *et al.*, 1983).

Since vinculin has previously been reported to be phosphorylated in chicken embryo fibroblasts exposed to tumor-promoting phorbol esters (Werth and Pastan, 1984), we have compared directly the relative increases in talin and vinculin phosphorylation in these fibroblasts exposed to PMA. Talin and vinculin were simultaneously immunoprecipitated from the same sample of ^{32}P-labeled cell lysates prepared from control (Figure 3, lane 1) and PMA-treated cells (Figure 3, lane 2). The autoradiograph shown in Figure 3 demonstrates that talin phosphorylation increases more dramatically in response to tumor promoter than does vinculin phosphorylation. It is important to note that in these co-immunoprecipitation experiments, to enable talin and vinculin samples run on the same gel to be analyzed simultaneously by autoradiography, only a fraction of the total cellular talin was immunoprecipitated. Consequently, the absolute level of phosphorylation of vinculin cannot be compared to that of talin. It is useful,

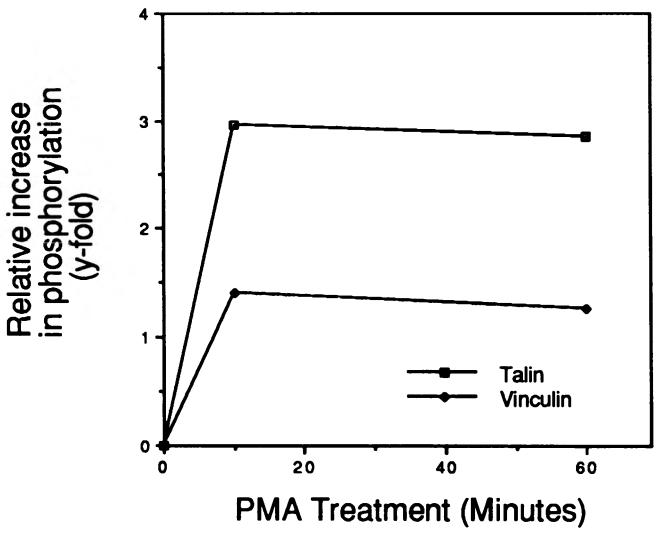


Figure 4. **Quantitative analysis of talin and vinculin phosphorylation in chicken embryo fibroblasts exposed to 50 ng/ml PMA.** Radioactive counts incorporated into talin and vinculin were quantitated by densitometry and are expressed as the mean relative increase over control levels.

however, to compare the relative increases in talin and vinculin phosphorylation by expressing immunoprecipitable radioactive counts in vinculin and talin from PMA-treated cells as a function of their control counts. As shown in Figure 4, both talin and vinculin exhibit increased levels of phosphorylation within 10 min of exposure to PMA, with talin phosphorylation increasing approximately two times more than that of vinculin.

Phosphorylation of talin and vinculin by purified protein kinase C

As discussed above, both talin and vinculin have been reported to be substrates for protein kinase C in vitro. Since we detected differences in the level of phosphorylation of vinculin and talin induced by exposure of cells to PMA, we directly compared the ability of purified protein kinase C to phosphorylate talin and vinculin. Talin was isolated from both avian smooth muscle (chicken gizzard) and human platelets; vinculin was isolated from avian smooth muscle. Equivalent amounts (by weight) of the purified proteins were incubated with purified protein kinase C and ^{32}P-ATP for 20 min at 30°C in the presence or absence of phosphatidyl serine and 1,2-diolein. As shown in Figure 5B, talin isolated from either avian smooth muscle (lane 1) or human platelets (lane 2) is phosphorylated by protein kinase C in the presence of phosphatidyl serine and 1,2-diolein. Some specific phosphorylation of vinculin (lane 3) was detected under these conditions (Figure 5B); however, talin phosphorylation was again more dramatic. Only a small amount of talin phosphorylation was detected in the absence of phosphatidyl serine and 1,2-diolein (Figure 5C, lanes 1 and 2). Lipid-independent phosphorylation of vinculin was not detected in this autoradiographic exposure, although some signal was visible with longer exposures (not shown). The lipid dependence of talin and vinculin phosphorylation illustrates that the phosphorylation that we observe in vitro occurs only under conditions believed to be prerequisite to protein kinase C's in vivo activity (Nishizuka, 1984). No phosphorylation of either talin or vinculin occurred in the absence of exogenously added protein kinase C; this demonstrates that the phosphorylation observed is not due to autophosphorylation or to any kinases contaminating the protein preparations. As expected for protein kinase C-mediated phosphorylation events, the phosphorylated amino acid residues were alkali sensitive (data not shown).

Phosphorylation of talin's 46 and 190–200 kDa proteolytic peptides by protein kinase C. Talin is a substrate for the calcium-dependent protease in vitro and in vivo (Fox *et al.*, 1983; Beckerle *et al.*, 1987). The calcium-dependent protease cleaves talin to generate two proteolytic peptides of ~46 and 190–200 kDa (Fox *et al.*, 1985; O'Halloran *et al.*, 1985; Beckerle *et al.*, 1987). The precise size of the large proteolytic peptide depends on the source of the talin; human platelet talin gives rise to a 200-kDa fragment, whereas chicken gizzard talin yields a 190-kDa fragment. When protein kinase C-phosphorylated avian smooth muscle talin is subjected to cleavage by the calcium-dependent

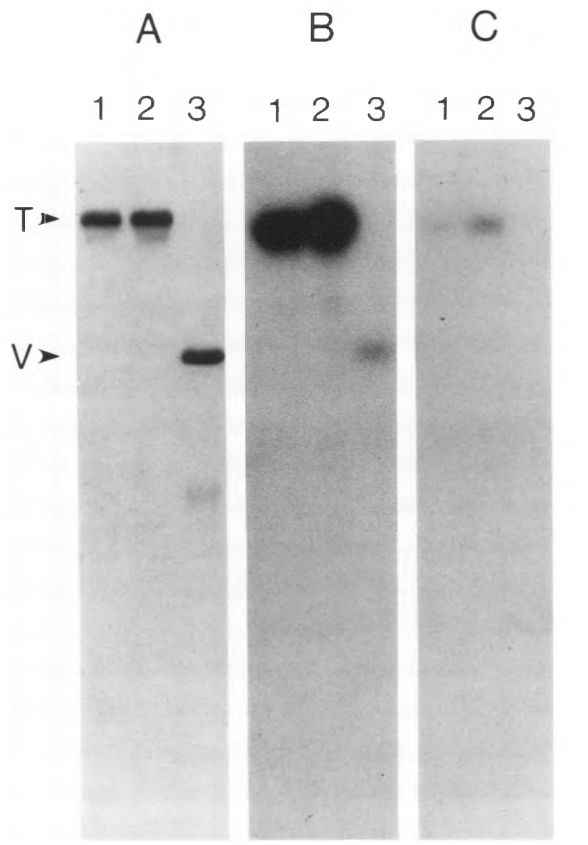


Figure 5. **Phosphorylation of talin and vinculin in vitro by purified protein kinase C.** (A) Coomassie blue-stained SDS-polyacrylamide gel of purified avian smooth muscle talin (lane 1), human platelet talin (lane 2), and avian smooth muscle vinculin (lane 3). (B) Autoradiograph of the gel in A. Proteins were phosphorylated by purified protein kinase C in the presence of γ^{32}P-ATP, calcium, phosphatidyl serine, and 1,2-diolein. (C) Phosphorylation of talin and vinculin by protein kinase C in the absence of phosphatidyl serine and 1,2-diolein (T, talin; V, vinculin).

protease, the phosphopeptide pattern shown in Figure 6, lane 2 is obtained. The majority of the label is associated with the 46-kDa proteolytic fragment of talin; however, the 190-kDa fragment is also labeled to some extent. This result confirms the fact that the in vitro labeling we observed is indeed due to phosphorylation of talin since cleavage of the labeled protein with the calcium-dependent protease results in the generation of proteolytic fragments characteristic of talin. Essentially identical results were obtained with human platelet talin (data not shown). These observations are also consistent with a previous report that talin is phosphorylated on multiple sites by protein kinase C (Litchfield and Ball, 1986). We have seen no evidence to suggest that protein kinase C-mediated phosphorylation of talin affects its susceptibility to cleavage by the calcium-dependent protease.

Effect of PMA on adhesion plaque integrity and morphology of chicken embryo fibroblasts

We have examined the overall morphology, actin filament organization, and talin distribution in chicken embryo fibroblasts in the presence and absence of PMA to determine whether the phosphorylation of talin we observed in PMA-treated cells correlates with any alteration in cell adhesion or cytoskeletal organization. Specifically, we have compared the distribution of talin and filamentous actin in cells cultured for up to 60 min in the presence or absence of 50 ng/ml PMA by indirect immunofluorescence (Figure 7). We have not detected any change in the organization of adhesion plaques or stress fibers when we compared control cells (Figure

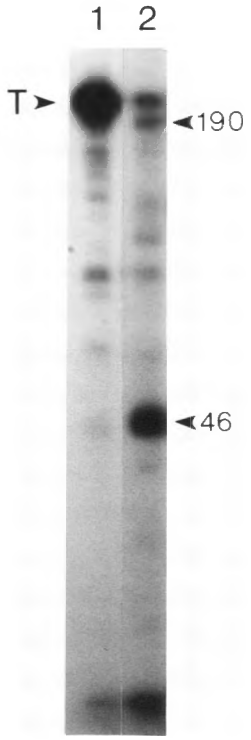
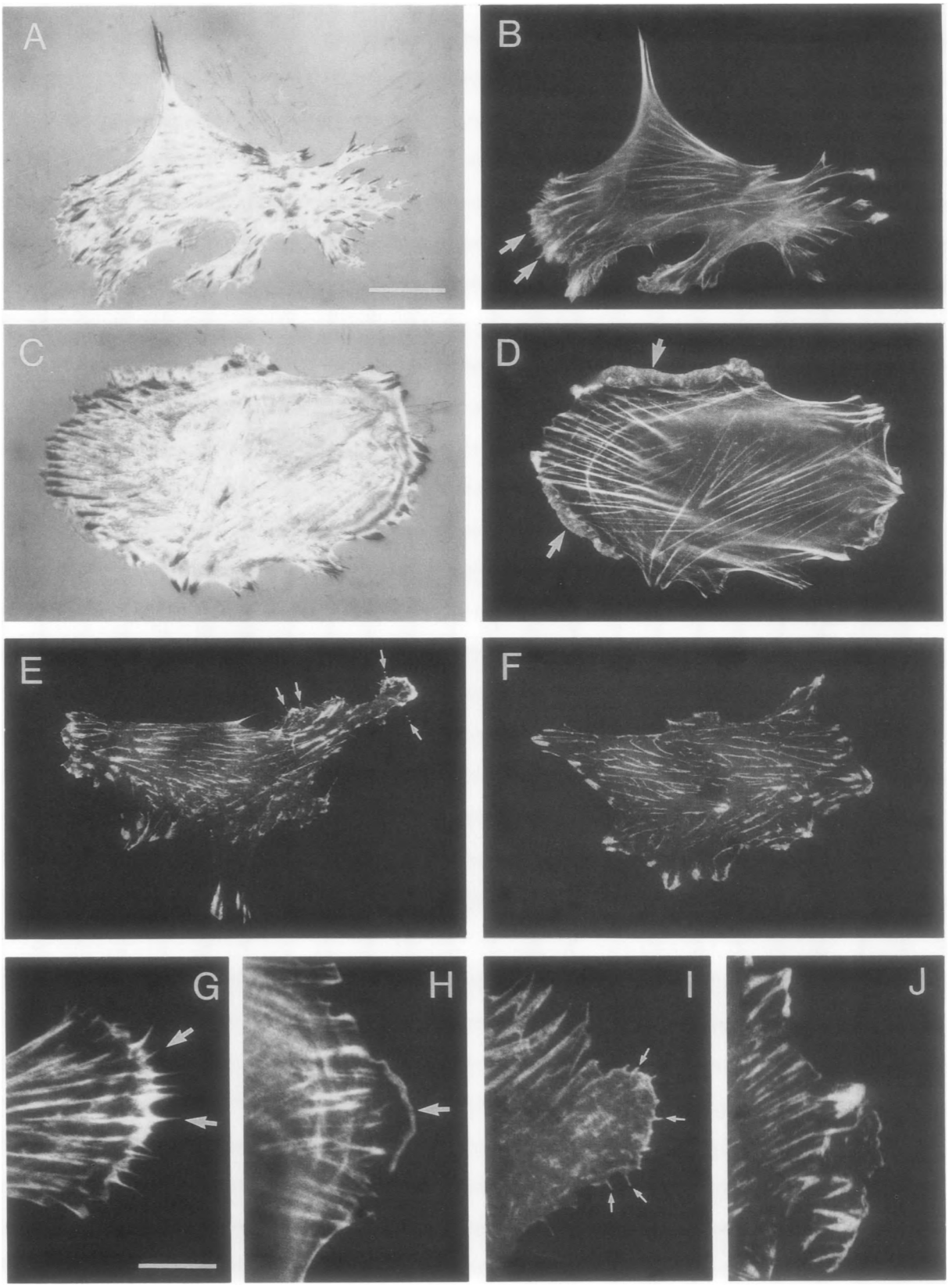


Figure 6. **Protein kinase C phosphorylates talin on multiple sites.** (Lane 1) Autoradiograph showing avian smooth muscle talin phosphorylation by purified protein kinase C. (Lane 2) Protein kinase C-phosphorylated talin was subjected to proteolysis by calcium-dependent protease II. The 190- and 46-kDa proteolytic products are indicated by the arrowheads. The proteolytic peptide map illustrates that there are at least two sites of protein kinase C-mediated phosphorylation on talin. The majority of the label is contained within the 46-kDa domain of talin (T, talin).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7, A and B) with PMA-treated cells (Figure 7, C and D). Likewise, talin was found in adhesion plaques and fibrillar streaks underlying cell-surface fibronectin (Burridge and Connell, 1983) in both control (Figure 7E) and PMA-treated (Figure 7F) cells. Overall, adhesion plaque and stress fiber morphology and organization appeared unperturbed by PMA-treatment.

However, we did observe one very interesting change in cell morphology and cytoskeletal organization in response to PMA treatment. Untreated chicken embryo fibroblasts typically exhibit numerous actin-rich fine filopodial extensions and short, rib-like actin filament bundles associated with lamellopodial extensions (Figure 7, B and G). In control cells, talin is found in association with these actin-rich microspikes and lamellopodia (Figure 7, E and I). The talin often appears to be specifically localized at the distal tips of the short actin filament bundles where no visible focal contact has yet formed. These actin- and talin-rich cell surface elaborations are reminiscent of the focal contact precursors described by Izzard (1988). Interestingly, cells exposed to PMA completely lacked these actin- and talin-rich components of cell surface elaborations (Figure 7, H and J). Areas of apparent lamellopodial extension were frequently visible in PMA-treated cells; however, these regions were typically devoid of organized actin filament bundles (Figure 7, D and H). Talin-rich cell surface extensions were also lacking in PMA-treated cells (Figure 7, F and J). Thus, in chicken embryo fibroblasts, exposure to 50 ng/ml PMA for up to 60 min has no detectable effect on established stress fiber arrays and adhesion plaques; however, there is a dramatic decline in the presence of apparent focal contact precursor structures.

If exposure to PMA perturbs a cell's ability to form new focal contacts, PMA would be expected to affect the efficiency of cell reattachment and spreading to a substratum after trypsinization. We have examined this possibility and have found that PMA causes a qualitative slow-down in cell spreading and focal contact development when added to trypsinized chicken embryo fibroblasts at the time of replating. The decline in plating efficiency noted in PMA-treated cells is most evident within 4 h after plating; we detected no obvious differences between control and PMA-treated cells after 6 h of reattachment time had elapsed.

Discussion

Talin is a high molecular weight phosphoprotein that is localized in adhesion plaques, ruffling edges and regions of the plasma membrane underlying cell-surface associated fibronectin (Burridge and Connell, 1983). Since it can interact with integrin (Horwitz *et al.*, 1986) and vinculin (Otto, 1983; Burridge and Mangeat, 1984), it has been postulated to be involved in bridging the gap between the membrane and cytoplasmic domains of adhesion plaques.

In this paper we have shown that talin is phosphorylated in vivo in chicken embryo fibroblasts exposed to the tumor-promoting phorbol ester, PMA. Talin phosphorylation increased an average of 3.0-fold over control levels within 10 min of exposure to 50 ng/ml PMA. When cells were maintained in the continuous presence of PMA, talin phosphorylation remained elevated to 2.9-fold above control levels for at least 60 min. Phosphoamino acid analysis of talin isolated from PMA-treated cells illustrated that the detectable label is in phosphorylated serine and threonine residues, a result consistent with the idea that PMA stimulates an increase in protein kinase C-mediated phosphorylation of talin. Vinculin phosphorylation also increased in cells exposed to PMA, rising to 1.4-fold above control values in 10 min and 1.3-fold above control values in a 60 min PMA treatment. The extent of increase in talin phosphorylation in response to PMA exceeded the increase in vinculin phosphorylation observed under the same conditions.

Figure 7. **Talin and actin distribution in control and PMA-treated chicken embryo fibroblasts.** (A and B) Control cells have well-defined adhesion plaques that can be visualized by interference reflection microscopy (A) and organized stress fibers that can be detected by Rhodamine phalloidin (B). Short bundles of actin filaments can be seen in the broad lamellopodia extending from the surface of the cell (B, large arrows). Chicken embryo fibroblasts exposed to PMA (50 ng/ml for 60 min) exhibit normal adhesion plaques (C) and stress fiber arrays (D), but lack the short bundles of actin filaments and microspikes that are found at the edges of untreated cells (compare regions marked with large arrows in B and D). Talin is found in adhesion plaques and in actin-rich microspikes extending from the cell surface in control cells (E, arrows). In PMA-treated cells, the talin organization in adhesion plaques appears to be unperturbed; however, the talin-rich microspikes are no longer visible (F). The actin and talin-containing surface protrusions of control cells are shown at higher magnification in G and I respectively (arrows). High magnification views of comparable regions of PMA-treated cells (H, actin; J, talin) reveals that these structures are absent in cells exposed to the tumor promoter (A–F, Bar = 20 μm; G–J, Bar = 10 μm).

Talin has previously been shown to be phosphorylated in normal chicken embryo fibroblasts. Talin isolated from untreated cells contains 0.07 mol phosphate/mole protein (DeClue and Martin, 1987). Consequently, talin isolated from PMA-treated cells would be expected to contain on average ~0.21 mol phosphate/mole protein. The significance of this level of phosphorylation for talin function in cells is difficult to assess. Avian talin is phosphorylated on multiple sites in vivo (DeClue and Martin, 1987) and in vitro by protein kinase C (this paper and Litchfield and Ball, 1986), so any given site is probably phosphorylated on only a small proportion of the molecules. However, as has been suggested previously (Pasquale *et al.,* 1986), an apparently substoichiometric level of phosphorylation could be significant if confined to a subpopulation of the cell's talin.

Recently the phosphorylation of talin in BSC-1 cells exposed to PMA has been studied (Turner *et al.,* 1989). BSC-1 cells exhibit a dramatic change in cell shape, cell adhesion, and cytoskeletal organization in response to PMA. Talin phosphorylation increases approximately threefold during the time when these morphological changes are occurring. Based on these results, it has been suggested that talin phosphorylation may be involved in promoting the disassembly of adhesion plaques in BSC-1 cells (Turner *et al.,* 1989). We have found that chicken embryo fibroblasts also exhibit a threefold increase in talin phosphorylation in response to exposure to PMA; however, there is little indication of an alteration in existing stress fiber or adhesion plaque organization that is triggered by exposure to PMA.

Although exposure to PMA did not detectably alter the organization of stress fibers and adhesion plaques in chicken embryo fibroblasts, it did result in a reproducible loss of certain actin and talin-rich structures. In particular, we observed a loss of fine, F-actin–containing cell surface filopodia and short, rib-like, orthogonally arrayed bundles of actin filaments that are normally found in cell surface lamellopodia. The structures that we have determined to be absent in PMA-treated chicken embryo fibroblasts are morphologically indistinguishable from the functional focal contact precursor structures described in detail by Izzard (1988). It is intriguing to consider the unifying possibility that a conserved effect of PMA on cells is the induction of a loss of focal contact precursors. If it were universally the case that PMA perturbed new focal contact formation without directly affecting the stability of existing adhesion plaques, the ultimate focal contact morphology displayed by cells exposed to PMA would be a reflection of the intrinsic, cell-type specific rate of focal contact turnover which could conceivably vary widely. Alternatively, there simply may not be a universal PMA effect on cells beyond its ability to activate protein kinase C. For example, different cell types from the same species have been reported to respond to PMA in apparently unique ways (Lin *et al.,* 1989).

The role of talin phosphorylation in generating the morphological changes we have observed in PMA-treated chicken embryo fibroblasts is not clear at this point. Although the increased talin phosphorylation we find in PMA-treated chicken embryo fibroblasts is temporally correlated with the changes in actin and talin distribution, we have no evidence that it is directly responsible for effecting those changes. In the future it will be important to determine the effect of phosphorylation on talin's biochemical properties, in particular its ability to interact with other adhesion plaque components such as vinculin and integrin.

Methods

Antibody production and characterization

Polyclonal antibodies against chicken gizzard talin were raised in rabbits (Beckerle *et al.,* 1986) and have been characterized previously. Rabbit anti-vinculin was generously provided by Keith Burridge, University of North Carolina, Chapel Hill, NC.

Protein purification

All proteins were purified by established procedures. Human platelet talin was isolated from outdated platelet-rich plasma by a modification (Beckerle *et al.,* 1986) of the method of Collier and Wang (1982). Avian smooth muscle vinculin and talin were purified from low ionic strength extracts of chicken gizzard according to previously published techniques (O'Halloran *et al.,* 1986). Protein kinase C was isolated from bovine brain by established methods (Parker *et al.,* 1984) and was a generous gift of Dr. Peter J. Parker, Ludwig Institute for Cancer Research, London, England. Calcium-dependent protease, Type II was purified from bovine heart (Croall and DeMartino, 1984) and was kindly provided by Dr. Dorothy Croall, University of Texas Health Science Center, Dallas, TX.

Radiolabeling and phorbol ester treatment of cells

Chicken embryo fibroblasts were isolated from 10- to 12-d-old chicken embryos. After at least two passages, cells were seeded heavily into 60 or 100 mm culture dishes and were allowed to grow until 80–90% confluent in complete Dulbecco's Modified Eagle's Medium (DMEM) supplemented with penicillin-streptomycin and 10% fetal bovine serum. Monolayers of cells were washed three times with phosphate-free modified Eagle's medium supplemented with glutamine, pyruvate, penicillin-streptomycin, and fetal bovine serum (1% or 4%, not dialyzed). Cells were labeled with ^{32}P-orthophosphate (0.5 mCi/ml) for 5 h in medium containing 1% fetal bovine serum or 16 h in medium containing 4%

fetal bovine serum. (There was no consistent variation in the conclusions derived from experiments using these two different labeling procedures; therefore we have combined the results from both sets of experiments for the analysis presented here.) PMA, dissolved in DMSO, was added at a final concentration of 50 ng/ml with a resulting DMSO concentration of 0.1%. Control cells were exposed to 0.1% DMSO for equivalent lengths of time. Control and experimental treatments occurred for the lengths of time indicated in the text during the last stages of the labeling procedure.

Immunoprecipitation

At the end of the labeling and treatment procedures, cell monolayers were washed rapidly with Tris-buffered saline or phosphate-free MEM until the counts present in the wash buffer declined (usually 5–6 washes over a 1–2 min period). Cells were then examined by phase contrast to assess cell viability and were harvested for immunoprecipitation of talin and/or vinculin as described previously (Beckerle *et al.*, 1986; Burridge and Connell, 1983). Immunoprecipitates were analyzed by SDS-PAGE on 10% polyacrylamide gels containing 0.13% bisacrylamide using the buffer conditions of Laemmli (1970). Following staining of the gels with Coomassie blue to evaluate whether a comparable amount of total protein was immunoprecipitated from both control and experimental lysates, the gels were dried and exposed for autoradiography at −70°C on Kodak X-Omat AR film using an intensification screen. After the initial autoradiographic exposure, some gels were rehydrated and the phosphoproteins were subjected to alkali hydrolysis according to previously published procedures (Cooper *et al.*, 1983) to evaluate whether the level of phosphotyrosine in talin was affected by PMA treatment.

Amino acid analysis

For phosphoamino acid analysis, the immunoprecipitated proteins resolved by SDS-PAGE and visualized by Coomassie blue staining were excised from the gel and digested with tolylsulfonyl phenylalanyl chloromethyl ketone-trypsin. The resulting peptides were subjected to acid hydrolysis in 6 N HCl at 110°C for 4 h as described previously (Cooper *et al.*, 1983); we chose this hydrolysis protocol because it increases the relative recovery of phosphothreonine residues. Phosphoamino acids were analyzed by two-dimensional high-voltage electrophoresis on thin layer chromatographic plates at pH 1.9 in the first dimension and pH 3.5 in the second dimension. Labeled amino acids were visualized by autoradiography. The marker amino acids phosphoserine, phosphothreonine, and phosphotyrosine were included in the sample and their positions were determined by ninhydrin staining (Hunter and Sefton, 1980).

In vitro phosphorylation

The ability of purified protein kinase C to phosphorylate talin and vinculin in vitro was assayed essentially as described previously (Ways *et al.*, 1986). Briefly, purified talin or vinculin (5–6 μg) was incubated with 0.06 U protein kinase C at 23°C for 20 min in a total reaction volume of 0.1 ml. The reaction mixture consisted of the above-mentioned proteins, 10 mM $MgCl_2$, 20 mM Tris HCl, pH 7.4, 5 mM $CaCl_2$, 0.01 mM unlabeled ATP, 2.5–5.0 μCi γ^{32}P-ATP ± 0.05 mg/ml phosphatidyl serine and 0.025 mg/ml 1,2-diolein. The reaction was initiated by addition of premixed labeled and unlabeled ATP and was stopped by addition of 50 μl Laemmli sample buffer followed by boiling of the samples for 3 min. For experiments in which protein kinase C-phosphorylated proteins were subjected to calcium-dependent proteolysis prior to SDS-PAGE, trifluoperazine was added to the reaction mixture to a final concentration of 100 μM after the initial incubation was complete; then either 0.34 μg calcium-dependent protease II or 5.0 μg leupeptin to block any endogenous protease, both in volumes of 5 μl, was added and the samples were incubated for an additional 5 min. Reactions were quenched by addition of Laemmli sample buffer. Labeled proteins were resolved by SDS-PAGE and visualized by autoradiography.

Indirect immunofluorescence and interference reflection microscopy

Chicken embryo fibroblasts grown on glass coverslips for ~18 h were subjected to experimental treatments and were fixed, permeabilized, and labeled for indirect immunofluorescence as described previously (Beckerle, 1986). Filamentous actin was visualized by staining of cells with Rhodamine-phalloidin (Molecular Probes, Junction City, OR). For interference reflection microscopy, coverslips were mounted on glass slides in a 1:1 mix of PBS and glycerol. Photomicrographs were taken on a Zeiss axiophot fluorescence microscope equipped with 63× Plan-apochromat and Antiflex-neofluor objectives.

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References

Beckerle, M.C., O'Halloran, T., Earp, S., and Burridge, K. (1985). Evidence for functional similarities between talin and platelet P235. J. Cell Biol. *101,* 411a.

Beckerle, M.C. (1986). Identification of a new protein localized at sites of cell-substrate adhesion. J. Cell Biol. *103,* 1679–1687.

Beckerle, M.C., O'Halloran, T., and Burridge, K. (1986). Demonstration of a relationship between talin and P235, a major substrate of the calcium-dependent protease in platelets. J. Cell. Biochem. *30,* 259–270.

Beckerle, M.C., Burridge, K., DeMartino, G.N., and Croall, D.E. (1987). Colocalization of calcium-dependent protease II and one of its substrates at sites of cell adhesion. Cell *51,* 569–577.

Burridge, Keith (1986). Substrate adhesions in normal and transformed fibroblasts: organization and regulation of cytoskeletal, membrane and extracellular matrix components at focal contacts. Cancer Rev. *4,* 18–78.

Burridge, K., and Connell, L. (1983). A new protein of adhesion plaques and ruffling membranes. J. Cell Biol. *97,* 359–367.

Burridge, K., and Mangeat, P. (1984). An interaction between vinculin and talin. Nature *308,* 744–746.

Chen, W.-T., Greve, J.M., Gottlieb, D.I., and Singer, S.J. (1985). Immunocytochemical localization of 140 kD adhesion molecules in cultured chicken fibroblasts and in chicken smooth muscle and intestinal epithelial tissues. J. Histochem. Cytochem. *33,* 576–586.

Collier, N.C., and Wang, K. (1982). Purification and properties of human platelet P235. J. Biol. Chem. *257,* 6937–6943.

Cooper, J.A., Sefton, B.M., and Hunter, T. (1983). Detection and quantification of phosphotyrosine in proteins. Methods Enzymol. *99,* 387–402.

Croall, D.E., and DeMartino, G.N. (1984). Comparison and purification of two calcium-dependent proteinases from bovine heart. Biochem. Biophys. Acta *788,* 348–355.

Damsky, C.H., Knudsen, K.A., Bradley, D., Buck, C.A., and Horwitz, A.F. (1985). Distribution of the cell substratum attachment (CSAT) antigen on myogenic and fibroblastic cells in culture. J. Cell Biol. *100,* 1528–1539.

David-Pfeuty, T., and Singer, S.J. (1980). Altered distributions of the cytoskeletal proteins vinculin and α-actinin in cultured fibroblasts transformed by Rous sarcoma virus. Proc. Natl. Acad. Sci. USA *77,* 6687–6691.

DeClue, J.E., and Martin, G.S. (1987). Phosphorylation of talin at tyrosine in Rous sarcoma virus-transformed cells. Mol. Cell. Biol. *7,* 371–378.

Fox, J.E.B., Reynolds, C.C., and Phillips, D.R. (1983). Calcium-dependent proteolysis occurs during platelet aggregation. J. Biol. Chem. *258,* 9973–9981.

Fox, J.E.B., Goll, D.E., Reynolds, C.C., and Phillips, D.R. (1985). Identification of two proteins (actin-binding protein and P235) that are hydrolyzed by endogenous Ca^{2+}-dependent protease during platelet aggregation. J. Biol. Chem. *260,* 1060–1066.

Herman, B., and Pledger, W.J. (1985). Platelet-derived growth factor induced alterations in vinculin and actin distribution in BALB/c 3T3 cells. J. Cell Biol. *100,* 1031–1040.

Horwitz, A., Duggan, K., Buck, C., Beckerle, M.C., and Burridge, K. (1986). Interaction of plasma membrane fibronectin receptor with talin—a transmembrane linkage. Nature *320,* 531–533.

Hunter, T., and Sefton, B.M. (1980). The transforming gene product of Rous sarcoma virus phosphorylates tyrosine. Proc. Natl. Acad. Sci. USA *77,* 1311–1315.

Izzard, C.S. (1988). A precursor of the focal contact in cultured fibroblasts. Cell Motil. Cytoskeleton *10,* 137–142.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (Lond.) *227,* 680–685.

Lin, Z., Eshleman, J., Grund, C., Fischman, D.A., Masaki, T., Franke, W.W., and Holtzer, H. (1989). Differential response of myofibrillar and cytoskeletal proteins in cells treated with phorbol myristate acetate. J. Cell Biol. *108,* 1079–1091.

Litchfield, D.W., and Ball, E.M. (1986). Phosphorylation of the cytoskeletal protein talin by protein kinase C. Biochem. Biophys. Res. Commun. *134,* 1276–1283.

Meigs, J.B., and Wang, Y.L. (1986). Reorganization of alpha-actinin and vinculin induced by a phorbol ester in living cells. J. Cell Biol. *102,* 1430–1438.

Mellström, K., Höglund, A., Nister, M., Heldin, C., Westermark, B., and Lindberg, U. (1983). The effect of platelet-derived growth factor on morphology and motility of human glial cells. J. Muscle Res. Cell Motil. *4,* 589–609.

Nishizuka, Y. (1984). Turnover of inositol phospholipids and signal transduction. Science *225,* 1365–1370.

O'Halloran, T., Beckerle, H. C., and Burridge, K. (1985). Identification of talin as a major cytoplasmic protein implicated in platelet activiation. Nature *317,* 449–451.

O'Halloran, T., Molony, L., and Burridge, K. (1986). Purification and assay of vinculin, metavinculin, and talin. Methods Enzymol. *134,* 69–77.

Otto, J.J. (1983). Detection of vinculin-binding proteins with an ^{125}I-vinculin gel overlay technique. J. Cell Biol. *97,* 1283–1287.

Parker, P.J., Stabel, S., and Waterfield, M.D. (1984). Purification to homogeneity of protein kinase C from bovine brain-identity with the phorbol ester receptor. EMBO J. *3,* 953–959.

Pasquale, E.B., Maher, P.A., and Singer, S.J. (1986). Talin is phosphorylated on tyrosine in chicken embryo fibroblasts transformed by Rous sarcoma virus. Proc. Natl. Acad. Sci. USA *83,* 5507–5511.

Schliwa, M., Nakamura, T., Porter, K.R., and Euteneuer, U. (1984). A tumor promoter induces rapid and coordinated reorganization of actin and vinculin in cultured cells. J. Cell Biol. *99,* 1045–1059.

Sefton, B.M., Hunter, T., Ball, E.H., and Singer, S.J. (1981). Vinculin: a cytoskeletal target of the transforming protein of Rous sarcoma virus. Cell *24,* 165–174.

Singer, I.I. (1979). The fibronexus: a transmembrane association of fibronectin-containing fibers and bundles of 5 nm microfilaments in hamster and human fibroblasts. Cell *16,* 675–685.

Turner, C.E., Pavalko, F.M., and Burridge, K. (1989). The role of phosphorylation and limited proteolytic cleavage of talin and vinculin in the disruption of focal adhesion integrity. J. Biol. Chem. *264,* 11 938–11 944.

Ways, D.K., Dodd, R.C., Gwynne, J.T., and Earp, H.S. (1986). Lipoprotein modulation of the intracellular localization of protein kinase C and alteration of phorbol ester-stimulated differentiation in the human monoblastic U937 cell line. Cancer Res. *46,* 6049–6053.

Werth, D.K., Niedel, J.E., and Pastan, I. (1983). Vinculin, a cytoskeletal substrate for protein kinase C. J. Biol. Chem. *258,* 11 423–11 426.

Werth, D.K., and Pastan, I. (1984). Vinculin phosphorylation in response to calcium and phorbol esters in intact cells. J. Biol. Chem. *259,* 5264–5270.