The Role of Phosphorylation and Limited Proteolytic Cleavage of Talin and Vinculin in the Disruption of Focal Adhesion Integrity*

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Chemical agents which activate specific kinases were employed to disrupt the stress fiber and focal adhesion organization of cells spread on a substratum. The phorbol ester 12-O-tetradecanoylphorbol-13-acetate, an activator of protein kinase C, promoted a rapid loss of stress fibers and focal adhesions from African green monkey kidney (BSC-1) cells. This was paralleled by an increase in the level of talin phosphorylation suggesting that this may play a role in the removal of talin from focal adhesions. Similar morphological changes were produced in the rat embryo fibroblast line (REF 52) by dibutyryl-cAMP, which stimulates protein kinase A. In contrast, however, the phosphorylation of talin was reduced in REF 52 cells when treated with dibutyryl cAMP. In untreated cells we found that the levels of vinculin phosphorylation were very low relative to the levels of talin phosphorylation and did not change following drug treatment in either cell line. Although limited proteolytic cleavage of cytoskeletal proteins represents a potential mechanism for focal adhesion disruption, we observed no proteolysis of talin or vinculin in response to either drug treatment.

In tissue culture, spreading cells often form transmembrane linkages between the extracellular matrix and the F-actin cytoskeleton in regions of the cell where the plasma membrane comes in closest proximity to the substratum. Using a variety of immunohistochemical and biochemical procedures several proteins have been localized to these specialized regions known as focal adhesions or focal contacts (Burrage et al., 1988). Among these are the integrins (Buck and Horwitz, 1987; Hynes, 1987; Ruoslahti and Pierschbacher, 1987), a family of glycoproteins, which span the plasma membrane and bind both extracellular matrix components, such as fibronectin and vitronectin (Horwitz et al., 1985; Akiyama et al., 1986; Buck and Horwitz, 1987) as well as talin (Horwitz et al., 1986), which is present in focal adhesions at the intracellular face of the plasma membrane (Burrage and Connell, 1983a, 1983b). Another cytoskeletal element, vinculin, interacts both with talin (Burridge and Mangeat, 1984) and, albeit weakly, with α-actinin (Otto, 1983; Wilkins et al., 1983; Burridge and Mangeat, 1984; Craig, 1985; Wachstock et al., 1987). In view of the additional affinity of α-actinin for F-actin (Maruyama and Ebashi, 1965; Burridge and Feramisco, 1981) a series of protein-protein interactions can be mapped out extending from the extracellular matrix through focal adhesions to the F-actin stress fibers within the cell. It seems likely, however, that other proteins are also involved in this linkage (for review see Burridge et al., 1988).

The importance of each of these interactions in the maintenance of a stable focal adhesion remains unclear. The integrity of these adhesions can be disrupted by a variety of physiological and pharmacological factors. During the cell cycle, spread cells in culture round up prior to division and subsequently respread forming new focal adhesions. Transformation by some viruses, such as Rous sarcoma virus, reveals a different focal adhesion phenotype (David-Pfeuty and Singer, 1980; Tarone et al., 1985). The oncogenes activated during transformation often exhibit potent tyrosine kinase activity (Hunter and Sefton, 1980; Gentry and Rohrschneider, 1984; Tarone et al., 1985). Observations that vinculin (Sefton et al., 1981), talin (Pasquale et al., 1986; DeClue and Martin, 1987), and integrin (Hirst et al., 1986) become phosphorylated at tyrosine residues in certain virally transformed cells led to the suggestion that these phosphorylation events could be responsible for the less stable nature of focal adhesions in these cells compared to those expressing a more normal phenotype. However, the importance of these changes is in question since the use of mutant virus strains which did not alter the morphology of cells produced similar tyrosine phosphorylation of vinculin (Rohrschneider and Rosok, 1983; Iwashita et al., 1983; Antler et al., 1985; Rohrschneider and Reynolds, 1985; Kellie et al., 1986; Nigg et al., 1986) and talin (DeClue and Martin, 1987).

Evidence suggesting that phosphorylation may be an important factor in the disruption of focal adhesions in "normal" cells has been obtained using chemical activators of specific kinases. In certain cell types the tumor-promoting phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA)1 induces rapid loss of stress fibers and a concomitant redistribution of vinculin (Schliwa et al., 1984; Kellie et al., 1985) and α-actinin (Meigs and Wang, 1986) from focal adhesions probably via the activation of protein kinase C (Nishizuka, 1984). The talin distribution under these conditions was not determined. Lamb et al. (1988) obtained similar disruption of stress fibers in REF 52 cells via the activation of the cyclic AMP (cAMP)-dependent protein kinase (A kinase) either by the use of stable synthetic analogues of cAMP or microinjection of the catalytic subunit of A kinase. The effect on the distribution of focal adhesion proteins was not investigated.

Cleavage of talin or vinculin by specific proteases represents

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1 The abbreviations used are: TPA, 12-O-tetradecanoylphorbol-13-acetate; DMEM, Dulbecco's modified Eagle's medium; TBS, Tris-buffered saline; b2cAMP, dibutyryl cyclic AMP; MIX, 3-isobutyl-1-methylxanthine; Me2SO, dimethyl sulfoxide; SDS, sodium dodecyl sulfate.
another potential mechanism for regulating focal adhesion organization. Talin is a preferred substrate in vitro for the calcium-dependent protease (CDP II) (Calpain II), being readily cleaved to stable fragments of 190 and 47 kDa in the case of chicken smooth muscle talin (O'Halloran and Barridge, 1986) or 200 and 47 kDa for platelet talin (P235) (Collier and Wang, 1982; Fox et al., 1985; O'Halloran et al., 1985; Beckerle et al., 1986). The idea that limited proteolysis of cytoskeletal proteins, including talin, may be important in regulating the organization of intracellular structures like focal adhesions is supported by data concerning platelet activation and aggregation by thrombin, in which a calcium-dependent proteolysis of both filamin and talin accompanies major cytoskeletal reorganization (Fox et al., 1985). Additionally, immunohistochemical studies have revealed a colocalization of Calpain II and talin to focal adhesions in a variety of cell types (Beckerle et al., 1987) providing circumstantial evidence that the cleavage of talin could be a factor in promoting focal adhesion and stress fiber disassembly.

We have attempted to answer biochemically some of the questions raised in the morphological studies described above. To this end, we investigated the state of phosphorylation of both talin and vinculin during focal adhesion disruption following specific kinase activation. Similarly, we have explored the role of proteolytic cleavage of these two proteins during this cytoskeletal reorganization.

MATERIALS AND METHODS

Cell Culture—African green monkey kidney cells (BSC-1) and rat embryo fibroblasts (REF-52) were grown in Dulbecco's modified Eagle's medium (DMEM, GIBCO) supplemented with 10% fetal calf serum (GIBCO), 50 units/ml penicillin, and 50 μg/ml streptomycin and 1% L-glutamine (as 10% CO2).

Fluorescence Microscopy—Cells for immunofluorescence were grown on glass coverslips for 24-48 h prior to use in containing 10% fetal calf serum. Cells were fixed in 3.7% formaldehyde (v/v) in phosphate-buffered saline (pH 7.4) for 10 min. They were then washed in TBS (150 mM NaCl, 0.1% NaN3, 50 mM Tris-HCl, pH 7.6) for 5 min, followed by permeabilization with 0.2% (v/v) Triton X-100 in TBS for 5 min, then washed an additional 5 min in fresh TBS. Cells were incubated in primary antibody (rabbit anti-talin or guinea pig anti-vinculin) diluted in TBS for 1 h at 37 °C. Excess antibody was removed by washing in 5 min in TBS. The cells were then incubated with secondary antibody (fluorescein-conjugated goat anti-rabbit Ig or goat anti-guinea pig Ig, Cappel Laboratories, Cochranville, PA) diluted 1:50 plus rhodamine-conjugated phalloidin (Molecular Probes, Inc., Eugene, OR) diluted 1:300 for 1 h at 37 °C. The coverslips were washed for 5 min in TBS, then rinsed briefly in deionized water, and mounted in Gelvatol (Monsanto). The cells were observed using a Zeiss IM-35 photomicroscope with a x63 oil immersion objective and photographed with a Tri-X film (Kodak).

Metabolic Labeling of Cells—Cells were grown for 24-48 h before radiolabeling. For phosphorylation experiments cells were grown with [32P]orthophosphate (Du Pont-New England Nuclear, carrier-free in water) at 0.75 mCi/ml or 2 or 4 at 37 °C in phosphate-free DMEM supplemented with 10% fetal calf serum that had been dialyzed against phosphate-free saline (0.15 M NaCl). [35S]Methionine-labeled cells were grown in DMEM containing 10% of the usual concentration of methionine and supplemented with 10% of dialyzed fetal calf serum and glutamine containing 50 μCi/ml [35S]methionine (Amersham Corp.) for 18 h at 37 °C.

Drug Treatment—Cells grown to approximately 50-75% confluence were treated with either TPA or a mixture of dibutyryl cyclic AMP (Bt2cAMP) and 3-isobutyl-1-methylxanthine (MIX), all obtained from Sigma. TPA, Bt2cAMP, and MIX were diluted into DMEM before use from 1 mg/ml stocks in MeSO4 stored at −20 °C. TPA was used at a final concentration of 100 ng/ml; Bt2cAMP and MIX were used at 2 mM. Control cells were treated with DMEM containing the same concentration of MeSO4 without drugs.

Phosphoamino Acid Analysis—[32P]-Labeled talin immunoprecipitated from TPA-treated BSC-1 cells electrophoresed on SDS gels and transferred to Immobilon (Millipore) membranes (Kamps and Sefton, 1989) was subject to phosphoamino acid analysis by hydrolysis in 5.7 N HCl for 60 min at 110 °C followed by electrophoresis and chromatography as described (Hunter and Sefton, 1980).

RESULTS

Immunofluorescence—BSC-1 cells spread on glass coverslips for 24-48 h in the presence of serum exhibited a network of short fine actin stress fibers (Fig. 1, b and d) which terminated at focal adhesions as characterized by the presence of both talin (Fig. 1a) and vinculin (Fig. 1c). Following treatment of these cells with the tumor promoter TPA, an activator of protein kinase C, the majority of the actin stress fibers had disassembled by 20 min (Fig. 1, f and h). This was accompanied by a concurrent loss of vinculin (as described previously by Schliwa et al., 1984) (Fig. 1g) and talin (Fig. 1e) from

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double labeling with thine ends. This disruption of the majority of stress fibers is observed in FIG. 2, a–h) or the tumor promoter (TPA) (i, j) on the distribution of talin, vinculin, and F-actin in REF 52 cells. In control cells, talin (a) and vinculin (c) are visualized in focal adhesions along with the majority of the F-actin stress fibers (j, h). All remaining stress fibers have vinculin and talin still present at their ends albeit in reduced amounts. Treatment of REF 52 cells with TPA (100 ng/ml for 20 min) did not alter the normal talin (i) or F-actin (j) distribution. Bar = 20 μm.

became progressively thinner with many of them being disrupted completely (Fig. 2, e–h). At no time was talin staining observed in the absence of vinculin or vice versa (data not shown) and, as was the case with BSC-1 cells treated with TPA, any stress fibers remaining had both talin and vinculin associated with their ends (Fig. 2, e–h). Unlike BSC-1 cells, the REF 52 cells demonstrated no change of stress fiber or focal adhesion organization following exposure to TPA (Fig. 2, i and j).

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FIG. 1. Effects of tumor promoter (TPA) (a–h) or the synthetic cAMP analogue dibutyryl-cAMP and methylisoxanthine (Bt2cAMP/MIX) (i, j) on the focal adhesion and stress fiber distribution in BSC-1 cells. Cells were treated with the respective drug for 20 min prior to fixation, permeabilization, and double labeling with talin or vinculin antisera and rhodamine phalloidin to visualize the F-actin. In control cells talin (a) and vinculin (c) are localized to focal adhesions into which stress fibers are inserted (b, d). TPA treatment (100 ng/ml for 20 min) causes the loss of the majority of talin (e) and vinculin (g) staining from adhesions and the disruption of accompanying stress fibers (f, h). Note that all remaining stress fibers still have talin and vinculin associated with their ends. Treatment of BSC-1 cells with Bt2cAMP/MIX (2 mM for 20 min) did not alter the normal talin (i) or F-actin (j) distribution. Bar = 20 μm.

focal adhesions. However, both talin and vinculin were always observed at the ends of any remaining stress fibers at all time intervals (Fig. 1, e and g). We did not detect a preferential depletion of vinculin staining over that of talin during the disruption of focal adhesions and stress fibers.

BSC-1 cells were also treated with a mixture of 2 mM Bt2cAMP and 2 mM MIX, both of which pass freely into cells, to activate the cAMP-dependent protein kinase (A kinase). This did not, however, alter the cell’s normal cytoskeletal morphology (Fig. 1, i and j) confirming previous observations (Schliwa et al., 1984).

In sharp contrast to these results, the stress fibers and focal adhesions of REF 52 cells were readily disrupted by incubation in Bt2cAMP/MIX as observed by Lamb et al. (1988). These cells normally have prominent stress fibers (Fig. 2, b and d) which terminate in extensive talin- and vinculin-containing focal adhesions (Fig. 2, a and c). During incubation with the Bt2cAMP/MIX both the stress fibers and the focal adhesions

FIG. 2. Effect of dibutyryl-cAMP (Bt2cAMP) (a–h) or the tumor promoter (TPA) (i, j) on the distribution of talin, vinculin, and F-actin in REF 52 cells. In control cells, talin (a) and vinculin (c) are localized in large focal adhesions into which stress fibers terminate (b, d). Following treatment with Bt2cAMP for 20 min most of the talin (e) and vinculin (g) is lost from the focal adhesions along with the majority of the F-actin stress fibers (j, h). All remaining stress fibers have vinculin and talin still present at their ends albeit in reduced amounts. Treatment of REF 52 cells with TPA (100 ng/ml for 20 min) did not alter the normal talin (i) or F-actin (j) distribution. Bar = 20 μm.

of the stress fibers was demonstrated by the following changes following treatment with either drug. With TPA, the talin stress fibers were always present (Fig. 2, a–h) and/or disrupted and never lost (Fig. 2, f–i). Unlike BSC-1 cells, the REF 52 cells demonstrated no change of stress fiber or focal adhesion organization following exposure to TPA (Fig. 2, i and j).
Following metabolic labeling with $[^{35}\text{S}]$methionine for 2 h cells were treated with TPA or Bt2cAMP for 20 min. Talin and vinculin were subsequently immunoprecipitated and compared to the talin and vinculin recovered from control cells. Lane 1, $^{125}$I markers (kilobaltons $\times 10^3$); lanes 2–7, BSC-1 cells; lane 2, talin from control cells; lane 3, talin from TPA-treated cells; lane 4, vinculin from control cells; lane 5, vinculin from TPA-treated cells; lanes 6 and 7, supernatants from control and TPA-treated cells respectively; lanes 8–13, REF 52 cells; lane 8, talin from control cells; lane 9, talin from Bt2cAMP/MIX-treated cells; lane 10, vinculin from control cells; lane 11, vinculin from Bt2cAMP/MIX-treated cells; lanes 12 and 13, supernatants from control and Bt2cAMP/MIX-treated cells, respectively. Note there is no change in the amount of talin or vinculin nor any increase in proteolytic fragments following either drug treatment.

Similarly, the amount of vinculin immunoprecipitated from the control and drug-treated cells was the same in all cases (Fig. 3, lanes 4 and 5 and 10 and 11). Similar analysis of $[^{35}\text{S}]$methionine-labeled BSC-1 or REF 52 cells treated with TPA or Bt2cAMP/MIX, respectively, revealed no detectable changes in the total protein profile (Fig. 3, lanes 6 and 7, BSC-1; lanes 12 and 13, REF 52). These data provide evidence that neither talin nor vinculin is proteolytically cleaved prior to their removal from focal adhesions under these experimental conditions.

**Phosphorylation**—Since both TPA and Bt2cAMP have the capacity to activate specific kinases (protein kinase C and cAMP-dependent kinase, respectively) the potential role of talin and/or vinculin phosphorylation in the disruption of focal adhesions was investigated following treatment of cells with these drugs.

Immunoprecipitation of talin from BSC-1 cells prelabeled with inorganic $^{32}\text{P}$ (see “Materials and Methods”) and subsequently treated with TPA for various lengths of time revealed a steady increase in the level of talin phosphorylation over a 20-min period (Fig. 4, lanes 2–7), a time course which paralleled focal adhesion disruption, as observed by immunofluorescence. Control cells treated with Me$_2$SO alone demonstrated basal levels of talin phosphorylation at all time points (Fig. 4, lanes 2, 4, and 6), indicating that the increase in talin phosphorylation observed in the presence of TPA (lanes 3, 5, and 7) was not an artifact attributable to the presence of Me$_2$SO. It should be noted that 4a-phorbol, an analogue of TPA which does not activate protein kinase C, caused no increase in talin phosphorylation above the Me$_2$SO control levels (data not shown). Examination of the total population of phosphorylated cellular proteins of cells incubated with TPA for 20 min (Fig. 4, lane 9) and control cells (Fig. 4, lane 8) revealed similar levels of protein phosphorylation, indicating that the TPA did not cause a general increase in phosphorylation levels. Similar analysis of talin immunoprecipitated from BSC-1 cells treated with Bt2cAMP/MIX (a case in which focal adhesion and stress fiber disruption was not observed) demonstrated that the level of talin phosphorylation remained constant after this treatment (Fig. 4, lanes 10 and 11).

While Bt2cAMP caused a disruption of stress fibers and the loss of talin and vinculin from focal adhesions in REF 52 cells, an increase in talin phosphorylation in these cells was not observed. On the contrary, a small but reproducible decrease in the level of talin phosphorylation was detected (Fig. 5, lane 5). This effect was specific for the Bt2cAMP/MIX since treatment of the REF 52 cells with TPA (which does not disrupt their cytoskeletal organization) did not alter the level of talin phosphorylation (Fig. 5, lanes 10 and 11).

Analysis of the vinculin immunoprecipitated from BSC-1 cells treated with TPA (Fig. 6, lane 3) or REF 52 cells treated with Bt2cAMP/MIX (Fig. 6, lane 5) revealed very low levels of phosphorylation similar to those found in untreated cells (Fig. 6, lanes 2 and 4). Similarly, no change in phosphorylation was observed in the vinculin immunoprecipitated from BSC-1 cells treated with Bt2cAMP/MIX or REF 52 cells treated with TPA (data not shown).

**Phosphopeptide Analysis and Extent of Talin Phosphorylation**—The increase in talin phosphorylation following TPA treatment was determined by scanning densitometry of autoradiographs of immunoprecipitates to be approximately 3-fold (data not shown). The extent of phosphorylation of talin was measured by scintillation counting of talin immunoprecipitated from BSC-1 cells labeled with $[^{32}\text{P}]$orthophosphate in medium of known specific activity. The amount of talin...
FIG. 5. Bt2eAMP/MIX treatment of REF 52 cells causes a small decrease in the level of talin phosphorylation while TPA has no effect. Lane 1, 125I markers; lanes 2 and 4, talin immunoprecipitated from control cells at 5 and 20 min; lanes 3 and 5, talin immunoprecipitated from Bt2eAMP/MIX-treated cells also at 5 and 20 min showing a small decrease in phosphorylation after 20 min of treatment in Bt2eAMP/MIX; lanes 6–9, supernatants corresponding to lanes 2–5; lanes 10 and 11, talin immunoprecipitated from REF 52 cells treated with TPA showing no change in phosphorylation levels after treatment with this drug.

**FIG. 6.** The levels of vinculin phosphorylation during focal adhesion disruption in BSC-1 cells treated with TPA and REF 52 cells treated with Bt2eAMP/MIX are low and unaffected by drug treatment. Lane 1, 125I markers; lanes 2 and 3, vinculin immunoprecipitated from BSC-1 control, and TPA-treated cells, respectively; lanes 4 and 5, vinculin immunoprecipitated from 32P-labeled REF 52 cells, control, and Bt2eAMP/MIX-treated cells, respectively.

was estimated by scintillation counting of talin immunoprecipitated from cells labeled with [35S]methionine. Talin from BSC-1 cells treated with TPA contained 0.15 mol of phosphate/mol of talin; control cells treated with Me2SO alone contained 0.06 mol of phosphate/mol of talin.

To determine if this increase was due to phosphorylation of a limited number of potential phosphorylation sites on the molecule, two-dimensional phosphopeptide maps of talin were generated. For these experiments, immunoprecipitated talin from both control and drug-treated cells was digested with trypsin. Two-dimensional peptide maps of the samples (Fig. 7) revealed a limited number of phosphopeptides in both cases.

![Phosphopeptide analysis of talin from control and TPA-treated BSC-1 cells.](image)

**FIG. 7.** Phosphopeptide analysis of talin from control and TPA-treated BSC-1 cells. Following drug treatment 32P-labeled talin was immunoprecipitated from lysed cells and digested with trypsin. The peptides were subjected to electrophoresis (left to right) followed by chromatography (bottom to top) on thin layer chromatography plates. * denotes the origin.

While four phosphopeptides exhibited approximately the same intensity (spots 4, 7, 10, and 11) and one clearly decreased (spot 5), there was a major increase in 4 phosphopeptides (spots 1–3 and 16) and 7 others showed minor increases (spots 6, 8, 9, 12–15) following treatment of the cells with TPA. Thus, it appears that the overall increase in talin phosphorylation we have observed is the result of increases in the phosphorylation levels of a selective subset of sites on the molecule. Phosphoamino acid analysis revealed that phosphorylation was restricted to serine residues (data not shown).

**DISCUSSION**

In an attempt to understand the mechanism which controls the integrity of focal adhesions and associated stress fibers within spread cells we have addressed in this paper two potentially relevant concepts, that of limited proteolytic cleavage and that of phosphorylation of two major focal adhesion components: talin and vinculin. Our data (summarized in Table I) suggest that the loss of talin and vinculin from focal adhesions, which parallels stress fiber disruption in two cell types following specific drug treatments, does not involve proteolytic cleavage of either protein. In contrast, the specific phosphorylation of talin by protein kinase C in BSC-1 cells treated with TPA may be an important factor in promoting the disassembly of focal adhesions and stress fibers. However, an alternative mechanism seems likely in the case of focal adhesion disruption in REF 52 cells treated with Bt2eAMP.

**Immunofluorescence**—Both the tumor promoter TPA and a membrane-permeable analogue of cAMP (Bt2eAMP) are known to stimulate specific protein kinases. While they are both capable of inducing focal adhesion and stress fiber disruption their effect is cell type-specific, with TPA causing disruption in BSC-1 cells and Bt2eAMP in REF 52 cells. The reason for this is unclear. However, in both cases the sequence of events leading to the loss of stress fibers and focal adhesions was similar. A gradual thinning of the stress fibers was accompanied by a decrease in the size of the talin and vinculin containing adhesions at their ends. Although a sequential loss of focal adhesion components can be induced by certain
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Table 1
Summary of drug treatment effects on stress fibers and focal adhesions

|                          | TPA          | db-cAMP     |
|--------------------------|--------------|-------------|
|                          | BSC-1        | REF 52      | BSC-1 | REF 52 |
| Focal adhesion and stress fiber disruption | Yes | No | No | Yes |
| Talin phosphorylation    | Increase     | No change   | No change | Decrease |
| Vinculin phosphorylation | No change    | No change   | No change | No change |
| Cleavage of talin or vinculin | None | None | None | None |

growth factors, such as platelet-derived growth factor (Herman and Pledger, 1985), this seems to represent an exceptional case. Our observations suggest that focal adhesion disassembly (stimulated via specific kinase activation) results from the simultaneous loss of talin and vinculin. This loss occurs gradually following kinase activation and probably commences at the periphery of the focal adhesion and progresses toward the center.

Phosphorylation—The increase in talin phosphorylation that accompanied the disruption of stress fibers and focal adhesions in BSC-1 cells following TPA treatment was possibly catalyzed directly by protein kinase C. TPA is known to activate this enzyme (Nishizuka, 1984), and talin has been shown to be a good substrate for protein kinase C in vitro (Beckerle et al., 1985; Litchfield and Ball, 1986). The absence of tyrosine phosphorylation on talin supports a direct phosphorylation of talin by protein kinase C rather than via a tyrosine kinase, as was observed in the phosphorylation of talin or vinculin in transformed cells (Sefton et al., 1981; Pasquale et al., 1986; DeClue and Martin, 1987).

The basal level of talin phosphorylation we have observed is similar to that reported by DeClue and Martin (1987) for talin from uninfected and Rous sarcoma virus-transformed chicken embryo fibroblast cells (0.07 mol of phosphate/mol of talin). Our results concerning the stoichiometry of talin phosphorylation in BSC-1 cells suggest that approximately 1 in 7 talin molecules (0.15 mol of phosphate/mol of talin) is phosphorylated following TPA treatment while about 1 in 16 (0.06 mol of phosphate/mol of talin) is phosphorylated in the absence of TPA. The elevated talin phosphorylation occurs on several peptides following treatment of cells with TPA, indicating an even lower stoichiometry for talin molecules phosphorylated at a particular site. One possible explanation for the low stoichiometry is that the phosphorylation stimulated by TPA treatment is confined to talin within a specific compartment, the focal adhesion, and that this is obscured by a large pool of soluble talin that is not phosphorylated. Undoubtedly, a pool of soluble talin exists, but our attempts to separate talin within the focal adhesions from that in the soluble pool have not been successful, due to the rapid dissociation of talin from focal adhesions in permeabilized cells and the loss of talin from these structures following TPA treatment. A second possible explanation for the low stoichiometry is if the phosphorylation occurs within focal adhesions resulting in the dissociation of talin from these structures and is followed by rapid dephosphorylation within the soluble pool. Since the disassembly of focal adhesions occurs over a 20-min time course or more and we do not know the rate of turnover of phosphates on talin, this would seem quite possible. Site-directed mutagenesis of cDNA clones of talin may enable the specific sites of phosphorylation responsible for disruption to be identified.

Whereas in BSC-1 cells TPA treatment led to disassembly of focal adhesions and a parallel increase in talin phosphorylation, with REF 52 cells following TPA treatment there was no significant disruption of focal adhesions and no increase in talin phosphorylation. This correlation of increased talin phosphorylation with focal adhesion disruption in response to TPA supports the hypothesis that the phosphorylation of talin by protein kinase C may contribute to the disruption of these structures.

In previous studies, investigators have concentrated on the possible role of vinculin phosphorylation in the regulation of cytoskeletal organization (Sefton et al., 1981; Werth and Pastan, 1984). The very low levels of vinculin phosphorylation observed in our studies, compared with that of talin, suggests that it is less likely to play a major role in regulating stress fiber and focal contact organization in the cell types employed in this study.

The disruption of the focal adhesions in REF 52 cells following treatment with BtCAMP/MIX is accompanied not by an increase in talin phosphorylation but rather by a decrease. Although dephosphorylation could be important in affecting the interactions of talin with other proteins, the low level of dephosphorylation leads us to suspect that an alternative mechanism is responsible for focal adhesion disassembly in response to elevated cAMP. Previous work with REF 52 cells demonstrated an increased phosphorylation of the myosin light chain kinase with an accompanying dephosphorylation of the myosin light chain when the cells were treated with BtCAMP or microinjected with the catalytic subunit of the cAMP-dependent protein kinase (Lamb et al., 1988). Myosin light chain dephosphorylation would be expected to diminish the force generating interaction between myosin and actin and consequently result in less tension in stress fibers. Since tension may contribute to their development and size (Burridge, 1981), the decrease in stress fiber tension may be the primary cause of their disruption in response to BtCAMP, with the loss of talin and vinculin from focal adhesions occurring secondarily.

Proteolytic Cleavage—Selective limited proteolytic cleavage of focal adhesion components has been suggested as one way in which the linkage of actin stress fibers with the plasma membrane could be disrupted (Beckerle et al., 1987). However, examination of immunoprecipitated talin and vinculin from BSC-1 cells treated with TPA or REF 52 cells treated with BtCAMP revealed no change in the level of proteolytic fragments of either protein nor a decrease in the amount of the intact proteins. This argues against proteolysis of talin or vinculin being a mechanism for disassembly of focal adhesions or stress fibers and continues to focus attention on other secondary modifications such as phosphorylation as a way to regulate the interaction of the various focal adhesion proteins.

Future studies should be directed toward ascertaining if the phosphorylation of talin by protein kinase C can be demonstrated to affect its interaction with vinculin or integrin in vitro.

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