Vinculin Phosphorylation in Response to Calcium and Phorbol Esters in Intact Cells*

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Vinculin phosphorylation in both chick embryo fibroblasts and Swiss 3T3 cells was increased by either calcium or biologically active phorbol esters. Increased phosphorylation of vinculin was noted as early as 10 min following phorbol 12-myristate 13-acetate treatment and was maximal at about 1 h. Maximal increases in phosphorylation were noted at approximately 100 nM phorbol 12-myristate 13-acetate. Phorbol 12,13-dibutyrate (80 nM), a less potent phorbol ester, resulted in smaller increases in vinculin phosphorylation than phorbol 12-myristate 13-acetate at equimolar concentrations. Phorbol, dibutyryl cAMP, and dibutyryl cGMP had no significant effect on phosphorylation. No correlation was found between vinculin phosphorylation and the morphological changes induced by phorbol esters. Tryptic peptide analysis of vinculin revealed multisite phosphorylation. Phosphorylation of only three of the peptides was significantly increased following phorbol 12-myristate 13-acetate treatment. Phosphoamino acid analysis revealed increases at both serine and threonine residues. The low level of phosphotyrosine present in control cells was not significantly increased by phorbol 12-myristate 13-acetate treatment. These findings combined with studies of vinculin phosphorylation by purified protein kinase C (Werth, D. K., Niedel, J. E., and Pastan I. (1983) J. Biol. Chem. 258, 11423–11426) suggest the hypothesis that protein kinase C may be involved in regulation of phosphorylation of vinculin, a cytoskeletal protein.

Phorbol esters have been shown to produce many biological effects (1), but the mechanisms responsible for their diverse effects are only beginning to be elucidated. Recently, it was shown that phorbol esters activate protein kinase C, the calcium, phospholipid-activated protein kinase (2, 3). Activation of protein kinase C with subsequent phosphorylation of specific substrate proteins could provide a mechanism by which phorbol esters produce multiple biochemical effects.

One of the biological effects of phorbol esters in cells is to produce morphological changes which mimic those seen in Rous sarcoma virus transformed cells (4, 5). One protein that has been postulated to have a role in the morphological changes seen in Rous sarcoma virus transformation is vinculin. Vinculin, a cytoskeletal protein, has been found to be a substrate for pp60*.

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The abbreviations used are: PMA, phorbol 12-myristate 13-acetate; pp60*, a 60,000-dalton phosphoprotein that is the product of the src gene of Rous sarcoma virus; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N',N'Tetraacetic acid; SDS, sodium dodecyl sulfate.

The transforming protein of the Rous sarcoma virus (6, 7). Using purified proteins, we have recently found that vinculin is a better substrate for protein kinase C than several other serine- and threonine-specific protein kinases (8). In addition, biologically active phorbol esters were found to stimulate vinculin phosphorylation by protein kinase C. These findings could establish a link between the morphological changes induced by phorbol esters and those seen following cellular transformation by viruses.

Studies involving phosphorylation with purified components allow detailed investigation of enzymatic properties, but the results of such studies may not accurately reflect physiological responses. Another approach to the study of protein phosphorylation is to metabolically label intact cells with 32P and examine phosphorylation in response to various agents. In the present study, we have examined vinculin phosphorylation in intact cells in response to phorbol esters, cyclic nucleotides, and calcium. We have also compared the effects of phorbol esters on cellular morphology with their effects on vinculin phosphorylation.

EXPERIMENTAL PROCEDURES

Cells and Radioactive Labeling—Chick embryo fibroblasts were prepared and grown at 39 °C as previously described (9). Third passage chick embryo fibroblasts were plated at a density of 8 × 10^6 cells/100-mm dish or 4.8 × 10^6 cells/60-mm dish and grown overnight. Cell monolayers were washed three times with Tris-buffered saline (0.15 M NaCl, 20 mM Tris-HCl, pH 7.2). Phosphoproteins were labeled by incubation with 0.5–1.0 μCi of [32P]orthophosphate/m of media in phosphate-free Dulbecco’s modified Eagle’s medium supplemented with 5% heated calf serum, 1 mM glutamine (2 mM), penicillin (50 units/ml), and streptomycin (50 μg/ml). Cells were labeled for 8 h at 37 °C. [35S]Methionine-labeled cells were prepared by incubation for 8 h at 37 °C with 250 μCi/ml of [35S]methionine in methionine-free Eagle’s media supplemented as for the 32P labeling.

Treatment with phorbol esters was performed for the time periods indicated during the last portion of the labeling period. Phorbol esters, dibutyryl cAMP, and dibutryl cGMP were dissolved in dimethyl sulfoxide. The final concentration of dimethyl sulfoxide was 0.2%. Control dishes were treated with the same concentration of dimethyl sulfoxide. Calcium concentration in the supplemented media (1.6 mM) was determined using a Perkin-Elmer model 5000 atomic absorption spectrophotometer. To obtain the desired calcium concentration, appropriate microliter amounts of stock solutions of 0.1 M EGTA and 0.05 M CaCl2 were added. Following the labeling and treatment periods, the cell monolayers were washed three times with ice-cold Tris-buffered saline containing 1% saponin and 1 mM EDTA. Cell lysates were prepared by adding modified RIPA buffer (Tris-buffered saline containing 1% Nonidet P-40, 0.5% deoxycholate, 1 mM EDTA, and 1% aprotonin) at a concentration of 8 × 10^6 cells/ml. The lysates were clarified by centrifugation at 160,000 × g for 15 min prior to immunoprecipitation.

Swiss 3T3 cells (ATCC-CCL92) were from American Type Culture (Rockville, MD). Cells were maintained in Dulbecco’s modified Eagle’s medium with 10% heated calf serum, 1 mM glutamine (2 mM), penicillin (50 units/ml), and streptomycin (50 μg/ml). Cells were plated at a density of 10^5 cells/100-mm dish. Labeling, treatment,
and preparation of cell lysates were as described above, except that the lysate concentration was 10⁶ cells/ml.

**Antisera and Immunoprecipitation**—Antiserum to purified chicken gizzard vinculin were prepared by methods similar to those previously described for actin (10). The antisera were purified by affinity chromatography using a column of vinculin (1.5 mg) coupled to cyanogen bromide-activated Sepharose 4B (Pharmacia) (11). For immunoprecipitation, 200 μl of cell lysate were precipitated with excess antibody preadsorbed to 50 μl of *Staphylococcus aureus* (10% suspension) in 1 ml of modified RIPA as described (12). After incubation for 30 min on ice, the immunoprecipitates were collected by centrifugation at 3000 × g for 10 min and washed successively with 1 ml of modified RIPA, 1 ml of modified RIPA with 2.5 mM KCl, and 1 ml of modified RIPA. The complexes were eluted by boiling in SDS gel sample buffer for 1 min and analyzed by SDS-polyacrylamide gel electrophoresis in 10% or 5-10% gradient gels according to the procedure of Laemmli (13). Gels containing [³²P]methionine-labeled proteins were fluorographed by the procedure of Bonner and Laskey (14).

Coomassie blue-stained gels were dried on cellophane membrane backing (Bio-Rad). The amount of vinculin was quantitated by densitometric scanning of the stained vinculin band. The radioactivity was quantified by excising the vinculin band and determining by scintillation counting in 10 ml of Aquasol (New England Nuclear).

**Tryptic Peptide Analysis**—Vinculin bands from SDS-polyacrylamide gels of the immunoprecipitates were excised, and tryptic digestion was performed as previously described (15). Separation of phosphorylated peptides was by electrophoresis in 30% formic acid in the first dimension on cellulose thin layer plates (Schleicher & Schüll) followed by chromatography in the second dimension in butanol:acetic acid:pyridine:water (60:12:40:48 v/v).

Cellulose chromatography plates were exposed to Kodak X-Omat film with a Chronex intensifying screen at ~70 °C. The individual peptides were marked by comparison with the autoradiograms and aspirated from the plate, and radioactivity was determined by scintillation counting.

**Phosphoamino Acid Analysis**—For phosphoamino acid analysis, 2 ml of cell lysates for each condition was immunoprecipitated with excess antibody. The [³²P]-labeled vinculin bands were excised from the gels and electroeluted for 12 h at 1 mA for 10 min and centrifuged at 3000 × g for 10 min. The wash was dissolved in 0.5 ml of 6 N HCl by heating at 100 °C for 1 min and hydrolyzed under N₂ for 2 h at 110 °C. The hydrolyzed material was lyophilized and redissolved in 0.5 ml of distilled water followed again by lyophilization to remove the acid.

Amino acid separation was performed at pH 3.5 as previously described (16). The individual amino acids were identified by comparison with standards detected by ninhydrin staining. Autoradiograms were obtained as described above, and radioactivity was determined by scintillation counting.

**Materials**—Vinculin was purified from chicken gizzards as previously described (17). Formalin-fixed *S. aureus* was prepared by the method of Kessler (18) and boiled in Tris-buffered saline containing 30% SDS and 10% 2-mercaptoethanol prior to use. Phorbol esters, dibutyryl cAMP, and dibutyryl cGMP were obtained from Sigma. [³²P]Orthophosphate (carrier-free, 10 mCi/ml) was obtained from New England Nuclear. [³⁵S]Methionine (1300 Ci/mmol) was from Amersham.

**RESULTS**

The present study utilized biosynthetic labeling of proteins in intact cells with [³²P], to ascertain whether factors reported to alter protein kinase C activity affect vinculin phosphorylation. Because of the many phosphoproteins present in the cell lysate, no clear increases in discrete proteins could be identified by one-dimensional separation of crude cell lysates by SDS-polyacrylamide gel electrophoresis. Therefore, immunoprecipitation of the cell lysates with antivinculin antisera was performed. The complexes were eluted by boiling in SDS gel sample buffer for 1 min and analyzed by 5-10% SDS-polyacrylamide gel electrophoresis. Therefore, immunoprecipitation was performed with vinculin labeled by [³⁵S]methionine during the short time of processing and extraction.

Vinculin phosphorylation in response to PMA—Phorbol esters, particularly PMA, have been reported to activate protein kinase C (2, 3) and stimulate vinculin phosphorylation by purified protein kinase C (8). Therefore, we examined whether treatment of intact chick embryo fibroblasts with PMA would activate protein kinase C and result in increased phosphorylation of vinculin. Fig. 2 demonstrates that exposure of the chick embryo fibroblasts to 50 ng/ml (80 nM) of PMA results in significantly increased phosphorylation of vinculin. B shows the autoradiogram of immunoprecipitates of [³⁵S]methionine-labeled cells treated in the same manner. No differences in the amount of vinculin precipitated by the antibody were found during the labeling and treatment period. Thus, the increased phosphorylation seen in response to phorbol esters represents increased protein phosphorylation.

Vinculin phosphorylation in response to PMA was rapid (Fig. 3). Significant increases in vinculin phosphorylation were noted in minutes and began to plateau around 1 h of PMA treatment. Again no changes were noted in the amount of vinculin labeled by [³⁵S]methionine during the short time periods of phorbol treatment (data not shown). Fig. 4 demonstrates the concentration dependence of vinculin phosphorylation in response to PMA treatment in both chick embryo fibroblasts (●) and Swiss 3T3 cells (○). Maximal increases in phosphorylation were noted around 100 nM. Even
very low concentrations (8 nM) resulted in small, but reproducible, increases in vinculin phosphorylation. To test the specificity of the effect of phorbol esters on vinculin phosphorylation, several other agents were examined (Table I). Dimethyl sulfoxide, the solvent used for these compounds, had no effect on vinculin phosphorylation at twice the final concentration used in the studies. Phorbol 12,13-dibutyrate, a less potent compound for protein kinase C activation, produced a small increase in vinculin phosphorylation, but not of the same magnitude as PMA at equimolar concentrations. Phorbol, an inactive compound, had no effect on vinculin phosphorylation. Two cyclic nucleotides, dibutyryl cAMP and dibutyryl cGMP, were also tested. Both had negligible effects on the phosphorylation of vinculin in the intact cells.

Vinculin Phosphorylation in Response to Calcium—Protein kinase C has an absolute requirement for Ca2+ and is reversibly activated by increasing Ca2+ concentrations. Fig. 5 shows the effect on vinculin phosphorylation of lowering the calcium concentration from 1.6 mM (lane 3) to 0.6 mM (lane 2) and to 0.2 mM (lane 1). Lowering the calcium concentrations produced decreases in vinculin phosphorylation. At both 0.2 mM Ca2+ and 0.6 mM Ca2+, PMA treatment resulted in increased vinculin phosphorylation (Fig. 2). However at 1.6 mM Ca2+, the addition of up to 160 nM PMA produced no further increases in vinculin phosphorylation (data not shown). Thus, the effect of PMA on vinculin phosphorylation was only seen at low calcium concentrations.

**Table I**

| Addition                   | Concentration | Vinculin phosphorylation % increase |
|----------------------------|---------------|-------------------------------------|
| Dimethyl sulfoxide         | 0.5%          | 100                                 |
| Phorbol 12-myristate 13-acetate | 8 nM        | 108                                 |
|                            | 20 nM         | 130                                 |
|                            | 80 nM         | 168                                 |
| Phorbol 12,13-dibutyrate   | 80 nM         | 118                                 |
| Phorbol (4β)               | 80 nM         | 100                                 |
| Dibutyryl cAMP             | 1 mM          | 104                                 |
| Dibutyryl cGMP             | 1 mM          | 98                                  |
concentration on vinculin phosphorylation. All procedures were done to determine whether phorbol ester treatment resulted in increased phosphorylation of vinculin (Fig. 4) and very dramatic morphological changes in the same time period in which changes in vinculin phosphorylation were noted (Fig. 8, A and B). However, as can be seen in Fig. 8, C and D, even the highest concentration of PMA tested produced no obvious morphological changes in chick embryo fibroblasts, even though comparable increases in vinculin phosphorylation were seen in these cells. Therefore, no simple correlation existed between increased phosphorylation of vinculin and the morphological change produced by PMA in these cells.

**DISCUSSION**

The present study demonstrates that increased phosphorylation of vinculin occurs in intact cells in response to both calcium and several biologically active phorbol esters (Figs. 2-5). Neither cyclic nucleotide (cAMP and cGMP) nor phorbol, a biologically inactive compound, significantly increased vinculin phosphorylation (Table I). These findings parallel results of experiments using purified components (8) in which both purified cAMP-dependent protein kinase catalytic subunit and cGMP-dependent protein kinase phosphorylated vinculin at a 100-fold lower rate than protein kinase C.

PMA has been shown to activate protein kinase C directly by decreasing the calcium concentration necessary for enzyme activation (2, 3). Therefore, at high calcium concentration (10^{-4} M) in the presence of phospholipid and/or diolein, no further effects of PMA on protein kinase C activity were found (2, 3). Previous studies in platelets have led to the
suggestion that PMA may also increase Ca\(^{2+}\) influx (2, 20), although in mouse thymocytes 10 nM PMA has been shown to decrease intracellular calcium (21). In both cell types, PMA resulted in increased vinculin phosphorylation in low calcium media but had no further effect on vinculin phosphorylation at high calcium concentrations. The present results do not allow us to distinguish the exact mechanism of the phorbol-induced increases in vinculin phosphorylation. Either mechanism, increased Ca\(^{2+}\) influx resulting in protein kinase C activation or direct interaction with protein kinase C to lower the \(K_a\) for Ca\(^{2+}\) activation, could have produced the increases in vinculin phosphorylation.

The finding that vinculin serves as a substrate for purified src kinase (22) and contains significantly increased levels of phosphotyrosine in Rous sarcoma virus-transformed cells (6) has led to the hypothesis that vinculin may be involved in the altered cytoskeletal structure of transformed cells. However, the role of vinculin in these morphological changes remains to be clarified. In view of previous reports that phorbol esters induce morphological changes which mimic those seen in transformed cells (4, 5), it was of obvious interest to compare the effects of phorbol esters on morphology with effects on vinculin phosphorylation. No morphological changes were observed in chick embryo fibroblasts at the time when significant increases in vinculin phosphorylation were observed. A previous study of the effect of PMA on actin-containing structures in chick embryo fibroblasts did show a loss of ordered actin structures resembling those seen in virally transformed cells (6). However, these changes were observed at 16 h of PMA exposure and required both RNA and protein synthesis. These discrepancies suggest that the effects of phorbol esters on morphology are complex, and vinculin phosphorylation alone was not sufficient to induce morphologic changes.

Two recent reports demonstrate that in chick embryo fibroblasts phorbol 12-myristate 13-acetate increases tyrosine phosphorylation on a protein or proteins (\(M_r = 40,000-43,000\)) (23, 24). The increased phosphorylation of vinculin seen in the present study occurred on serine and threonine residues (Fig. 7). Protein kinase C has been shown to phosphorylate both of these residues, but not tyrosine in vinculin and other substrates. In agreement with a previous report (6), vinculin from normal cells did contain low levels of phosphotyrosine. No distinct increases in phosphotyrosine were found in vinculin from phorbol-treated cells. Because of the low level of phosphotyrosine, small changes might have gone undetected. However, comparable techniques were used in a study of Rous sarcoma virus-transformed chick embryo fibroblasts and revealed significant increases in phosphotyrosine in vinculin. In addition, small increases in phosphotyrosine were detected in several other proteins (6).

The tryptic peptide analysis reveals a complex pattern of phosphorylation (Fig. 6). The findings indicate that multiple protein kinases are involved in the phosphorylation of vinculin. Two major and several minor phosphorylated peptides were found in purified vinculin phosphorylated by protein kinase C (8). One of the major peptides and one of the minor peptides phosphorylated in purified vinculin correspond with the peptides whose phosphorylation was increased by PMA and calcium in intact cells. However, the other major phosphate-containing peptide from purified vinculin did not correspond to any of the phosphorylated peptides seen in the present study. These findings are not surprising in view of the multiplicity of sites which can be phosphorylated. Discrepancies between phosphorylation sites in intact tissues or cells when compared to purified proteins have been reported for other proteins (25) and vinculin (6, 8). We have recently shown that vinculin may undergo a conformational change by binding to anionic phospholipids (26). Thus, data obtained with purified vinculin may not correspond exactly to results obtained in intact cells. These differences and the reports of increased tyrosine phosphorylation following PMA treatment (23, 24) may indicate that phorbol esters affect other protein kinases or phosphatases. Therefore, it is not possible to establish the exact identity of the kinase responsible for the increased phosphorylation of vinculin seen following treatment with calcium or phorbol esters.

The significance of multisite phosphorylation is not very well understood. Myosin light chain kinase has been shown to be a substrate for cAMP-dependent protein kinase with phosphorylation occurring on two separate peptides. Phosphorylation of one site decreased the affinity of the enzyme for its activator, calmodulin. Phosphorylation of the other
site did not affect binding of calmodulin (27). Differential effects on the activity of glycogen synthase following site-specific phosphorylation has also been reported (28). The differential phosphorylation of vinculin on specific sites (Fig. 6) suggests that phosphorylation of different sites regulates different functions.

Vinculin has been shown to be able to bind F actin (29, 30) and is proposed to link actin microfilament bundles to the membrane. An actin-independent interaction of vinculin with the cell membrane has also been demonstrated (31). Thus, vinculin may be involved in several types of molecular interactions. Identification of the enzymes responsible for vinculin phosphorylation and its probable regulation are necessary prerequisites to the understanding of the role of vinculin in transmembrane interactions. The present findings that increases in vinculin phosphorylation occur in intact cells in response to factors which activate protein kinase C, coupled with data obtained with purified proteins (8), suggest that protein kinase C, the calcium, phospholipid-activated protein kinase may directly or indirectly regulate vinculin phosphorylation.

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REFERENCES

1. Blumberg, P. M. (1980) CRC Crit. Rev. Toxicol. 8, 153–234
2. Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U., and Nishizuka, Y. (1982) J. Biol. Chem. 257, 7847–7851
3. Niedel, J. E., Kühn, L. J., and Vandenbark, G. R. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 36–40
4. Rifkin, D. B., Crowe, R. M., and Pollack, R. (1979) Cell 18, 361–368
5. Parkinson, E. K., and Emmerson, A. (1982) Carcinogenesis 3, 522–531
6. Sefton, B. M., Hunter, T., Ball, E. H., and Singer, S. J. (1981) Cell 24, 165–174
7. Ito, S., Richert, N. D., and Pastan, I. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 4628–4631
8. Worth, D. K., Niedel, J. E., and Pastan, I. (1983) J. Biol. Chem. 258, 11423–11426
9. Vogt, P. K. (1969) in Fundamental Techniques in Virology (Habel, K., and Saltzman, N., eds) pp. 198–211, Academic Press, New York
10. Willingham, M. C., Yamada, S., Davies, P. J., Rutherford, A., Gallo, M., and Pastan, I. (1981) J. Histochem. Cytochem. 29, 17–37
11. Yamada, K. M. (1978) J. Cell Biol. 78, 520–541
12. Richert, N. D., Davies, P. J., Jay, G., and Pastan, I. (1979) J. Virol. 31, 695–706
13. Laemmli, U. K. (1970) Nature (Lond.) 227, 680–685
14. Bonner, W. M., and Laskey, R. A. (1974) Eur. J. Biochem. 46, 83–88
15. Shih, T. Y., Williams, D. R., Werks, M. O., Moryak, J. M., Vass, W. C., and Scolnick, E. A. (1978) J. Virol. 24, 45–55
16. Blithe, D. L., Richert, N. D., and Pastan, I. H. (1982) J. Biol. Chem. 257, 7135–7142
17. Feramisco, J. R., and Burridge, K. (1980) J. Biol. Chem. 255, 1194–1199
18. Kessler, S. W. (1975) J. Immunol. 115, 1617–1624
19. Kikkawa, U., Takai, Y., Minakuchi, R., Inohara, S., and Nishizuka, Y. (1982) J. Biol. Chem. 257, 13341–13348
20. Kaibuchi, K., Takai, Y., Sawamura, M., Hoshijima, M., Fujikura, T., and Nishizuka, Y. (1983) J. Biol. Chem. 258, 6701–6704
21. Tsien, R. Y., Pozzan, T., and Rink, T. J. (1982) Nature (Lond.) 295, 68–71
22. Richert, N. D., Blithe, D. L., and Pastan, I. (1982) J. Biol. Chem. 257, 7143–7150
23. Bishop, R., Martinez, R., Nakamura, K. D., and Weber, M. J. (1983) Biochem. Biophys. Res. Commun. 115, 536–543
24. Gilmore, T., and Martin, G. S. (1983) Nature (Lond.) 306, 487–490
25. Graziani, Y., Erikson, E., and Erikson, R. L. (1983) J. Biol. Chem. 258, 6344–6351
26. Ito, S., Werth, D. K., Richert, N. D., and Pastan, I. (1983) J. Biol. Chem. 258, 14626–14631
27. Conti, M. A., and Adelstein, R. S. (1981) J. Biol. Chem. 256, 3178–3181
28. Cohen, P. (1982) Nature (Lond.) 296, 613–620
29. Jockusch, B. M., and Isenberg, G. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 3005–3009
30. Wilkins, J. A., and Lin, S. (1982) Cell 28, 83–90
31. Avnur, Z., Small, V. J., and Geiger, B. (1982) Isr. J. Med. Sci. 18, 1a
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