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Phosphorylation of Vinculin in Human Platelets Spreading on a Solid Surface

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Abstract Vinculin is a cytoskeletal protein believed to be involved in linking microfilaments to the cell membrane. It is a substrate for the Ca^{2+}- and phospholipid-dependent protein kinase C. We show here that when human platelets attach and spread on a solid surface, the α isoforms of vinculin become phosphorylated at serine and/or threonine residues. Phosphorylation is dependent on adhesion to a surface, since suspended, unattached platelets can produce filopodia but no phosphorylation of vinculin. Phosphorylation is also dependent on actin polymerization, as it does not occur when platelets had been pretreated with cytochalasin B. Most likely, protein kinase C is responsible for the phosphorylation of vinculin, since phosphorylation also occurs when platelets are treated with a phorbol ester, which activates protein kinase C, and is blocked by treatment with a staurosporine derivative which inhibits this enzyme. These results suggest that phosphorylation plays a role in anchoring vinculin at sites of microfilament-membrane interaction. © 1992 Wiley-Liss, Inc.

Key words: cytoskeleton, phosphorylation, platelet, vinculin, protein kinase C

Cytoskeleton-membrane interactions play a key role in biological phenomena as diverse as cell motility and transformation. Phosphorylation of proteins linking the cytoskeleton to the cell membrane is suspected to contribute to the regulation of these interactions. While both in vitro and in vivo phosphorylation of several linkage proteins has been demonstrated, it has been difficult to correlate these modifications with either the assembly or disassembly of linkage sites [Niggli and Burger, 1987]. Vinculin, a 116 kD protein, is implicated in binding microfilaments to the cell membrane [Geiger, 1979; Price et al., 1987]. It can be phosphorylated by both serine and tyrosine kinases: phorbol esters which stimulate the serine kinase protein kinase C (PKC) increase the phosphorylation of vinculin in chicken embryo fibroblasts and in Swiss 3T3 cells [Werth and Pastan, 1984]; in addition, both PKC and the tyrosine kinase $pp60^{src}$ phosphorylate vinculin in vitro [Werth et al., 1983; Ito et al., 1983]. Treatment with phorbol esters and transformation of fibroblasts with Rous sarcoma virus which carries $pp60^{src}$ can lead to changes of cell shape and cell attachment typical for the transformed phenotype. It has consequently been postulated that phosphorylation of vinculin might lead to the disruption of membrane-microfilament linkage sites [Sefton et al., 1981]. However, this notion has been challenged by authors who found no correlation between phosphorylation of vinculin and disruption of the cytoskeletal system [Nigg et al., 1986; Turner et al., 1989].

In human platelets activated by contact with a solid surface, cytoskeletal modifications are irreversible: after attachment to the surface, filopodia grow out, and veils invade the space between them [Allen et al., 1979; Karlsson et al., 1984]. Platelets are therefore better suited to studies of the biochemical changes associated with the assembly of substrate attachment sites than cells where assembly at one site might be accompanied by simultaneous breakdown at another site. We show here that attachment of platelets to a surface and consequent spreading is correlated with phosphorylation of vinculin. Phosphorylation depends on intact microfilaments. However, it does not occur when platelets are activated by thrombin in suspension, i.e., when they form filopodia without attachment. These results suggest that phosphorylation of vinculin by protein kinase C plays a role in anchoring this protein in platelet-substrate attachment sites.

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METHODS

Platelets

Blood from healthy volunteers was drawn into siliconized glass tubes containing citrate (10 mM final concentration). After centrifugation (60g, 15 min), the platelet rich plasma was brought to 8 mM EDTA and the platelets were pelleted at 550 g for 15 min and resuspended in Hepes buffered Tyrode's solution (HTS, 137 mM NaCl, 2.6 mM $MgCl_2$, 5.5 mM glucose, 15 mM Hepes, pH 7.4) to give a concentration of about 10^8 platelets/ml.

Microscopy

Platelets spread on coverslips were observed by differential interference contrast (DIC) microscopy using a Plan-Neofluar 100× objective and a Zeiss Axiovert 35 microscope. The video signal from the Hamamatsu C2400-07 camera was enhanced digitally with Image 1 software (Universal Imaging Corporation, West Chester, PA). Photographs were taken directly from the screen.

Immunofluorescence

Platelets adhering to glass cover-slips were fixed with 3.7% formaldehyde for 5 min, extracted with 0.1% Triton X-100, and stained with a monoclonal antivinculin antibody (BioMakor) and FITC coupled rabbit antimouse IgG together with rhodamine labelled phalloidin. Photographs were taken directly from the microscope.

In Vivo Phosphorylation of Vinculin

Platelets suspended in HTS were loaded with 1 mCi/ml of [^{32}P]orthophosphoric acid (Amersham) for 2 h at 37°C. After washing, 150 μl of the suspension (approximately 1.5×10^7 platelets) were placed on glass coverslips (22 × 22 mm) previously coated with 50 μg/ml of bovine fibronectin. Twenty minutes later, adhesion and spreading were complete. After rinsing the coverslips with phosphate buffered saline, adhering platelets were dissolved in sample buffer. Control platelets were maintained in suspension under the same conditions.

Gel Electrophoresis and Quantitation

Equal amounts of total platelet proteins were separated by two-dimensional gel electrophoresis according to O'Farrell [1975]. Gels were exposed for autoradiography and silverstained. Vinculin was identified by its position and isoform pattern. Decoration of platelet proteins blotted onto nitrocellulose filters with a monoclonal antibody against chicken gizzard vinculin (BioMakor) and a specific, polyclonal antibody against human platelet vinculin confirmed the identification (data not shown). The density of the autoradiographic spots corresponding to the phosphorylated α isoforms of vinculin was scanned with a Shimadzu CS-930 scanner.

In Vitro Phosphorylation of Vinculin

Chicken gizzard vinculin was isolated as described by Feramisco and Burridge [1980] and further purified by hydroxylapatite chromatography [Wilkins and Lin, 1986]. PKC was purified from calf brain according to Wooten et al. [1987]. The kinase was pure as judged by SDS gel electrophoresis and silverstaining, and was recognized by a specific antibody against PKC (data not shown); 5 μg of chicken gizzard vinculin were phosphorylated in a reaction mixture containing 5 mM $MgCl_2$, 1.2 mM $CaCl_2$, 10 μg of phosphatidylserine, 5 μl of PKC, 2 μCi of [^{32}P]ATP (3,000 Ci/mmol), and 20 mM Hepes, pH 7.5, at 30°C for 15 min. The phosphorylated protein was subjected to two-dimensional gel electrophoresis, blotted onto nitrocellulose, and autoradiographed. Autoradiographs were compared to vinculin phosphorylated with cold ATP, blotted after two-dimensional gel electrophoresis, and decorated with the same monoclonal antibody against chicken gizzard vinculin (BioMakor) used for immunofluorescence.

Reagents

Cytochalasin B, 12-O-tetradecanoylphorbol-13-acetate (TPA), and thrombin were obtained from Sigma Chemical Co. (St. Louis, MO). Rhodamine labelled phalloidin was from Molecular Probes, OR). Okadaic acid was from Moana BioProducts, Honolulu, Hawaii, and CGP 41 251 was a gift from Dr. T. Meyer, CIBA-GEIGY, Basel.

RESULTS

When platelets make contact with glass coverslips, they become activated and spread, until they cover an area of about 12.5 to 70 μ^2. Spreading can be subdivided into elongation of filopodia and extension of veils between filopodia [Allen et

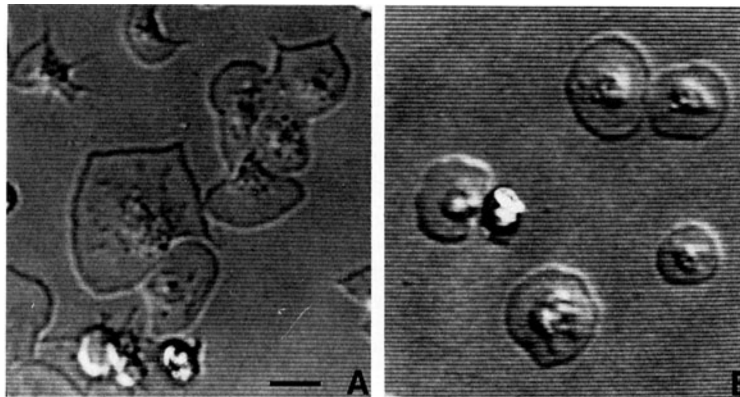


Fig. 1. Differential interference contrast image of platelets spread on a coverslip. **A:** No treatment. **B:** Platelets pretreated for 30 min with 10 μg/ml of cytochalasin B. The calibration bar is 5 μ long.

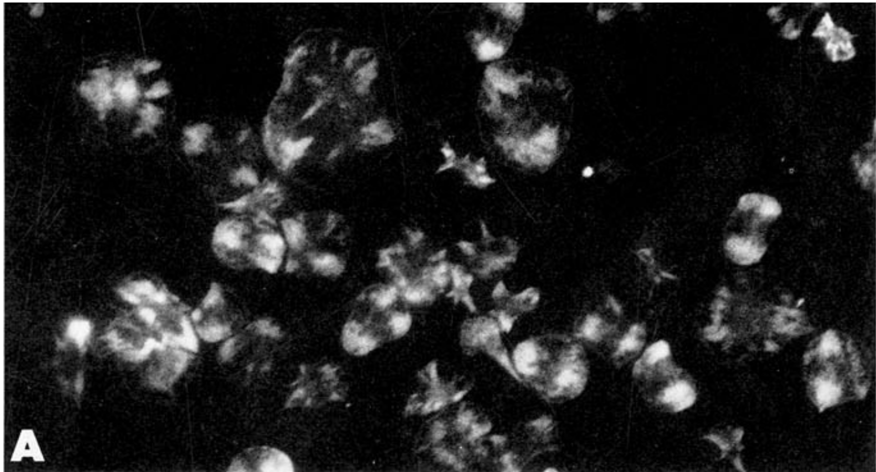
al., 1979; Karlsson et al., 1984]. As a result, the fully spread platelet has a typical morphology (Fig. 1A). Seen through the electronmicroscope, filopodial structures are characterized by bundles of microfilaments [White, 1987]. Pretreatment of platelets with 10 μg/ml of cytochalasin B for 30 min does not prevent them from attaching to the coverslip nor from spreading, despite the fact that no filopodia are formed. The lack of filopodia produces a rounder, less jagged outline of the spread platelets (Fig. 1B).

In fully spread platelets, vinculin is localized at the ends of the microfilament bundles, where, after fixation and subsequent extraction with detergents, it can be detected by immunofluorescence [Fig. 2A,B, and Rosenfeld et al., 1985]. However, no specific staining of vinculin is detected in spread platelets which have been pretreated with cytochalasin, indicating that in this case vinculin had not been incorporated into the cytoskeleton, and was consequently removed by the extraction with nonionic detergent, (Fig. 2C). In this case, the formation of microfilament bundles was equally depressed (Fig. 2D).

We next studied the changes of in vivo phosphorylation of vinculin after spreading of platelets on coverslips. Platelets were loaded with [^{32}P]orthophosphoric acid and allowed to attach and spread on fibronectin coated glass cover slips. After 30 min, adhering platelets were dissolved in sample buffer and their proteins were analyzed by two-dimensional gel electrophoresis. The α and β isoforms of vinculin are easily identified by their position on two-dimensional polyacrylaminde gels. Autoradiographs show that after attachment to cover-slips and spreading, the α isoforms of vinculin are phosphorylated (Figs. 3, 4A,G). In control platelets maintained in suspension, the level of vinculin phosphorylation is very low (Figs. 4D, 5). Phosphorylation is not affected when platelets are attaching to the coverslips in medium containing 2 mM EGTA (Fig. 5). The identity of the phosphorylated protein was confirmed when autoradiography and decoration with a specific monoclonal antibody against vinculin were performed with the same sample blotted onto nitrocellulose (Fig. 6).

Thrombin induces a shape change and the formation of long filopodia in platelets kept in suspension. Interestingly, no phosphorylation of vinculin is observed under these conditions (Fig. 4E). When, in addition to the stimulation provided by the surface of the cover-slip, thrombin is present, phosphorylation of vinculin does not exceed the level observed without thrombin (Figs. 4B, 5). This demonstrates that elongation of filopodia without attachment to a surface is not sufficient to trigger the phosphorylation of vinculin and that phosphorylation of vinculin is not necessary for generating filopodia. Neither is attachment of platelets to a solid surface sufficient for phosphorylation of vinculin. It seems to also depend on actin polymerization, since in platelets spreading after pretreatment with cytochalasin B vinculin is not phosphorylated (Figs. 4C, 4F, 5).

Vinculin can be phosphorylated at either serine/threonine by PKC, or at tyrosine by pp60^{src} [Werth et al., 1983; Ito et al., 1983]. Both kinases are present in platelets in large amounts. The following results indicate that PKC might be involved in vinculin phosphorylation in spreading platelets. i) The phosphate group is



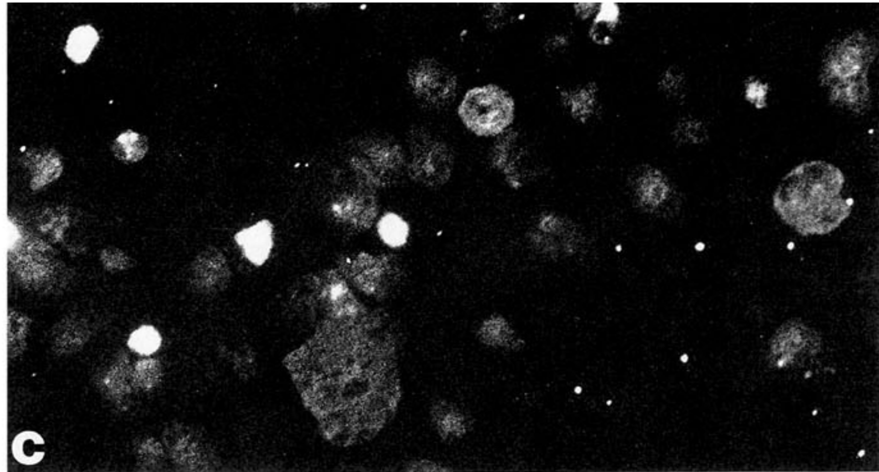


Fig. 2. Double staining of spread platelets. **A** and **B:** No treatment. **C** and **D:** Platelets pretreated for 30 min with 10 μg/ml of cytochalasin B. The platelets were fixed, extracted with triton X-100 and decorated with a monoclonal antibody against vinculin followed by FITC-labelled second antibody (A and C) and with rhodamine-phalloidin (B and D). The calibration bar is 10 μ long.

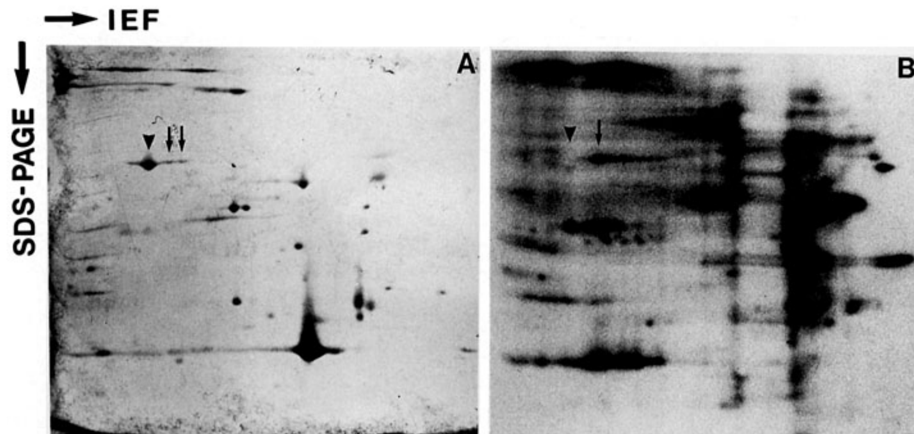


Fig. 3. Two-dimensional gel electrophoresis of proteins from platelets adhering to fibronectin coated coverslips. **A:** Silver-stained gel. **B:** Autoradiograph. Positions of the β isoform (arrowhead) and of the α isoforms (arrows) of vinculin are indicated.

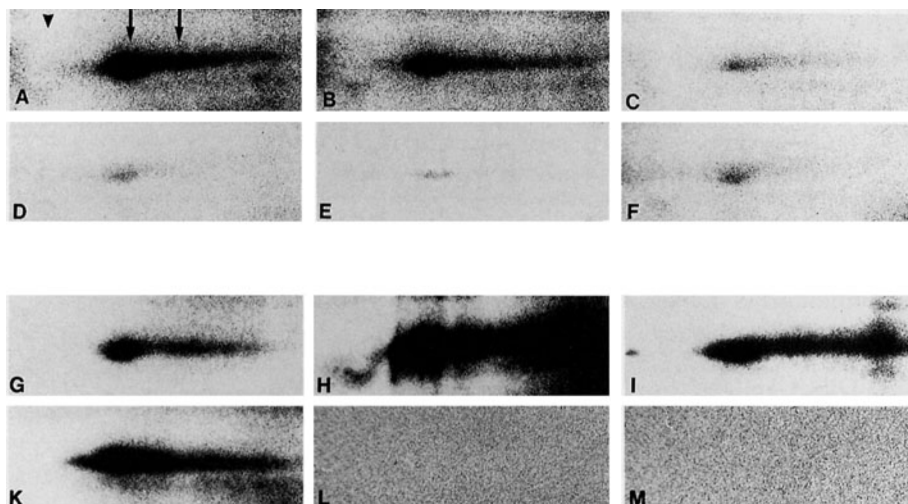


Fig. 4. Phosphorylation of vinculin in platelets. Autoradiographs of the areas of two dimensional gels carrying vinculin are shown. In **A,** the arrowhead indicates the position of the β isoform, the arrows point to the α isoforms. **A–C, G, H, K–M:** Platelets adhering to fibronectin-coated coverslips. **D–F** and **I:** Platelets incubated in suspension. A, D and G: Untreated platelets; B and E: 0.1 units/ml of thrombin was added at the beginning of the experiment. C and F: Platelets pretreated for 30 min with 10 μg/ml of cytochalasin B; H and I: 100 ng/ml of TPA added at the beginning of the experiment; K: platelets pretreated with 2.5 μM okadaic acid; L: platelets pretreated for 30 min with CGP 41 251; M: gel shown in panel G treated with 1 M NaOH for 1 h at 65°C. A–F and G–M are from two different experiments.

released by treatment with 1 M NaOH (Fig. 4M). Under these conditions, tyrosine-phosphate bonds are stable. ii) Okadaic acid, an inhibitor of the serine phosphatases 1, 2A, and 2B [Cohen et al., 1990], enhances phosphorylation of vinculin (Figs. 4K, 5). iii) TPA, an activator of PKC, markedly enhances the phosphorylation of vinculin in spread platelets (Figs. 4H, 5). Phosphorylation also occurs when platelets are treated with TPA in suspension (Figs. 4I, 5). iv) CGP 41 251, a staurosporine derivative which inhibits PKC [Meyer et al., 1989], prevents phosphorylation of vinculin (Figs. 3L, 4). However, treatment with CGP 41 251 has far reaching effects, since it also inhibits the formation of filopodia and spreading (data not shown).

Only the α isoforms of vinculin are phosphorylated. We therefore sought to establish, whether phosphorylation of vinculin by PKC in vitro would generate a similar phosphorylation pattern. When vinculin purified from chicken gizzard is phosphorylated by calf brain PKC, only the minor α isoforms are labeled (Fig. 7). Phosphorylated bands with a higher apparent mole-

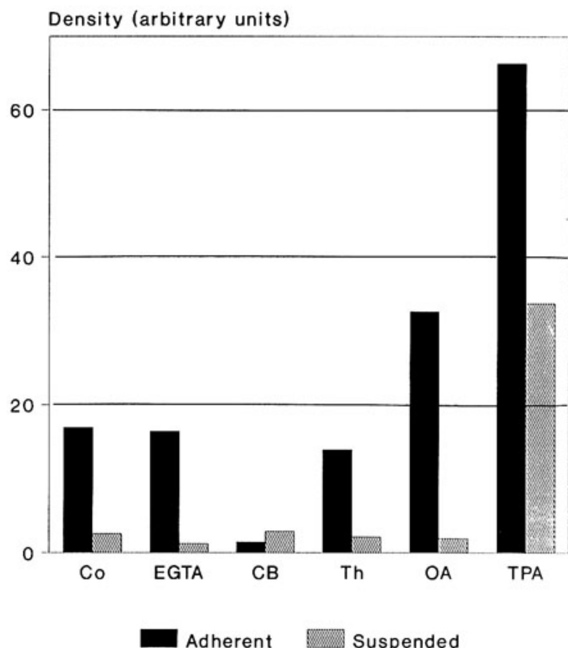


Fig. 5. Phosphorylation of vinculin in platelets. Densitometry scans of the phosphorylation signal of α isoforms of vinculin. Co, control; CB, cytochalasin B; Th, thrombin; OA, okadaic acid. The results of one representative experiment are shown.

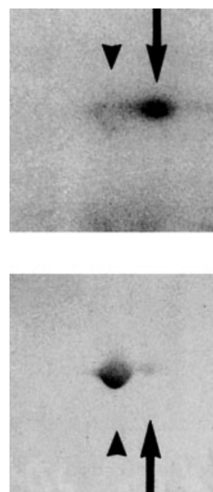


Fig. 6. Identification of vinculin in two-dimensional gels. **Top panel:** Autoradiography. **Bottom panel:** Immunodecoration of the same nitrocellulose with a monoclonal antibody against vinculin. Arrowheads: β-isoform. Arrows: α-isoform.

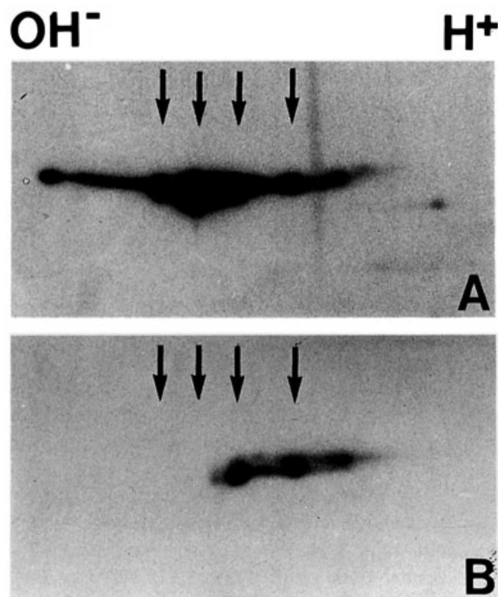


Fig. 7. In vitro phosphorylation of chicken gizzard vinculin by PKC. **A:** Immunoblot of phosphorylated vinculin. **B:** Fluorograph of phosphorylated vinculin. The isoforms indicated by the arrows are from left to right: v, β, α_1, and α_2.

cular weight are always observed in vitro. However, this phenomenon is never detected in vinculin phosphorylated in vivo.

DISCUSSION

When platelets spread on a surface, microfilament bundles are laid down which resemble stress fibers found in nucleated tissue culture cells [White, 1987]. In platelets which had been fixed and then extracted with nonionic detergents, staining by immunofluorescence shows that vinculin is localized in these microfilament structures [Fig. 2A,B, and Rosenfeld et al., 1985]. Platelets which had been pretreated with cytochalasin B lack microfilament bundles. In this case, the vinculin is extracted by the detergent (Fig. 2C,D). It therefore appears that vinculin becomes incorporated into the actin cytoskeleton upon spreading of platelets on surfaces.

Vinculin is a substrate for both serine and tyrosine kinases [Werth et al., 1983; Ito et al., 1983; Nigg et al., 1986]. The results of the present work show that the α isoforms of vinculin become phosphorylated upon spreading of platelets on coverslips coated with fibronectin. Fibronectin, and hence integrin type receptors, seem not to be required since identical results are obtained with uncoated glass coverslips (data not shown). Phosphorylation is dependent on both attachment to the coverslip and assembly of microfilaments: when platelets are stimulated in suspension by thrombin which causes the formation of filopodia containing microfilament bundles, phosphorylation of vinculin is not observed (Fig. 5). However, the need for surface attachment can be bypassed by the acti-

vation of PKC with phorbol esters (see below). On the other hand, attachment and spreading alone are not sufficient in the absence of microfilament polymerization. This is shown by the results obtained with platelets pretreated with cytochalasin which spread on coverslips without forming radial microfilaments (Fig. 1B) and which exhibit no increase in vinculin phosphorylation (Fig. 5).

It has recently been claimed that vinculin of platelets spreading on surfaces is not phosphorylated [Nachmias and Golla, 1991]. However, no data are shown. Moreover, one-dimensional SDS-polyacrylamide electrophoresis of immuno-precipitated material was used. Possibly, the antibody did not precipitate the phosphorylated α-isoform. Alternatively, the small signal might have been obscured by a large background on one-dimensional electrophoresis, even after immunoprecipitation. In any case, our results clearly show that the phosphorylated protein detected in two-dimensional gels is identical with the α isoform of vinculin (Fig. 6).

Phosphorylation of vinculin in platelets seems to affect serine and/or threonine residues. This is inferred from the fact that the phosphate group can be removed by treatment with 1 N NaOH under conditions where tyrosine-phosphate bonds are stable (Fig. 4M). Moreover, okadaic acid, which inhibits the serine phosphatases 1, 2A, and 2B [Cohen et al., 1990], increases the phosphorylation of vinculin in platelets spreading on coverslips, but not in control platelets maintained in suspension (Fig. 5). The latter observation also suggests that inhibition of phosphatase activity is not sufficient for inducing the increase in vinculin phosphorylation.

Possibly the Ca^{2+}- and phospholipid-dependent protein kinase PKC is responsible for the phosphorylation of vinculin. The staurosporine derivative CGP 41 251 has been reported to specifically inhibit PKC in vitro and in vivo [Meyer et al., 1989]. Figures 4L and 5 show that this compound also inhibits the phosphorylation of vinculin in platelets. Finally, the phorbol ester TPA, a potent activator of PKC in vivo, dramatically increases the phosphorylation of vinculin, even when platelets are treated in suspension (Figs. 4H, 4I, 5).

When chicken gizzard vinculin is phosphorylated in vitro by PKC isolated from calf brain, only the α isoforms are phosphorylated, as is observed with platelet vinculin in vivo (Fig. 7). In addition to the 116 kD form, phosphorylated bands with a higher apparent molecular weight are found. A shift to a higher apparent molecular weight is not uncommon in strongly phosphorylated proteins both in vitro and in vivo [Viereck and Matus, 1990; Viereck et al., 1989]. This more slowly migrating form of vinculin is not related to metavinculin which was absent from the purified vinculin preparation and which differs from vinculin by an insert of 68 additional residues [Gimona et al., 1988]. Although we looked for phosphorylated high molecular weight forms in platelets and in chicken embryo fibroblasts, we never found any (data not shown). Possibly, in the case of vinculin the phenomenon is an in vitro phenomenon.

Several attempts have been made to relate the phosphorylation of vinculin with either its incorporation in sites of cytoskeleton-membrane linkages or with its release from these structures. No conclusive results have been obtained, although one of the earliest reports has shown that phosphorylated α isoforms are enriched in focal contacts left behind on plastic Petri dishes after removing the cytoplasm and the plasma membranes of fibroblasts [Geiger, 1982]. Treatment of cells with phorbol esters that activate PKC has given conflicting results. Whereas some cell types respond to the esters with a disruption of focal contacts [Schliwa et al., 1984], others display increased adhesiveness and expression of focal contacts [Järvinen et al., 1987]. But most investigators have observed no concomitant change in the phosphorylation of vinculin [Turner et al., 1989]. In platelets, on the other hand, attachment and spreading are irreversible: we never observed retraction of attached parts or migration of a platelet. Our results therefore allow us to correlate the incorporation of vinculin into adhesive cytoskeletal structures with phosphorylation at serine and/or threonine residues. Whether phosphorylation is the cause or the result of incorporation of vinculin into focal contacts can not be decided based on the results reported.

The molecular details of the interactions of vinculin within microfilaments and membrane remain unclear. Geiger et al. [1981] have found that vinculin is closely apposed to the lipid bilayer, and we have demonstrated the interaction of vinculin with a photolabel buried within the bilayer [Niggli et al., 1986, 1990]. Interestingly, we found that the residues phosphorylated by PKC in vitro are located in the carboxyterminal

tail of the molecule (data not shown). The tail also contains a PKC phosphorylation site at position 913, and, based on circumstantial evidence, was proposed to carry a membrane binding domain [Milam, 1985; Bendori et al., 1989]. Possibly, phosphorylation of the tail of vinculin leads to a *conformational* change enhancing direct or indirect association with the lipid bilayer, thereby stabilizing microfilament-plasma membrane attachment sites.

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