A Lipid-regulated Docking Site on Vinculin for Protein Kinase C*

Received for publication, October 17, 2001, and in revised form, December 7, 2001
Published, JBC Papers in Press, December 10, 2001, DOI 10.1074/jbc.M110008200

Wolfgang H. Ziegler‡, Ulrich Tigges, Anke Zieseniss, and Brigitte M. Jockusch

From the Department of Cell Biology, Zoological Institute, Technical University of Braunschweig, D-38092 Braunschweig, Germany

During cell spreading, binding of actin-organizing proteins to acidic phospholipids and phosphorylation are important for localization and activity of these proteins at nascent cell-matrix adhesion sites. Here, we report on a transient interaction between the lipid-dependent protein kinase Ca and vinculin, an early component of these sites, during spreading of HeLa cells on collagen. In vitro binding of protein kinase Ca to vinculin tail was found dependent on free calcium and acidic phospholipids but independent of a functional vinculin tail. During cell spreading, critical phosphorylation of vinculin and/or vinculin interaction partners, thereby affecting the formation of cell adhesion complexes.

Cell spreading is a complex process involving receptor-mediated adhesion, membrane protrusion, and the formation of discrete cell-matrix adhesion sites linked to a reorganization of the actin cytoskeleton (1–3). The modulation of proteins by protein kinase C (PKC)-dependent phosphorylation and by phosphoinositide binding is critically involved in the regulation of these phenomena (4–7). Both processes are part of signaling cascades but are not independent of each other and participate in other signaling pathways. PI4,5P$_2$, the major phosphoinositide involved, is not only a precursor of the second messengers diacylglycerol, inositol 3,4,5-trisphosphate, and phosphatidylinositol 3,4,5-trisphosphate but is also critically involved in the organization of the actin cytoskeleton at the plasma membrane (8–10). Its local concentration and accessibility control multiple aspects of the cytoskeleton-plasma membrane linkage via ERM proteins (11), assembly and disassembly of actin filaments (12–14), and of protein complexes bundling these filaments and connecting them to transmembrane receptors. Because of the heterogeneity and complexity of their protein composition, structure and composition of these complexes are only partially understood (2). Essential components include vinculin, talin, α-actinin, and filamin (15), which have all been proposed to be regulated in their conformation and ligand binding activity by PI4,5P$_2$ (16–19).

Vinculin, an early and essential component of nascent cell-matrix adhesions (20), has been shown to associate with talin, α-actinin, and filamin, and members of the vasodilator-stimulated phosphoprotein/Ena and ponsin/ArgBP52/vinexin families (15). The function of vinculin in cell adhesions is not fully understood. By bringing multiple actin-organizing proteins to close proximity, vinculin may act as an adaptor protein in addition to its structural role in the binding and cross-linking of membrane-apposed actin filaments. The atomic structure of the entire vinculin molecule is still not known, but the overall structure as revealed by electron microscopy shows a tadpole-like molecule whose tail can be folded and attached to the more globular head portion (21). The vinculin tail, whose atomic structure has been resolved (22), consists of a bundle of five helices (H1-H5) in antiparallel orientation, with N- and C-terminal extensions emerging from the same side of the bundle. It contains two interaction sites for acidic phospholipids, one located at the surface of helices H2-H3 (23), and a second one comprising a C-terminal hydrophobic hairpin (22). In the closed vinculin conformation that is supposed to reflect the cytosolic moiety, binding sites for talin and α-actinin at the vinculin head, for F-actin at the vinculin tail, and for vasodilator-stimulated phosphoprotein and others at the proline-rich hinge between head and tail are not accessible (20, 24–26). Both PI4,5P$_2$ binding to and phosphorylation of the tail are proposed to alter vinculin conformation and, thus, expose protein ligand-binding sites (15, 16, 27, 28). Hence, PI4,5P$_2$-regulated protein kinases seem good candidates to execute this change in vinculin conformation and activity, and PKC has been shown in vitro to accept the vinculin tail as a substrate (27, 29).

PKC is a lipid-regulated serine/threonine kinase (30, 31) whose functional involvement in cell adhesion and spreading has been documented by pharmacological studies in numerous cellular systems (4, 5, 32, 33). PKC isotypes α, δ, and ε have been implicated in the formation and maintenance of cell-matrix adhesion sites (34–37). There are mainly three (classes of) proteins that have been identified as PKC binding partners in this location; the receptors for activated protein kinase C (RACKs), syndecan 4, and the transmembrane-4 superfamily (tetraspansins) (38–40). All three bind to integrin β-chains, and the latter two are transmembrane proteins that function as

* This work was financially supported by the German Research Council. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed. E-mail: w. ziegler@tu-bs.de.

The abbreviations used are: PKC, protein kinase C; PI4,5P$_2$, phosphatidylinositol 4,5-bisphosphate; DSP, dithiothreitol/memipimidyl propionate; Vt, vinculin tail; MBP, maffose-binding protein; BiPro, birch profilin tag; Ni-NTA, Ni$^{2+}$-nitrotetroxaetic acid complex; TBST, Tris-buffered saline with Tween 20; MALDI, matrix-assisted laser desorption ionization; MS, mass spectroscopy; ELISA, enzyme-linked immunosorbent assay; MARCKS, myristoylated alanine-rich protein kinase C substrate.
integrin co-receptors (41, 42). Binding partners and substrates of PKC directly involved in the actin organization of cell adhesion complexes, however, are less well defined (43). Although talin, vinculin, and filamin are considered potential PKC in vivo substrates (27, 44, 45), their role in PKC-dependent cell adhesion processes is still not clear.

In this report, we present evidence for a physical interaction of PKCα with vinculin during cell spreading. The docking of the enzyme depends on the C-terminal lipid-binding site of the vinculin tail, and PI4,5P2 binding capacity correlates with phosphorylation of adjacent serine residues. These results suggest a possible mechanism whereby PI4,5P2-induced conformational changes in vinculin may result in PKCα binding and subsequent phosphorylation of vinculin and/or vinculin interaction partners as required for the formation of cell-matrix adhesion complexes.

**Experimental Procedures**

**Cloning, Mutagenesis, and Graphic Representation—Constructs used** in this study were amplified according to standard PCR protocols, cloned in pCR-blunt (Invitrogen), and verified by DNA sequencing. Bovine brain actin (a gift of Dr. Peter J. Parker, Imperial Cancer Research Fund, London) was equipped with either an N-terminal His tag or a birch-profilin epitope tag (BiPro tag (46)) and cloned into pcDNA3 (Invitrogen). An ATP binding-deficient K368M mutant (47) was generated by site-directed mutagenesis according to the manufacturer's instructions using the QuikChange kit (Stratagene). Vinculin fragments encoding the tail domain (amino acids 858–1066) were equipped with an N-terminal FLAG tag and cloned into pQE-30 (Qiagen) that contains a His tag. To remove potential phosphorylation sites at Ser-941/Thr-43, Ser-999, Thr-1050/Thr-55, and Thr-1062 single or double alanine mutants of Vt-(858–1062) were generated, as described above. For the analysis of the lipid dependence of the PKC-vinculin interaction, mutants Vt-(R1060Q/K61Q), Vt-(T1062E), and the deletion/insertion complexes, however, are less well defined (43). Although talin, vinculin, and filamin are considered potential PKC in vivo substrates (27, 44, 45), their role in PKC-dependent cell adhesion processes is still not clear.

In this report, we present evidence for a physical interaction of PKCα with vinculin during cell spreading. The docking of the enzyme depends on the C-terminal lipid-binding site of the vinculin tail, and PI4,5P2 binding capacity correlates with phosphorylation of adjacent serine residues. These results suggest a possible mechanism whereby PI4,5P2-induced conformational changes in vinculin may result in PKCα binding and subsequent phosphorylation of vinculin and/or vinculin interaction partners as required for the formation of cell-matrix adhesion complexes.

**Experimental Procedures**

**Cloning, Mutagenesis, and Graphic Representation—Constructs used** in this study were amplified according to standard PCR protocols, cloned in pCR-blunt (Invitrogen), and verified by DNA sequencing. Bovine brain actin (a gift of Dr. Peter J. Parker, Imperial Cancer Research Fund, London) was equipped with either an N-terminal His tag or a birch-profilin epitope tag (BiPro tag (46)) and cloned into pcDNA3 (Invitrogen). An ATP binding-deficient K368M mutant (47) was generated by site-directed mutagenesis according to the manufacturer's instructions using the QuikChange kit (Stratagene). Vinculin fragments encoding the tail domain (amino acids 858–1066) were equipped with an N-terminal FLAG tag and cloned into pQE-30 (Qiagen) that contains a His tag. To remove potential phosphorylation sites at Ser-941/Thr-43, Ser-999, Thr-1050/Thr-55, and Thr-1062 single or double alanine mutants of Vt-(858–1066) were generated, as described above. For the analysis of the lipid dependence of the PKC-vinculin interaction, mutants Vt-(R1060Q/K61Q), Vt-(T1062E), and the deletion/insertion complexes, however, are less well defined (43). Although talin, vinculin, and filamin are considered potential PKC in vivo substrates (27, 44, 45), their role in PKC-dependent cell adhesion processes is still not clear.

In this report, we present evidence for a physical interaction of PKCα with vinculin during cell spreading. The docking of the enzyme depends on the C-terminal lipid-binding site of the vinculin tail, and PI4,5P2 binding capacity correlates with phosphorylation of adjacent serine residues. These results suggest a possible mechanism whereby PI4,5P2-induced conformational changes in vinculin may result in PKCα binding and subsequent phosphorylation of vinculin and/or vinculin interaction partners as required for the formation of cell-matrix adhesion complexes.
a cooled CCD camera (Roper Scientific, Tucson, AZ) and the MetaMorph® software (Visitron, Puchheim, Germany).

**PI4,5P₂ Binding Assays**—Binding of the Vt C terminus to PI4,5P₂ micelles was tested as described (55). Briefly, Micronel ELISA plates (Greiner, Frickenhausen, Germany) were coated with rH proteins (50 pmol each), blocked with 1% bovine serum albumin in phosphate-buffered saline, and incubated for 2 h with increasing amounts of PI4,5P₂ micelles (10–500 pmol), prepared as described in Huttelmaier et al. (20). After intensive washing, bound PI4,5P₂ was detected using a 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS) as substrate, and absorbance was measured in an ELISA reader. To compensate for variations in protein adsorption and development between assays, we normalized the values using the highest absorption as reference. The value of each data point is reported as the mean ± S.D. from three independent experiments.

**RESULTS**

**PKCα and Vinculin Form Complexes in Vivo**—Because PKC effects on cell spreading have been observed during the early phase and flattening of cells (4, 5), we studied a possible association of vinculin with PKC during the formation of focal adhesion structures at early (compare with Huttelmaier et al. (20)) and late time points during spreading. HeLa cells transfected with PKCα were subjected to SDS-PAGE and immunoblotting. As shown in Fig. 1, vinculin precipitated with His-tagged PKCα was precipitated from lysates of spreading cells using Ni-NTA-agarose (Fig. 1A, precipitate). PKCα was precipitated using a BiPro-specific monoclonal antibody (4A6) (46). Cells were analyzed 15 min (Fig. 1A) and 16 h after plating (immunoprecipitate (IP)), hc indicates the 50-kDa heavy chain of the BiPro-specific antibody. Note that co-precipitation of PKCα and vinculin was obtained by both experimental approaches in spreading cells, and actin was not part of the complex.

**FIG. 1. Interaction of vinculin and PKCα is demonstrated by in vivo chemical cross-linking.** PKCα-transfected HeLa cells and vector-transfected controls were allowed to adhere on collagen for 10–15 min or overnight. Subsequently, cells were treated with a membrane-permeant cross-linker (DSP) for 15 min and lysed. PKCα was precipitated from cleared supernatants. After addition of DSP, precipitated proteins were analyzed in a Western blot. The membrane was probed sequentially with antisera specific to vinculin, PKCα, and actin (as indicated). A, His-tagged PKCα was precipitated from lysates of spreading cells using Ni-NTA-agarose. F, precipitate. B, BiPro-tagged PKCα was precipitated using a BiPro-specific monoclonal antibody (4A6) (46). Cells were analyzed 15 min (Fig. 1A) and 16 h after plating (immunoprecipitate (IP)). hc indicates the 50-kDa heavy chain of the BiPro-specific antibody. Note that co-precipitation of PKCα and vinculin was obtained by both experimental approaches in spreading cells, and actin was not part of the complex.

**Vinculin Tail Phosphorylation by PKCα Depends on the C Terminus**—We postulated that vinculin phosphorylation by PKCα depends on the PI4,5P₂-mediated interaction between the kinase and its substrate. The vinculin tail structure reveals two acidic lipid-interacting areas, with a basic ladder at the H2-H3 helical hairpin and a basic collar connected to the C-terminal hydrophobic hairpin (15, 22) that both could support the lipid-regulated interaction with PKCα. To clarify which of these sites might be involved, in vitro phosphorylation assays with recombinant PKCα and deletion fragments of the vinculin tail were performed. We used vinculin tail constructs tagged with MBP that had previously been shown to bind to actin like

that are also located in the vinculin tail. If the role of phospholipids in complex formation between PKCα and vinculin would be confined to unmasking such sites, PKCα should bind to the vinculin tail even in the absence of phospholipids. On the other hand, the regulatory domain of PKCα that consists of the C1 and C2 modules also binds phospholipids (57, 58), and the C2 module requires a free Ca²⁺ concentration of ~100 μM. To analyze the conditions for the interaction between vinculin and PKCα in more detail, we performed overlay experiments with purified smooth muscle vinculin or recombinant vinculin tail (Vt-(858–1066)) and in vitro translated PKCα using PI4,5P₂ as an acidic phospholipid. As shown in Fig. 2, PKCα binding to both vinculin and vinculin tail was observed only when these proteins were preloaded with PI4,5P₂, indicating that the sole exposure of a PKC-binding site on the vinculin tail without PI4,5P₂ is insufficient for PKC recognition. Remarkably, the addition of the lipid to the PKCα-containing solution used for the overlay experiments did not result in PKC binding (data not shown). Furthermore, even with PI4,5P₂-preloaded vinculin proteins, the interaction was effectively blocked by addition of the Ca²⁺ chelator EGTA (Fig. 2). These results strongly suggest a prominent role of the regulatory PKCα-C2 module in the lipid-lipid-protein interaction with vinculin. This was in contrast to smooth muscle α-actinin, which also binds to acidic phospholipids (18) and PKCα, but the interaction was found independent of the free Ca²⁺ concentration in this assay (Fig. 2). When an ATP binding-deficient PKCα mutant (47) was used that is characterized by a defectively folded kinase domain, the interaction with vinculin or vinculin tail was not reduced (Fig. 2). Taken together, these data allow for the conclusion that the contact between vinculin and PKCα is mediated by the PKC regulatory domain and not by the kinase domain.

**Vinculin Tail Phosphorylation by PKCα Depends on the C Terminus**—Early studies show that in vitro phosphorylation of vinculin by PKCα requires acidic phospholipids (27, 29) that bind to the vinculin tail (56). This requirement could derive from the lipid-regulated head-to-tail interaction in vinculin, masking potential PKC binding and/or phosphorylation site(s)
PKCα and Vinculin

PKCα and Vinculin

PKCα

PKCα(ATP–)

EGTA

- + + + +

- + + + +

vinculin

Vt(858–1066)

α-actinin


cosedimentation and microscopic assays as well as in low shear viscometry (49, 54, 64). As shown in Fig. 5, actin binding and actin to the vinculin tail can mutually affect each other (16, 18).

As it had been shown previously that the binding of PI4,5P2 and actin to the vinculin tail can mutually affect each other (16, 18), we tested the actin binding of the presumed PI4,5P2 binding site in syndecan 4 that is essential for PKC docking to this protein (60). PI4,5P2 binding to syndecan 4 induces the oligomerization of the short cytoplasmic tail (61), which is also known for the vinculin tail (20). In addition, both syndecan 4 (amino acids 183–202) and the vinculin C terminus (1053–1066) do not contain the consensus motif described for PI4,5P2 binding in actin binding proteins, (K/R)(X)_5–6(K/R)(X)_5–6(K/R) (62) (Fig. 4C). As shown in Fig. 4D, we constructed two point mutants with amino acid substitutions and one deletion mutant. The mutant Vt-(R1060Q/K61Q) was expected to display reduced or no binding of the negatively charged phosphoinositol ring (Fig. 4D), whereas the Vt-(T1062E) mutant should mimic the effect of a phosphorylation on Thr-1062. The deletion mutant Vt-(858–1052) was generated to probe the importance of the vinculin C terminus for PKC binding.

As it had been shown previously that the binding of PI4,5P2 and actin to the vinculin tail can mutually affect each other (16, 18), we tested the actin binding of the presumed PI4,5P2 binding mutants to exclude general effects on folding and ligand binding properties. The mutants Vt-(R1060Q/K61Q) and Vt-(858–1052) were tested for their interaction with F-actin in cosedimentation and microscopic assays as well as in low shear viscometry (49, 54, 64). As shown in Fig. 5, actin binding and bundling was unaffected in standard high and low speed cen-

FIG. 2. The PKC regulatory domain mediates binding to the vinculin tail. Vinculin, Vt-(858–1066), and α-actinin were preincubated with PI4,5P2 where indicated and spot-blotted onto nitrocellulose.Overlay assays were performed with in vitro-translated 32P-labeled PKCα (wild type) or an ATP binding-deficient (ATP–) mutant. EGTA (5 mM) was used to reduce free Ca2++. The autoradiographs show that the binding of PKC to vinculin or Vt-(858–1066) required both PI4,5P2 and calcium, suggesting the involvement of the PKC C2-module in binding. Conversely, unchanged binding properties of the ATP binding-deficient PKC suggest that the kinase domain was not involved.

FIG. 3. C-terminal deletion of the vinculin tail interferes with phosphorylation by PKC. MBP fusion proteins of Vt-(893–1066), Vt-(893–985), and Vt-(1016–1066) were purified from bacterial extracts. Equimolar amounts of protein were subjected to phosphorylation by recombinant PKCα. Proteins were separated by SDS-PAGE, and phosphorylated products are revealed by autoradiography. Note that MBP alone was not phosphorylated, whereas a faint PKC autophosphorylation signal can be seen (PKC).

PKC Phosphorylates Serines 1033 and 1045 in Helix H5—PKC binding to vinculin during cell spreading is expected to have two major consequences, localization of the activated kinase to a specific site and phosphorylation of the binding partner(s). To further dissect the involvement of vinculin with respect to defining potential phosphorylation sites and analyzing the lipid binding properties of the C-terminal arm, a number of Vt mutants were generated by site-directed mutagenesis (Fig. 4A). Serine and threonine residues within a PKC consensus motif (59) were selected from accessible loop regions and the C terminus of the vinculin tail structure to generate phosphorylation site-deficient Vt proteins (Fig. 4, A and B). Mutant proteins included alanine substitutions of Ser-941/Thr-43 and Ser-999 from loop regions as well as Thr-1050/Thr-55 and Thr-1062 from the C terminus. However, phosphorylation assays with the corresponding recombinant Vt proteins and PKCα did not demonstrate a reduction of phosphate incorporation as compared with the wild type Vt protein (1.5 ± 0.3 mol of phosphate/mol of Vt). This result renders it rather unlikely that any of the potential phosphorylation sites selected (Fig. 4B) contribute markedly to the overall phosphorylation. In contrast, when proteolytic peptides of phosphorylated vinculin tail were generated using Lys-C or Glu-C endopeptidase and subsequently analyzed by MALDI and electrospray ionization time-of-flight mass spectrometry, peptides containing phosphorylated Ser-1033 and Ser-1045 were detected. The identity of these peptides and their phosphorylation sites were verified by MS/MS sequencing (Fig. 4B). Both sites are located in helix H5. This is consistent with our results on the phosphorylation of the MBP-Vt-(1016–1066) polypeptide.

C-terminal Mutations in the Predicted PI4,5P2-binding Site Do Not Interfere with Actin Binding—To estimate the importance of the lipid-binding site at the vinculin C terminus for PKC-vinculin interactions, we generated appropriate vinculin mutants. The selection of relevant mutants was based on the characterization of the PI4,5P2-binding site in syndecan 4 that is essential for PKC docking to this protein (60). PI4,5P2 binding to syndecan 4 induces the oligomerization of the short cytoplasmic tail (61), which is also known for the vinculin tail (20). In addition, both syndecan 4 (amino acids 183–202) and the vinculin C terminus (1053–1066) do not contain the consensus motif described for PI4,5P2 binding in actin binding proteins, (K/R)(X)_5–6(K/R)(X)_5–6(K/R) (62) (Fig. 4C). As shown in Fig. 4D, we constructed two point mutants with amino acid substitutions and one deletion mutant. The mutant Vt-(R1060Q/K61Q) was expected to display reduced or no binding of the negatively charged phosphoinositol ring (Fig. 4D), whereas the Vt-(T1062E) mutant should mimic the effect of a phosphorylation on Thr-1062. The deletion mutant Vt-(858–1052) was generated to probe the importance of the vinculin C terminus for PKC binding.

The wild type vinculin tail and are thus probably in a native conformation (49). MBP itself was not phosphorylated by PKC. As shown in Fig. 3 and consistent with earlier results from our and other groups (27, 29), a Vt-(893–1066) fragment comprising the five-helix bundle (H1–H5) and the C terminus was readily phosphorylated by PKC. A C-terminal deletion fragment, Vt-(893–985), was not phosphorylated (Fig. 3), although this protein contained the lipid binding domain in helices H2–H3 and several consensus motifs for PKC phosphorylation sites. In contrast, the short C-terminal construct Vt-(1016–1066) comprising helix H5 and the C terminus, retained some phosphate incorporation (Fig. 3). This was ~20–30% of the value obtained for the intact tail, as judged by densitometric analysis of the signal. This indicated that at least some of the phosphorylation sites are located at the very C terminus of the vinculin tail (amino acid residues 1016–1066) and that this part also includes the main binding site for the kinase.

FIG. 4. The PKC regulatory domain mediates binding to the vinculin tail. Vinculin, Vt-(858–1066), and α-actinin were preincubated with PI4,5P2 where indicated and spot-blotted onto nitrocellulose. Overlay assays were performed with in vitro-translated 32P-labeled PKCα (wild type) or an ATP binding-deficient (ATP–) mutant. EGTA (5 mM) was used to reduce free Ca2++. The autoradiographs show that the binding of PKC to vinculin or Vt-(858–1066) required both PI4,5P2 and calcium, suggesting the involvement of the PKC C2-module in binding. Conversely, unchanged binding properties of the ATP binding-deficient PKC suggest that the kinase domain was not involved.
trifugation assays (Fig. 5A), low speed data not shown), in actin-bundle formation of the co-polymers (Fig. 5B), and in low shear viscometry (data not shown). These results suggest that both mutant proteins are in their native conformation.

Deletion of the Presumed PI4,5P2-binding Region at the Vinculin C Terminus Results in Reduced Affinity for PI4,5P2—The Vt C-terminal arm seems critical for the binding to PI4,5P2- and phosphatidylserine-containing multilamellar vesicles. This was shown in cosedimentation assays using a Vt-(879–1051) mutant (22). For our deletion mutant Vt-(858–1052), we tested PI4,5P2-binding as compared with wild type Vt using an ELISA assay (Fig. 5C). Vt proteins (50 pmol each) were adsorbed to ELISA plates and incubated with 5–500 pmol of PI4,5P2 vesicles in a total volume of 100 μl. Lipid binding was monitored with a PI4,5P2-specific antibody as described earlier (55). As shown in Fig. 5C, the deletion mutant bound less lipid at lower PI4,5P2 concentrations than wild type, suggesting that the affinity of this mutant protein for lipids is reduced. However, the overall capacity of lipid binding was comparable for both proteins when saturation levels of PI4,5P2 were reached.
PKC Binding to Vt Mutants of the C-terminal PI4,5P_2-binding Site Is Reduced—The mutants described and characterized above were used in phosphorylation assays to estimate relative binding affinities of PKC for the lipid-binding site of the Vt. Vt proteins were preincubated with PI4,5P_2 and analyzed for phosphorylation in a PKC-mixed micelle assay (52). There was no difference in the total amount of phosphate incorporated between these mutants and wild type Vt protein (1.5 mol of phosphate/mol of Vt protein). However, when the initial rate of phosphate incorporation was tested using a low enzyme to substrate ratio, a significant difference was observed. Variance analysis was performed to compare incorporation rates using data from four independent experiments. All substrate proteins showed a linear time dependence of phosphate incorporation (R^2 > 0.97). As shown in Fig. 6, Vt-(R1060Q/K61Q) and the deletion Vt-(858–1052) exhibited only 50%, and Vt-(T1062E) exhibited 75% of wild type phosphorylation rate. Hence, deletion of or mutations in the presumptive lipid-binding motif at the vinculin C terminus affects the kinase activity, suggesting a possible mechanism for a regulated interaction between both proteins, PKC and vinculin, as a function of local PI4,5P_2 availability.

DISCUSSION

In this paper, we have analyzed the interaction of vinculin, a prominent component of focal contacts, and PKC, a Ser/Thr kinase that is known to be essential for cell adhesion and spreading of fibroblasts and other cell types (1). The interaction between PKC and vinculin was monitored at an early time point (15 min) after seeding of HeLa cells, when adhesion has been completed, and the initial phase of spreading is induced. Engagement of integrins by a suitable substrate is accompanied by a rapid activation and translocation of PKC to the plasma membrane (4, 65). To trap a presumedly labile and transient interaction of the kinase with vinculin at the membrane, we used a dual strategy. First, we expressed a PKC construct to increase the number of kinase molecules available for complex formation with endogenous vinculin. Second, we used a cross-linking protocol to preserve membrane-associated complexes formed in situ that could be disintegrated during cell lysis. The usefulness of DSP cross-linking in stabilizing such complexes has previously been shown in several studies (20, 66, 67). The validity of both strategies is also demonstrated by the fact that the PKC-vinculin complexes identified were confined to spreading but were not seen with fully spread cells.
Although PKC activation upon cell adhesion and spreading has been known for almost a decade, downstream events involving specific PKC-substrate interactions and the mechanism(s) underlying these time point- and site-specific interactions are poorly understood. The conformation and activation of both PKC and vinculin both depend on acidic phospholipids (31, 68). Therefore, a mechanism controlling local availability of these lipids at the plasma membrane must be important for a regulated interaction. Local fluctuations of phospholipids may be regulated by GAP43-like proteins. These proteins accumulate at PI4,5P₂-enriched rafts, where they co-distribute with PI4,5P₂ and promote its retention and clustering. Changes in actin dynamics are correlated with alterations in raft organization by GAP43-like proteins (7, 10). The myristoylated alanine-rich protein kinase C substrate (MARCKS), a widely expressed member of this family, possibly acts as a key regulator in cell spreading. Sequestering of acidic phospholipids, phosphatidylserine, and PI4,5P₂ in membrane domains by MARCKS is abolished by PKC-dependent phosphorylation (61, 74). Oligomerization of the syndecan-4 tail provides a Ca²⁺-dependent binding site for the PKC regulatory domain. In our assay, vinculin and its tail fragment were membrane-adsorbed after preincubation with PI4,5P₂. With this protocol a Ca²⁺-dependent interaction with PKC was observed that resisted intensive washing with TBST. Earlier, Hyatt et al. (72) performed an overlay analysis with membrane-bound vinculin and a partially purified PKC fraction and observed a reversible interaction. The complex required the continuous presence of lipids and Ca²⁺ throughout the washing and staining procedure or, alternatively, a fixation step after kinase addition. Fixation resulted in a residual binding of PKCα even in the absence of Ca²⁺. The apparent discrepancy between the results obtained by both procedures can be explained by the different mode of lipid application. The addition of the lipid to membrane-bound vinculin will loosen its intramolecular head-to-tail bonding and allow a lipid-mediated binding of the kinase. In contrast, pretreatment of soluble vinculin with PI4,5P₂ results in the formation of vinculin tail oligomers (20) that bind the lipid tightly, which probably reflects the in vivo situation (56, 73). A similar effect of the state of oligomerization on the binding of PKC to a lipid-presenting partner was observed for syndecan-4. The cytoplasmic tail of syndecan-4 binds to and activates PKCα only in its lipid-bound, oligomeric conformation (61, 74). Oligomerization of the synde-
PKCα and Vinculin

The oligomeric vinculin tail serves as a PKC-docking site, which leads to the phosphorylation and activation of vinculin and/or vinculin binding partners. Increased PI4,5P2 attracts lipid-binding proteins, e.g., MARCKS, displacing them from PI4,5P2 rafts. Locally increased PI4,5P2 attracts lipid-binding proteins, e.g., vinculin and induces conformational changes and/or oligomerization. Lipid-bound oligomeric vinculin tail serves as a PKC-docking site, which leads to the phosphorylation and activation of vinculin and/or vinculin binding partners (see “Discussion,” last paragraph).

In a mixed micelle assay, we show that binding of PKC and subsequent phosphorylation require and are probably restricted to the C-terminal part of the vinculin tail (985–1066). This corroborates earlier reports that located the main PKC phosphorylation site(s) to the vinculin tail (27, 29). Surprisingly, some of the sites in Vt with a PKC consensus motif, Ser-941 and Ser-999, predicted to be attractive sites for PKC phosphorylation (15) as well as the C-terminal threonine residues (Thr-1050, -1055, -1062) were not phosphorylated in our in vitro assay. In contrast, serines 1033 and 1045 in helix H5, adjacent to the PKC-binding site, were phosphorylated, and we have preliminary data indicating one to two further sites in helices H2, H3 (23) and amino acids 1053–1066, representing the hydrophobic finger at the C terminus (22), differ in their capacity to attract the kinase. Ser-1033 and -1045 both lie within the binding site for the vinculin head (1009–1066) that has been determined by x-ray structure analysis (22) the phosphorylation sites identified so far appear to be readily accessible for the kinase. Ser-1033 and -1045 both lie within the binding site for the vinculin head (1009–1066) (24, 27, 75) and the C-terminal binding site for F-actin (1016–1066) (49). Conversely, engagement of vinculin head or actin-binding sites on the Vt might modulate PKC-dependent phosphorylation. This concept is supported by an early study showing that talin, a ligand of the vinculin head domain, can increase PKC-dependent vinculin phosphorylation, whereas binding of vinculin to F-actin decreases phosphorylation (76).

Remarkably, the two acidic phospholipid-binding sites in vinculin tail, amino acids 916–970 in helices H2, H3 (23) and amino acids 1053–1066, representing the hydrophobic finger at the C terminus (22), differ in their capacity to attract PKC. Although constructs including these sites individually, MBP-Vt-(893–985) and MBP-Vt-(1016–1066), both perform homotypic interactions (49), bind to PI4,5P2 (20), and contain PKC consensus phosphorylation sites (Ref. 59 and this study), only the short Vt-(1016–1066) peptide is accept as a PKC substrate. This striking difference may result from the configuration by which the lipid moiety is bound to the respective peptide, thereby influencing the capacity of the PKC regulatory domain to bind the Vt-lipid complexes. This notion is supported by our kinetic analysis of the phosphorylation of Vt-(858–1066) C-terminal lipid-binding site mutants. Mutants that were designed to reduce binding of the negatively charged head group of PI4,5P2 showed a reduced phosphorylation rate (25–50%), although the overall organization and activity was not affected, as judged by their capacity to interact with F-actin. Furthermore, the effect on the phosphorylation rates not simply reflects a reduced oligomerization of the vinculin tail mutants, since the deletion mutant Vt-(858–1052) shows an increased capacity to oligomerize upon addition of PI4,5P2 as compared with Vt-(wild type). The syndecan 4/PKCβ binding might again serve as a model. For this pair, a biophysical analysis suggests that PI4,5P2 mediates their interaction, and PKC binding affinity mainly derives from its interaction with the nositol head group (60). The reduced initial rate of phosphorylation observed for lipid-binding site mutants of Vt suggests that PI4,5P2 binding to the C terminus can control interaction kinetics with PKCα. Given the low number of PKC molecules in cells as compared with vinculin, a lipid-induced rise in binding affinity may provide a mechanism for efficient phosphorylation of vinculin at the time point when focal contacts are forming.

Although there are some reports on vinculin phosphorylation in cells (77, 78), it has proven difficult to establish conditions of phosphorylation. There are, however, two reports analyzing specific activation events that provide evidence for a PKC-dependent phosphorylation and/or major subcellular redistribution of vinculin in adherent cells. During Ca2+-induced junctional sealing in Madin-Darby canine kidney cells, a PKC inhibitor-sensitive redistribution of vinculin was observed. Phosphorylation of Ser/Thr residues on vinculin immunoprecipitated from cell extracts was dependent on PKC activity (79).

FIG. 7. Putative model for a conformational activation of vinculin (*Vinc). I, upon cell-matrix adhesion PKC is translocated to and activated (*) at the membrane, where the kinase phosphorylates GAP43-like proteins, e.g., MARCKS, displacing them from PI4,5P2 rafts. II, locally increased PI4,5P2 attracts lipid-binding proteins, e.g., vinculin and induces conformational changes and/or oligomerization. III, lipid-bound oligomeric vinculin tail serves as a PKC-docking site, which leads to the phosphorylation and activation of vinculin and/or vinculin binding partners (see “Discussion,” last paragraph).

2 W. H. Ziegler, unpublished observation.
Treatment of confluent epithelial cells (Int 407) with the inflammatory mediator leukotriene D4 resulted in a dissociation of vinculin from a complex containing α-catenin and an increased localization to focal contacts. These effects were mimicked by the PKC activator 12-O-tetradecanoylphorbol 13-acetate (TPA) and blocked by the specific inhibitor bisindolylmaleimide I (GF109203X) (80).

So far, the mode of regulation of vinculin and its function in model cell adhesion sites have escaped a comprehensive analysis. Our results indicate a lipid-dependent mechanism for the release of the inhibitory vinculin head-tail interaction by induction of membrane-associated PKC activity. The principles of this model are outlined in Fig. 7. Spreading-induced activation of PKC at the plasma membrane leads to the phosphorylation of GAP43 family proteins, like MARCKS. Phospho-MARCKS has a reduced binding affinity for acidic phospholipids and is released from PI4,5P2-enriched rafts. Cytosolic vinculin gains access to the surface of rafts, where PI4,5P2 binding releases the head-tail interaction, thereby activating cryptic binding and homo-oligomerization sites in the molecule. Upon oligomerization of the vinculin tail, a docking site for PKC is provided. PKC phosphorylation of the vinculin tail and/or vinculin binding partners is expected to regulate the incorporation of vinculin in nascent cell adhesion complexes.

Because of the large number of acidic phospholipid binding molecules involved in the regulation of the membrane-anchored actin cytoskeleton (2, 10), such a model may be expanded to other proteins.

Acknowledgments—We are grateful to P. Parker for the gift of the PKCa cDNA and to S. Huttelmaier and K. Schluter for providing Vt protein and actin, respectively. We thank E. Saxinger for technical assistance, U. Beutling and S. Hermann for assistance with mass spectroscopy, and D. Critchley, J. Norman, and S. Illenberger for stimulating discussion.

REFERENCES

1. Clark, E., and Brugge, J. S. (1995) Science 268, 233–239
2. Jockusch, B. M., Bubeck, P., GeHL, K., Kroemer, M., Moscher, J., Rothkugel, M., Rudiger, M., Schluter, K., Stanke, G., and Winder, J. (1995) Annu. Rev. Cell Biol. 11, 379–416
3. Yamada, K. M., and Geiger, B. (1995) Curr. Opin. Cell Biol. 9, 76–85
4. V互补, R., and Russinol, E. (1995) J. Biol. Chem. 270, 21465–21469
5. Delfiatti, P., Venturino, M., Gulino, D., Duperray, A., Boquet, P., Fiorentini, C., Volpe, G., Palmieri, M., Silengo, L., and Taron, G. (1997) J. Biol. Chem. 272, 26746–26754
6. Myat, M. M., Anderson, S., Allen, L. A., and Aderem, A. (1997) Curr. Biol. 7, 611–614
7. Lau, X., Fukami, K., Thibon, M., Golub, T., Frey, D., and Caroni, P. (2000) J. Cell Biol. 149, 1455–1472
8. Hinchliffe, K. (2000) Curr. Biol. 10, 104–115
9. Raucher, D., Staufer, T., Chen, W., Shen, K., Guo, S., York, J. D., Sheetz, M. P., and Karp, C. (1997) J. Biol. Chem. 272, 21616–21624
10. Ohno, S., Konno, Y., Akiy, Y., Yano, A., and Suzuki, K. (1996) J. Biol. Chem. 271, 6266–6270
11. Guex, N., and Peitsch, M. C. (1997) Electrophoresis 18, 2714–2723
12. Huttelmaier, S., Bubeck, P., Rudiger, M., and Jockusch, B. M. (1997) Eur. J. Biophys. 267, 127–134
13. Hurley, J. H., and Meyer, T. (2001) Curr. Opin. Cell Biol. 13, 146–152
14. Cho, W. (2001) J. Biol. Chem. 276, 32407–32410
15. Critchley, D. R. (2000) J. Biol. Chem. 276, 14616–14621
16. Steinle, P. M., Hoffert, J. D., and Bishop, L. J. (1997) J. Biol. Chem. 272, 952–960
17. Horowitz, A., Murakami, M., Gao, Y., and Simons, K. (1999) Biochemistry 38, 15871–15877
18. Ohno, S., Woods, A., and Couchman, J. R. (1999) J. Biol. Chem. 274, 18414–18420
19. Harbeck, B., Huttelmaier, S., Schluter, K., Jockusch, B. M., and Illenberger, S. (2000) J. Biol. Chem. 275, 30817–30825
20. Chun, J. S., and Jacobson, B. S. (1993) J. Biol. Chem. 268, 275–281
21. Hinck, L., Natke, I., S., Parkhoff, J., and Nelson, W. J. (1994) Trends Biochem. Sci. 19, 538–542
22. Weiss, E. E., Kroemer, M., Rudiger, A. H., Jockusch, B. M., and Rudiger, M. (1998) J. Biol. Chem. 273, 755–764
23. Critchley, D. R., Holt, M. R., Barry, S. T., Priddle, H., Henningham, L., and Norman, J. (1999) Biochem. Soc. Symp. 65, 79–99
24. Glaser, M., Wasko, S., Bazer, C. A., Boguslavsky, V., Rashidzada, W., Morris, A., Rebbeck, M., Scarlata, S. F., Runnels, L. W., Prestwich, G. D., Chen, J., Aderem, A., A., and McLaughlin, S. (1996) J. Biol. Chem. 271, 26187–26193
25. Seykora, J. T., Myat, M. M., Allen, L. A., Ravetch, J. V., and Aderem, A. (1996) J. Biol. Chem. 271, 18797–18802
26. McLaughlin, S., and Aderem, A. (1995) Trends Biochem. Sci. 20, 272–276
27. Hyatt, S. L., Liao, L., Chapline, C., and Jaken, S. (1994) Biochem. Biophys. Res. Commun. 208, 2538–2543
28. Kuhn, F., Endo, T., Inamura, M., and Takenawa, T. (1994) J. Biol. Chem. 269, 1518–1522
29. Horowitz, A., and Simons, K. (1998) J. Biol. Chem. 273, 5245–5251
30. Were, G. J., Dunn, S. D., and Bell, E. H. (2001) J. Biol. Chem. 276, 11729–11734
31. Kawamoto, S., and Hidaka, H. (1984) Biochem. Biophys. Res. Commun. 118, 1150–1156
32. Setton, B. M., Hunter, T., Ball, E. H., and Singer, S. J. (1981) Cell 24, 165–174
33. Werth, D. K., and Paskin, I. (1984) J. Biol. Chem. 259, 5264–5270
34. Perez-Moreno, A., Avila, A., Isla, S., Sanchez, S., and Gonzalez-Mariscal, L. (1998) J. Cell Sci. 111, 3563–3571
35. Massoumi, R., and Sjolander, A. (2001) J. Cell Sci. 114, 1925–1934
