Acidic phospholipids inhibit the intramolecular association between the N- and C-terminal regions of vinculin, exposing actin-binding and protein kinase C phosphorylation sites

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Chick vinculin polypeptides expressed in Escherichia coli as glutathione S-transferase (GST) fusion proteins have been used to identify the sites involved in the intramolecular association between the 90 kDa N-terminal head and the 30 kDa C-terminal tail region of the vinculin molecule. Fusion proteins spanning vinculin residues 1–258 and 1–398, immobilized on glutathione–agarose beads, were shown to bind a C-terminal vinculin polypeptide spanning residues 881–1066 (liberated from GST by thrombin cleavage). However, the C-terminal polypeptide did not bind to a fusion protein spanning residues 399–881 or to itself. Binding was dependent on residues 167–207 within the N-terminal polypeptide, a sequence also essential for talin binding. Conversely, the 90 kDa head polypeptide was shown to bind to residues 1029–1036 in the tail region of vinculin. The association of head and tail was inhibited by acidic, but not neutral, phospholipids. Pre-incubation of vinculin with acidic phospholipids exposed the binding site for F-actin and a phosphorylation site for protein kinase C. The phosphorylation site was located in the tail region of the vinculin molecule. These results raise the possibility that acidic phospholipids play a role in regulating the activity of vinculin and therefore the assembly of both cell-cell and cell–matrix adherens-type junctions.

INTRODUCTION

Vinculin is a highly conserved 117 kDa cytoskeletal protein containing 1066 amino acids [1] found in both cell-cell and cell–extracellular matrix adherens-type junctions. In such junctions it is thought to be one of a number of interacting proteins which link the cytoplasmic face of adhesion receptors of the cadherin or integrin families to the actin cytoskeleton. Rotary shadowing electron microscopy suggests that vinculin is comprised of a globular head and an extended tail [2], although the tail is not always visible. The globular head region contains the N-terminus of the protein, a talin-binding region within residues 1–258 [3,4], and three 112-residue repeats of unknown function (Figure 1). Evidence for an α-actinin-binding site between residues 1 and 107 has recently been presented [5]. A proline-rich region, which spans residues 837–878 and contains two sites for V8-protease [6], is thought to separate the globular head from the extended tail. The C-terminal tail region has been shown to contain a binding site (residues 893–1016) for F-actin [7] and for paxillin (residues 978–1000) [8], another protein localized to focal adhesions.

Recent studies have provided compelling evidence for an intramolecular association between the tail region of vinculin and the globular head [9,10]. The tail has also been shown to bind acidic phospholipids [11]. In the present study, we have defined the regions responsible for the intramolecular association within the vinculin molecule and have shown that this association is relieved by acidic phospholipids exposing both an F-actin-binding site and a site recognized by protein kinase C (PKC).

MATERIALS AND METHODS

Rat brain PKC (1000 units/mg), isopropyl β-D-thiogalactoside and PtdIns4P and Ptd(4,5)P2, supplied as the diammonium salts, were obtained from Calbiochem (Nottingham, U.K.). Phosphatidylserine was obtained from Lipid Products (S. Nutfield, Surrey, U.K.). Ampicillin, rabbit muscle actin, thrombin, PMSF, V8-protease–agarose beads, brain lipids, glutathione–agarose beads and phorbol 12-myristate 13-acetate (PMA) were from Sigma (Poole, Dorset, U.K.). [γ-32P]ATP with a specific activity of 111 TBq per mmol was from Amersham International (Little Chalfont, Bucks., U.K.).

Abbreviations used: GST, glutathione S-transferase; PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C.

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Expression of vinculin polypeptides as glutathione S-transferase (GST) fusion proteins

All of the chick vinculin fusion proteins used in this study, with the exception of one spanning residues 881–1036, have been described previously [4]. This latter fusion protein was generated by PCR using a cDNA encoding vinculin residues 881–1066 as template, and primers containing a 5′ BamHI site and a 3′ EcoRI site. The PCR product was cloned into BamHI–EcoRI-cut pGEX-2T (Pharmacia). The identity of the constructs used throughout this study was confirmed by double-strand sequencing using primers 5′ and 3′ to the pGEX cloning site. GST-fusion proteins expressed in *Escherichia coli* (strain JM101) were purified from 250 ml cultures by standard methods [12]. Briefly, after induction with isopropyl β-D-thiogalactoside, cells were harvested by centrifugation, resuspended in 5 ml of PBS, and lysed by sonication and the solubilized fusion proteins purified by adsorption on glutathione-agarose beads. Beads were washed thoroughly with PBS to remove contaminating beads. Beads were washed thoroughly with PBS to remove contaminating *E. coli* proteins, and 1 mM PMSF was added to all buffers immediately before use.

Purification of vinculin and production of vinculin polypeptides by limited proteolysis

Vinculin was purified to homogeneity from adult chicken gizzard as described by Evans et al. [13]. The 90 kDa N-terminal head fragment of vinculin was produced by incubation (3 h at 37 °C) of intact vinculin with V8-protease immobilized on agarose beads, as described by Price et al. [6]. A C-terminal vinculin tail polypeptide (residues 881–1066) was expressed as a GST-fusion protein in *E. coli* and the vinculin polypeptide was liberated by thrombin cleavage whilst the fusion protein was still attached to glutathione-agarose. Cleavage was carried out in 50 mM Tris/HCl, pH 7.5, containing 150 mM NaCl/2.5 mM CaCl₂ and an enzyme-to-substrate ratio of 1:500. Digestion was carried out at 37 °C for up to 2 h with continual rotation of the sample.

Binding of vinculin polypeptides to vinculin fusion proteins

Vinculin polypeptides were incubated (30 min at 37 °C) with vinculin GST-fusion proteins bound to glutathione–agarose with continuous inversion. Incubations were performed in PBS containing 0.1% (w/v) BSA to eliminate non-specific protein–protein interactions. The concentration of each fusion-protein bound to the agarose beads was estimated by SDS/PAGE, and washing with Coomassie Blue, and approximately the same amount of each fusion protein was used in each assay. After incubation, the beads were pelleted in a microfuge, the supernatants removed and the pellets washed once with PBS/0.1% BSA (1.5 ml) and a further two times with PBS. Proteins bound to glutathione–agarose were dissolved by boiling in electrophoresis sample buffer and were analysed by SDS/PAGE [14].

Preparation of liposomes

Lipid stocks were dissolved in chloroform and kept at −20 °C. For liposome preparation, lipids were dried under vacuum for 30 min before sonication (2 × 5 min) in a buffer containing 20 mM Tris/HCl (pH 7.2)/0.2 mM EGTA (10 mg of lipid/ml). The clear dispersions were centrifuged at 100000 g for 20 min and the supernatant incubated with the vinculin tail polypeptide for 20 min at room temperature at a molar ratio of 2:1 (lipid:tail). Lipid–tail mixtures so prepared were used directly in the binding assay described above.

Actin co-sedimentation assay

Rabbit muscle G-actin (Sigma) was dissolved in buffer A [10 mM Tris/HCl (pH 8)/0.2 mM ATP/0.2 mM dithiothreitol/0.2 mM CaCl₂] at a protein concentration of 5 mg/ml. Assays were carried out in airfuge tubes (Beckman) and contained 5 µl of actin, 10 µl of 10× buffer A, 5 µl of buffer B (60 mM MgCl₂/2 mM NaCl) and 10–20 µg of vinculin in a final volume of 100 µl. Samples were mixed by pipetting, incubated at room temperature for 60 min, then centrifuged at 100000 g for 30 min. The resulting supernatants and pellets were analysed by SDS/PAGE. Where appropriate, intact vinculin was pre-incubated (20 min at room temperature) with lipid prepared as described above, and any aggregates removed by centrifugation at 100000 g before use in an actin sedimentation assay.

Phosphorylation of vinculin and vinculin polypeptides

Vinculin (1–2 mg/ml) was incubated with PKC (0.11 units/ml final concentration), 140 µM ATP (approx. 3000 c.p.m./nmol), 5 mM MgCl₂ and 0.1 µg/ml PMA at 37 °C. At the times indicated, aliquots were mixed with SDS/PAGE sample buffer and were analysed in SDS gels. In some cases, intact vinculin was pre-incubated (20 min at room temperature) with various lipids before use in phosphorylation assays *in vitro*.

RESULTS

Vinculin residues 1–258 contain a binding site for the C-terminal region of the molecule

To define the site within the 90 kDa N-terminal globular head region of vinculin that is recognized by the C-terminal tail region of the molecule, we first expressed a fusion protein spanning...
The results show that fusion proteins containing residues 881–1066 were responsible for the head–tail interaction, a series of C-terminal deletion mutants based on the fusion protein 881–1066 were constructed (Figure 1). The ability of these GST-fusion proteins to bind to the 90 kDa vinculin polypeptide estimated from SDS/PAGE is close to that predicted from the deduced amino acid sequence.

Identification of the binding site for the N-terminal region of vinculin in the C-terminal tail region of the molecule

To further define the region in the C-terminal tail of vinculin responsible for the head–tail interaction, a series of C-terminal deletion mutants based on the fusion protein 881–1066 were constructed (Figure 1). The ability of these GST-fusion proteins to bind to the 90 kDa vinculin polypeptide generated by V8-protease cleavage of vinculin was assayed by co-sedimentation. The results show that fusion proteins containing residues 881–1066 and 881–1036 were both able to bind the 90 kDa vinculin head fragment (Figure 3). However, removal of a further eight amino acid residues to yield a GST-fusion protein spanning residues 881–1028 totally abolished binding. The GST-fusion proteins 881–1021, 881–1012 and 881–1000 were also unable to bind the head domain (results not shown). We conclude that the sequence between residues 1028 and 1036 is essential for binding of the C-terminal region of vinculin to the N-terminal region of the molecule.

Effect of lipids on the vinculin head–tail interaction

The C-terminal tail region of the vinculin molecule has recently been shown to contain a binding site for acidic phospholipids [11]. The effect of pre-incubating the free tail polypeptide (residues 881–1066) with various phospholipids on its ability to bind to a fusion protein spanning vinculin residues 1–258 was therefore investigated. Pre-incubation of the vinculin tail polypeptide with phosphatidylserine or brain lipids for 20 min markedly inhibited head–tail binding (Figure 4), as did PtdIns4P and Ptd(4,5)P2 (results not shown). In contrast, the neutral lipid phosphatidylcholine (Figure 4) or phosphatidylethanolamine (results not shown) had no effect on the interaction. Quantitative analysis of the effects of various lipids on the vinculin head–tail interaction revealed that mixed brain lipids were more effective than either phosphatidylserine, PtdIns4P or Ptd(4,5)P2 alone (Figure 4), raising the possibility that the effect is either due to a combination of acidic phospholipids or that a novel phospholipid present in brain has a greater potency than any of the individual phospholipids used in this study.

Acidic phospholipids expose the F-actin-binding site in vinculin

To establish whether binding of acidic phospholipids to the C-terminal tail region of vinculin caused any change in the activity

Figure 3 Identification of the head-binding region in the tail domain of vinculin

Vinculin fusion proteins spanning residues 881–1066, 881–1036 and 881–1028 adsorbed on glutathione–agarose beads were incubated in the presence (+) or absence (−) of the 90 kDa N-terminal vinculin head domain generated from vinculin by V8-protease cleavage. Binding of the 90 kDa polypeptide to the fusion proteins was assayed by co-sedimentation and SDS/PAGE. Molecular-mass markers (kDa) are shown on the left. Although the truncated fusion proteins are less stable than that spanning residues 881–1066, the size of each of the largest fusion proteins estimated from SDS/PAGE is close to that predicted from the deduced amino acid sequence.

Figure 4 Effect of phospholipids on binding of an N-terminal vinculin fusion protein (residues 1–258) to the C-terminal tail polypeptide

The vinculin C-terminal tail polypeptide (residues 881–1066) was pre-incubated (20 min) with phosphatidylserine (PS) (lane B), phosphatidylinerine and phosphatidylcholine (PS + PC) (lane C), mixed brain lipids and phosphatidylcholine (BL + PC) (lane D), BL alone (lane E) and PC alone (lane F) before assaying binding to a fusion protein spanning vinculin residues 1–258 by co-sedimentation and SDS/PAGE. The stained gel was analysed by densitometry. Binding in the absence of lipid (lane A) (100%) was compared with that in the presence of PS (20%), PS + PC (33%), BL + PC (21%), BL (10%) and PC (33%). Similar results were obtained in three separate experiments. In additional experiments, PtdIns4P and Ptd(4,5)P2 were found to inhibit the vinculin head–tail interaction to 35% and 21% of control values respectively, whereas phosphatidylethanolamine (94%) had little or no effect on the interaction. Control experiments showed that acidic lipids had no effect on the integrity of the tail polypeptide (results not shown).
Effect of lipids on binding of vinculin to F-actin

Intact vinculin was pre-incubated (20 min) with the indicated lipid (PC, phosphatidylcholine; PS, phosphatidylserine; Ptd(4,5)P_2) and any aggregates were removed by centrifugation at 100,000 g before the addition of the G-actin and buffer B as described in the Materials and methods section. After 60 min, F-actin was sedimented by centrifugation at 100,000 g and the resultant pellets and supernatants were analysed by SDS/PAGE. Molecular-mass markers (kDa) are shown on the left. Acidic phospholipids did not increase the amount of vinculin recovered in the pellet in the absence of F-actin (results not shown).

Figure 7 Phosphorylation of vinculin fusion proteins by PKC

Purified vinculin fusion proteins spanning residues 1–398 (A), 399–881 (B) and 881–1066 (C) were incubated with rat brain PKC and [γ^32P]ATP for 15 min in phosphorylation buffer and the products of the reaction analysed by SDS/PAGE and autoradiography. The positions of PKC and of the various fusion proteins are indicated. The degree of purity of the purified fusion proteins can be seen in Figure 2.
between the 90 kDa N-terminal head region and the 30 kDa C-terminal tail region of the molecule. This association is inhibited by acidic phospholipids which expose a cryptic F-actin-binding site and a PKC phosphorylation site which is located within the vinculin tail. We have previously shown that residues 1–258 contain the talin-binding site(s) within vinculin [3,4], and binding of the vinculin tail to this region shows the same characteristics as talin binding in that vinculin residues 167–207 appear to be essential for the interaction. This result is consistent with the observation that the vinculin tail and talin compete for binding to the globular head domain of vinculin [9]. Unfortunately, it has not proved possible to further define the talin-binding site within residues 1–258, as all deletions tested to date, including those made according to the exon structure of the vinculin gene [17], inactivate tail binding [4]. Whether the vinculin tail and talin bind to exactly the same site in vinculin, or whether the tail sterically inhibits talin binding, remains to be resolved.

The C-terminal residues (1029–1036) which are important in binding the head region of vinculin are close to the actin-(893–1016) [7] and paxillin-binding (978–1000) [8] binding sites and a focal adhesion targeting sequence (1000–1028) [8] within the molecule (Figure 1). Head–tail association has been shown to inhibit actin binding [10]. The vinculin tail has also recently been shown to bind acidic phospholipids [11]. Modelling studies suggest potential lipid-binding sites between residues 935–978 and 1020–1040 [18]. The fact that such lipids inhibit the interaction between the acidic N-terminal region of the protein (isoelectric point 5.4) and the basic tail (isoelectric point 9.7) exposing the actin-binding site, provides one possible mechanism for regulating the intramolecular association within the vinculin molecule and the availability of the binding sites for other cytoskeletal proteins such as talin and actin. The finding that the head–tail interaction is inhibited by both phosphatidylserine and Ptd(4,5)P₂, is consistent with previous reports that vinculin binds to a number of acidic phospholipids [19,20], although recent experiments suggest that it binds to vesicles containing Ptd(4,5)P₂ to a greater extent than those containing other acidic lipids [11]. Interestingly, the activity of several other actin-binding proteins has also been reported to be regulated by Ptd(4,5)P₂ [21] and a Ptd(4,5)P₂ binding sequence has been identified in gelsolin [22]. Whether the effects of acidic phospholipids on the structure of vinculin are of physiological relevance remains to be established.

Vinculin has been shown to be labelled by protein-activatable hydrophobic probes incorporated into both phospholipid vesicles [20] and cultured fibroblasts [23], suggesting that vinculin does indeed bind to phospholipids in vivo. Cellular vinculin has also been reported to be associated with Ptd(4,5)P₂, the amount of which decreases in response to growth factors such as platelet-derived growth factor [24], which are known to lead to transient disruption of focal adhesions [25].

The assembly of focal adhesions in response to agents such as lysophosphatidic acid and bombesin [26], as well as ligand binding to integrins (S. T. Barry, A. J. Ridley, H. M. Flinn, M. J. Humphries and D. R. Critchley, unpublished work), is dependent on rho, a small GTP-binding protein. Interestingly, rho has been shown to regulate a phosphatidylinositol 4-phosphate 5-kinase in mammalian cells [27], raising the possibility that Ptd(4,5)P₂ does indeed play a role in mediating the assembly of vinculin-containing focal adhesions. Other molecules which might regulate the intramolecular head–tail interaction in vinculin include paxillin. Paxillin is rapidly and heavily phosphorylated by both tyrosine and serine/threonine [28] protein kinases during the assembly of focal adhesions, and this could increase its affinity for vinculin. The paxillin-binding site in vinculin (residues 978–1000) [8] is close to that for the N-terminal region of vinculin (residues 1029–1036), and paxillin binding could sterically inhibit the intramolecular association within vinculin.

Phosphorylation of vinculin itself may also play a role in regulating the vinculin head–tail interaction. The observation that the vinculin associated with the detergent-insoluble cytoskeleton is more heavily phosphorylated than that in the soluble compartment [29] is consistent with the view that phosphorylation is important in controlling the function of vinculin. Vinculin has been reported to be phosphorylated by PKC in vitro [15], and vinculin phosphorylation in both chick embryo fibroblasts and Swiss 3T3 cells has been shown to increase upon treatment with phorbol esters and calcium-elevating agents, although the levels of phosphorylation observed in these experiments were low [16]. Our studies show that vinculin is not a good substrate for various PKC isoforms, at least in vitro, but that phosphorylation is significantly increased by acidic phospholipids. Using recombinant proteins we have shown that the major PKC phosphorylation site is within the basic tail region of the molecule. Inspection of the vinculin tail sequence reveals several serine and threonine residues in the correct context for PKC phosphorylation sites (Arg/LysXXSer/ThrXXArg/Lys), as well as potential sites for cAMP-dependent protein kinase, cGMP-dependent protein kinase, AMP-activated protein kinase, casein kinase II and proline-dependent protein kinases. Cyanogen bromide cleavage of the vinculin tail polypeptide, phosphorylated in vitro by PKC in the presence of [γ³²P]ATP, liberated a single labelled polypeptide of 8 kDa. A polypeptide of this size can only be generated by cleavage at methionine residues 933 and 1007. This region (residues 934–1006) contains two serines (Ser-941 and Ser-999) within the context of PKC phosphorylation sites, but we have yet to establish whether one or both of these sites are used. We have been unable to demonstrate any phosphorylation of vinculin by casein kinase II or the AMP-activated kinase. Unfortunately, because the proportion of total cellular vinculin that is phosphorylated is low, it has not proved possible to map the site(s) that are phosphorylated in vitro. Nevertheless, these results raise the possibility that binding of acidic lipids to vinculin relieves the intramolecular association within the molecule, and that this conformation is stabilized by subsequent phosphorylation, allowing vinculin to bind to other cytoskeletal components. Analysis of the effects of acidic phospholipids and protein phosphorylation on the kinetics of the head–tail interaction in vinculin should help resolve this hypothesis.

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