Vol. 210, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 5, 1995 Pages 159-164

THE CARBOXY-TERMINAL TAIL DOMAIN OF VINCULIN CONTAINS A CRYPTIC BINDING SITE FOR ACIDIC PHOSPHOLIPIDS

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Received March 23, 1995

Using a gel filtration assay, we have characterized the binding of acidic phospholipids to vinculin. Vinculin binds phosphatidyinositol (K_d ~5 μM) in a reversible, saturable manner in low ionic strength buffers. This interaction is inhibited substantially at 100 mM NaCl and therefore may not be of physiological interest. In contrast, the carboxy-terminal 30-kDa fragment of vinculin, produced by *S. aureus* V8 protease cleavage, binds acidic phospholipids more tightly than the intact protein, and in a manner insensitive to 100 mM NaCl. Re-addition of the 95-kDa head fragment to the tail restores salt-sensitivity to the tail-lipid interaction. These data indicate that under physiologic ionic conditions, the intramolecular head-tail interaction in vinculin masks a high affinity acidic phospholipid binding site present in the tail domain.

Genetic experiments (1,2) demonstrate that vinculin is essential to the formation and function of adherens junctions (AJs), specialized sites of force transduction between the actin-based cytoskeleton and the extracellular environment (3-5). Insight into the the role of vinculin in these structures and the mechanism by which vinculin is recruited from the cytoplasm to newly forming junctions has been obtained through the discovery that vinculin contains an F-actin binding site in its carboxy-terminal, 30-kDa tail domian (6,7) that is masked in the intact protein by an intramolecular interaction between the tail and the amino-terminal, 95-kDa head domain (7). The head-tail interaction also reduces the affinity of vinculin for talin by at least five-fold (8), and may have a similar effect on the interaction of vinculin with α-actinin (9). These observations suggest that after activation to expose cryptic ligand binding sites, vinculin is recruited to junctions by the enhanced affinity of the activated molecule for other junctional components. Activated vinculin could also tether microfilaments to the adherens junction. Although vinculin associates *in vitro* with several other AJ proteins, including talin (10), the existence *in vivo* of these specific intercatiuons has not been demonstrated.

In addition to protein ligands, vinculin also binds anionic phospholipids (11), and can be labelled by photoactivatable hydrophobic probes incorporated into acidic phospholipid vesicles

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Abbreviations:AJ, adherens junction; PI, phosphatidylinositol; PC, phosphatidylcholine; PS, phosphatidylserine; PA, phosphatidic acid; PIP_2, phosphatidylinositol 4,5-*bis*phosphate; TEEA, 10 mM TrisHCl (pH 7.5), 1 mM EGTA, 0.1 mM EDTA, 3 mM NaN_3; BME, β-mercaptoethanol; TCA, trichloroacetic acid; SDS, sodium dodecylsulfate.

0006-291X/95 $5.00

(12). Interestingly, vinculin can also be labelled by such probes *in vivo*, following probe incorporation into primary chicken embryo fibroblasts (13). Vinculin labelled in these cells following photoactivation is predominantly associated with the crude membrane fraction. Although it remains to be determined whether this labelled pool of vinculin corresponds to AJ-associated protein, these observations suggest that the vinculin-lipid interaction may be physiologically relevant, and that vinculin might act as a direct link between F-actin and anionic phospholipids, major components of the cytoplasmic leaflet of the plasma membrane (14). To understand better the role of vinculin-phospholipid binding, we have attempted to characterize this interaction quantitatively using a gel filtration assay, and to delineate the region of vinculin responsible for lipid association.

Materials and Methods

Protein purification. Chicken smooth muscle vinculin and its *Staphylococcus aureus* V8 protease fragments were purified as described (8,15-17).

Phospholipid vesicles. Pure phospholipids were obtained from Avanti Polar Lipids (Alabaster,AL) and stored at (-)20° C in chloroform under N_2, except for PIP_2 (Calbiochem; La Jolla,CA), which was resuspended in H_2O and stored frozen at (-)20° C. Vesicles were prepared by rehydration, in octylglucoside solutions, of thin phospholipid films produced by evaporation of chloroform under a stream of N_2, followed by detergent removal to allow spontaneous vesicle formation (18). Dried phospholipid was resuspended in 10 mM TrisHCl (pH 7.5), 100 mM NaCl, 3% (wt/vol) *n*-octyl-β-D-glucopyranoside (Calbiochem), and dialyzed against 10 mM TrisHCl (pH 7.5), 100 mM NaCl and against 10 mM TrisHCl (pH 7.5) to remove detergent. When PIP_2 was included, this lipid was added in aqueous solution directly to the detergent-lipid mixture before dialysis. [^{3}H]Phosphatidylcholine (Dupont-NEN; Boston,MA) was routinely added to 0.1% (wt/wt) to serve as a tracer. Phospholipid concentrations were determined by the method of Ames (19).

Gel filtration. Proteins and [^{3}H]-labelled lipid vesicles were incubated as described in the figure legends in 10 mM TrisHCl (pH 7.5), 1 mM EGTA, 0.1 mM EDTA, 3 mM NaN_3 (TEEA) with 0.1% β-mercaptoethanol (BME) and with or without added NaCl or KCl as indicated. Phospholipid-bound and free protein were separated by gel filtration through a Sepharose 4B (Pharmacia Biotech; Piscataway,NJ) column (1 x 8 cm). Lipid vesicles elute relatively rapidly (< 10 min) in the void volume of this column. Phospholipid-containing fractions were identified by liquid scintillation counting. Because phospholipids interfere with several common protein assays, protein in column fractions was quantified using the method of Schaffner and Weissman (20). Briefly, protein was precipitated in 10% trichloroacetic acid (TCA), 1% sodium dodecyl sulfate (SDS) and spotted onto a Millipore (Bedford,MA) filter under vacuum. Spotted proteins were stained with Amido Black (J.T. Baker Chemical; Phillipsburg, N.J.), and the bound dye eluted and quantified spectrophotometrically at 630 nm.

Results and Discussion

The interaction of vinculin with acidic phospholipids can be demonstrated by gel filtration chromatography of vinculin-lipid mixtures in low ionic strength buffers (11). Vinculin partially co-elutes with vesicles composed of anionic phospholipids such as phosphatidylinositol (PI), while little to no protein coelutes with vesicles composed of phosphatidylcholine (PC), a neutral phospholipid (Fig. 1). The interaction of vinculin with anionic phospholipids is dependent upon ionic strength, and is inhibited approximately 80% at 100 mM NaCl or KCl (Fig. 2a). Under low ionic strength conditions, vinculin associates rapidly (within 10 min, data not shown) with PI vesicles to form a stable but reversible complex having a half-life of ~2 h (Fig. 2b). Vinculin binds PI with an apparent dissociation constant (K_d) of ~5 μM and a stoichiometry at saturation of 1 mol

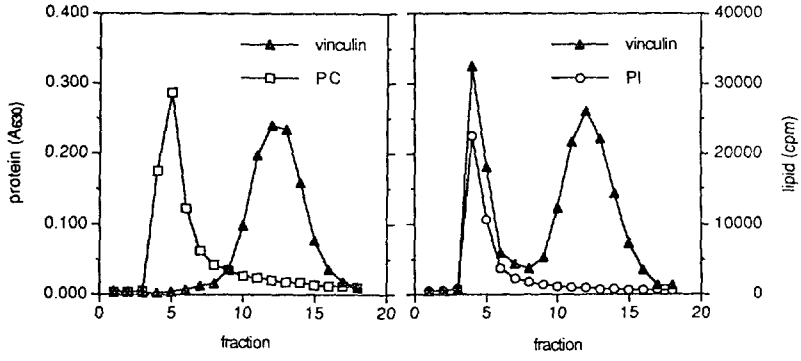

Figure 1. Gel filtration chromatography of vinculin in the presence of phospholipids. Vinculin (5 μM) was incubated with phosphatidylinositol (PI) or phosphatidylcholine (PC) vesicles (99.9% phospholipid:0.1% [^{3}H]-PC) at 25° C for 2 h in TEEA, 0.1% BME. Final phospholipid concentration was 500 μM. The reaction was applied to a 1 x 8 cm Sepharose 4B column and chromatographed in TEEA. Fractions were analyzed for lipid content by liquid scintillation counting and for protein by the method of Schaffner and Weissman (20).

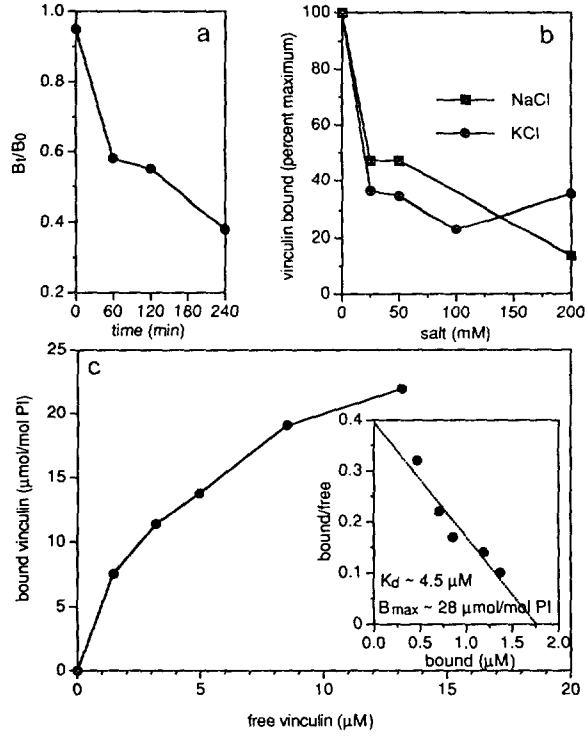

Figure 2. The interaction of vinculin and PI is reversible, saturable and ionic-strength dependent. *a.* Vinculin (3 or 5 μM) was incubated (2 h, 25° C) with PI vesicles in TEEA, 0.1% BME to which the indicated concentrations of NaCl or KCl were added. Separation and quantification of bound and free protein were as described. *b.* Vinculin (5 μM) and PI (1000 μM) were incubated (2 h, 25° C) in TEEA, 0.1% BME, and lipid-bound vinculin was separated from free protein by Sepharose 4B chromatography. Fractions containing lipid-bound protein were pooled and incubated at 25° C. At the indicated times, an aliquot of the vinculin-PI complexes was rechromatographed on Sepharose 4B. The total amount of vinculin reapplied to the column (B_0) and the amount remaining bound to PI (B_t) at time t were determined. *c.* Vinculin was incubated (2 h, 25° C) at various concentrations with PI vesicles (63 μM PI) and bound and free protein separated and quantified as described. *inset.* Data were analyzed by the method of Scatchard (24) to determine the apparent dissociation constant (K_d) and stoichiometry at saturation (B_{max}).

vinculin/30 mol PI (Fig. 2c). These observations extend earlier findings (11,12) to demonstrate that vinculin interacts electrostatically with acidic phospholipids in a reversible, saturable manner.

Comparison of the amount of vinculin co-eluting with various phospholipids (Fig. 3) indicates that vinculin does not distinguish significantly between the major classes of anionic phospholipid, such as PI, phosphatidylserine (PS) and phosphatidic acid (PA), essentially in agreement with earlier reports (11,12). However, we find that vinculin binds more extensively to vesicles containing phosphatidylinositol 4,5-*bis*phosphate (PIP_2), indicating a preference for polyphosphoinositides (Fig. 3). This observation provides *in vitro* evidence to support the recent observation that vinculin may bind PIP_2 *in vivo* (21). Interestingly, polyphosphoinositides have been found to regulate the activities of a number of actin binding proteins (22).

Vinculin can be cleaved by *S. aureus* V8 protease to produce 95-kDa head and 30-kDa tail fragments (17). Analysis of the ability of these domains to interact with acidic phospholipids indicates that the 30-kDa tail domain binds better to anionic phospholipids than does intact vinculin, and that its binding is relatively insensitive to 100 mM NaCl (Fig. 4, lanes 2-5 *vs* lanes 10-13). The 95-kDa head fragment interacts weakly with PI vesicles in a salt-sensitive manner (Fig. 4, lanes 6-9). Re-addition of the 95-kDa head to the 30-kDa tail renders the interaction of the 30-kDa tail with PI vesicles ionic strength sensitive (Fig. 4, lanes 14-17). This result indicates that binding of the 95-kDa head to the 30-kDa tail (8) alters the properties of the lipid binding site in the tail so as to mask this binding site under more physiologic ionic strength conditions (100 mM NaCl). This masking effect of the head-tail interaction on acidic phospholipid binding is similar to its effect on binding of the tail domain to F-actin (7) and on binding of the head domain to the focal adhesion protein talin (8).

Curiously, the 30-kDa tail appears to enhance association of the 95-kDa head with PI vesicles under low ionic strength conditions (Fig. 4, lanes 6,7 *vs* lanes 14,15). This effect was also observed when the 30-kDa domain was incorporated into proteoliposomes *via* freeze-thaw of

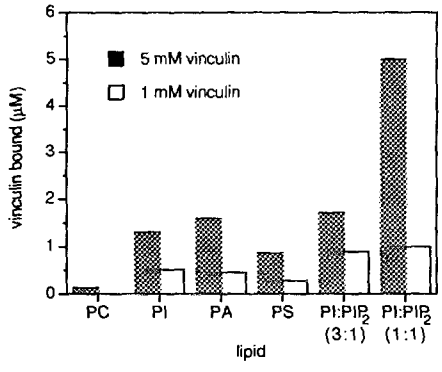

Figure 3. Vinculin binds PIP_2 preferentially compared to other acidic phospholipids. Vinculin (1 or 5 μM) was incubated (3 h, 25° C) with the indicated phospholipid vesicles (500 μM total phospholipid) in TEEA, 0.1 % BME. Bound and free protein were separated and quantified as described. Vinculin binds somewhat better to 3:1 PI:PIP_2 vesicles than to pure PI vesicles and binds quantitatively to 1:1 PI:PIP_2 vesicles. Mixed composition vesicles were utilized here because in the absence of other phospholipids, PIP_2 spontaneously forms micelles (rather than vesicles) which cannot be separated from the protein by this gel filtration assay.

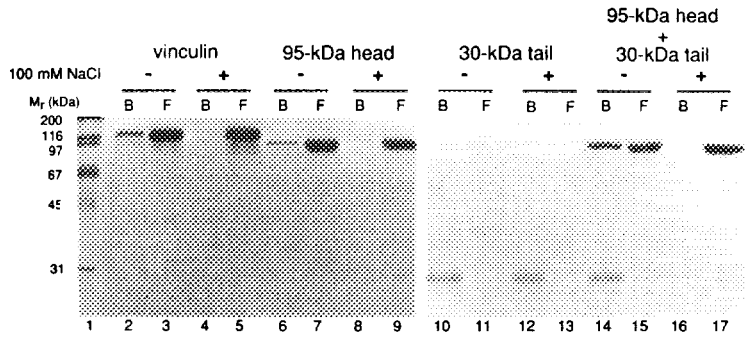

Figure 4. An acidic phospholipid binding site in the carboxy-terminal tail domain of vinculin is masked in the intact protein at physiologic ionic strength. Vinculin or its 95-kDa head or 30-kDa tail domains were incubated (3 h, 25° C) individually or in the indicated combination at 6 μM with PI vesicles (600 μM PI). Incubations were performed in TEEA, 0.1% BME with or without 100 mM NaCl as indicated. Lipid-bound and free protein were separated by gel filtration. Equal aliquots of the pools of PI-bound (B) and free (F) protein were adjusted to 10% TCA. Precipitated protein was recovered by centrifugation (30 min, 12,000*g*) and analyzed by SDS-polyacrylamide gel electrophoresis (25) on a 12 % polyacrylamide gel.

lipid-protein mixtures, a procedure used to study the interaction of talin with anionic phospholipids (23). The 95-kDa head fragment incorporated more efficiently into 30-kDa domain-PI proteoliposomes formed during freeze-thaw than into liposomes containing PI alone (data not shown). We interpret these data to indicate that in this reconstitution experiment the head domain does not competitively inhibit acidic phospholipid binding to the tail, but rather alters the sensitivity of the binding site to ionic strength. Under low ionic strength conditions a triple complex of 95-kDa head:30-kDa tail:phospholipid is possible, whereas at high ionic strength (100 mM NaCl) the affinity of the tail for lipid is sufficiently reduced by the head-tail interaction that no protein-lipid complex forms under the conditions tested. It is possible that in intact vinculin, where the two domains are linked covalently through the peptide backbone, the head domain is more effective at masking the phospholipid binding site in the tail under low ionic strength conditions. This interpretation provides an explanation for the relatively low affinity, salt-sensitive acidic phospholipid binding observed with the intact protein compared to the free tail fragment.

In conclusion, this report demonstrates that the carboxy-terminal tail domain of vinculin contains a high-affinity acidic phospholipid binding site, in addition to a site or sites mediating F-actin binding (6,7) and crosslinking (7). The ability of the tail domain of vinculin to interact both with anionic phospholipids and with microfilaments is modulated under physiological buffer conditions by the intramolecular head-tail interaction (7, this work). We have postulated that this intramolecular interaction regulates the affinity of vinculin for its ligands and thereby its recruitment into adherens junctions (7). Assembly of vinculin into AJs is envisioned to require activation of the protein *via* disruption of the head-tail association through the action of cellular signalling events. Based on the observations reported here, we anticipate that in an activated state vinculin can directly mediate F-actin bundling and attachment to the plasma membrane.

Acknowledgments

This work was supported by grants from the National Institutes of Health and the Muscular Dystrophy Association.

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