Conformational Change in the Vinculin C-terminal Depends on a Critical Histidine Residue (His-906)*

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Vinculin is a cytoskeletal protein involved in the attachment of the actin cytoskeleton to the plasma membrane at some types of cell junctions (1). Although the precise role of vinculin within adhesions is not clear, it is critical for mammalian development (2) and is highly conserved from nematode to human (3). It is a multidomain protein with a 90-kDa globular head and a 30-kDa tail connected by a proline-rich domain (4). Each of these domains has been shown to have binding sites for several other proteins (e.g. talin, vasodilator-stimulated phosphoprotein, actin), and thus vinculin may function as an integrator or sensor (reviewed in Refs. 5–8). In cells vinculin is found in soluble form as well as bound in the dense assemblies of proteins at adhesion sites (9). Its incorporation into adhesion sites has been shown to be dependent upon its interaction with other proteins (10), and it is thought that interactions between its N- and C-terminal domains control its binding proclivities (11–14). Thus the observed tight association of the N-terminal and C-terminal domains within the soluble protein acts to mask binding sites for several adhesion and signaling proteins. This self-association has been shown to be decreased byPIP2-1 (15, 16), implicating this lipid in the regulation of vinculin location and activity.

The interaction between the N- and C-terminal domains is of great interest because it is key to understanding vinculin regulation. The interacting regions of the domains have been mapped to small oppositely charged sequences (15, 17), but how binding might be turned on and off is not known. Conformational changes in the C-terminal domain have been detected by alterations in limited proteolysis patterns or cross-linking (18, 19), suggesting that the domain undergoes structural rearrangements in response to lipids or upon actin binding. Circular dichroism spectroscopy also points to conformational changes in the domains upon binding to each other (17). Despite the recent determination of the three-dimensional structure of the C-terminal domain showing a mainly α-helical bundle (19), the nature of such conformational changes is not clear. Because lipid binding sites have been mapped to the C-terminal (20–22), it may be changes in this domain that result in vinculin activation by altering the binding site for the N-terminal domain. In this model dissociation of the N-terminal would then expose binding sites in both domains, but further evidence is necessary to clarify the events.

A few observations point to a key role for histidine residues in the N-terminal-C-terminal interaction. First, proteolytic cleavage of native vinculin left the head and tail regions of the molecule still tightly associated, but they could be separated at lower pH (pH 5) (23). Our own work has shown a pH-sensitive interaction between the expressed N-terminal (amino acids 1–266) and C-terminal (amino acids 877–1066) domains, with half-maximal inhibition occurring between pH 6 and 7 (17). Second, truncation of the last 15 residues from the C-terminal domain left a molecule that could not bind lipid at neutral pH but could do so at pH 5.5 (19). Thus at least two properties of vinculin change in the pH range where histidine side chains become protonated. Third, there are several highly conserved histidine residues in both the N- and C-terminal domains.

With the aim of understanding vinculin regulation, we have previously expressed in bacteria and characterized the two terminal domains of the molecule (17). Here we report studies investigating the potential role of histidine residues in the interaction between them. Evidence gleaned from chemical modification and mutagenesis of two histidines in the C-terminal implicates histidine 906 as a key player in conformational changes that affect lipid binding and oligomerization as well as interdomain binding. This points to ionic interactions as a driving force in C-terminal conformational flexibility.

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1 The abbreviations used are: PIP2, phosphatidylinositol 4,5-bisphosphate; DEPC, diethylpyrocarbonate; PI, phosphatidylinositol; MES, 4-morpholineethanesulfonic acid; apoE, apolipoprotein E.
EXPERIMENTAL PROCEDURES

Recombinant Proteins—Recombinant DNA techniques were from Sambrook et al. (24). Vinculin constructs V-(1–266) and V-(877–1066) were cloned, expressed, and purified as described (17). Site-directed mutagenesis of His-906 and His-1026 to alanine was performed using a polymerase chain reaction protocol (25). The mutagenic primers used were CAGACAGCTGCTGATAGAAC for H906A and CAGACAGCTGCTGCGCCGAC for H1026A. The resulting polymerase chain reaction products were cloned, sequenced, and placed in pGEX-KG (26). To make the double H906A,H1026A mutant, restriction fragments containing the individual mutations were ligated together. Proteins were induced, isolated, and cleaved from their glutathione S-transferase partner as described (17).

Chemical Modification of Histidine Residues—Vinculin domains V-(1–266) and V-(877–1066) (25 μM) were reacted with DEPC (0.5 mM) in 20 mM MES, pH 6.5, 75 mM NaCl, pH 7.0, at 20 °C for 10, 30, and 60 min prior to quenching with 2 mM imidazole. Following dialysis to remove unreacted DEPC, the number of modified residues was determined by measuring the increase in absorbance at 240 nm (ε = 3200 M⁻¹ cm⁻¹) (27). There was no significant difference in the degree of DEPC modification between the 10- and 60-min reactions.

Circular Dichroism—The far-UV spectra of vinculin domains were measured using a Jasco-J810 spectropolarimeter. Solutions contained 2.5 μM V-(877–1066), V-(877–1066/H906A), V-(877–1066/H1026A), or V-(877–1066/H906A + H1026A) in 20 mM MES, pH 7.0, 25 mM NaCl, 3 mM MgCl₂, and 1 mM EGTA. Spectra were measured between 190 and 260 nm.

The effect of PI on the spectra of each construct was determined. Solutions contained 2.5 μM V-(877–1066), V-(877–1066/H906A), V-(877–1066/H1026A), or V-(877–1066/H906A + H1026A) and 100 μM PI. PI in buffer alone did not give measurable spectra within the 190–260 nm range. Determination of the α-helical content of the constructs was determined using the CDNN deconvolution program (28).

Solid-phase Protein-binding Assay—Protein-protein interactions were measured using a solid-phase binding assay as described (17). Binding was performed in 20 mM MES, pH 7.0, 50 mM NaCl, 3 mM MgCl₂, 1 mM EGTA.

The activity of the DEPC-modified domains was determined by competition assay. V-(877–1066) was coated on the wells of a 96-well plate, and binding of 125I-labeled V-(1–266) was determined in the presence of the DEPC-modified proteins. The Ki of DEPC-modified protein and PI inhibition of the interaction between V-(1–266) and V-(877–1066) or V-(877–1066/H906A) was calculated using the Cheng-Prusoff equation (29): K_i = IC_{50}/(1 + [DEPC]-labeled V-(1–266)/K_i).

The effect of phospholipids on protein adsorbed to the 96-well plates was determined by coating wells with unlabelled protein at 15 μg/mL and 125I-labeled protein at trace concentrations for 2 h. Wells were then blocked with 5% bovine serum albumin in Tris-buffered saline for 2 h. After blocking, the amount of labeled protein remaining on the plate was not significantly different from control wells incubated for 10 h with buffer alone.

Size Exclusion Gel Filtration Chromatography—Size exclusion chromatography of the various proteins was performed using a 24-mL Superose 12 HR column (Amersham Pharmacia Biotech). Samples of 25 μg were run at 0.5 mL/min in 10 mM Tris-HCl, pH 7.5, 75 mM NaCl, 1 mM EDTA, and 1 mM dithiothreitol.

A sample of each fraction was run on an SDS-polyacrylamide gel (15%), and the intensity of the band was measured using densitometry (Alpha Innotech Corp.). Data were plotted as the percentage of the total protein eluting from the column measured in each fraction.

Phospholipid Binding Assay—PI (Doosan-Serdy Research Laboratories) was dissolved at 10 mg/ml in chloroform. PI was dried down from a chloroform stock under nitrogen gas and suspended in 20 mM MES, pH 6.8, 1 mM EGTA, and 75 mM NaCl. The lipid suspension was sonicated (5 × 2 min) on ice.

Vinculin domains were labeled using IODO-GEN (Pierce, Brockville, Ontario) as described (30), and lipid binding was assayed using a gel filtration column. Protein samples were incubated with PI vesicles for 30 min at room temperature before 100-μl samples were loaded onto Ultrogel AcA 34 columns (8 × 0.5 cm) (LKB, Bromma, Sweden). Binding was performed in 20 mM MES buffer, pH 6.8, 75 mM NaCl, 3 mM MgCl₂, 1 mM EGTA. Fractions were collected in 200-μl aliquots. Each fraction was counted in a Wallac model 1470 γ-counter. Elution patterns of each protein were determined in the presence and absence of phospholipid. Phospholipid content in each fraction was measured using a detergent-dye solubilization assay as described (31).

RESULTS

Effect of DEPC Modifications on V-(1–266)-V-(877–1066) Binding—To directly probe for the role of histidine residues in the N-C domain interaction, each domain was treated separately with DEPC, which reacts specifically with histidine residues to produce carboxyethylhistidine. The extent of reactions was quantified by measuring the increase in absorbance of the modified peptides at 240 nm (ε = 3200 M⁻¹ cm⁻¹) (27). Maximal modification occurred within 10 min, and histidine residues in V-(1–266) were found to be 48 ± 15% modified (2.4 ± 0.6 of 5 histidine residues) whereas construct V-(877–1066) was found to be 90 ± 18% modified (>1.4 residues out of 2 modified).

The effect of this modification on the structures of the proteins was examined using circular dichroism. For both V-(1–266) and V-(877–1066) there were measurable differences in the determined secondary structure. The unmodified V-(1–266) was calculated to be 64% α-helical, which decreased to 59% after DEPC, whereas the V-(877–1066) was found to be 43% helical before and 37% after DEPC treatment (data not shown).

Interaction of the modified domains was measured using a solid-phase binding assay (Fig. 1). Unmodified V-(877–1066) was coated on 96-well assay plates, and binding of 125I-labeled V-(1–266) was measured in the presence of variable concentrations of DEPC-modified V-(1–266), V-(877–1066), or the unmodified domains. The measured K_i values for the modified proteins were 61 ± 14 nM for V-(1–266) compared with 87 ± 20 nM for the unmodified construct and 367 ± 45 nM for the modified V-(877–1066) compared with 70 ± 15 nM for the unmodified construct. These results provide strong evidence for the importance of histidine(s) in the C-terminal domain.
Effect of Histidine Mutations on V-(877–1066) Activity and Conformation—Comparison of the vinculin sequences from four different species and one meta-vinculin sequence shows that the two histidines (His-906 and His-1026 from the human vinculin sequence) in the tail domain are totally conserved although they are not found in the related catenins. To examine the role of these histidines, three mutant constructs were made, expressed, and purified: the single mutants V-(877–1066/H906A) and V-(877–1066/H1026A), as well as the double mutant V-(877–1066/H906A,H1026A), for comparison with the wild type tail domain.

The effect of the histidine mutations on the ability of the tail domains to bind to V-(1–266) was determined using a solid-phase binding assay. At pH 7.0, V-(877–1066) bound to V-(1–266) with a $K_d$ of 90 ± 30 nM, whereas the mutants V-(877–1066/H906A), V-(877–1066/H1026A), and V-(877–1066/H906A,H1026A) bound with $K_d$ values of 110 ± 20, 80 ± 10, and 130 ± 30 nM, respectively (Fig. 2A). Thus, surprisingly, at pH 7.0, there was no significant difference between the binding affinities of the wild type vinculin tail and the mutants. Because the $pK_a$ of histidine is near 6, we also checked lower pH values. At pH 5.5 V-(877–1066) bound to V-(1–266) with a $K_d$ of 680 ± 110 nM showing a large decrease in binding affinity but with no loss in total binding capacity. This finding is consistent with the observation that low pH allows separation of the vinculin head and tail (23). The mutant proteins containing the His-906 mutation, however, did not show the decreased affinity at lower pH (Fig. 2B). The mutant C-terminal domains with the His-906, His-1026, or both mutations bound to V-(1–266) with $K_d$ values of 120 ± 30, 470 ± 80, and 210 ± 40 nM, respectively.

Therefore, the H906A mutation and not the H1026A seems to confer resistance to the loss of affinity at pH 5.5.

To establish that the changes in binding affinity induced by the DEPC treatment resulted from His-906 modification, the H906A mutant was treated with DEPC. This modified protein was then tested for its ability to compete with V-(877–1066) for binding to V-(1–266). The $K_i$ values for the competition of V-(877–1066), V-(877–1066/H906A), and the DEPC-modified V-(877–1066/H906A) in this assay were 85 ± 20, 140 ± 35, and 115 ± 30 nM, respectively (data not shown). Thus the H906A mutation protected the C-terminal domain from loss of binding affinity for V-(1–266) by DEPC treatment.

The effect of the two single mutations and the double mutation on the structure of the vinculin tail domain was studied using circular dichroism (Fig. 3). At pH 7.0 there was little difference between the measured circular dichroism spectra of the histidine mutant and the unmutated V-(877–1066). At pH 5.5, however, the spectra showed measurable changes. In the wild type, ellipticity at 208 nm was decreased at pH 5.5, causing the ratio $[\theta_{222}] / [\theta_{208}]$ to increase from 0.87 at pH 7.0 to 1.07 at pH 5.5 (Fig. 3A). This change was also observed for the H1026A mutant but not for V-(877–1066/H906A) (Fig. 3B and C). To define more precisely the pH dependence of the conformational transition, circular dichroism spectra were collected over a range of pH values. The conversion between $[\theta_{222}] / [\theta_{208}] < 1$ to $>1$ occurs at pH ~6.5 (Fig. 4), which suggests that this conversion, like the conformational change in general, is reliant upon the histidine residue. The H906A mutant showed no, or very little, change, strongly supporting this idea.

Gel Filtration Chromatography of V-(877–1066) and Histi-
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**Fig. 4.** Change in the $\theta_{208}$ and $\theta_{222}$ of V-(877–1066) and histidine mutants with pH. Circular dichroism spectra were collected for V-(877–1066) (A), V-(877–1066/H906A) (B), and V-(877–1066/H1026A) (C) between 200 and 260 nm. Spectra were collected over a pH range of 4.5–8.0. Data are plotted as the $\theta_{208}$ (○) and $\theta_{222}$ (●).

**Fig. 5.** Gel filtration chromatography of V-(877–1066) and histidine mutants at pH 7.0 and 5.5. Elution profiles of V-(877–1066) (A), V-(877–1066/H906A) (B), and V-(877–1066/H1026A) (C) at pH 7.0 (○) and at pH 5.5 (●). Data are presented as the percentage of total protein that eluted from the column as measured by densitometry of fractions run on SDS-polyacrylamide gel electrophoresis. Standards used to calibrate the column are indicated (IgG, 160 kDa; bovine serum albumin, 66.2 kDa; β-lactoglobulin, 35 kDa; and cytochrome c, 12.4 kDa).

**Fig. 6.** Phospholipid binding of V-(877–1066/H906A). Elution profiles of 125I-labeled V-(877–1066/H906A) (50 ng) in the presence (○) or absence (●) of PI vesicles. The elution volume of PI vesicles was determined using a detergent solubilization assay and is presented as the percent of total PI measured eluting from the column (●).

**Fig. 7.** Circular dichroism of both the wild type (not shown) and similarity in binding, the H906A mutant did not undergo lipid-induced changes in its circular dichroism spectrum (Fig. 7). Circular dichroism of both the wild type (not shown) and

dine Mutants—To examine possible consequences of the conformational changes seen at low pH, gel filtration profiles of V-(877–1066) and each of the histidine mutants were studied under different pH conditions (Fig. 5). V-(877–1066) was found, at physiological pH, to elute with a molecular weight ($M_r$) of ~25,000, which is the approximate $M_r$ of the monomeric species. However, upon lowering the pH to 5.5, the eluting peak was shifted, indicating a $M_r$ of >100,000. Similar profiles were obtained when examining V-(877–1066/H1026A) (Fig. 5C). The elution profile of V-(877–1066/H906A), however, was much less affected by acidic pH (Fig. 5B).

**Effect of Histidine Mutations on Lipid Inhibition of Head-Tail Binding**—It is evident that both activity and conformational changes induced by lowered pH were abrogated by mutation of histidine 906 to a nonionizable residue. To test the possibility that this residue may also affect the activity and conformational changes induced by vinculin ligands, phospholipid was tested for its ability to alter the conformation and activity of V-(877–1066/H906A).

Using a gel filtration-based lipid-binding assay, V-(877–1066/H906A) was found to interact with PI vesicles (Fig. 6), like the wild type domain. Sixty-six percent of the mutant co-eluted with lipid, compared with 63% of the wild type. Despite this
H1026A mutant (Fig. 7) showed a roughly 20% decrease in the absolute value of the ellipticity in the 208–222 nm range in the presence of 100 mM PI. In contrast, the H906A spectrum remained unaltered in the presence of the lipid. These spectral changes were different from those seen at low pH (Fig. 3), but they still depended on the presence of histidine 906.

Because acidic lipids, notably PI and PIP2, inhibit N-C interactions, it was of interest to determine whether the interaction between the H906A mutant and V-(1–266) would be similarly affected. A range of concentrations of PI was included in an N-C binding assay (Fig. 8). Although lipid could interfere with H906A-N-terminal interactions, 5–10-fold higher concentrations were required. The \( K_i \) of PI inhibition was determined to be 104 \( \pm \) 1.5 mM, in contrast to the \( K_i \) of 17 \( \pm \) 1.4 mM determined for the PI inhibition for V-(1–266) binding to wild type V-(877–1066).

**DISCUSSION**

Efforts to purify the tightly associated N- and C-terminal domains of vinculin after proteolytic cleavage of native vinculin first led to the application of low pH (pH 5.0) to separate the domains (23). Later work made it clear that the N-C interaction is a major regulatory mechanism and controls the binding activities of the molecule (12). Inside cells, the lipid PIP2 is likely the factor that causes dissociation of the two domains (15, 16), but the mechanism has not been studied. One possibility would be a simple competition for binding to the C-terminal domain between the N-terminal and the lipid; however, different conformations of the C-terminal have been detected by limited proteolysis suggesting more complicated pathways. Several factors pointed to histidines as possible key residues, and our experiments reported here have amply confirmed their importance.

Our initial experiments showed that DEPC, a specific modifier of histidine residues in proteins (32), inhibited the N-C interaction by modifying residues in the C-terminal. This limited the possible targets to two histidines at positions 906 and 1026. These residues are located in quite different regions of the C-terminal crystal structure (Fig. 9). Because we had mapped the binding site for the N-terminal domain to residues 1009–1036 (17), we anticipated that His-1026 would be a target and used mutagenesis to alanine to test this idea. Surprisingly, neither the H906A nor the H1026A mutant showed defects in binding to the N-terminal at neutral pH, raising the possibility that DEPC was reacting with some other residue. To clarify the situation, DEPC treatment of the mutants was performed, and this showed that His-906 was indeed the important target. Thus, DEPC modification of histidine 906 inhibited binding to the N-terminal domain whereas a different modification (to alanine) did not. Possibly the increase in size of the histidine side chain resulting from chemical modification changed the structure enough to prevent binding. In support of this idea, the circular dichroism spectra indicate a somewhat different conformation after treatment. When a smaller side chain was substituted, no such conformational change occurred, and binding to the N-terminal domain could take place. One possibility is that electronic repulsion between the protonated histidine and other nearby positively charged residues may be a driving force for conformational change that is mimicked by the steric repulsion due to the larger size of the histidine side chain after DEPC treatment.

To look for a possible role of this histidine in conformational dynamics we examined the effects of pH on normal and mutant C-terminal domains. Circular dichroism spectra showed clear...
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There was little change in the 222 nm region that reflects the amount of $\alpha$-helix in the protein. Rather, ellipticity is decreased at 208 nm, changing the 208/222 ratio, often taken to indicate a rearrangement of $\alpha$-helices (33). Interestingly, one consequence of the change in conformation is a shift to a larger apparent size by gel filtration, probably corresponding to an oligomerization although a change in the shape of the molecule could contribute to it. Oligomerization of the C-terminal in the presence of phospholipids has been detected by cross-linking, and specific regions prone to oligomerization have been identified. One possibility is that the molecule "opens up" as postulated by Bakolitsa et al. (19) to expose its more hydrophobic interior that could then interact to form oligomers. Structural determination of the low pH-induced conformation will be needed to answer this question.

Because the H906A mutant no longer undergoes conformational changes at low pH, protonation of His-906 in the wild type must be a key step, and the charged side chain then results in changes in the binding site for the N-terminal, some distance away in the molecule. The environment of His-906 in the crystal structure gives few obvious clues to a mechanism for this effect. The histidine is found in a basic region including arginines 903 and 910 and lysine 924 (Fig. 9). Phenylalanine 885 and aspartate 907 are also close neighbors. One interesting feature of the crystal structure is the presence of a sulfate ion in a pocket formed by His-906, Arg-910, and Lys-1061. This ion is likely a substitute for the natural ligand, possibly a phosphate or a carboxyl group. The area could provide a binding surface for negatively charged lipids. Nearby negative charge would also be expected to increase the $pK$ of the histidine from the value of 6.0 for the free amino acid, possibly explaining the pH of 6.5 for the conformational change. Protonation of the histidine in a positively charged environment could lead to electrostatic strain that triggers shifts in the relative position of the helices.

The reduced sensitivity of the H906A mutant to binding inhibition by PIP2 indicates that the histidine plays a role in phospholipid effects as well. Because binding to PIP2 by the mutant is normal, loss of the initial interaction is not an explanation. The lack of change in the circular dichroism spectrum of the mutant compared with the wild type protein in the presence of PIP2 suggests that a requisite conformational change does not occur in the mutant. The characteristics of the lipid-induced change in circular dichroism seen in the wild type C-terminal are different from those induced by low pH. Thus His-906 appears to be important for two different conformational changes. There is some evidence that the vinculin C-terminal also has a different conformation when bound to actin (18, 19). It will be of interest to determine whether the H906A mutation affects that interaction as well.

Reduction of pH has been reported to mimic the effect of phospholipid binding to other proteins. In particular, apolipoprotein E (apoE) undergoes conformational changes at low pH analogous to those that occur upon its interaction with phospholipid (34). This example may be of particular relevance as the N-terminal portion of apoE is structurally similar to the vinculin C-terminal (19). The helices of the apoE four-helix bundle are believed to reorient, resulting in an extended conformation with increased phospholipid binding and insertion activity, and a parallel model has been proposed for vinculin. There are, however, no histidines in this region of apoE comparable with histidine 906 in vinculin so the mechanism must be different. Lower pH may affect a variety of interhelix interactions and, in apoE, allow expression of conformational flexibility. In fact, it has been proposed that the negatively charged, polar head groups on acidic lipids increase the electrical surface potential in membranes, which leads to a decrease in the surface pH (35). Thus a decrease in pH will have similar effects on lipid binding for some proteins, with similar intramolecular mechanisms. Further experiments will be needed to see if this idea is applicable here, although the different circular dichroism spectra of lipid-bound versus low pH C-terminal suggests that the conformations are different.

In summary, it is clear that His-906 plays a key role in the conformational changes of the vinculin C-terminal domain. In turn, the overall conformation and activity of vinculin is controlled by these changes. Thus the H906A mutant will likely be useful in examining how the protein functions and how it is regulated in its natural environment.

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