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Intramolecular interactions in vinculin control α-actinin binding to the vinculin head

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Abstract Using blot overlay techniques we have investigated the interaction of vinculin with α-actinin. We show that an α-actinin binding site is located in the 90 kDa vinculin head and confirm a vinculin binding site in the C-terminal rod of α-actinin, as recently reported by McGregor et al. [(1994) Biochem. J. 310, 225–233]. The isolated vinculin head binds much more strongly to α-actinin than intact vinculin. Using a proteolytic 81 kDa head fragment, we show that vinculin residues 1–107 are required for α-actinin binding. Antibodies directed against vinculin residues 808–850 inhibit the vinculin–α-actinin binding, suggesting that this sequence is directly involved in, or topographically related to, the α-actinin binding site.

Key words: Vinculin; α-Actinin; Intramolecular interaction; Cytoskeleton

1. Introduction

Microfilament-associated cell–cell and cell–matrix junctions both contain α-actinin and vinculin [2,3] at their cytoplasmic faces. In vitro, both proteins are known to interact with other components of the junctional complexes. In the case of α-actinin which forms homodimers in solution, a direct binding to actin, zyxin, nebulin, clathrin, β1-integrins and to vinculin has been documented (for review see [4]). On the other hand, vinculin has been shown to bind to talin [5,6], paxillin [7], α-actinin [1,8–10], actin [11–13], and also to itself [1,5,10,14].

While the biological role of α-actinin resides probably mainly in its F-actin-crosslinking capacity, the function of vinculin in adherens junctions is less clear. However, it is well documented that it plays a crucial role in the organisation and maintenance of cellular morphology and adhesiveness [15–17]. The in vivo existence of several independent binding sites for junctional proteins in vinculin has been shown by the targetting of non-overlapping fragments to adhesion sites [18]. These findings have raised the question on how specific vinculin-ligand interactions might be regulated. An attractive hypothesis in this context is based on the assumption that the vinculin molecule displays a certain degree of flexibility, allowing for the controlled exposure of discrete ligand binding sites. Intramolecular mobility has been postulated from dynamic light-scattering data [19], and Johnson and Craig have shown a strong association of vinculin head and tail domains as derived by V8 proteolytic cleavage [20]. This interaction influences the binding affinity for talin, and probably that for phospholipids [21] and for actin [13].

Here, we have characterized the binding site of α-actinin in the vinculin head and demonstrate that the vinculin tail domain in intact vinculin molecules greatly influences the binding affinity for α-actinin, possibly by head–tail interactions.

2. Materials and methods

Vinculin and α-actinin were purified from turkey gizzard according to Feramisco and Burridge [22]. α-Actinin (2 mg/ml in buffer B (20 mM Tris-acetate, 0.1 mM EDTA, 0.2 mM DTE, pH 7.6)) was treated with thermolysin (Sigma) at 5% by weight for 45 min at 37°C. Thermolysin was inactivated by the addition of EGTA to a final concentration of 2 mM. The N-terminal 27 kDa fragment and the C-terminal 53 kDa fragment were purified by ion-exchange chromatography as described [23].

5 mg of endoproteinase Glu-C (V8 from *Staphylococcus aureus*; ICN Biochemicals) was immobilized on a 1 ml HiTrap column (Pharmacia) according to the manufacturer's instructions. Vinculin (0.5 mg/ml in buffer B) was incubated with 10 μg of immobilized V8 protease/ml for 90 min at 37°C as recently described [20]. The 90 kDa vinculin head and the 29/27 kDa tail fragments were purified from digests by ion-exchange chromatography as described [24]. 81 kDa vinculin head fragments were prepared by incubating purified 90 kDa head (0.5 mg/ml in buffer B) with 3 μg/ml V8 for 60 min at 37°C basically as described [20].

For blot overlays, mixtures containing equal amounts of α-actinin and each of the purified fragments were separated by SDS-PAGE and blotted onto nitrocellulose filters. After blocking with 5% dried milk in PBS, blots were overlayed with vinculin or its purified fragments (0.4 mg/ml in buffer B) for 3 h at room temperature. Bound vinculin fragments were detected by monoclonal antibodies As8, 1E4 and 1A10 that recognize the N-terminal 90 kDa and 81 kDa fragments or by monoclonal antibody 4E7 directed against the C-terminal 27 kDa vinculin tail fragment as indicated. Blots were developed with Horseradish peroxidase-labelled rabbit anti-mouse secondary antibodies (Sigma) and enhanced chemoluminescence (ECL, Amersham).

Protein concentrations were determined according to Bradford [25].

For N-terminal analysis, purified protein was blotted onto PVDF membranes (Immobilon P, Millipore) and sequenced, using an Applied Biosystems gas-phase sequenator (model A470) equipped with an on-line PTH-amino acid analyser.

For mass spectrometric investigations proteins were extensively dialyzed against water and analyzed by matrix-assisted laser desorption ionisation (MALDI) mass spectrometry using a Bruker REFLEX MALD/TOF with sinapinic acid as matrix.

3. Results

3.1. Characterization of ligand probes

The experiments described below were carried out with the following probes (Fig. 1A): (i) intact gizzard vinculin; (ii) V8-cleaved vinculin, comprising a large N-terminal head domain ('90 kDa head') and a smaller fragment derived from it ('81 kDa head fragment'), and two C-terminal tail fragments ('29/27 kDa tail'). The preparation used here contained the 29 kDa piece as

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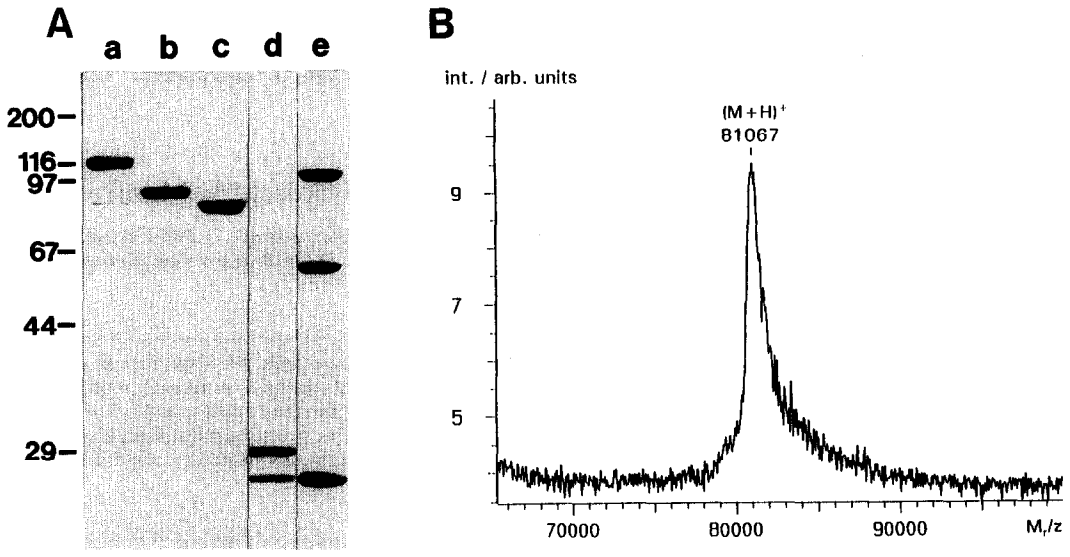

Fig. 1. Protein characterization by SDS-PAGE (A) and mass spectrometry (B). (A) Coomassie-stained gel of probes. Lanes: a, vinculin (116 kDa); b, vinculin head (90 kDa); c, vinculin 81 kDa head fragment; d, vinculin tail domain (29/27 kDa); e, α-actinin and the thermolysin derived 53 kDa and 27 kDa fragments. (B) Mass spectrum of the 81 kDa vinculin head fragment. The molecular mass of the molecular ion $(M + H)^+$ is indicated.

the major tail fragment; (iii) intact gizzard α-actinin; (iv) thermolysin-cleaved α-actinin, comprising an N-terminal fragment harbouring the actin-binding site ('27 kDa fragment') [26], and a 53 kDa C-terminal fragment ('α-actinin rod').

The vinculin 90 kDa head has been shown to contain amino acid residues 1–857, corresponding to a calculated mass of 92.7 kDa [27], while the 81 kDa head fragment has not been characterized so far. Therefore, we analyzed it (Fig. 1A, c) by N-terminal sequencing and by mass spectrometry. Fig. 1B shows the MALDI mass spectrum yielding a molecular weight of 81.070 ± 100 Da. N-Terminal sequencing showed that this fragment starts at position 108 of the vinculin amino acid sequence. From these data, it can be deduced that this degradation product of the 90 kDa head contains vinculin residues 108–850 with a calculated molecular mass of 81,087 Da. Analysis of the 29/27 kDa tail fragments by N-terminal sequencing revealed the starting point at residues 851 and 858, respectively, as already reported [27], but this does not exclude an additional C-terminal degradation. MALDI mass spectrometry of the isolated 29 kDa tail fragment yielded a molecular weight of 24,100 ± 50 Da (not shown). This is in perfect agreement with a calculated molecular weight of 24,052 Da, corresponding to vinculin residues 851–1066. Thus, the 29 kDa tail fragment contains the intact vinculin C-terminus.

3.2. *Analysis of ligand interaction*

Mixtures containing equal amounts of α-actinin and its purified 53 and 27 kDa fragments were separated by SDS-PAGE, blotted onto nitrocellulose filters and probed with vinculin and with the 90 kDa and 29/27 kDa fragments either alone or in combination (Fig. 2). To localize the vinculin probes on α- actinin we used the monoclonal antibody As8, which recognizes intact vinculin as well as the 90 kDa and the 81 kDa head fragments in Western blotting experiments (not shown). In the case of intact vinculin as a probe, only a very weak interaction with α-actinin was detected (Fig. 2, b) which is in agreement with earlier observations [1,8–10]. In contrast, the isolated 90 kDa head (Fig. 2, c) strongly decorates α-actinin and the C-terminal 53 kDa α-actinin rod, but not the 27 kDa α-actinin N-terminal fragment. The binding of the vinculin 90 kDa head to the α-actinin rod is in accordance with a recent report [1] showing that the binding site of vinculin resides within α-actinin residues 713–749. The 29/27 kDa tail of vinculin showed no interaction with either α-actinin or its proteolytic fragments, as shown in Fig. 2, d.

Johnson and Craig reported a strong intramolecular interaction of vinculin heads and tails [20] which might account for different affinities for α-actinin. when vinculin and its 90 kDa

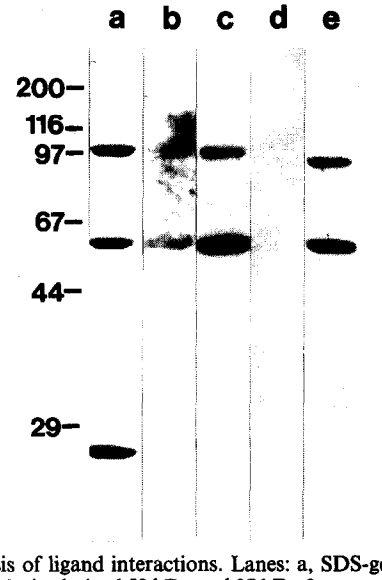

Fig. 2. Analysis of ligand interactions. Lanes: a, SDS-gel of α-actinin and the thermolysin-derived 53 kDa and 27 kDa fragments stained with Coomassie brilliant blue; overlays with vinculin (b), the vinculin 90 kDa head (c), the vinculin 29/27 tail (d) and a mixture containing equal amounts of head and tail domains (e). Antibodies used to detect bound vinculin fragments: As8 directed against the vinculin head domain (b,c,e); 4E7 directed against the vinculin tail domain (d).

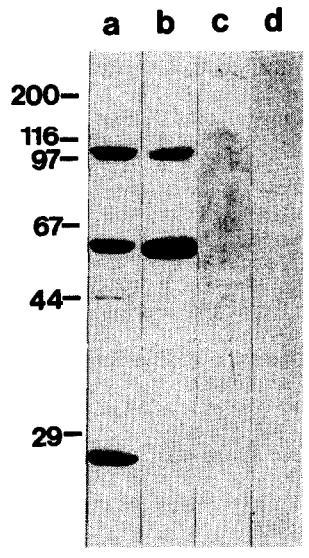

Fig. 3. Characterization of the α-actinin binding site on vinculin. Lanes: a, SDS-gel of α-actinin and the thermolysin derived 53 kDa and 27 kDa fragments stained with Coomassie brilliant blue; overlays with the vinculin 90 kDa head (b) and the 81 kDa head fragment (c), both detected with antibody As8. (d) Inhibition of the binding of the vinculin 90 kDa head to α-actinin after incubation with antibody 1E4.

head fragment are compared (see Fig. 2). To address this question, blots were probed with a mixture of both vinculin fragments (Fig. 2, e). A strong binding to α-actinin and its 53 kDa fragment was detected, similar to the result seen with the vinculin 90 kDa head alone.

3.3. Characterization of the α-actinin binding site on vinculin

We then used two different approaches to define the α-actinin binding site within the vinculin head more precisely. First, we tested the ability of a truncated vinculin head fragment to interact with α-actinin in blot overlays (Fig. 3, c). For this purpose, we used the vinculin fragment containing residues 108–850 as characterized above. Using monoclonal antibody As8 as marker for succesful binding, as described above for the 90 kDa head, no binding of the 81 kDa head fragment could be detected. Thus, this shortened head fragment had lost its ability to bind to α-actinin.

In a second approach we tried allowed vinculin antibodies to compete for the binding of the vinculin 90 kDa head to α-actinin. The monoclonal antibodies 1E4 and 1A10 succesfully inhibited the interaction between the vinculin 90 kDa head and α-actinin and its rod domain, as shown in Fig. 3, d for 1E4. In contrast, antibody As8, which was used as a control, does not interfere with this binding under identical conditions (Fig. 3, b). As8 has been mapped to vinculin residues 220–587, while both 1E4 and 1A10 map to vinculin residues 808–850 (our own unpublished results). We thus could demonstrate that this sequence is directly involved in, or topographically related to, the α-actinin binding site.

4. Discussion

We have analyzed the interaction of vinculin and α-actinin using proteolytic fragments and blot overlay techniques. We could show that the binding site on vinculin is located in the N-terminal head and not in the C-terminal tail. Within the head, vinculin residues 1–107 were found to be necessary for α-actinin binding. Our finding that the binding site of the vinculin head is located on the C-terminal α-actinin rod contradicts earlier results of Pavalko and Burridge [23] who located the vinculin binding site to the α-actinin 27 kDa fragment, but is in full agreement with a report of McGregor et al. [1] demonstrating that vinculin binds to α-actinin residues 713–749.

Interestingly, two antibodies binding to vinculin residues 808–850 strongly interfere with the binding of α-actinin, possibly by steric hindrance. This argues for a close structural proximity of vinculin residues 1–107 which are necessary for α-actinin binding and vinculin residues 808–850. A verification of this image will have to await future structural work on vinculin.

The intact vinculin molecule demonstrates a much weaker interaction with α-actinin and its rod domain than the isolated 90 kDa vinculin head. The weak interaction of intact vinculin described here is in accordance with earlier observations and the determination of a K_d of 1.3×10^{-5} for the vinculin–α-actinin interaction in solution [1,8–10]. The finding that intact vinculin molecules and isolated fragments behave differently in ligand binding is not without precedence in the literature. Already in 1984 Burridge and Mangeat reported that vinculin heads bind more strongly to talin than intact vinculin [6], and the finding that acidic phospholipids bind much more strongly to isolated vinculin tail fragments than to intact molecules indicates an analogous situation for lipid ligands [21]. The simplest explanation for these observations is that vinculin heads and tails interfere with the binding of partner molecules. The work of Johnson and Craig [20] unequivocally showed a strong intra-

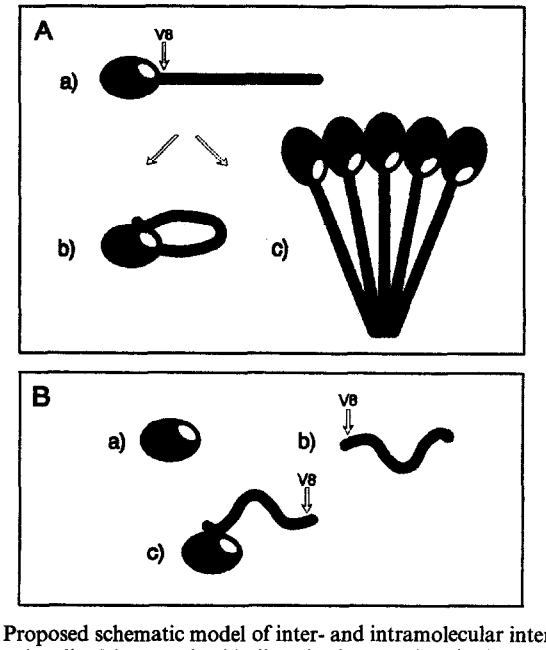

Fig. 4. Proposed schematic model of inter- and intramolecular interactions in vinculin. The putative binding site for α-actinin is shown as a white patch and V8 cleavage sites are indicated. (A) a: extendend vinculin molecule with dimensions and head to tail proportions roughly corresponding to vinculin as seen in the electron microscope [14]. b: vinculin molecule with intramolecular head to tail interaction. c: 'parachute-like' aggregates as seen in the electron microscope [14]. (B) a: isolated vinculin 90 kDa head. b: isolated vinculin 29/27 tail. c: interaction of the vinculin 90 kDa head and tail fragments in solution.

molecular interaction between heads and tails residing in vinculin tail residues 1013–1043.

Our model presented in Fig. 4 tries to combine current data on vinculin structure, flexibility and its interaction with α-actinin, used here as a model ligand. Fig. 4Aa shows a hypothetical vinculin molecule roughly corresponding to the extended shape as seen in the electron microscope [14]. Fig. 4Ab depicts a vinculin molecule with intramolecular head-to-tail interaction. This structure is based on light scattering data by Eimer et al., who showed that vinculin in solution is an asymmetric, elongated molecule [19], and crosslinking data from Johnson and Craig proving an intramolecular interaction [20]. The particle length provided by Eimer et al. [19] is consistent with such a structure containing a tail simply folded backwards. In contrast, the parachutes in Fig. 4A(c) described by Milam [14] might be explained by the aggregation of the elongated molecules shown in Fig. 4A(a). Fig. 4B shows the V8 cleavage products after biochemical purification: the 90 kDa head (a) where the α-actinin binding site is fully exposed and (b) the isolated 29 kDa tail. The structure in Fig. 4Bc proposes the situation after V8 cleavage, but without further separation: an isolated tail fragment associated with the head. Since amino acid residues 1013–1043 of the vinculin tail were found essential for head-to- tail association [20] it seems likely that the very carboxy-terminal position of the tail associates with the head, at some distance from the α-actinin-binding site. All data combined favour such a model, explaining the influence of the tail on binding ligands to the head, as reported here for α-actinin. Conversely, the presence of the head in such a molecule would influence ligand binding to the tail, as observed by Johnson and Craig [21] and Menkel et al. [13]. Proof of the validity of this model will have to await more detailed structural studies.

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