VASP interaction with vinculin: a recurring theme of interactions with proline-rich motifs

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Abstract VASP (vasodilator-stimulated phosphoprotein), a protein associated with microfilaments at cellular contact sites, has been identified as a ligand for profilin and zyxin, two proteins also involved in microfilament dynamics and organization at these regions. Here, we report that VASP also directly binds to vinculin, another component of adherens junctions. Competition experiments with a vinculin-derived peptide showed that a proline-rich motif, located in the hinge region that connects vinculin's head and tail domains, is involved in VASP binding. The same motif is present in zyxin but the interactions of VASP with vinculin and zyxin differ in detail. Hence, this motif may be recognized by VASP in different ways when presented in distinct cellular sites.

Key words: ActA; Focal adhesion; Focal contact; Microfilament regulation; Zyxin

1. Introduction

VASP, an established substrate of both cAMP- and cGMP-dependent protein kinases, is present in high concentrations in human platelets and in lower abundance also in a wide variety of other mammalian cells and tissues [1]. In tissue culture cells, VASP is associated with focal adhesions, microfilaments, cell-cell contacts and the leading edge [2–4]. More recently, the gene product of the *Drosophila* gene *enabled* (*ena*), which has been discovered as a dose-dependent suppressor of *abl*- and *disabled (dab)*-dependent phenotypes [5], and its mammalian homologue Mena ([6]; mammalian enabled) have been identified as members of the novel Ena-VASP protein family which share extended sequence similarity and an overall domain organization originally described for VASP [4–6]. Additional clues with respect to VASP function came from investigations of the actin-based motility of the intracellular cytopathogenic bacterium *Listeria monocytogenes*. Intracellular *Listeria* exploit the host cell actin regulatory system causing actin to polymerize at their backpole. This leads to actin tail formation and propels the bacteria through the host cell cytoplasm eventually forming cell surface protrusions capable of infecting neighboring cells [7]. VASP [8], Mena [6], and also profilin [9] differ from all the other actin-associated proteins tested so far as they are concentrated at one pole of the motile bacteria, i.e. at the base of the actin tail, where actin polymerization is thought to take place. VASP interacts directly both with profilin [10] and with the listerial surface protein ActA [8], a protein that is both essential and sufficient for intracellular listerial motility and host cell F-actin recruitment, respectively [7,11]. More recently, the direct interaction of Mena with both profilin and ActA has also been demonstrated [6]. Independently, microsequencing [3] and additional sequence analysis (M. Reinhard and U. Walter, unpublished results) of a VASP binding protein purified from porcine platelets using ^{32}P-labeled VASP in blot overlays revealed that this VASP binding protein is the porcine equivalent of human zyxin (GenBank accession number X94991). The similar VASP binding features of ActA and zyxin led to the suggestion that ActA and zyxin (a possible cellular ActA analogue) may recruit profilin/profilactin to distinct subcellular sites via VASP [3,8] which may be involved in the initiation and/or augmentation of spatially confined actin polymerization at these sites [3,8,12]. Whereas profilin interaction with VASP involves the poly-L-proline binding site of profilin and the GP_5 repeats containing central proline-rich VASP domain [4,10], the Mena N-terminus containing the conserved EVH1 (Ena-VASP homology 1) domain is sufficient for the interaction with the proline-rich ligands ActA and zyxin [6]. Deletion analysis of mitochondrial targeted ActA transfected into eukaryotic cells revealed that the proline repeats of ActA are required for the VASP interaction and efficient actin recruitment, whereas the proline-rich region and VASP binding are not essential for basal actin accumulation [11].

Vinculin is a major component of all types of adherens junction [13,14]. It is also an essential protein, as modulation of vinculin levels leads to drastic alteration of cellular adhesion [15]. The tadpole-shaped vinculin has long been known to interact with a variety of other focal adhesion proteins, the binding sites for which have been mapped either to the globular vinculin head, e.g. in the case of talin and α-actinin, or to the C-terminal vinculin tail, e.g. for actin and paxillin [15]. The vinculin head is capable of folding back to the tail domain [16], thus affecting the interaction with other vinculin ligands [17,18]; this has stimulated intensive research in this putative regulatory mechanism of protein-protein interactions. The hinge region between the vinculin head and tail domains contains a proline-rich region. The similarity of this sequence element to the proline motifs of ActA [21] and zyxin prompted us to investigate the possibility of a direct VASP-vinculin interaction. Here we report that vinculin harbors a functional VASP binding site. Our results demonstrate that the VASP-vinculin interaction joins the recurring theme of VASP interactions with proline-rich ligands, although it differs in detail from the binding characteristics reported for the couples VASP/zyxin and VASP/ActA.

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Abbreviations: EVH1, Ena-VASP homology 1; EVL, Ena-VASP-like; MBP-Vinc8, fusion protein of maltose binding protein and vinculin (aa 809–1063); Mena, mammalian enabled (Ena); VASP, vasodilator-stimulated phosphoprotein

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2. Materials and methods

2.1. Protein purification

Zyxin and VASP were purified from porcine platelets [3]. Vinculin was purified from chicken and turkey gizzard, and the MBP-Vinc8 fragment was expressed and purified as described [19].

2.2. Peptide synthesis

The human vinculin peptide EPDFPPPPPDLEQLR was synthesized in N-terminally acetylated and C-terminally amidated form, purified (>95%), and verified by HPLC (VYDAC RP C-8) and mass spectrometry (Genosys Biotechnologies, Cambridge, UK). The peptide content was estimated to be 60%. The peptide stock solution was prepared in PBS and neutralized by the addition of Tris base.

2.3. Binding assays

[^{32}P]VASP overlays were performed as described previously [3]. Solid phase binding assays were done essentially according to published protocols [10]. Briefly, for coating native vinculins and MBP-Vinc8 were added directly to removable microtiter wells and diluted to final concentrations of 2.5 and 5 μg/ml, respectively. After blocking nonspecific binding sites with 3% (w/v) BSA in PBS, VASP was added as a ligand and diluted with BSA solution to a final concentration of 1.3 μg/ml. The wells were washed with PBS, and bound VASP was detected by incubating the wells with a specific VASP antiserum followed by [^{125}I]protein A (each in 3% BSA). All incubation volumes were 400 μl, except for the peptide competition and subsequent incubation steps, which were done in 200 μl assays. For competition experiments, VASP was added to the microtiter wells either without or together with the vinculin peptide at a final concentration of 300 μM.

3. Results

3.1. VASP binding to vinculin in solid phase binding assays

To test the possible interaction of VASP and vinculin in vitro, removable microtiter wells were coated with vinculin purified from chicken and turkey gizzard. After blocking nonspecific binding sites with BSA, VASP was added as a soluble ligand. Bound VASP was detected with a polyclonal antiserum followed by ^{125}I-labeled protein A. As shown in Fig. 1,

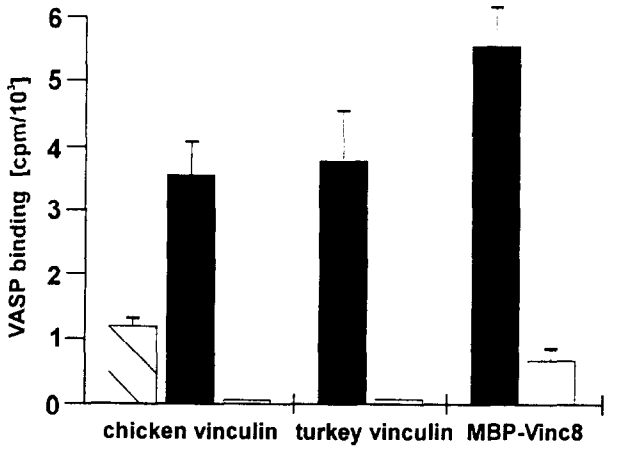

Fig. 1. VASP interaction with vinculin in solid phase binding assays. Removable microtiter wells were coated with vinculin (2.5 μg/ml) purified from chicken or turkey gizzard or with the recombinant vinculin fragment MBP-Vinc8 (5.0 μg/ml). After blocking nonspecific binding sites, VASP was added as a soluble ligand. Bound VASP was detected by a specific antiserum followed by [^{125}I]protein A (black columns). Wells without coating (hatched column) or without addition of VASP (white columns) served as controls. The numbers given are means and standard deviations of triplicates of one out of several independent experiments.

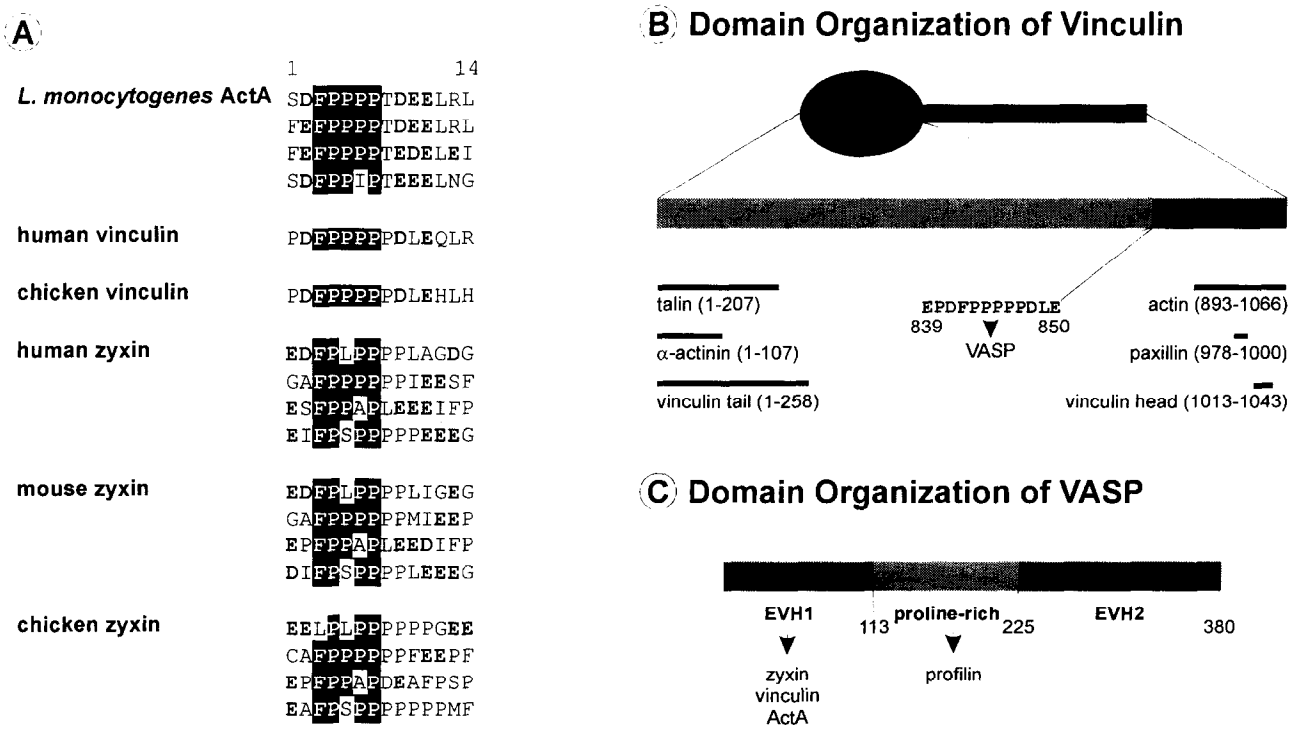

Fig. 2. Overview of the binding motifs and domains involved in the VASP-vinculin interaction. A: Alignment of the VASP binding motif of human vinculin [25] to the proline-rich repeats of ActA [20,21], chicken zyxin [22], mouse zyxin (GenBank accession number X99063), and human zyxin (GenBank accession number X94991). The FPPPP core motif is boxed and acidic residues on both sides are marked in bold. B: Mapping of the binding sites of vinculin binding proteins to different vinculin domains (for review, see [18,33]). Numbers given in parentheses refer to amino acid positions in the vinculin sequence. The VASP binding motif is located in the putative hinge region between the globular head and elongated tail. C: Domain organization of VASP [4,6] and assignment of binding activities for profilin [10] and – by inference from the corresponding Mena data [6] – for ActA, zyxin, and vinculin. Amino acid numbering refers to human VASP [4].

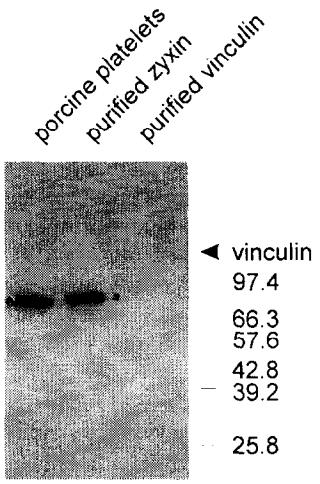

Fig. 3. Comparison of VASP binding to zyxin and vinculin in [^{32}P]VASP overlays. Total protein from porcine platelets (20 μg), zyxin purified from porcine platelets (~80 ng) and vinculin purified from chicken gizzard (1 μg) were separated by SDS-PAGE and blotted onto nitrocellulose. The membrane was stained with Ponceau S, blocked and overlaid with [^{32}P]VASP in blocking medium. After washing with detergent containing PBS, bound VASP was detected by autoradiography. The positions of molecular mass standards (in kDa) and of vinculin (as determined by Ponceau S staining) are marked in the right margin.

VASP bound significantly and specifically to vinculin-coated wells. However, significant VASP binding required vinculin concentrations for coating in the range of μg/ml, which exceeds the zyxin concentrations necessary for significant VASP binding with an identical assay system [3] by about two orders of magnitude. Therefore, it was mandatory to rule out that the results obtained might be due to minor zyxin contamination present in the vinculin preparations. To this end, two different approaches were chosen. First, vinculin was captured by coating the microtiter wells with a monoclonal vinculin antibody (hVin-1) and tested for VASP binding as described above. Again, VASP bound specifically to antibody-captured vinculin (not shown), indicating that zyxin contamination was not a problem. Second, the solid phase binding assays were repeated using a recombinant vinculin fragment (MBP-Vinc8) comprising vinculin amino acids 809–1063 fused to maltose binding protein [19]. VASP binding to the recombinant protein purified from *E. coli* was similar to that obtained with native vinculin (Fig. 1), confirming the results obtained with vinculin purified from natural sources and ruling out the possible contribution of zyxin contamination. At the same time, these results indicate that the N-terminal portion of vinculin (aa 1–808) is not required for the VASP interaction.

3.2. *VASP interaction with vinculin, zyxin, and ActA: a common principle*

VASP has been shown to interact in vitro directly with ActA [8] and zyxin [3], which both share related proline-rich elements [20–22] that are characterized by an FPPPP core motif flanked by acidic residues on both sides (Fig. 2A). Since VASP recruitment by ActA in intact cells is dependent on the presence of these proline-rich repeats [11], it is reasonable to assume that these motifs directly mediate VASP binding. Therefore we reasoned that a similar proline-rich motif present in the putative hinge region of vinculin (Fig. 2B) might be responsible for the VASP interaction. To test our hypothesis, we asked whether the ActA- and zyxin-related vinculin peptide Ac-EPDFPPPPPDLEQLR-NH_2 would be able to compete with vinculin for VASP binding in a solid phase binding assay. For this purpose, VASP was added either together with or in the absence of the peptide to MBP-Vinc8-coated microtiter wells. The VASP-vinculin interaction was markedly decreased to 33% residual binding in the presence of the peptide, indicating that the proline-rich sequence element in vinculin is indeed involved in VASP binding.

3.3. *VASP-vinculin and VASP-zyxin interactions: similar but different*

In vitro interactions of VASP with zyxin and ActA have been demonstrated not only by solid phase binding assays but also in blot overlays with [^{32}P]VASP [3,8]. Interestingly, zyxin was the only protein present in total platelet extracts that was labeled with [^{32}P]VASP in the overlay assay [3]. In particular, there was no protein related in size to vinculin although platelets are known to contain substantial amounts of vinculin [23]. These results are seemingly at variance with the results described above.

To resolve this issue, we readdressed the problem using overlays of purified vinculin and zyxin, which had been separated by SDS-PAGE and blotted onto nitrocellulose. Efficient protein transfer was monitored by Ponceau S staining of the blot membrane. Although vinculin was present in huge excess over zyxin, not even a faint signal could be detected in the overlay assay of vinculin, whereas even nanogram amounts of zyxin yielded a strongly labeled band. Fig. 3 shows a side-by-side comparison of total platelet protein, purified zyxin, and vinculin assayed in a [^{32}P]VASP overlay. These results demonstrate that the absence of VASP binding to vinculin in this type of assay is not caused by the absence or limited avail-

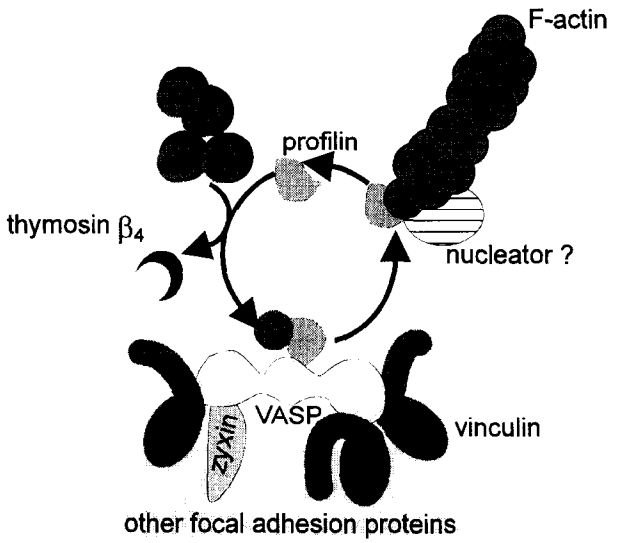

Fig. 4. A model of the concerted action of vinculin and zyxin in VASP recruitment and actin polymerization. Binding of VASP to zyxin and vinculin is envisaged to have two primary functions: (a) consolidation by crosslinking of the meshwork of protein-protein interactions and providing additional docking points; (b) local increase of the concentration of polymerization-competent profilactin complexes, thus supporting an efficient elongation of actin filaments. The depicted addition of profilactin to the barbed ends of actin filaments is only one example out of several conceivable scenarios (for a discussion of additional mechanisms see [12,15]).

ability of vinculin on the blot membrane. Instead, the observed results reflect an inherent difference between the interactions of VASP with zyxin/ActA and vinculin.

4. Discussion

In this report we present evidence that a proline-rich motif previously identified in zyxin and ActA is a functional VASP binding element also in vinculin. In addition, we show that the VASP interactions with zyxin and ActA on the one hand and vinculin on the other hand differ. These differences are obvious both in the solid phase binding assays (where vinculin is required for coating in markedly higher concentrations than zyxin) and in blot overlays (where only zyxin and ActA display detectable VASP binding). We would like to suggest that these results reflect the fact that vinculin harbors only a single FPPPP motif [24,25] whereas both zyxin [22] and ActA [20,21] each contain up to four copies of this identified [11] VASP binding motif (Fig. 2A), which might increase the affinity for VASP. Such an effect might be enhanced in VASP overlays of SDS-denatured vinculin as compared to the native vinculin in solid phase binding assays, where oligomerization of vinculin may compensate for the single binding site per polypeptide chain. Interestingly, affinity chromatography with a fusion protein comprising the N-terminal EVH1 of the VASP family member Mena also resulted in a significantly lower retention efficiency for vinculin than for zyxin [6]. Although a direct Mena-vinculin interaction has not yet been shown, this observation, together with our present results, suggests that the vinculin FPPPP motif represents a general binding site for the EVH1 domain of the VASP family proteins (Fig. 2C).

Whereas all of the known binding sites for vinculin ligands map to either the N-terminal vinculin head or the C-terminal vinculin tail (Fig. 2B; for a review see [15,18]), the VASP binding element is unique as it is localized at the putative hinge region, i.e. at the flexible connection between the vinculin head and tail domains [24,25]. This might be a crucial position to regulate vinculin's conformation and accessibility of ligand binding sites. In the light of recent observations that vinculin binding to F-actin [19,26], talin [27], and α-actinin [28] is affected by an intramolecular interaction between the vinculin head and tail, it will be interesting to investigate whether binding of VASP or VASP-related proteins either induces or depends on a specific vinculin conformation.

Vinculin localization at focal adhesions appears to be independent of the interaction with VASP family proteins, as microinjection of a proline-rich ActA peptide displaces VASP [11] and Mena [6] but not noticeable amounts of vinculin from focal adhesions. Moreover, vinculin fragments lacking the VASP binding site are also targeted to focal adhesions [29]. Vice versa, VASP localization to stress fibers and the leading edge [2–4] probably does not depend on vinculin, as the co-localization of both proteins is mainly restricted to focal adhesions and cell-cell contacts [2–4,13,14].

At these sites, vinculin and zyxin may act in concert to recruit VASP and related proteins, such as Mena. VASP, due to its tetrameric structure [4], may stabilize by cross-linking homo- or hetero-oligomeric complexes comprising vinculin and/or zyxin, respectively, and thus initiate the accumulation of further vinculin and zyxin ligands such as F-actin and α-actinin. The profilin binding capacity of VASP and Mena [10,6] in turn might supply polymerization-competent profilactin complexes, allowing efficient actin polymerization. These events are schematically depicted in Fig. 4.

Support for a vinculin function as depicted in the model has recently come from work on *Shigella*, which – like *Listeria* spp. – has evolved an actin-based motility system [30]. Unlike *Listeria* however, no direct interaction of VASP with any surface protein could be detected [8]. Instead, the *Shigella* VirG/IcsA protein was reported to bind vinculin [31], which in turn might serve to recruit VASP or other VASP family proteins. The observation that microinjection of a peptide, comprising a VASP binding motif of ActA, arrested *Shigella* actin tail formation and intracellular motility [32] despite the lack of similar proline-rich sequence stretches in the VirG/IcsA protein, is also consistent with such an idea.

In conclusion, we have shown that vinculin harbors a functional VASP binding site, which not only joins a recurring theme of interactions between VASP family proteins and ligands containing proline-rich sequence motifs, but which also has some unique features. Future studies will have to elucidate the concerted function of these phosphoproteins in the regulation of the microfilament system.

4.1. Note added during revision

A communication has recently appeared which also demonstrates the direct interaction of VASP and vinculin [N.P.J. Brindle et al. (1996) Biochem. J. 318, 753–757].

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