RESEARCH COMMUNICATION

The focal-adhesion vasodilator-stimulated phosphoprotein (VASP) binds to the proline-rich domain in vinculin

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In mammalian cells vasodilator-stimulated phosphoprotein (VASP) is localized to focal adhesions and areas of dynamic membrane activity where it is thought to have a role in actin-filament assembly. The proteins responsible for recruiting VASP to these sites within the cell are not known. The bacterial protein ActA binds VASP via a proline-rich motif that is very similar to a sequence in the proline-rich region of the focal-adhesion protein vinculin. We have examined the ability of VASP, synthesized using an *in vitro* transcription/translation system, to bind to a series of vinculin peptides expressed as glutathione S-transferase fusion proteins, and have shown that it binds specifically to the proline-rich region in vinculin. Using immobilized peptides corresponding to the two proline-rich motifs within this domain, the VASP-binding site was localized to proline-rich motif-1 (residues 839–850). Binding to this motif was not affected by the phosphorylation state of VASP. The C-terminal region of VASP, which is known to be important in targeting VASP to focal adhesions, was shown to be required for binding. These results identify vinculin as a VASP-binding protein likely to be important in recruiting VASP to focal adhesions and the cell membrane.

INTRODUCTION

Vasodilator-stimulated phosphoprotein (VASP) is a 380-amino-acid protein with a predicted molecular mass of 39.8 kDa [1]. In its native state it exists as a homo-tetramer and is associated with focal adhesions, microfilaments and lamellipodia [1–3]. In epithelial cells it is found at cell–cell junctions [1,4]. Recent evidence suggests that VASP may have an important role in regulating microfilament assembly and cytoskeletal organization. On entry into the cytoplasm of a cell, the bacterium *Listeria monocytogenes* recruits VASP and other host-cell proteins to its surface by specific binding of VASP to the bacterial surface protein ActA [5]. Following VASP recruitment, a cloud of actin forms around the bacterium and microfilament assembly is initiated. The filaments are orientated with the growing barbed end adjacent to one pole of the bacterial surface, and it is thought that growth of these filaments provides the motive force for movement of the bacteria [6,7]. VASP is located between the bacterial surface and the growing barbed end of the filament, consistent with a role in filament assembly [5]. Inhibition of VASP binding to ActA ectopically expressed within cells inhibits the ability of this protein to induce actin-filament formation [8]. VASP has been shown to bind to profilin [9], a G-actin-binding protein known to promote filament formation [10]. In mammalian cells both VASP and profilin localize to areas of highly dynamic membrane activity, including lamellipodia and microspikes [2,11,12], again consistent with a role in filament formation. Interestingly, it has been shown that this localization is regulated by cdc42Hs, a member of the family of Rho GTP-binding proteins [12]. Expression of a constitutively active form of CDC42Hs in HeLa cells induces a dramatic reorganization of F-actin and VASP into cortical microspike structures.

The cellular analogues of ActA responsible for localization of VASP to focal adhesions and lamellipodia are not known. However, recent data strongly suggest that the focal-adhesion protein zyxin may be a VASP-binding protein [3]. Binding of VASP to ActA requires the presence of a proline-rich domain in the bacterial protein [8]. This domain contains four copies of a proline-rich motif [13,14]. Microinjection of peptides based on this motif inhibit filament assembly induced by *L. monocytogenes* and cause depletion of VASP from focal adhesions and loss of actin staining in lamellipodia [8]. The focal-adhesion protein vinculin contains a proline-rich domain that includes a sequence very similar to the ActA proline-rich motif [15]. Vinculin is known to contain binding sites for talin [16], paxillin [17] and F-actin [18], and can also bind α-actinin [19]. This ability to interact with several cytoskeletal proteins, together with its localization to focal adhesions and the presence of an ActA proline-rich motif, make vinculin an attractive candidate for a cellular VASP-binding analogue of ActA. Here we show that VASP can indeed bind to the proline-rich domain of vinculin and suggest that this interaction is important in actin-filament assembly and focal-adhesion stability.

MATERIALS AND METHODS

Materials

Protein phosphatase 2A purified from bovine heart was obtained from Boehringer Mannheim UK Ltd. (Lewes, East Sussex, U.K.). The catalytic subunit of cAMP-dependent protein kinase (cAK), purified from porcine heart, and cGMP were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.), and recombinant bovine cGMP-dependent protein kinase (cGK) isoform 1α was obtained from Calbiochem (Nottingham, U.K.). The sources of all other materials are indicated or were as previously described [20].

Abbreviations used: cAK, cAMP-dependent protein kinase; cGK, cGMP-dependent protein kinase; FP4, FPPPP sequence; GST, glutathione S-transferase; IVT/T, *in vitro* transcription/translation; PP2A, protein phosphatase 2A; VASP, vasodilator-stimulated phosphoprotein.

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Production of radiolabelled VASP

Nucleotides 254–1431 of the published VASP sequence [1], which includes the entire coding region, was amplified by reverse transcription and PCR from human endothelial cell RNA using the 5′ primer CATGAGCGAGACGGTCATCT and the 3′ primer GGAAAGGAGAAGCGGGTCTT. PCR was performed for 30 cycles of 94 °C for 30 s and 68 °C for 2 min, using *Taq* DNA polymerase with 3′–5′ proofreading exonuclease activity (Life Technologies Ltd., Paisley, Scotland, U.K.). The VASP amplification product was ligated into the pCR3 vector (Invitrogen, NV Leek, The Netherlands), and the sequence was confirmed by restriction-endonuclease mapping and sequencing using a 373A ABI automated DNA sequencer. Radiolabelled VASP was produced from this plasmid using T7 RNA polymerase in a rabbit reticulocyte lysate *in vitro* transcription/translation (IVT/T) system (Promega Ltd., Southampton, U.K.), in the presence of L-[4,5-^{3}H]leucine with a specific radioactivity of 5.18 TBq/mmol (Amersham International plc, Amersham, Bucks., U.K.), as per the manufacturer's instructions. To produce full-length and truncated radiolabelled VASP, the plasmid was linearized with *Xho*I or *Bst*EII respectively, purified using Qiaquick DNA-purification spin columns (Qiagen Ltd., Dorking, Surrey, U.K.), and used for IVT/T.

Generation of chick vinculin–glutathione S-transferase (GST) fusion proteins

The chick vinculin fusion proteins used in this study have been described before [16], with the exception of GST–vinculin 836–940, which was generated by PCR from a chick vinculin cDNA template and primers containing a 5′ *Bam*HI site and a 3′ *Eco*RI site. The PCR product was cloned into the vector pGEX 2T, and the construct was sequenced to confirm its identity, using primers 5′ and 3′ to the pGEX cloning site. GST fusion proteins expressed in *Escherichia coli* (strain JM101) were purified from 500 ml cultures using standard methods. Briefly, after induction with isopropyl β-D-thiogalactoside, cells were harvested by centrifugation, resuspended in 10 ml of PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 1.8 mM KH_2PO_4, pH 7.3)/1 % Triton X-100/1 mM PMSF, and lysed by sonication. Solubilized fusion proteins were adsorbed on to glutathione–agarose beads, which were then washed thoroughly with PBS to remove contaminants.

Actigel-coupled vinculin peptides

Peptides corresponding to proline-rich motif-1 (amino acids 839–850), and proline-rich motif-2 (amino acids 859–879) of chick vinculin were synthesized on an ABI 431A peptide synthesizer and purified by HPLC. Peptides were coupled to Actigel (Sterogene Bioseparations Inc., Arcadia, CA, U.S.A.) in 0.1 M KH_2PO_4, pH 7.0, according to the manufacturer's instructions, washed in 16 vol. of 0.5 M NaCl, and stored at 4 °C in Tris-buffered saline (50 mM Tris/HCl, pH 7.5, and 100 mM NaCl).

Phosphorylation of VASP

Phosphorylation and dephosphorylation of VASP was carried out essentially as described by Reinhard et al. [9]. Briefly, VASP produced by IVT/T was incubated for 30 min at 30 °C in 50 mM Tris (pH 7.2)/150 mM NaCl/1 mM dithioerythritol/2.5 mM $MgCl_2$/0.1 mg/ml BSA/100 μM ATP, in the presence of 80 ng of protein phosphatase 2A (PP2A), 980 ng of cGK and 2 mM cGMP, or 250 ng of cAK. The reaction volume was 50 μl. The extent of dephosphorylation and phosphorylation was monitored by examining the mobility shift of VASP after SDS/PAGE and fluorography, as described below. Aliquots of reaction mix containing extensively dephosphorylated or phosphorylated forms of VASP were used in binding studies.

Binding of radiolabelled VASP to vinculin fusion proteins and peptides

GST fusion protein (25 μg) adsorbed to glutathione–agarose beads, or vinculin-derived peptide coupled to Actigel, was incubated with 5 μl of rabbit reticulocyte lysate containing radiolabelled VASP in 0.5 ml of binding buffer (50 mM Tris/HCl, pH 7.5, 100 mM NaCl and 0.1 mM 4-(2-aminoethyl)benzenesulphonyl fluoride) containing 1 mg/ml BSA for 2 h at 4 °C with continuous rotation. Beads were washed five times in binding buffer containing 0.1 % Tween 20. Bound proteins were then eluted into Laemmli sample buffer (reducing) and separated by SDS/PAGE [21]. Proteins were detected by staining with Coomassie Brilliant Blue. For fluorography, gels were fixed for 15 min in 45 % methanol/10 % acetic acid, washed in water, incubated in 1 M sodium salicylate for 30–45 min [22], dried and exposed to X-ray film at −80 °C.

RESULTS

VASP binds to the proline-rich domain of vinculin

Deletion analysis of ActA expressed ectopically in PtK2 cells has demonstrated that amino acids 265–422 of the protein are required for VASP binding [8]. This 158-amino-acid sequence is rich in prolines and includes the sequence FPPPP (FP4), which is flanked by acidic residues and is repeated three times. IactA has a similar motif and is also able to bind VASP (Figure 1). The focal-adhesion protein vinculin contains a proline-rich region that also includes an FP4 motif (Figure 1). These similarities and the focal location of VASP prompted us to investigate whether VASP binds directly to vinculin.

To test this, a series of GST–vinculin fusion proteins corresponding to different regions of vinculin, including the proline-rich region, were constructed. The ability of these proteins to bind radiolabelled VASP, produced in an IVT/T system, is shown (Figure 2). Radiolabelled VASP was observed after SDS/PAGE as a doublet with apparent molecular masses of 48 and 53 kDa under the conditions used in this study (Figures 2 and 3). This is similar to the behaviour of purified VASP, where

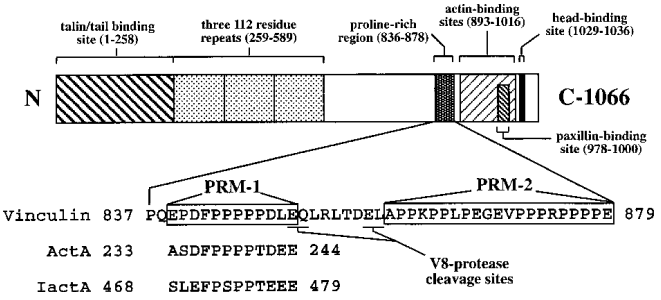

Figure 1 Domain structure of vinculin and sequence similarities to ActA and IactA

The relative positions of binding sites for talin, paxillin and F-actin, as well as the proline-rich domain and V8-protease cleavage sites within the vinculin molecule, are shown. Amino acid numbering includes the initiating methionine. Similarities between the deduced amino acid sequences in the proline-rich domain of chick vinculin [15], ActA of *L. monocytogenes* [13,14] and IactA of *L. ivanovii* [27] are also shown.

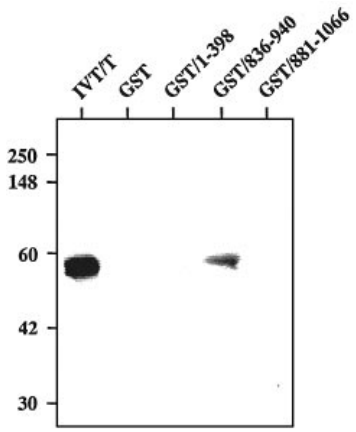

Figure 2 VASP binds to the proline-rich domain of vinculin

Radiolabelled VASP produced by IVT/T was incubated with glutathione–agarose-bound GST or GST–vinculin fusion proteins containing residues 1–398, 836–940 or 881–1066 for 2 h at 4 °C. Bound protein was collected by centrifugation, washed extensively, eluted by boiling in electrophoresis sample buffer and analysed by SDS/PAGE. Binding of radiolabelled VASP was detected after fluorography, as described in the Materials and methods section. Radiolabelled VASP before incubation with fusion proteins (IVT/T) is included for comparison. Mobilities of molecular-mass markers (kDa) are indicated on the left-hand side.

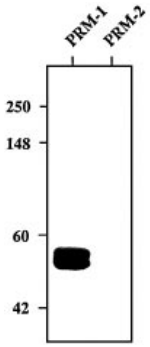

Figure 3 VASP binds to vinculin proline-rich motif-1

Radiolabelled VASP produced by IVT/T was incubated with agarose-coupled peptides corresponding to vinculin proline-rich motif-1 (PRM-1) or proline-rich motif-2 (PRM-2) for 2 h at 4 °C. Bound protein was collected by centrifugation and analysed as described in the legend to Figure 2. Mobilities of molecular-mass markers (kDa) are indicated on the left-hand side.

apparent molecular masses of 46 kDa and 50 kDa, depending on their phosphorylation state, have been reported [23]. VASP bound to the vinculin fusion protein contained residues 836–940, but did not bind to GST alone or to fusions containing residues 1–398 or 881–1066 (Figure 2). This suggests that vinculin residues 836–880 contain a VASP-binding site. This region contains the FP4 motif as well as another proline-rich sequence.

In order to determine more precisely which vinculin residues are involved in binding VASP, peptides corresponding to the two proline-rich sequences between 836 and 880 were synthesized and coupled to agarose beads. These peptides are designated proline-rich motif-1 (839-EPDFPPPPPDLE-850) and proline-rich motif-2 (859-APPKPPLPEGEVPPPRPPPPE-879). [^{3}H]VASP bound to proline-rich motif-1 but did not bind to proline-rich motif-2 (Figure 3).

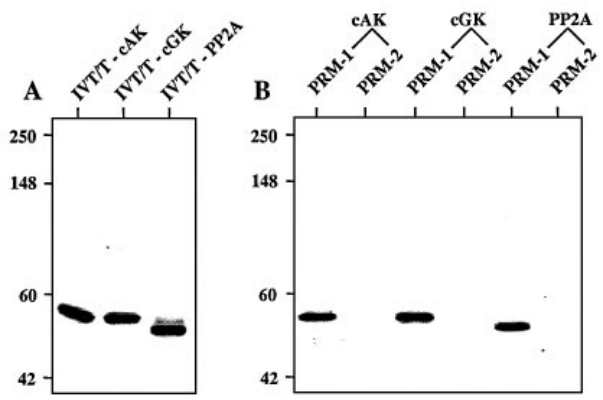

Figure 4 Effect of phosphorylation on the binding of VASP to vinculin proline-rich motif-1

Radiolabelled VASP produced by IVT/T was incubated with cAK, cGK and cGMP, or PP2A for 30 min at 30 °C. (**A**) Mobility of VASP after incubation with cAK, cGK and PP2A. (**B**) Extensively phosphorylated or dephosphorylated forms of VASP were incubated with agarose-coupled peptides corresponding to vinculin proline-rich motif-1 (PRM-1) or proline-rich motif-2 (PRM-2) for 2 h at 4 °C. Bound protein was collected by centrifugation and analysed as described in the legend to Figure 2. Mobilities of molecular-mass markers (kDa) are indicated on the left-hand side of each panel.

Effects of VASP phosphorylation state on binding to proline-rich vinculin peptides

Phosphorylation of VASP is associated with inhibition of platelet aggregation and inhibition of endothelial cell contraction [4,24]. The way in which phosphorylation modulates VASP function is not known. To investigate the possibility that interaction of VASP with vinculin could be regulated by phosphorylation, the phosphorylation state of VASP was manipulated using cAK, cGK and PP2A. These enzymes have previously been shown to regulate VASP phosphorylation *in vitro* and *in vivo* [23–25]. On treatment with PP2A, VASP exhibited increased mobility in SDS/PAGE, whereas cAK and cGK decreased mobility (Figure 4). The shift of untreated VASP from a doublet to the apparent higher or lower mobility forms on treatment with the phosphatase or kinases respectively is similar to that seen with VASP purified from platelets, and indicates that the IVT/T product is a mixture of phosphorylated and dephosphophosphorylated VASP. This mobility shift is used as an indicator of the VASP phosphorylation state [26].

The two immobilized vinculin proline-motif peptides were incubated with extensively phosphorylated and dephosphorylated VASP (Figure 4). Phosphorylated and dephosphorylated forms of VASP bound equally well to proline-rich motif-1. VASP did not bind to proline-rich motif-2 in either the phosphorylated or dephosphophosphorylated forms.

C-terminal truncation of VASP abolished its ability to bind vinculin

When human VASP is expressed in hamster kidney cells, it co-localizes with vinculin to focal adhesions [1]. However, a C-terminal truncated form of VASP, consisting of residues 1–284, is diffusely distributed and is not recruited to focal adhesions. This led to the suggestion that the C-terminal domain of VASP, which includes a repetitive mixed-charge cluster sequence, is important in targeting the protein to the focal adhesion [1]. At least one way in which VASP is targeted to the focal adhesion is

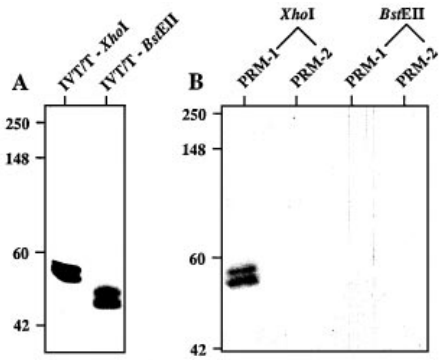

Figure 5 Effect of C-terminal truncation of VASP on binding to vinculin proline-rich motif-1

(**A**) Full-length [^{3}H]VASP and C-terminal truncated VASP lacking residues 326–380 were produced by IVT/T from plasmids linearized with *Xho*I and *Bst*EII respectively and resolved by SDS/PAGE. (**B**) These VASP polypeptides were incubated with agarose-coupled peptides corresponding to vinculin proline-rich motif-1 (PRM-1) or proline-rich motif-2 (PRM-2) for 2 h at 4 °C. Bound protein was collected by centrifugation and analysed as described in the legend to Figure 2. Mobilities of molecular-mass markers (kDa) are indicated on the left-hand side of each panel.

likely to be via its ability to bind vinculin. Thus, the C-terminal residues in VASP could be involved in binding to vinculin. This was investigated by comparing the ability of full-length and truncated VASP to bind to proline-rich motif-1 of vinculin (Figure 5). As before, full-length VASP bound to the vinculin proline-rich motif-1 peptide coupled to agarose beads, whereas the truncated form of VASP, consisting of residues 1–325 and lacking the C-terminal repetitive charge sequence, was incapable of binding to proline-rich motif-1 (Figure 5).

DISCUSSION

Here we show that VASP binds to the proline-rich domain of the focal-adhesion protein vinculin; furthermore, we refine the binding site to a sequence containing the FP4 motif also found in the VASP-binding protein ActA of *L. monocytogenes*. The VASP-binding site on vinculin lies within proline-rich motif-1, as shown by direct binding of VASP to this peptide. It is noteworthy that native vinculin is very sensitive to V8-protease cleavage at two sites between proline-rich motif-1 and -2 [15]. This implies that these regions are likely to be exposed on the surface of the protein and suggests that VASP may have direct access to proline-rich motif-1 in native vinculin. Vinculin proline-rich motif-1 consists of an FP4 motif, with an additional proline, flanked by acidic residues. Three copies of an almost identical motif (Figure 1) and one of a similar motif (350-DFPPIPTEEE-359) are present in the VASP-binding domain of ActA [13,14]. The presence of several copies of this motif in the ActA sequence may account for the ability of this protein to deplete VASP from endogenous VASP-binding proteins. Seven copies of a similar sequence are present in the VASP-binding protein IactA of *L. ivanovii* [27]. VASP has recently been shown to bind to a protein that, based on immunological and isoelectric data, appears to be related to chicken zyxin, another focal-adhesion protein [3]. It is noteworthy that chicken zyxin [28] also contains a copy of the FP4 motif (102-FPPPPPPFEE-111) as well as a related sequence (121-FPSPPPPPPMFDE-134). The FP4 motif is distinct from other proline-rich motifs that bind SH3 and WW domains [29] or motifs that bind profilin [9]. Thus, the FP4 motif and corresponding proline-rich binding domain in VASP may be novel modules mediating protein–protein interactions.

Immunolocalization studies have shown VASP to be concentrated in focal adhesions, where it co-localizes with vinculin [1,2]. The present data suggest that targeting of VASP to these structures is mediated, at least in part, by its ability to bind vinculin.

It has already been shown that deletion of residues 284–380 of VASP, which includes a cluster of repeated charged residues, prevents recruitment of the protein to focal adhesions in BHK21 cells [1]. Here, we demonstrate that removal of the charged cluster, residues 326–380, prevents binding of VASP to the vinculin proline-rich motif-1. This is consistent with targeting of VASP to focal adhesions being mediated via binding to vinculin. Furthermore, these data indicate that the charge cluster sequence is involved in binding to the vinculin proline-rich domain. This may be a direct interaction between residues in this sequence and the vinculin sequence. Alternatively, this sequence may have a role in maintaining features of VASP secondary or tertiary structure critical for vinculin binding.

VASP is a substrate for cAK and cGK, both *in vitro* and in whole cells [23,24]. Phosphorylation occurs on Ser-157 and -239 and Thr-278 [1,23], although the basal level of threonine phosphorylation is not increased by activators of these cyclic nucleotide-dependent kinases in platelets. Phosphorylation of Ser-157 causes a marked conformational change in VASP, which results in decreased mobility of the protein in SDS/PAGE [23], as observed in the present study. VASP phosphorylation on Ser-157 correlates with inhibition of platelet aggregation [24], binding of platelet integrin IIb/IIIa to its ligand fibrinogen [26] and endothelial cell retraction [4]. Potentially, interaction between VASP and vinculin could also be regulated by phosphorylation of VASP. However, we found no evidence in the present study that the phosphorylation state of VASP affected its ability to interact with the proline-rich motif of vinculin.

The demonstration that VASP plays a role in actin nucleation, together with the finding that VASP is located in focal adhesions, lamellipodia and microspikes in mammalian cells, suggests that this protein could be important in regulating actin-filament formation in areas of highly dynamic membrane activity. It is possible that cellular analogues of ActA with VASP-binding activity located at the plasma membrane could act to recruit VASP and thereby initiate microfilament assembly leading to ruffling and lamellipodial extension. It is of interest that vinculin has been observed at the leading edge of newly forming lamellipodia in fibroblasts activated by platelet-derived growth factor or after microinjection with active Rac1 [11]. The present data suggest that vinculin at these locations could act to recruit VASP and thereby initiate filament assembly. VASP binding to vinculin could also have a role in actin-filament assembly at focal adhesions.

A further functional consequence of the VASP–vinculin interaction demonstrated here may be to modulate the ability of vinculin to associate with other proteins. It has recently been shown that interaction between N- and C-terminal sequences within vinculin results in the protein adopting a closed conformation that prevents binding of F-actin, talin and α-actinin [20,30]. Binding of VASP to the proline-rich domain of vinculin could disrupt this conformation and permit binding of these cytoskeletal proteins. Potentially, this could stabilize the focal adhesion and allow coupling to F-actin stress fibres. This hypothesis is currently being tested.

We are grateful to S. T. Barry for providing the GST-836-940 construct. We thank the Medical Research Council, the British Heart Foundation and the Yamanouchi

Research Institute U.K. for their financial support. N.P.J.B. and J.E.D. also thank Professor P. R. F. Bell for support.

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1 July 1996/22 July 1996; accepted 24 July 1996