

Short communication

**ZYXIN-VASP INTERACTIONS ALTER ACTIN REGULATORY
ACTIVITY IN ZYXIN-VASP COMPLEXES**JACOB GRANGE[§], JAMES D. MOODY[§], MARC P. A. ASCIONE
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Abstract: Cell-cell and cell-substrate adhesions are sites of dramatic actin rearrangements and where actin-membrane connections are tightly regulated. Zyxin-VASP complexes localize to sites of cell-cell and cell-substrate adhesion and function to regulate actin dynamics and actin-membrane connections at these sites. To accomplish these functions, zyxin recruits VASP to cellular sites via proline-rich binding sites near zyxin's amino terminus. While the prevailing thought has been that zyxin simply acts as a scaffold protein for VASP binding, the identification of a LIM domain-VASP interaction could complicate this view. Here we assess how zyxin-VASP binding through both the proline rich motifs and the LIM domains alters specific VASP functions. We find that neither individual interaction alters VASP's actin regulatory activities. In contrast, however, we find that full-length zyxin dramatically reduces VASP-mediated actin bundling and actin assembly. Taken together, these results suggest a model where zyxin-VASP complexes occur in complex organizations with suppressed actin regulatory activity.

Key words: Actin, VASP, Zyxin, Adhesion, Migration, Cytoskeleton

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Abbreviation used: VASP – vasodilator stimulated phosphoprotein

INTRODUCTION

Actin reorganization events are fundamental to migration and cell adhesion. At focal adhesions, actin is organized into a thick cable that abuts the membrane [1-2]. Though actin at mature cell-cell contacts is organized differently than at focal adhesions, structures similar to focal adhesions are transiently observed as cell-cell junctions are assembled [3-5]. The interface between actin cables and adhesion sites contains zyxin and VASP [6-7]. Zyxin binds VASP through a series of four proline-rich (FPPPPP) motifs [7-9], as well as through the LIM domain region [10]. Cells lacking zyxin no longer localize VASP to focal adhesions [11], an effect that also results from zyxin mislocalization [12], suggesting that zyxin localizes VASP to adhesion sites. It has been suggested that zyxin-VASP complexes regulate local actin dynamics and mediate actin connections to adhesion systems. Consistent with this, defects in stress fiber reinforcement following stretch stimuli are also observed in cells lacking zyxin [13], highlighting a critical role for VASP in organizing actin at focal adhesions. Only fragments of zyxin containing VASP binding sites initiate actin assembly when expressed in cells [14]. Further, zyxin function in driving accelerated cell-cell junction formation and resistance to epithelial scattering depends on proline-rich VASP binding sites [4, 15]. How zyxin and VASP activity at adhesion sites is regulated is less clear, though zyxin LIM domains have been implicated here. Access of binding partners, including VASP, to zyxin is regulated by a LIM domain-mediated head-tail interaction within the zyxin molecule [10].

Since zyxin can bind VASP through either the FPPPPP motifs or the LIM domains, it is possible that zyxin-VASP complexes might link to actin in different ways. Alternately, it is possible that different zyxin-VASP interactions alter the biochemical activities of zyxin-VASP complexes. In vitro biochemical assays are employed here to assess VASP's actin regulatory activities in the presence or absence of LIM domain- and proline-rich repeat-mediated zyxin interactions. We find that full-length zyxin, which can bind to VASP via two domains simultaneously, inhibits VASP's actin regulatory functions. However, neither interaction alone alters VASP's actin regulatory properties. Based on the results presented, a model for regulation of VASP availability at adhesion sites is proposed.

MATERIALS AND METHODS

Protein purification

Proteins used here were purified from bacteria and dialyzed into Buffer A (100 mM NaCl, 20 mM Tris, 0.5% (v/v) Triton X-100, 10 µg/ml antipain, 10 µg/ml leupeptin, 10 µg/ml pepstatin A, 1 mM PMSF, 1 mM DTT, pH 7.4) and their concentration determined by Bradford method [16].

Actin polymerization assays

Pyrene actin assays were performed as described by Mullins and Machesky [17]. Fluorescence of a 9:1 actin:pyrene-actin mixture (Cytoskeleton) was monitored in a fluorometer cuvette following initiation of polymerization by addition of Mg^{2+} ions. Critical concentration assays were performed as described by Mullins and Machesky [17]. A 5 μ M 9:1 actin:pyrene-actin mixture was allowed to polymerize for 2 hours at room temperature. The resulting actin filaments were supplemented, as indicated, with 250 nM VASP and/or 500 nM GST-zyxin LIM region fusion protein. The mixture was then diluted into F- or G-actin conditions, and incubated overnight at room temperature and in the dark, and fluorescence measured for each concentration of each sample. In these experiments, each data point was generated in quadruplicate. Intersection of linear fits to the averaged data points for each condition, using Microsoft Excel, revealed the critical concentration for actin under each condition.

Actin sedimentation assays

Actin (Cytoskeleton) was diluted into to a final concentration of 1 μ M in buffer containing 100 mM NaCl, 5 mM $MgCl_2$, 1 mM ATP, and 1 mM DTT. Additional proteins were added and actin was allowed to polymerize at room temperature for 1 hour. VASP was added in increasing amounts to a final concentration that reached as high as 500 nM. Zyxin fusion proteins were added to a final concentration of 500 nM. Actin bundles were obtained by differential centrifugation at 10,000 x g for 7.5 minutes. Actin filaments were subsequently isolated by differential centrifugation at 100,000 x g for 7.5 minutes. Fractions were separated in SDS-polyacrylamide gels and proteins visualized with GelCode Blue (Pierce).

RESULTS AND DISCUSSION

Since zyxin binds VASP through multiple motifs, zyxin could modulate the actin regulatory functions of VASP in unpredictable ways. We sought to define how zyxin might alter VASP function using biochemical assays that measure VASP's specific actin regulatory functions. Since VASP is an obligate tetramer, VASP has intrinsic actin filament bundling activity [18]. We reasoned that a single zyxin molecular could crosslink multiple VASP molecules together, thus increasing actin bundling. To assess this, we examined VASP-mediated actin bundle formation in the presence and absence of full-length GST-zyxin fusion protein. Actin can be separated by differential centrifugation into monomeric G-actin, individual filaments in a high speed pellet, and bundles of actin filaments in a low speed pellet. When actin polymerizes in the absence of VASP, forming individual filaments, actin is not observed in the low speed pellet fraction, only in the high speed pellet fraction (Fig. 1A). When VASP is present and actin bundles are formed, actin and VASP are mainly observed in the low speed pellet fraction. To detect changes in VASP's actin bundling

activity, actin filaments were incubated with increasing concentrations of VASP and the actin bundle fraction isolated by low speed differential centrifugation. If VASP activity in actin bundling were altered by zyxin binding, we would expect to see VASP appear in the low speed pellet fraction at lower or higher concentrations. As expected, a concentration dependent effect on actin bundle formation is observed (Fig. 1B). Surprisingly, the presence of GST-zyxin dramatically reduces VASP's actin bundling activity compared to VASP alone; less actin and VASP is observed in pellet fractions derived from samples containing zyxin than in samples that lacked zyxin. This shows that the presence of zyxin reduces actin bundling by VASP and suggests that a zyxin-VASP

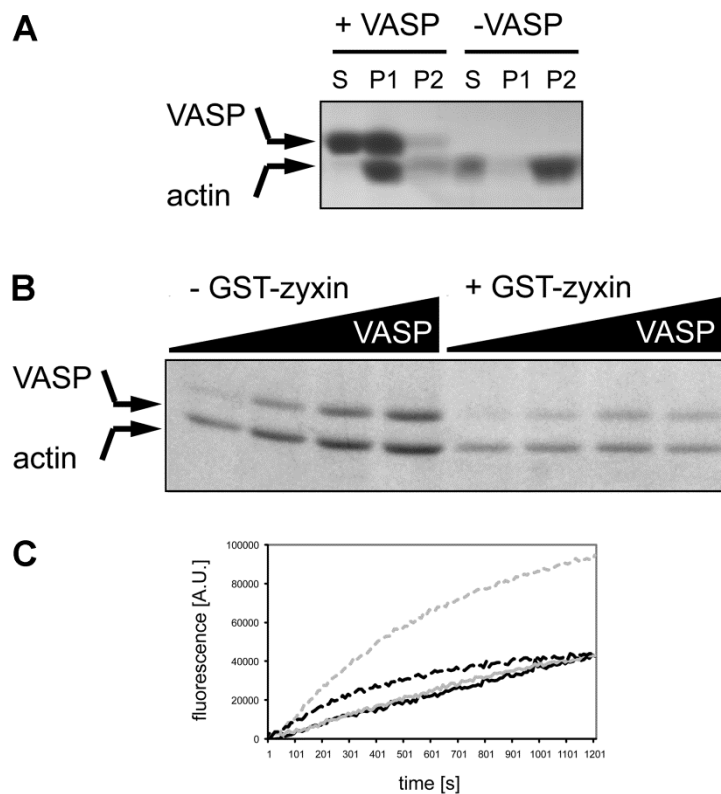


Fig. 1. Inhibition of VASP availability by full-length zyxin. A – Actin filaments, incubated with or without VASP, were separated into low speed pellet (P1), a high speed pellet (P2), and supernatant (S) fractions by differential centrifugation. Fractions were separated in SDS-polyacrylamide gels and proteins visualized with GelCode Blue. B – Low speed pellet fractions were generated following incubation of actin filaments with or without GST-zyxin and increasing amounts of VASP. Fractions were separated in SDS-polyacrylamide gels and proteins visualized with GelCode Blue. C – Pyrene actin assays containing actin alone (grey), actin and VASP (dotted grey), actin and GST-zyxin (black), and actin with both VASP and GST-zyxin (dotted black) were performed. VASP concentration was 500 nM and that of GST-zyxin 1 μ M.

interaction prevents VASP from participating in actin dynamics. To confirm this unexpected result, we measured the effect of full-length zyxin on VASP activity using pyrene actin assays (Fig. 1C), which measures actin nucleation and polymerization. Under the conditions used, VASP dramatically increases actin assembly above that observed for actin alone. GST-zyxin alone has minimal effect on actin assembly above actin alone. However, when GST-zyxin is added to assays containing VASP, actin assembly is comparable to the amount seen in assays containing actin alone and is significantly reduced from the amount seen when VASP is present without zyxin. Clearly, VASP availability in contributing to actin bundling and assembly *in vitro* is inhibited by the presence of full-length zyxin. Zyxin is thought to recruit VASP to specific cellular sites and allow VASP to direct actin rearrangements there. Consistent with this, expression of zyxin or zyxin fragments containing the proline rich repeats stimulate local actin assembly [14]. Since we see a reduction in VASP participation in actin dynamics in the presence of full-length zyxin, we wondered whether zyxin LIM domain-VASP binding might reduce VASP activity. This might explain why expression of zyxin LIM domains in cells did not result in local actin assembly [14]. We therefore sought to determine whether zyxin LIM domain altered the behavior of VASP in pyrene actin assays (Fig. 2A). GST-zyxin LIM region protein alone does not alter actin assembly at any concentration tested. Addition of VASP to pyrene actin assays greatly increases the rate of actin assembly compared to that of actin alone; the maximal rate of assembly is achieved essentially immediately. However, unlike as was observed for full-length zyxin, addition of GST-zyxin LIM region protein did not alter actin assembly in the presence of VASP (Fig. 2B). Thus, the zyxin LIM-VASP interaction has no effect on VASP's activity in regulating actin assembly.

We sought to further examine the effect of LIM domain binding on VASP's activity at barbed ends. VASP binds to actin barbed ends, allowing continued actin polymerization in the presence of capping protein [19-20]. In order to examine the effect of LIM domain binding on this activity, the critical concentration of actin polymerization was measured in the presence of purified proteins. The direct effects of VASP or zyxin LIM domains are negligible (Fig. 2C, E). In our hands, the critical concentration of actin alone is 0.208 μM , similar to an expected result of around 0.1 μM [17]. In the presence of VASP it is 0.146 μM and in the presence of GST-zyxin LIMs 1-3 it is 0.255 μM , values that are within experimental variation. Although a significant increase in the critical concentration would be expected for a barbed end binding protein such as VASP [21], our results are consistent with those already reported that also show no effect of VASP on actin depolymerization from barbed ends, despite barbed end binding [17]. In a separate experiment, we measured the critical concentration in the presence of both VASP and GST-zyxin LIMs 1-3 (0.060 μM) to be nearly identical to that of actin alone (0.055 μM , Fig. 2D, F). Clearly, actin barbed ends are not affected by VASP molecules participating in an interaction with zyxin LIM domains.

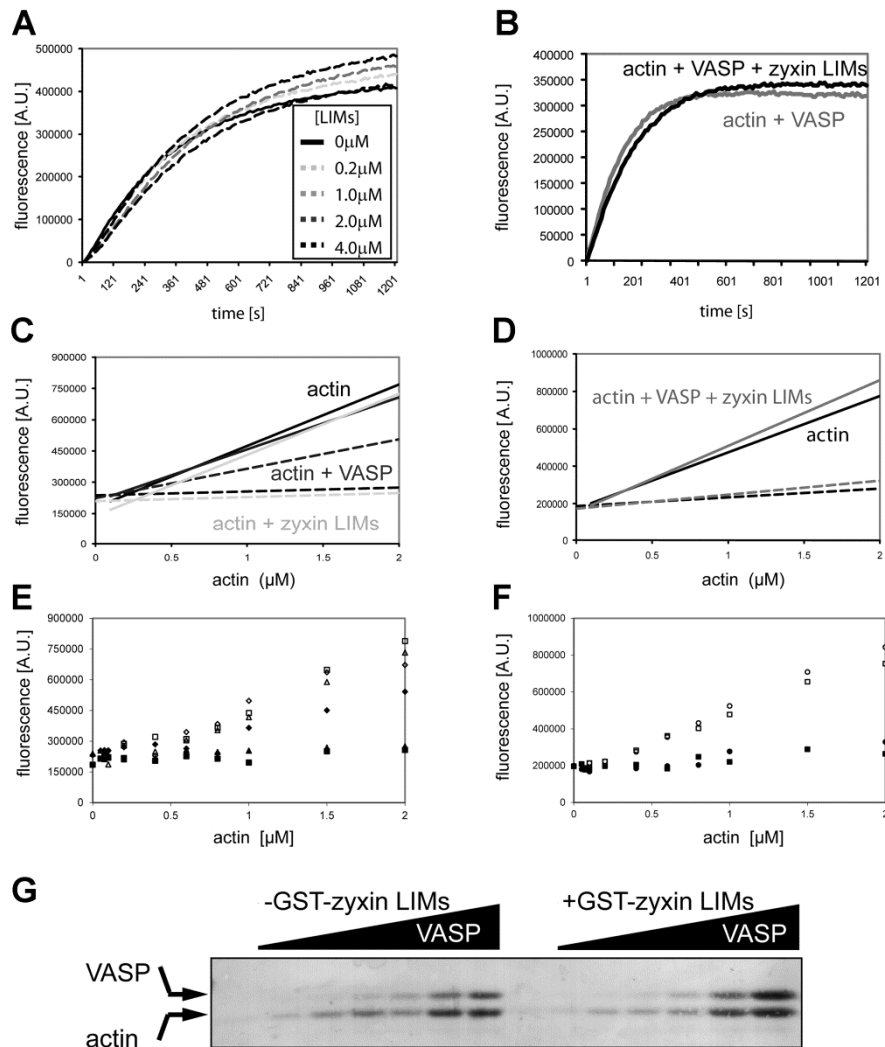


Fig. 2. Zyxin LIM domain binding permits VASP activity. A-B – Pyrene actin assays containing the indicated proteins were performed. For B, VASP and GST-zyxin LIMs 1-3 concentrations were 500 nM and 2 μM , respectively. C-F – Line fits (C-D) and scatter plots (E-F) of critical concentration of actin measurements in the presence of purified protein. Solid lines represent fits to measurement in polymerizing conditions, dotted lines represent fits to measurements in non-polymerizing conditions. The intersect of solid and dotted lines is the critical concentration of actin polymerization for the relevant condition. For the scatter plots, open and closed markers indicate taken for F-actin and G-actin conditions and the marker denotes the proteins present: squares, actin alone; triangles, actin and VASP; diamonds, actin, VASP, and GST-zyxin LIMs 1-3; circles, actin and GST-zyxin LIMs 1-3. G – Low speed pellet fractions were generated following incubation of actin filaments with or without zyxin LIM domain fusion protein and increasing amounts of VASP. Fractions were separated in SDS-polyacrylamide gels and proteins visualized with GelCode Blue.

We finally sought to determine whether zyxin LIM domain binding would alter actin bundling by VASP. We isolated bundles of actin filaments from samples where the VASP concentrations was increased, resulting in the increased detection of VASP and actin in those fractions (Fig. 2G). When precisely the same experiment was performed in the presence of GST-zyxin LIM region protein, actin bundling is observed with precisely the same VASP concentration dependence. This indicates that zyxin LIM binding does not alter the actin bundling activity of VASP. Clearly LIM domain binding does not increase VASP-mediated actin bundling. And while one might conclude that VASP binding to actin and LIM domains in a mutually exclusive, the VASP-LIM interaction would still be expected to alter bundling activity; competitive binding by the LIM domain protein would reduce the level of VASP available to bind F-actin when the LIM domain fusion protein is present. Not only would this be expected to reduce VASP-mediated actin bundling, but it would be expected to reduce the effect of VASP on actin assembly in pyrene actin assays in Fig. 2B also. Since neither of these effects is observed, we must conclude that VASP-LIM binding allows VASP-actin interactions, without affecting the result of those interactions.

Since the zyxin LIM domain interaction does not alter VASP activity *in vitro*, we next sought to determine if a zyxin fragment containing proline-rich motifs could alter VASP's actin bundling activity. The presence of a GST zyxin fusion protein containing proline rich domains and lacking LIM domains (zyxin Δ LIM-GST) did not alter the concentration dependence of actin bundle formation (Fig. 3).

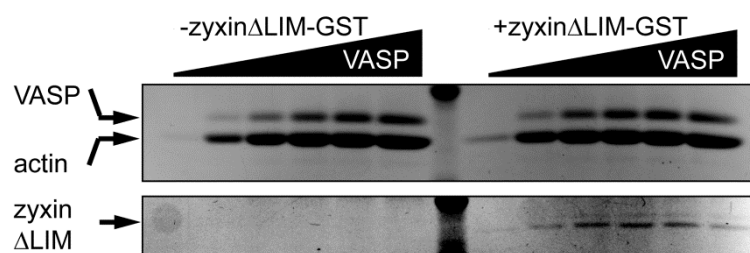


Fig. 3. Binding by zyxin FPPPPP motifs does not alter VASP-dependent actin bundling. Low speed pellet fractions were generated following incubation of actin filaments with or without a zyxin fusion protein lacking LIM domains (GST-zyxin Δ LIM) and increasing amounts of VASP. Fractions were separated in SDS-polyacrylamide gels and proteins visualized with GelCode Blue.

Zyxin Δ LIM-GST was also detected in the bundle fraction, showing that VASP can simultaneously bind actin filaments and zyxin FPPPPP motifs. Clearly zyxin binding via the FPPPPP motif does not prevent VASP-actin binding and allows a different complex architecture for linking zyxin to actin filaments.

That neither the LIM domain region nor the FPPPPP motif interaction can alter VASP-mediated actin bundling alone, while the presence of both interactions in full-length zyxin blocks VASP activity in this process, suggests that both interaction must act together when full-length zyxin reduces VASP participation in actin dynamics. That full-length zyxin is observed to drive actin assembly in cells [14] indicates that the suppression of VASP function in actin related processes can be relieved, possibly by breaking one zyxin-VASP interaction within zyxin-VASP complexes. The occurrence of active and inactive zyxin-VASP complexes could allow for rapid control of VASP activity at cellular sites, a level of cellular control that would extend beyond zyxin's widely accepted mechanism of controlling VASP's ability to participate in local actin dynamics by simple recruitment alone. One possible mechanism for releasing VASP activity is VASP phosphorylation, which specifically blocks LIM-mediated zyxin-VASP interactions [10]. Phosphorylation of zyxin could also alter organization within zyxin-VASP complexes. Zyxin phosphorylation at serine 142 by AKT does allow LIM domain interactions with acinus S [22]. Further, expression of a phosphomimetic zyxin S142 mutant generates identical phenotypes as those observed with expression of a zyxin mutant lacking LIM domains [23]. However zyxin-VASP complexes are regulated, zyxin appears to be capable of doing more than passively recruiting VASP to cellular sites where it is needed for actin reorganization.

Acknowledgements. J.G. was supported by a BYU Cancer Center summer undergraduate research internship. Thanks to Dorothy Schafer, who provided VASP expression constructs.

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