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Norio Takizawa  
*University of Massachusetts Medical School*

Tara C. Smith  
*University of Massachusetts Medical School*

Thomas Nebl  
*University of Massachusetts Medical School*

*See next page for additional authors*

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Supervillin modulation of focal adhesions involving TRIP6/ZRP-1

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1Department of Cell Biology, and 2Department of Physiology, University of Massachusetts Medical School, Worcester, MA 01605
3Huntsman Cancer Institute, University of Utah, Salt Lake City, UT 84112

Introduction

Cell–substrate contacts, called focal adhesions (FAs), are dynamic in rapidly moving cells. We show that supervillin (SV)—a peripheral membrane protein that binds myosin II and F-actin in such cells—negatively regulates stress fibers, FAs, and cell–substrate adhesion. The major FA regulatory sequence within SV (SV342-571) binds to the LIM domains of two proteins in the zyxin family, thyroid receptor–interacting protein 6 (TRIP6) and lipoma-preferred partner (LPP), but not to zyxin itself.

SV and TRIP6 colocalize within large FAs, where TRIP6 may help recruit SV. RNAi-mediated decreases in either protein increase cell adhesion to fibronectin. TRIP6 partially rescues SV effects on stress fibers and FAs, apparently by mislocating SV away from FAs. Thus, SV interactions with TRIP6 at FAs promote loss of FA structure and function. SV and TRIP6 binding partners suggest several specific mechanisms through which the SV–TRIP6 interaction may regulate FA maturation and/or disassembly.

Correspondence to Elizabeth J. Luna: Luna@umassmed.edu

S.J. Palmieri’s present address is Department of Infection and Immunity, The Walter and Eliza Hall Institute of Medical Research, VIC 3050, Australia.

Abbreviations used in this paper: ATCC, American Type Culture Collection; AV, archvillin; CAS, Crk-associated substrate; ERK, extracellular factor-regulated kinase; ds, double-stranded; FA, focal adhesion; LPA, lysophosphatidic acid; LPP, lipoma-preferred partner; PTP, protein tyrosine phosphatase; SmAV, smooth muscle AV; SV, supervillin; TRIP, thyroid receptor–interacting protein; ZRP-1, zyxin-related protein 1.

The online version of this article contains supplemental material.
SV negatively regulates FA structure and function

EGFP-SV at low levels overlaps with vinculin at or near FAs on the basal surfaces of CV1 (Wulfkuhle et al., 1999) and COS7 (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200512051/DC1) cells. This overlap is more pronounced at large, mature FAs in the center and posterior of the cell than at newly formed FAs at the cell periphery (Fig. S1, arrow). SV also localizes along associated stress fibers; this signal increases disproportionately with increasing amounts of EGFP-SV (Wulfkuhle et al., 1999).

SV targeting to large FAs apparently reduces the number of these structures (Fig. 1). Large vinculin-labeled FAs are abundant in COS7 cells that overexpress EGFP (Fig. 1 A, a and b), but most large vinculin spots are lost in cells that overexpress EGFP-SV (Fig. 1 A, c and d). We find an ∼1:1 ratio of EGFP-SV to endogenous SV in lysates from preparations with ∼20% transfected cells (Fig. 1 B), indicating that the average level of EGFP-SV in the transfected cells is approximately five times greater than endogenous SV, for a total of approximately six times more SV than in untransfected cells. We normalized the amount of fluorescence in cells transfected with EGFP alone or EGFP-SV so that cells with approximately sixfold overexpression of SV exhibit ∼50% of maximal fluorescence intensity (Fig. 1 C). The number of large FAs (≥10 μm²) in cells expressing EGFP-SV with ∼30–90% of maximal fluorescence are less than half those observed in cells expressing equivalent amounts of EGFP alone or in untransfected cells (Fig. 1, C and D). Thus, even an approximately threefold increase in SV levels down-regulates the number of large FAs.

Cell–substrate adhesion also correlates inversely with SV levels (Fig. 1, E and F). After preparative FACS, COS7 cells expressing EGFP-SV contain ∼6.3-fold more SV than cells expressing EGFP (Fig. 1 E, top) and are significantly less adherent to fibronectin-coated coverslips (Fig. 1 E, bottom). Conversely, cells with ∼10% of endogenous levels of SV adhere more tightly to fibronectin (Fig. 1 F, bottom). Collectively, these results show an inverse correlation between SV levels and FA function, although any increases in the number and/or size of mature FAs after SV knockdown lack statistical significance (unpublished data).

Identification of FA regulatory sequences

To identify SV sequences responsible for loss of FA integrity, we quantified the number of total and large FAs in COS7 cells expressing different EGFP-tagged SV constructs (Fig. 2).
Full-length SV (Fig. 2 A, SV1-1792) reduces the number of large vinculin foci (Fig. 1 A–D and Fig. 2 E), but has relatively little effect on the number of the more abundant, smaller foci near cell peripheries (Fig. 2 B, b). The result is the absence of a statistically significant effect on the number of total FAs per cell (Fig. 2 C). The SV N terminus (SV1-830) decreases the number of both total and large FAs, whereas the SV C terminus (SV830-1792) exhibits effects similar to those of full-length SV (Fig. 2, B, C, and E). Thus, a sequence with major impact on FA stability resides within the SV N terminus.

Further dissection of N-terminal SV sequences identifies SV1-174 and SV342-571 as FA disruption sequences (Fig. 2, B, D, and F). Both of these sequences significantly reduce the number of large FAs (Fig. 2 F), but only SV342-571 significantly reduces the total number of vinculin foci per cell (Fig. 2 D).

COS-7 cells transfected with SV or SV N-terminal sequences also exhibit decreased numbers of large F-actin.
bundles (Fig. 3). Although full-length SV with its three F-actin–
binding sites (Chen et al., 2003) increases the number of thin
actin structures (Wulfkuhle et al., 1999), SV overexpression
greatly decreases the number and size of large, straight ac-
tin bundles (Fig. 3 A, compare b with a). However, most SV-
expressing cells still contain at least one large basal F-actin bundle
(Fig. 3 B). Because most of these bundles exhibit periodic
staining for α-actinin (Wulfkuhle et al., 1999) and myosin II
(unpublished data), we refer to them here as “stress fi bers,” al-
though they are smaller and less well organized than are stress
fibers in fi broblasts.

Consistent with their effects on FA structure (Fig. 2),
SV1-830 and SV342-571 signifi cantly decrease the number of
COS7 cells containing at least one stress fi ber, as compared
with cells expressing EGFP alone or other EGFP-SV fragments
(Fig. 3, A–C). SV1-830 and SV342-571 also decrease the num-
ber and thickness of stress fi bers in CV1 cells (unpublished
data). Thus, SV342-571 contains a site that is primarily respon-
sible for the morphological effects of SV and SV1-830 on FAs
and stress fi ber organization and function.

In support of this conclusion, SV342-571 reduces the con-
tractility of CV1 cells (Fig. 4 and Table I). Significant percentages
of cells expressing EGFP only or other EGFP-SV N-terminal se-
quencies deform fl exible silicon substrates, forming “wrinkles”
(Fig. 4 and Table I). In contrast, wrinkles are absent from the areas
around most CV1 cells expressing SV342-571 (Table I),
even cells near untransfected cells that are actively wrinkling the
substrate, thus, demonstrating its local deformability (Fig. 4 C,
arrow vs. arrowhead). Thus, SV342-571 contains sequences ca-
pable of reducing FA structure and function.

Identification of SV342-571 binding partners

We hypothesized that a previously unknown interaction medi-
ated the effects of SV342-571 on FAs. The only known binding
partner for SV342-571 is F-actin (Fig. 2 A), but F-actin also
binds to SV171-342 and SV570-830 (Chen et al., 2003), nei-
ther of which affects FA structure and function to the same ex-
tent (Figs. 2–4 and Table I). To identify candidate interactors
with SV342-571, we undertook an undirected yeast two-hybrid
screen. Because SV343-570, SV171-343, and SV343-830 all
self-activated reporter gene expression (unpublished data),
our bait plasmid encoded SV171-830. 11 prey proteins were
identifi ed and confi rmed by directed yeast two-hybrid assays
(Table II). These proteins were further screened using baits en-
coding either SV171-343 or SV570-830. Only the longer plas-
mid (SV171-830) containing SV343-570 sequences interacted
strongly with the prey proteins.

Two interactors, TRIP6/ZRP-1 and Tctex-1/DYNLT1, ac-
count for a majority of the clones identifi ed in these screens
(Fig. 5). Ten independent clones encode all three C-terminal
LIM domains of TRIP6. Another clone encodes the fi rst two
LIM domains of LPP, suggesting that two or more LIM domains
are required for binding. Six independent clones encode full-
length Tctex-1, which is a dynein light chain (DYNLT1).

We confirmed binding interactions with TRIP6 and
Tctex-1 using pull-down assays with GST fusion proteins con-
taining SV343-571 (Fig. 6). V5-tagged TRIP6 LIM domains
(Fig. 6 A) and V5-tagged full-length Tctex-1 (Fig. 6 B) copellet

Table I. Quantifi cation of cell deformability

<table>
<thead>
<tr>
<th>EGFP construct</th>
<th>Wrinkled cell</th>
<th>Total cells</th>
<th>%</th>
<th>No. expts</th>
</tr>
</thead>
<tbody>
<tr>
<td>SV1-174</td>
<td>15</td>
<td>28</td>
<td>54%</td>
<td>5</td>
</tr>
<tr>
<td>SV171-342</td>
<td>10</td>
<td>28</td>
<td>36%</td>
<td>4</td>
</tr>
<tr>
<td>SV342-571</td>
<td>2</td>
<td>31</td>
<td>6%</td>
<td>5</td>
</tr>
<tr>
<td>SV570-830</td>
<td>18</td>
<td>61</td>
<td>30%</td>
<td>4</td>
</tr>
<tr>
<td>EGFP only</td>
<td>20</td>
<td>29</td>
<td>69%</td>
<td>4</td>
</tr>
</tbody>
</table>

Pooled number of wrinkled cells, total cells, and percentages of wrinkled cells from 4–5 experiments with sparsely spread CV1 cells expressing the designated EGFP chimeric proteins. No. expts, number of experiments.
with glutathione-Sepharose beads containing GST-tagged SV171-830, SV343-571, SV343-571, and SV343-830 (Fig. 6, A and B, lanes 2, 5, 7, and 8). Neither V5-tagged protein cosediments with beads containing GST only or SV171-342 (Fig. 6, A and B, lanes 3 and 4). A low-affinity interaction with SV570-830 is observed for Tctex-1 (Fig. 6 B, lane 6). No proteins reactive with the anti-V5 antibody are detectable in control yeast extracts (Fig. 6, A and B, lane 9), confirming the binding specificities of TRIP6 and Tctex-1 for SV342-571.

Binding of SV342-571 to TRIP6 or Tctex-1 is also observed in doubly transfected COS7 cells. Myc-tagged TRIP6 LIM domains and Tctex-1 cosediment with GST-tagged EGF-P-SV342-571, but not with EGF-P-GST alone (Fig. 6 C, lane 3 vs. 4). Notably, neither Myc-tagged LIM domains from zyxin nor Flag-tagged, full-length TRIP6 cosediments with EGFP-SV342-571-GST. Coimmunoprecipitation of full-length proteins was precluded by the inextractability of full-length SV (Pestonjamasp et al., 1997; Nebl et al., 2002).

The TRIP6 LIM domains and Tctex-1 bind directly to overlapping sequences within SV343-571 (Fig. 6, D–F). Purified, bacterially expressed hexahistidine (6×His)-tagged TRIP6 LIM domains (Fig. 6 D) and 6×His-Tctex-1 (Fig. 6 E) cosediment with glutathione–Sepharose beads containing pre-bound, purified GST-SV343-571 (Fig. 6, D and E, lane 2), but not with beads bound to GST alone (Fig. 6, D and E, lane 4). Increasing amounts of 6×His-tagged Tctex-1 compete with 6×His-tagged TRIP6 LIM domains for binding to a fixed, limiting amount of GST-SV343-571 (Fig. 6 F).

Point mutagenesis provides further support for competition between TRIP6 and Tctex-1 for binding to SV343-571. Conversion of the highly conserved SV residues Arg-426 and Tyr-427 to alanines (RY/AA) coordinately reduces binding of GST-SV343-571-RY/AA to both TRIP6 and Tctex-1 by ~70% (Fig. 6 G). Similarly, mutagenesis of these residues in EGF-P-SV343-571 reduces this protein’s effects on stress fiber and FA structure (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200512051/DC1), demonstrating the functional importance of the TRIP6/Tctex-1–binding site within SV342-571.

**Physiological relevance of the SV–TRIP6 interaction**

To determine whether the SV interaction with TRIP6 and/or with Tctex-1 is involved in SV-mediated changes in FA structure and function, we colocalized these proteins in A7r5 (Fig. 7) and COS7 (Fig. 8) cells. Endogenous levels of the relatively abundant SmAV (Gangopadhyay et al., 2004) partially colocalize with endogenous vinculin (Fig. 7, b–d) and TRIP6 (Fig. 7, j–l) in A7r5 cells on fibronectin (Fig. 7, a, e, and i). The SmAV signal is a subset of that for vinculin and TRIP6 in FAs, but stops short of the labeling for these two proteins at cell edges. This result is consistent with a role for the SV–TRIP6 interaction in regulating adhesion, but is uninformative about Tctex-1 because we could not detect Tctex-1 in A7r5 cells (unpublished data).

![Figure 5. TRIP6/ZRP-1 and Tctex-1/DYNLT1 are the prey proteins obtained most frequently in yeast two-hybrid screens with SV171-830 as bait.](https://www.jcb.org/cgi/content/full/jcb.200512051/DC1)
Figure 6. SV343-571 binds to both TRIP6 and Tctex-1 in GST pull-down assays. Immunoblots with anti-V5 antibody of extracts (A and B, lane 1) from yeast expressing V5-tagged TRIP6 LIM domains (A) or V5-tagged full-length Tctex-1 (B). Specifically bound proteins were eluted with glutathione from glutathione–Sepharose columns with prebound GST-tagged SV proteins (~1.0 nmol), as shown. V5-immunoreactive proteins were absent from control (Con) extracts (A and B, lane 9). (C) Immunoblots with anti-tag antibodies, as shown on the left, of COS-7 cell lysates (lanes 1 and 2) or lysate proteins eluted from glutathione–Sepharose (lanes 3 and 4). The COS-7 cells coexpressed either EGFP-SV342-571-GST (lanes 1 and 3, top) or EGFP-GST (lanes 2 and 4, top) and the tagged constructs indicated on the right. Although Flag-tagged full-length TRIP6 (second row) and Myc-tagged zyxin LIM domains (fourth row) bound neither GST protein, the Myc-tagged LIM domains of TRIP6 (third row) and Myc-tagged Tctex-1 (fifth row) each cosedimented with EGFP-SV342-571-GST, but not with EGFP-GST. Direct binding in vitro of 6×His-tagged TRIP6 LIM domains (D) and Tctex-1 (E) to purified GST-SV343-571 (D and E, lane 2). Supernatants (S; lanes 1, 3, and 5) and pellets (P; lanes 2, 4, and 6) from incubations with GST-SV343-571 and 6×His-proteins (lanes 1 and 2); GST alone and 6×His-proteins (lanes 3 and 4); or GST-SV343-571 alone (lanes 5 and 6). (F) Competition assay showing that increasing amounts of 6×His-Tctex-1 decreased binding of 6×His-TRIP6 LIM domains to GST-SV343-571. Bound proteins were detected with Coomassie blue (CBB) or immunoblotting with anti-6×His antibodies (His-Ab). TRIP6 also modulates adhesion of human A549 lung carcinoma cells to fibronectin (Fig. 10, A and B). RNAi-mediated knockdowns of TRIP6 (hTR1, hTR2), but not Tctex-1 (hTx), significantly increase adhesion (Fig. 10, A and B). This effect is comparable to that observed upon SV knockdown (hSV) and is consistent with the reported inverse correlation between TRIP6 levels and the number of large FAs (Guryanova et al., 2005).
TRIP6 sequences also partially reverse losses of stress fibers and large FAs induced by SV sequences capable of binding to these proteins (Fig. 10, C and D). Exogenously co-expressed TRIP6 LIM domains, but not zyxin LIM domains or Tctex-1, reduce the effects of SV domains, EGFP-SV1-830 and EGFP-SV342-571, on stress fibers in COS7 cells (Fig. 10 C). Full-length TRIP6 partially rescues the effects of full-length EGFP-SV (Fig. 10 D) and EGFP-SV1-830, although not that of SV342-571 (Fig. 10 C). The basis for the TRIP6-mediated rescue may be mislocalization of TRIP6–SV complexes because cells with high expression levels of both TRIP6 and EGFP-SV (Fig. 10 E) exhibit increased colocalization of TRIP6 and SV in protrusions lacking vinculin (Fig. 10 E, a–d, arrows, vs. Fig. 8 A). Rescued FAs contain TRIP6, but are largely devoid of EGFP-SV (Fig. 10 E, arrowheads). As expected, TRIP6 has no effect on the phenotype of point (SV-RY/AA) and deletion (SVΔ343-561) mutants of full-length SV that have reduced or no binding to TRIP6, respectively (Fig. 10, D and E [e–h]). Consistent with the observations that multiple SV sequences affect FA structure (Fig. 2, E and F), full-length SV mutants deficient in binding to TRIP6 induce a loss of large FAs in both the presence and absence of TRIP6 (Fig. 10 D). This result emphasizes the importance of additional SV-binding partners.

**Discussion**

We show that SV down-regulates FA structure and function, and that the mechanism involves interactions with TRIP6. Decreased levels of either protein increase cell adhesion to fibronectin. Increased SV levels decrease cell adhesion, as well as the number of stress fibers and large, mature FAs. Although more than one region of SV deleteriously affects FAs, the SV sequence with the largest effect on FA structure and function is SV342-571, which binds directly to Tctex-1 and the C-terminal LIM domains of TRIP6. SV and TRIP6 colocalize at mature FAs, and optimal SV recruitment to FAs requires binding to TRIP6. TRIP6 and the TRIP6 LIM domains partially rescue disruptive effects of SV sequences on FAs and stress fibers. Specificity is indicated by the lack of effect of Tctex-1 or the zyxin LIM domains on SV phenotypes. Thus, binding to SV342-571 is necessary, but not sufficient, to reverse SV effects on FAs.

The TRIP6 N terminus may shield the C-terminal LIM domains from SV342-571 in the absence of a regulatory signal, as has been proposed for other LIM domain proteins (Kadomatsu and Beckerle, 2004; Lai et al., 2005). No direct interaction between SV sequences and the TRIP6 N terminus was detected in either yeast two-hybrid or pull-down assays. Nevertheless, full-length TRIP6 rescues the disruptive effects of longer SV proteins, implying the possibility of regulatory cross-talk between the TRIP6 N terminus and SV sequences other than SV342-571.
Observations that zyxin influences motility, adhesion, and stress fiber formation (Golsteyn et al., 1997; Hoffman et al., 2006) are reminiscent of those observed upon overexpression of SV sequences. However, SV342-571 does not bind zyxin. In conjunction with other recent observations (Petit et al., 2005), these results suggest that the members of the zyxin protein family have overlapping, but distinguishable, functions.

The loss of adhesion induced by SV overexpression apparently represents a gain of function because this phenotype is opposite that observed after SV knockdown. An SV-induced negative effect on large FAs is supported by the localization of SV with large FAs, which are structures that undergo dynamic remodeling (Ballestrem et al., 2001; Carragher and Frame, 2004). SV-mediated loss of large FAs also fits with the absence or reduced prevalence of large FAs and stress fibers in cells that contain relatively high amounts of endogenous SV, e.g., carcinomas and neutrophils (Pestonjamasp et al., 1997; Pope et al., 1998). Carcinomas and hematopoietic cells also express TRIP6 and/or LPP (Daheron et al., 2001; Xu et al., 2004), which is consistent with a physiological role for interactions with SV at dynamic FAs. Neutrophil FAs must be highly dynamic because they turn over rapidly during immune responses when little or no integrin is synthesized (Zhang et al., 2004).

Although the rescue of the SV phenotype by the TRIP6 LIM domains may be attributable to a simple dominant-negative effect on TRIP6 function (Kassel et al., 2004), the rescue by full-length TRIP6 is more interesting. In cells that overexpress both TRIP6 and wild-type SV, both proteins mislocalize into cell protrusions, sequestering SW away from FAs. Full-length SV proteins deficient in binding to TRIP6 still reduce the number of mature FAs, although these SV mutants are largely absent from FAs; TRIP6 localization at residual (or new) FAs is essentially unaffected. Thus, the FA equilibrium is disturbed by SV mutants that either contain the TRIP6-binding site out of...
context or contain the other SV FA-targeting sequences in the absence of high-affinity binding to TRIP6.

The simplest interpretation of these results is that TRIP6 and SV, together with other associated proteins, act during a FA assembly/disassembly cycle to control the rate of FA turnover. In this working model, TRIP6 helps recruit SV to FAs; SV then, directly or indirectly, either blocks later stages in FA maturation and/or increases the rate of FA turnover. When SV or TRIP6 levels are limiting, cell-substrate adhesion increases because FAs are locked “on.” Increasing amounts of the limiting protein decrease cell adhesion by increasing the rate of maturation of adhesive nascent FAs into less adhesive mature FAs and/or by increasing the rate of FA disassembly. In this model, TRIP6 overexpression helps restore the balance between FA assembly and disassembly in SV-overexpressing cells by (a) accelerating the formation of new FAs, (b) sequestering SV and proteins required for FA disassembly away from FAs, and/or (c) promoting the recycling of SV-associated proteins that are required for FA assembly. Overexpressed full-length SV that is deficient for binding to TRIP6 may disrupt FAs by interacting with other proteins involved in FA turnover in such a way that they become rate limiting for FA reassembly.

This model is consistent with the conflicting observations about the role of TRIP6 during cell migration. The prediction is that cellular responses to exogenous changes in TRIP6 are dependent on endogenous TRIP6 levels, relative to the levels of SV and other interactors in the proposed FA disassembly pathway, and on cell type–specific regulation.

We suggest that the SV–TRIP6 interaction demarcates a FA subdomain, perhaps a signaling scaffold, which controls FA integrity and/or turnover. One possibility is that the SV–TRIP6 interaction brings other SV-binding proteins into proximity with other TRIP6 partners. In addition to TRIP6, the SV N terminus (SV1-830) binds to the S2 subdomain of nonmuscle myosin II, to F-actin, and to the plasma membrane (Wulfkuhle et al., 1999; Chen et al., 2003). SV binding to TRIP6 may recruit myosin II in stress fibers into the vicinity of TRIP6-binding proteins that destabilize FAs. Candidate TRIP6 interactors include CAS/Casl (Yi et al., 2002), c-Src (Xu et al., 2004), Crk (Lai et al., 2005), the LPA2 receptor (Xu et al., 2004), the tyrosine phosphatase PTPN13/PTP-BL/FAP-1, and the adaptor protein RIL/PDLIM4 (Cuppen et al., 2000), which is known to increase stress fiber dynamics (Vallenius et al., 2004). In agreement with this hypothesis, both SV and the LPA–TRIP6–CAS pathway positively regulate ERK signaling (Gangopadhyay et al., 2004; Lai et al., 2005), a process implicated in FA turnover (Webb et al., 2004).

Alternatively, binding to TRIP6 may displace a positive regulator of FA stability through steric hindrance or binding to a shared site on the TRIP6 C terminus. For instance, SV may disrupt the interaction of TRIP6 with endoglin/CD105, which is a transmembrane component of the TGF-β complex that promotes stress fiber formation and the localization of TRIP6 and zyxin to FAs (Conley et al., 2004; Sanz-Rodriguez et al., 2004). Finally, we cannot exclude the possibility that SV indirectly influences FA structure through potentiation of TRIP6 effects in the nucleus. Despite its lack of a canonical NLS, TRIP6 can accumulate in the nucleus and modulate transcription (Kassel et al., 2004; Li et al., 2005). The TRIP6 LIM domains are sufficient for both SV binding (Fig. 6) and nuclear transport (Wang and Gilmore, 2001). SV contains functional NLS (Wulfkuhle et al., 1999) and, like TRIP6, is implicated in steroid hormone signaling (Ting et al., 2002). Thus, some of the effects reported here might be caused by changes in transcriptional activity induced by an SV–TRIP6 complex.

In summary, we show that the myosin II– and actin-binding protein SV regulates FA function through binding to TRIP6, a LIM domain–containing protein associated with signaling scaffolds that control cell motility. This is the first evidence for a myosin II–binding protein at FAs and for an explicit role for TRIP6 in adhesion. The direct binding of SV and TRIP6 suggest several specific, testable hypotheses by which TRIP6-associated scaffolds may control FA function. The SV–TRIP6 interaction may provide a “missing link” for actin-independent attachment of myosin II to the membrane at FAs and insight into molecular mechanisms for FA disassembly and/or recycling.

Materials and methods

Chemicals, proteins, and expression vectors

Chemicals were obtained from Sigma-Aldrich, Calbiochem-Novabiochem, Fisher Scientific, or VWR International, Inc., unless otherwise noted. EGFP-tagged SV constructs, purified GST-tagged SV proteins, and Flag-tagged murine TRIP6 were previously described (Wulfkuhle et al., 1999; Yi et al., 2002; Chen et al., 2003). Vectors encoding His-tagged TRIP6 LIM domains or Tctex-1 were generated by excising the insert from the pYESTrp prey vector using the vector KpnI and Xhol sites and ligating in-frame into the corresponding sites of the pET30a bacterial expression vector. The TRIP6 insert encoded amino acids 265–476, and the Tctex-1 insert was full length. Proteins tagged with 6×His were expressed and purified as previously described (Takizawa et al., 2003).

The mammalian expression vector pCMV-Myc (BD Biosciences and CLONTECH Laboratories, Inc.) was modified by inserting a HindIII restriction site into the EcoRI site in the multiple cloning site and used to create vectors encoding N-terminal Myc-tagged proteins. Full-length Tctex-1 (aa 1–113), the TRIP6-LIM domains (aa 265–476), and zyxin-LIM domains (aa 361–562) were ligated in frame between the introduced HindIII and the endogenous XhoI restriction sites. Sequences encoding full-length Tctex-1 or the TRIP6 LIM domains were excised from isolated pYESTrp library prey vectors. The sequence encoding the zyxin LIM domains was obtained by PCR using a murine zyxin cDNA in plBluecript-5′(+) vector as template. All vectors were confirmed by end sequencing.

EGFP-SV lacking the TRIP6/Tctex-1–binding site (EGFP-SVΔ343-561) was created by deleting the coding sequence for aa 343–561, converting Ser 343 to a tyrosine. PCR was used to introduce an AgeI site upstream of the codon for Gly 542 in double-stranded DNA (dsDNA) that included the unique endogenous EcoRV restriction site after the codon for Asp 830 (Wulfkuhle et al., 1999). The PCR product was digested with AgeI and EcoRV and ligated into similarly cut plBluecript II SK (−) (Wulfkuhle et al., 1999), replacing the codons for 343–560 with those for 361–562. SVΔ343-561 was then transferred into the mammalian expression vector EGFP-C1 (BD Biosciences and CLONTECH Laboratories, Inc.) with KpnI and Xhol.

Bovine SV sequences with reduced TRIP6/Tctex-1 binding were created by alanine replacement mutagenesis using the QuickChange Site-Directed Mutagenesis kit (Stratagene), following the manufacturer’s instructions and using the following PAGE-purified primers: sense: 5′-ATGAAATGCGCTGCTGCTAAACCCACGGCC-3′, and antisense: 5′-CGGCTGGGTTTGAGCGCAGCACTAT-3′. Modified DNA segments were sequenced in full.

Cell culture

COS7-2 cells (Wulfkuhle et al., 1999), an SV40-transformed derivative of monkey kidney epithelial CV-1 cells, and rat A7r5 aorta cells (American
Type Culture Collection (ATCC) were grown in DME with 10% FCS. CV-1 cells (ATCC) were maintained in MEM Alpha (Invitrogen) with 10% FCS. A549 human lung carcinoma cells (ATCC) were grown in Ham’s F12K medium, 2 mM L-glutamine, and 10% FCS. Cells were transfected using Effectene Transfection Reagent (QIAGEN). Populations of 100% transfected cells were obtained by FACS 48 h after transfection with plasmids encoding EGFP or EGFPSV.

Immunofluorescence microscopy

Methods for indirect immunofluorescence microscopy have been previously described (Chen et al., 2003). In brief, cells transfected for 24 h were fixed with 4% paraformaldehyde in PBS in the presence of 1 mM MgCl₂ and 1 mM EGTA for 10 min and permeabilized with 0.1% Triton X-100 in PBS for 5 (COS7-2) or 10 min (AV-7) before immunostaining. Cells were stained for vinculin (mouse clone hVIN1; 1:200; Sigma-Aldrich), Flag (rabbit polyclonal; 1:100; Sigma-Aldrich), Myc (mouse clone 9E10; 1:1,000; ATCC), and zyxin (rabbit polyclonal B71; 1:100; Hoffman et al., 2003), or mouse anti-TRIP6, clone 16; 1:100; BD Biosciences), or Alexa Fluor 350 (Invitrogen). Slides were analyzed at room temperature with a 100× Plan-Neofluar oil immersion objective (NA 1.3) on a fluorescence microscope (Axioskop; both Carl Zeiss Microlmaging, Inc.) with a charge-coupled device camera (Retiga Exi; Retiga Corp.) and Openlab software (Improvement). The cross-adsorbed secondary antibodies, conjugated with Alexa Fluor 350, purified rabbit polyclonal H340; 1:100; Nebl et al., 2002; Oh et al., 2003). Alexa Fluor 350 images were treated identically to show antibody specificity. Because of the high background noise in Alexa Fluor 350 images (Fig. 7, a, e, and i; Reynolds et al., 2004) and two dsRNA treatments over 4 d. Cells at ~60% confluence were transfected with 10 mM dsRNA and Lipofectamine 2000 as recommended (Invitrogen), split 1:3 after 2 d, retransfected 8–10 h after splitting, and used in experiments after another 36–40 h of growth.

The same procedure was used to target human TRIP6, Tctex-1, and SV in A549 cells, hTR1(5′-AGGACGAGAGGUGGUGUCAAGUUAU3′), and hSV (5′-AGGACGAGAGGUGGUGUCAAGUUAU3′) and 2,467–2,491 (dsRNA 2472 sense: 5′-GCGUUGAGAAGAGGUGUCAAGUUAU3′). Two candidate RNAi sequences (http://jura.wi.mit.edu/siRNAext; Reynolds et al., 2004). Two dsRNAs were rehydrated using Stealth duplexes (Invitrogen) and used to deplete COS7 cell SV. These dsRNAs corresponded to coding nucleotides 666–690 (dsRNA 668 sense: 5′-AGGACGAGAGGUGGUGUCAAGUUAU3′ and 5′-CCCCUGUGAGCACGGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGA-
Yeast two-hybrid screens

A bait plasmid encoding bovine SV residues 171–830 was constructed in pHybLex/Zeo, transformed into the EGY48/pSH18-34 strain of S. cerevi-
siae, and used to screen a Hela cell library in the prey vector pYESTrip (Hybrid Hunter Premade cDNA Library and Two-Hybrid System; Invitrogen) as previously described [Chen et al., 2003]. In brief, a library of ~10^3 × 10^8 primary transformants was screened approximately four times on selection medium (ura, trp, leu, Raff/GAL; +2200). Large colonies grown on induction medium (ura, trp, leu, Raff/GAL; +2200) were picked after 24 or 48 h and tested for β-galactosidase activity on modified induction medium (ura, trp, Raff/GAL; +2200). Out of 506 initial colonies, 172 passed both the leucine autotrophy and β-galactosidase expression tests. Interacting prey vectors were segregated by growth for three generations on –trp medium, recovered, electrooporated into XL1 Blue cells (Stratagene), and sequenced using the pYESTrip forward primer. Sequences with an open reading frame were verified by retransformation into yeast containing the pHybLex/BSV171-830 bait vector. Nonspecific interactions were eliminated by control trans-
formations into yeast with pHybLex/Zeo (empty bait). To localize potential binding sites, confirmed clones were transformed into yeast strains contain-
ing either pHybLexA-BSV171-342 or pHybLexA-BSV570-830, and as-
sayed for leucine autotrophy and β-galactosidase activity.

Pull-down assays

Yeast cells (300 μl packed cell vol) expressing VS-tagged bait proteins af-
after a 6-h induction were washed with ice-cold SLB (25 mM TrisCl, pH 7.5, 1 mM DTT, 2 mM EDTA, 150 mM NaCl, 30% glycerol), and protease inhibitors (1 μM aprotanin, 2 μM AILM, 1 mM benzamidine, 10 μM E64, 1 μM leupeptin, 1 μM pepstatin A, 1 mM PMSF). Yeast were lysed with 400 μl 0.2% Triton X-100 and SLB by vortexing three times at maximum speed for 30 s with 0.5 mm glass beads (Biospec Products Inc., Bartlesville, OK). For GST pull-down assays, COS7-2 cells (10^6 cells) were lysed with 1 ml Hybrid Hunter Premade cDNA Library and Two-Hybrid System; Invitrogen) pHybLex/Zeo, transformed into the EGY48/pSH18-34 strain of Yeast two-hybrid screens binding sites, confirmed clones were transformed into yeast strains contain-
ing pHybLex/Zeo (empty bait). To localize potential binding sites, confirmed clones were transformed into yeast strains contain-
ing pHybLexA-BSV171-342 or pHybLexA-BSV570-830, and as-
sayed for leucine autotrophy and β-galactosidase activity.

Immunoblots

Glutathione-activated beads nitrocellulose membranes (Protran BA85; Schleicher & Schuell BioScience, Inc.). Endogenous proteins in mammalian cell lysates were analyzed after extraction and precipitation with 10% TCA (Takizawa et al., 2003). Blot strips were stained with antibodies against SV (rabbit polyclonal H340; 1:10,000), β-actin (mouse clone AC-74; 1:3,000; Sigma-Aldrich), zyxin (rabbit polyclonal B71; 1:5,000), TRIP6/ZIP1 (rabbit polyclonal B65; 1:2,000; and mouse monoclonal C.16; 1:2,000), or Tctex-1 (rabbit polyclonal R2505; 1:25; King et al., 1996). R2505 was provided by S.M. King (University of Connecticut Health Center, Farmington, CT).

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Online supplemental material

Fig. S1 shows EGFP/SV overlap with vinculin-stained FAs at the basal surface of a COS7 cell. Fig. S2 shows that the RI/AA mutant of EGFP-
SV342-571 exhibits a less deleterious phenotype than EGFP/SV342-571 on stress fibers and large FAs. Fig. S3 shows the specificities of the anti-
bodies used in this study on COS7 and A7r5 cells. Fig. S4 shows that TRIP6 remains at FAs after SV knockdown. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200512051.

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