Relief of Autoinhibition Enhances Vta1 Activation of Vps4 via the Vps4 Stimulatory Element*§

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The endosomal sorting complexes required for transport (ESCRTs) impact multiple cellular processes including multivesicular body sorting, abscission, and viral budding. The AAA-ATPase Vps4 is required for ESCRT function, and its full activity is dependent upon the co-factor Vta1. The Vta1 carboxyl-terminal Vta1 SBP1 Lip5 (VSL) domain stimulates Vps4 function by facilitating oligomerization of Vps4 into its active state. Here we report the identification of the Vps4 stimulatory element (VSE) within Vta1 that is required for additional stimulation of Vps4 activity in vitro and in vivo. VSE activity is autoinhibited in a manner dependent upon the unstructured linker region joining the amino-terminal microtubule interacting and trafficking domains and the carboxyl-terminal VSL domain. The VSE is also required for Vta1-mediated stimulation of ESCRT-III subunits Vps60 and Did2. These results suggest that ESCRT-III binding to the Vta1 microtubule interacting and trafficking domains relieves linker region autoinhibition of the VSE to produce maximal activation of Vps4 during ESCRT function.

The Endosomal Sorting Complexes Required for Transport (ESCRT-0, -I, -II, and -III) are multiprotein complexes that facilitate “reverse topology” membrane fission events (1) involved in the diverse cellular processes of multivesicular body (MVB) sorting, autophagy, exosome formation, viral budding, and cytokinesis (for review, see Refs. 1–9). The ESCRT machinery is conserved in fungi and throughout higher eukaryotes (10), and the yeast MVB sorting pathway has provided a tractable system for understanding ESCRT function. The early ESCRTs (ESCRT-0, -I and -II) appear to be recruited to endosomal membranes as preformed complexes, where they recognize and sequester ubiquitinated MVB cargoes into microdomains (for review, see Ref. 11). ESCRT-II also facilitates the assembly and function of ESCRT-III and associated proteins to deform the endosomal membrane and facilitate vesicle budding into the MVB (12–14). ESCRT-III assemblies dynamically into a membrane-deforming polymer from cytosolic monomers of structurally related proteins, including the core subunits Vps20/CHMP6, Snf7/CHMP4, Vps2/CHMP2, and Vps24/CHMP3 (15–19) as well as the accessory subunits Did2/Fti1/CHMP1, Ist1/hIst1, and Vps60/CHMP5 (20–28). The polymerization of ESCRT-III promotes the recruitment of the AAA-ATPase Vps4/SKD1 along with its co-factor Vta1/SBP1/LIP5 and Vps60 (15, 29). A complex series of interactions between ESCRT-III subunits and Vps4-Vta1 coordinates ESCRT-III assembly and disassembly to permit efficient ESCRT function (for review, see Ref. 30).

Vps4 ATP hydrolysis is required for the disassembly of ESCRT-III, and dysfunction of Vps4 ATP hydrolysis results in the stabilization of ESCRTs on endosomal membranes (31, 32). Vta1 promotes Vps4 function in vivo and can enhance Vps4 ATP hydrolysis and Vps4 disassembly of ESCRT-III in vitro (20, 32–36). Both Vps4 and Vta1 harbor amino-terminal microtubule interacting and trafficking (MIT) domains, which recognize elements within ESCRT-III subunits (MIT-interacting motifs, or MIMs) and facilitate recruitment of Vps4 and Vta1 to ESCRT-III (29, 37–42). In some cases, MIM interactions with the MIT domains of Vps4 or Vta1 enhance the ATP hydrolysis activity of the Vps4-Vta1 complex (43, 44). In addition, a general stimulatory mechanism has been described wherein ESCRT-III subunits promote Vps4 ATP hydrolysis activity through an acidic region distinct from the MIM elements (45).
Autoinhibition of the Vta1 VSE

Thus, ESCRT-III appears to promote Vps4 activity through recruitment to the site of function, by a general acidic region mechanism, and via specific MIM interactions with the MIT domains of the Vps4-Vta1 complex.

Binding of the ESCRT-III subunit Vps60 or Did2 to the Vta1 MIT domains enhances Vta1 stimulation of Vps4, although the mechanism of this enhancement is not understood (43, 44, 46, 47). The Vta1 carboxyl-terminal 40 amino acids (Vta1 SBP1 Lip5 domain, or VSL) are highly conserved throughout eukaryotes and mediate Vta1imerization and interaction with Vps4 (35, 37, 48). (Figs. 1A and 6 depict the locations of the ESCRT-III and Vps4 binding regions of Vta1.) Vta1 reduces the apparent $K_{m}$ of Vps4 ATP hydrolysis and lowers the concentration of Vps4 required to observe oligomeric Vps4 by size exclusion chromatography, consistent with a role facilitating or stabilizing Vps4 oligomerization (35). The VSL domain alone is capable of enhancing Vps4 ATP hydrolysis activity (35, 37); however, Vta1 also enhances Vps4 function through additional mechanisms. Vps4 stimulation by full-length Vta1 is greater than the activity observed with the VSL domain alone, and the addition of Vta1 enhances the Vps4 maximal ATP hydrolysis rate ($V_{max}$) to a level greater than the $V_{max}$ observed with Vps4 alone (35). Maximal stimulation of Vps4 ATP hydrolysis by Vta1 requires residues beyond the VSL domain but does not require the Vta1 MIT domains, suggesting that unappreciated Vta1-Vps4 interactions occur via the unstructured linker region connecting the MIT and VSL domains (43). Vps60 and Did2 have been proposed to function as allosteric regulators of Vta1 through binding the Vta1 MIT domains, possibly by stabilizing the linker region autoinhibits this VSE. The VSE residues require the Vta1 MIT domains, suggesting that unappreciated Vta1-Vps4 interactions occur via the unstructured linker region connecting the MIT and VSL domains (43). Vps60 and Did2 have been proposed to function as allosteric regulators of Vta1 through binding the Vta1 MIT domains, possibly by affecting reorganization of the linker region (37, 43). Another possibility is that Vta1-bound Vps60 or Did2 directly stimulates Vps4, in which case the enhanced stimulation observed is a combination of Vta1 and direct ESCRT-III stimulation of Vps4.

To understand how Vta1 and ESCRT-III coordinate Vps4 ATPase activity, studies were undertaken to address the mechanisms by which Vta1 stimulates Vps4 beyond its impact on oligomerization. Truncation and site-directed mutagenesis studies identified a Vps4 stimulatory element (VSE) within the Vta1 linker region. Deletion of amino-terminal portions of the linker region generated a hyperactive form of Vta1, suggesting that the linker region autoinhibits this VSE. The VSE residues also participate in Vta1-dependent enhancement of Vps4 activity by the ESCRT-III subunits Vps60 and Did2. These observations suggest that the binding of ESCRT-III subunits Vps60 and Did2 to the Vta1 MIT domains induces conformational changes in the Vta1 linker region that promote VSE activation of Vps4 in addition to the stimulation afforded by Vta1 VSL-Vps4 association.

EXPERIMENTAL PROCEDURES

Plasmids and Strains—The generation of pET28-Vta1 has been described previously (35). pRS415-Vta1 Promoter-mCherry plasmid was generated by PCR-amplifying the 264-bp Vta1 promoter with 3’ Nhel and BamHI sites and subcloning this fragment into the Nhel and BamHI sites of pRS415 along with the Nhel, SalI mCherry fragment from pmCherry-C3. Mutagenesis of Vta1 was performed using the Gene Tailor Site-Directed Mutagenesis system (Invitrogen) with a PBS-Vta1 template. Vta1 mutants were cloned into the BamHI and Xhol sites of pET28b or pGST-parallel1 and the BglII and Xhol sites of pRS415-Vta1 Promoter-mCherry. All cloned PCR products and mutant plasmids were sequenced to exclude unexpected mutations. All Vta1 mutants examined in this study exhibited equivalent expression levels in bacteria and yeast (data not shown), as assessed by Coomassie Blue staining or Western blotting with anti-Vta1 polyclonal antiserum (35). The BY4742 vta1Δ::NEO strain was obtained from Open Biosystems.

Protein Expression and Purification—Protein expression was performed in the BL21-DE3 bacterial strain at 30 °C for 4 h (GST fusions) or 22 °C for 14–20 h (His$_{6}$ fusions) with 0.5 mM isopropyl 1-thio-β-D-galactopyranoside. His$_{6}$-Vta1 fusion proteins were purified by Ni$^{2+}$-affinity chromatography (5 ml of HiTrap Chelating FF or Ni$^{2+}$-nitrilotriacetic acid resin), treated with thrombin (optional), incubated with ATP to dissociate chaperones, and subjected to anion exchange (Bioscale Q2) or size exclusion (Superdex 75 HiLoad 16/60) chromatography. Purification of GST fusion proteins (pGST Did2 and pGST Vps60 (43)) included treatment with ATP to minimize co-purifying chaperones and contaminating ATPase activities, and these GST fusion proteins were used in ATPase assays still bound to the glutathione-Sepharose 4B resin. Vps4 was purified as described previously (31).

Biochemical Analyses—Analysis of carboxypeptidase S (CPS) transport to the vacuole by pulse-chase immunoprecipitation was performed as described previously (15). Representative scans of the PhosphorImager plates are presented in supplemental Fig. 2A. The significances of differences in maturation rates were assessed by two-way analysis of variance tests using Prism5 (GraphPad). Subcellular fractionation was performed as described previously (15). Briefly, 5 OD equivalents of spheroplasted yeast were resuspended at 10 OD/ml in lysis buffer (50 mM Tris, pH 7.5, 200 mM sorbitol, 2 mM EDTA with protease inhibitors), lysed by 15 strokes in a Dounce homogenizer, and subjected to a 10 min, 13,000 × g spin at 4 °C to separate the S13 and P13 fractions. Samples (0.04 OD equivalents) were resolved by SDS-PAGE and Western blotted for Smf7 (pAb, 1:5000), phosphoglycerate kinase (mAb, 1:2000) (Innventogen), Pep12 (mAb, 1:2000) (Innventogen), and Vta1 (pAb, 1:1000) (35). Western blots were developed using both film and the UVP Autochemi System (Upland, CA), and quantitation was performed using ImageQuant software (GE Healthcare). Representative blots are presented in supplemental Fig. 2B.

ATPase Assay—Measurement of Vps4 ATPase activity was performed as described previously (31, 35, 43). Vps4 (500 nM) was combined with Vta1 proteins (100 nM–20 μM) in ATPase reaction buffer (0.1 M KOAc, 20 mM Hepes, and 5 mM MgOAc, pH 7.5) in a total of 18 μl at 30 °C. Reactions were initiated by the addition of ATP to 2 mM. 1–μl samples were removed at various time points after ATP addition (e.g. 4, 8, and 12 min for low Vps4 activities and 1 min 20 s, 2 min 40 s, and 4 min for high Vps4 activities) and resolved by thin layer chromatography using precoated polyethyleneimine-cellulose TLC glass plates (Merck) and developing buffer (0.75 M KPO$_{4}$, pH 3.5). Plates were dried and exposed to PhosphorImager screens for 12–16 h and processed using the Storm 840 system (GE Healthcare), and ADP and ATP signals were quantitated using ImageQuant software.
software. An example of this analysis is presented in supplemental Fig. 1. Rates of ATP hydrolysis were assessed using data points with <40% ATP hydrolysis to prevent error due to substrate inhibition and were expressed as ADP molecules generated (ATP hydrolyzed) per Vps4 molecule per min (ADP/Vps4/min). For analysis of GST fusion proteins, ~1.5 µg of GST fusion proteins (~1.5 µM in a final volume of 20 µl) bound to glutathione-Sepharose 4B resin was equilibrated in ATPase buffer. Residual buffer was aspirated, and 500 nM Vps4 with or without 2 µM Vta1 was added in a total of 18 µl of ATPase reaction buffer. ATP addition, time point collection, and sample processing were then performed as described for the untagged proteins. Data were analyzed with Excel (Microsoft) to determine ATP hydrolysis rates and Prism 5 (GraphPad) to determine kinetic and statistical parameters.

**Live Cell Imaging**—Yeast cells grown in minimal media were used for fluorescence microscopy. Images were captured using an Olympus IX70 fluorescence microscope (Center Valley, PA) with eGFP filters and a digital camera (CoolSnap HQ; Photometrics, Tucson, AZ) and were deconvolved using Delta Vision software (Applied Precision, Issaquah, WA).

**RESULTS**

The VSE Facilitates Maximal Vta1 Stimulation of Vps4—Vta1 impacts ESCRT function by positively modulating the activity of the AAA-ATPase Vps4. The Vta1 VSL (Vta1/SBP1/LIP5) domain at the carboxyl terminus (residues 290–330) is necessary and sufficient for Vps4 binding and can enhance the ATPase activity of Vps4 (35, 37). However, stimulation by the VSL domain is less robust than the activity of full-length Vta1, suggesting that additional Vta1-Vps4 interactions are relevant for enhancement of Vps4 activity. Deletion of the Vta1 amino-terminal MIT domains did not impair Vps4 stimulation (Fig. 1A and Ref. 43), implicating residues 165–290 linking the MIT and VSL domains as the region of interest required for full Vta1 stimulation of Vps4. Truncations within this region identified a fragment spanning residues 267–330 (Vta1(267–330)) that exhibited robust stimulatory activity (Fig. 1A and data not shown). Titration of full-length Vta1, Vta1(267–330), and Vta1(VSL)(290–330) indicated similar concentration-dependent stimulation of Vps4 ATPase activity (half-maximal stimulation with 1.14 ± 0.46 µM, 0.88 ± 0.2 µM, and 0.46 ± 0.35 µM, respectively) although the maximal enhancement by Vta1(VSL) was reduced (Fig. 1B and supplemental Fig. 1). These results suggested that Vta1, Vta1(267–330), and Vta1(VSL) exhibit similar apparent affinity for Vps4, although the stimulatory effects of these associations are distinct. These observations are consistent with previous results indicating that the VSL domain is the primary determinant of association with Vps4 and is the domain responsible for enhancing Vps4 oligomerization (35). Furthermore, these results suggested that an additional Vps4 stimulatory element within Vta1 residues 267–290 contributes to enhanced Vps4 ATPase activity.

Vta1 residues 267–290 contain a hydrophobic patch followed by mixed charged residues without identified secondary structure (267–281) as well as a region of mixed charged and hydrophobic residues that forms a flexible α-helix(281–290) adjacent to the VSL domain (Fig. 2A) (37). To determine the potential contributions within this region, additional Vta1 truncations were generated, and their stimulation of Vps4 ATPase activity was assessed. 500 nM Vps4 displayed a specific activity of 24 ADP/Vps4/min, and this increased to 134 ADP/Vps4/min with the addition of 10 µM Vta1 (5.5-fold stimulation, Fig. 2B). Interestingly, addition of 10 µM Vta1(267–330) further enhanced hydrolysis to 241 ADP/Vps4/min (10-fold stimulation) whereas 10 µM Vta1(275–330) stimulated 500 nM Vta1 VSL domain, wild type Vta1, or Vta1(267–330). Specific activity is expressed as ADP generated per Vps4 molecule per min (ADP/Vps4/min). Error bars, S.E.
aspect of Vta1-mediated Vps4 activation. To examine these possibilities, titration of Vta1 and Vta1(275–330) was performed in the presence of 500 nM Vps4 (Fig. 2C). Vta1 and Vta1(275–330) exhibited similar concentration dependence with half-maximal stimulation at 1 and 5 μM, respectively. In contrast, the Vps4 ATPase activity with 10 μM Vta1 or Vta1(275–330) was potentiated from 146 ADP/Vps4/min (Vta1) to 303 ADP/Vps4/min (Vta1(275–330)). These results suggested that increased Vps4 stimulation by Vta1(275–330) was not a result of enhanced affinity for Vps4 (Fig. 2C) and that additional contacts within the 275–290 region promote Vps4 ATPase activity.

To support the conclusion that residues beyond the VSL domain participate in Vps4 stimulation, alanine scanning mutagenesis of the 275–290 region was performed in the context of full-length Vta1. Although truncation of residues 275–279 and 280–284 reduced the stimulation of the Vta1 truncations (Vta1(275–330), Vta1(280–330), and Vta1(285–330), Fig. 2B), replacement of the corresponding residues with alanines (Vta1(275–280Ala) “A”, Vta1(281–285Ala) “B”), or residues 283–287 (Vta1(283–287Ala) “C”) did not disrupt the ability of full-length Vta1 to stimulate 500 nM Vps4 to wild type levels (Fig. 2D). By contrast, mutation of residues 286–290 compro-
mised stimulatory activity with 10 μM Vta1(286–290Ala) (“D”) enhancing 500 nM Vps4 activity to 32 ADP/Vps4/min, comparable with stimulation by the VSL domain alone. These results suggested that the carboxyl-terminal portion of the flexible helix proximal to the VSL domain participates in stimulating Vps4 ATPase activity.

The VSL domain forms a dimer with two surfaces (yellow residues, Fig. 3A) that interact with the Vps4 domains (37, 48). The structure of the Vta1 VSL in complex with the Vps4 domain (48) suggests that the flexible helix (281–290, not present in the co-crystal, but modeled in Fig. 3A) may be positioned in proximity to the Vps4 AAA domain to stimulate ATPase activity. To further address the determinants within the flexible helix mediating stimulation of Vps4, single point mutations were generated and evaluated for enhancement of Vps4 ATPase activity. Threonine 285, lysine 286, and serine 292 were converted to alanine residues consistent with the alanine scanning approach, whereas the hydrophobic amino acids leucine 284, isoleucine 287, methionine 288, and alanine 291 were replaced with negatively charged glutamic acid residues to disrupt potential hydrophobic interactions. (Arginine 290, indicated in blue in Fig. 3A, forms a salt bridge important for Vta1 dimerization (35, 37) and was excluded from this survey.)
Autoinhibition of the Vta1 VSE

Vta1(T285A), Vta1(K286A), Vta1(A291E), and Vta1(S292A) (10 μM; green residues in Fig. 3A) enhanced 500 nM Vps4 ATPase activity similarly to wild type Vta1 (149 ADP/Vps4/min, Fig. 3B and data not shown). By contrast, 10 μM Vta1(I287E) enhanced Vps4 ATPase activity to 111 ADP/Vps4/min whereas 10 μM Vta1(L284E) and Vta1(M288E) stimulated Vps4 ATPase activity to only 69 and 75 ADP/Vps4/min, respectively (Fig. 3B; red residues in Fig. 3A). Triple mutation of these three residues (Vta1(L284E,I287E,M288E)) exhibited a further reduction in Vps4 stimulatory activity (37 ADP/Vps4/min), comparable with levels observed with the VSL domain alone (Fig. 3B). These results suggested that the surface of the flexible helix defined by Leu-284, Met-288, and (to a lesser extent) Ile-287 participates in Vps4 stimulation in addition to the VSL domain itself. Whereas the VSL domain sequence (yeast residues 290–330) is well conserved from yeast to plants to mammals, the sequence of the flexible helix has diverged across evolution. As a result, we will refer to this surface of the flexible helix as a distinct feature of Vta1: the Vps4 stimulatory element (VSE).

The VSE Contributes to ESCRT-III Enhancement of Vta1-dependent Vps4 Stimulation—The ability of Vta1 to stimulate Vps4 ATPase activity is further enhanced by the interaction of ESCRT-III subunits Vps60 or Did2 with the MIT domains within Vta1 (43). The mechanism of this enhancement is not understood but has been suggested to involve conformational changes within the linker region of Vta1. The identification of the VSE led to the hypothesis that this element of the linker region participates in the enhanced stimulation of Vps4 by Vta1 upon ESCRT-III binding. To investigate the involvement of the VSE in transducing ESCRT-III activation of Vta1, ATPase activity of the Vta1 VSE mutants was measured in the presence of GST-Vps60 or GST-Did2. Incubation of 2 μM Vta1 with 1.5 μM GST-Vps60 enhanced 500 nM Vps4 activity from 120 ADP/Vps4/min (Vps4, Vta1 alone) to 238 ADP/Vps4/min, an activity consistent with the 2–3-fold enhancement reported previously (43). Similar GST-Vps60 potentiation of Vta1(S292A) was observed (Fig. 4A), consistent with the ability of this mutant to stimulate Vps4 in a manner equivalent to wild type (Figs. 3C and 4A). In contrast, Vta1(L284E), Vta1(I287E), Vta1(M288E), and Vta1(L284E,I287E,M288E) displayed reduced ESCRT-III-stimulated activity upon incubation with GST-Vps60 (28, 67, 42, and 17% of wild type activity, respectively). Normalizing these activities to stimulation observed with addition of GST alone (1.3-, 1.6-, 1.5-, 1.1-fold enhanced stimulation, respectively, compared with 2.0-fold for wild type; Fig. 4A) indicated diminished ESCRT-III enhancement of these VSE mutants in addition to the deficits in intrinsic Vps4 stimulation. A similar pattern of enhancement was observed with addition of GST-Did2 (Fig. 4B). The severity of defects in enhancement correlate with the relative deficits in intrinsic Vps4 stimulation exhibited by these Vta1 mutants. This correlation supports the hypothesis that the VSE mediates enhanced Vps4 stimulation by Vta1 upon Vps60 or Did2 binding.

Changes in the conformation of the linker region have been proposed to mediate Vps60 and Did2 enhancement of Vta1 stimulation, and the truncation studies identified a fragment (Vta1(275–330)) that may mimic this enhanced conformation. The role of the VSE in Vps4 stimulation by Vta1(275–330) was therefore evaluated. Point mutations I287E and M288E were generated in the context of Vta1(275–330) (Vta1(275–330: I287E), Vta1(275–330: M288E)), and their abilities to stimulate 500 nM Vps4 were tested. Whereas 10 μM Vta1(I287E) exhibited 74% intrinsic Vps4 stimulatory activity (Fig. 3B), a more severe reduction in Vps4 stimulation was observed with 10 μM Vta1(275–330:I287E) (57% of Vta1(275–330) stimulation, Fig. 3B). 10 μM Vta1(275–330:M288E) exhibited 50% intrinsic Vps4 stimulatory activity (Fig. 3B). These results indicated that the
enhanced stimulatory activity of Vta1(275–330) was dependent upon the VSE, analogous to the VSE dependence of ESCRT-III enhanced Vta1 activity (Fig. 4, A and B). This similarity supported the model that Vta1(275–330) recapitulates the ESCRT-III-induced conformation of Vta1 relevant for enhanced Vps4 stimulation. These results further support the hypothesis that the VSE mediates enhanced Vps4 stimulation by Vta1 upon ESCRT-III binding.

The VSE Contributes to Vta1 Function in Vivo—Vta1 promotes Vps4 function in MVB sorting. To evaluate whether the VSE contributes significantly to this process in vivo, VSE mutants were reexpressed in vta1/H9004 yeast, and MVB cargo sorting and membrane association of the Vps4 substrate ESCRT-III were addressed as indicators of Vta1 function. First, the localization of the MVB cargo GFP-CPS was observed in vta1Δ cells transformed with vector, wild type VTA1, or vta1 mutants. Loss of Vta1 confers a defect in MVB sorting, with GFP-CPS localized to the limiting membrane of the vacuole (Fig. 5A). This defect was complemented by reintroduction of wild type VTA1, but vta1(L284E) or vta1(L284E,I287E,M288E) only partially restored GFP-CPS localization (Fig. 5A). These observations suggested that a functional VSE is required for VTA1 function in yeast.

The maturation of endogenous CPS was evaluated as an additional indicator of MVB pathway function. CPS undergoes proteolytic conversion concurrent with delivery into the lumen of the vacuole, allowing kinetic analyses by pulse-chase immunoprecipitation experiments (supplemental Fig. 2A). Loss of VTA1 function results in a kinetic delay in the conversion of CPS from precursor to mature forms (p value < 0.001; Fig. 5B).
Expression of wild type Vta1 or the Vta1 mutants that retain wild type Vps4 stimulation in vitro (Vta1(S292A), Vta1(S292D)) in vta1Δ cells restored CPS maturation to the kinetics apparent in wild type cells. Expression of the VSE mutants Vta1(I287E) and Vta1(M288E) also restored CPS maturation to wild type levels, although a subtle delay was apparent that did not reach statistical significance. By contrast, expression of Vta1(L284E) or Vta1(L284E,I287E,M288E) was unable to restore wild type CPS maturation kinetics (p value < 0.05), although CPS maturation was more rapid than in cells devoid of Vta1 (p value < 0.01; Fig. 5B). These observations with both GFP-CPS localization and CPS maturation suggested that partial Vta1 function in MVB sorting is retained in the absence of a functional VSE, consistent with the abilities of the VSL domain alone and Vta1(L284E,I287E,M288E) to facilitate residual Vps4 stimulation in vitro. This analysis supported the model that the VSE contributes to Vta1 function in MVB sorting.

Vta1 facilitates MVB sorting through promoting Vps4 activity, and loss of Vps4 activity results in membrane accumulation of ESCRT-III. To more directly evaluate Vps4 function in the presence of these Vta1 mutants, the membrane accumulation of the ESCRT-III subunit Snf7 was examined (supplemental Fig. 2B). In wild type cells, Vps4-mediated disassembly of ESCRT-III results in predominantly soluble Snf7. However, loss of Vta1 perturbed the recycling of ESCRT-III resulting in Snf7 membrane accumulation consistent with previous results (47% in the soluble fraction in vta1Δ cells; Fig. 5B and Ref. 35). Expression of wild type Vta1 prevented membrane accumulation with 65% of Snf7 in the soluble fraction (Fig. 5C). By contrast, cells expressing the VSE mutants Vta1(L284E) or Vta1(L284E,I287E,M288E) exhibited decreased amounts of soluble Snf7 (55 and 54% of Snf7 in the soluble fraction, respectively) compared with wild type Vta1 (Fig. 5C). Together, these results supported the conclusion that the VSE contributes to Vta1 function to promote ESCRT-III recycling and permit efficient MVB sorting.

**DISCUSSION**

Vps4 hydrolyzes ATP to disassemble membrane-bound ESCRT-III polymers. Loss of Vps4 function results in the accumulation of ESCRT machinery on membranes and dysfunction in MVB formation, cytokinesis, and budding viral particles (15, 31, 49–54). The critical role of Vps4 in these processes underscores the importance of understanding the regulatory mechanisms that control the localization, timing, and magnitude of its activity. In the present study, we have demonstrated that yeast Vta1 stimulates Vps4 through an additional surface (VSE) proximal to the VSL domain. This VSE contributes to ESCRT-III-enhanced Vta1-mediated stimulation of Vps4 and impacts Vta1 function in vivo.

ESCRT-III association with Vta1 promotes Vta1 stimulation of Vps4 (43, 44). However, the mechanism of this enhancement has been unclear. The ESCRT-III subunits Did2 and Vps60 bind to the Vta1 amino-terminal MIT domains (37). Recent structural analysis of these associations revealed that ESCRT-III binding does not effect significant conformational changes within the MIT domains (46, 47, 55). Removal of the Vta1 MIT domains similarly did not enhance Vta1 stimulation of Vps4 (43). However, additional deletion of portions of the linker region generated a Vta1 peptide (Vta1(275–330)) with enhanced stimulation of Vps4 above the level observed with full-length Vta1. These observations indicate that the linker region

**FIGURE 5. VSE contributes to Vta1 function in vivo.** A, the MVB cargo GFP-CPS was visualized by fluorescence microscopy in living vta1Δ cells transformed with vector or plasmids expressing Vta1 or Vta1 point mutants. B, pulse-chase immunoprecipitation was performed on endogenous CPS in vta1Δ cells transformed with vector or plasmids expressing Vta1 or Vta1 point mutants. CPS maturation was quantitated using a PhosphorImager and plotted relative to time zero. CPS maturation kinetics in cells expressing Vta1(L284E) or Vta1(L284E,I287E,M288E) was significantly different (*, p < 0.05) from cells lacking Vta1 or cells expressing wild type Vta1. B, subcellular fractionation of the ESCRT-III subunit Snf7 was performed on vta1Δ cells transformed with vector or plasmids expressing Vta1 or Vta1 point mutants. Snf7 fractionation in cells expressing Vta1(L284E) or Vta1(L284E,I287E,M288E) was significantly different (*, p < 0.05) from cells lacking Vta1 or cells expressing wild type Vta1. Error bars, S.E.
Autoinhibits enhanced stimulation of Vps4 and suggest a mechanism whereby ESCRT-III binding to the Vta1 MIT domains relieves this linker region autoinhibition (although not through altering the conformation of the MIT domains) (Fig. 6).

Additional amino-terminal deletions and scanning mutagenesis served to define the VSE within the linker region. Interestingly, the smallest truncation, Vta1(285–330), exhibited Vps4 stimulation comparable with full-length Vta1 and greater than stimulation afforded by the VSL domain alone (residues 290–330), whereas Vta1(275–330) stimulated Vps4 to levels comparable with the enhanced stimulation induced by ESCRT-III association with Vta1. Investigation by alanine scanning mutagenesis revealed a stimulation defect with mutation of residues 286–290 (Fig. 2). This portion of the linker region is present in the VSL domain structure as a flexible helix (37). Therefore, surfaces of this flexible helix were further examined through site-directed mutagenesis. Nonconservative mutations of Leu-284 and Met-288 displayed the strongest defects in intrinsic Vps4 stimulatory activity, whereas mutation of Ile-287 generated a more modest defect (Fig. 3B). When combined into a triple mutant, Vta1(L284E,I287E,M288E), the ATPase stimulation defect was exacerbated to an extent comparable with the VSL domain alone. Mutation of Ala-291 or Ser-292, residues one turn further down the helix, did not perturb Vps4 stimulation or CPS sorting kinetics (Fig. 5B and data not shown). Thus, Met-288 defines the carboxyl-limit of the VSE contributing surface residues. This analysis suggests a particular surface (VSE, residues Leu-284, Ile-287, Met-288) of the flexible helix preceding the VSL domain contributes to Vps4 stimulation. Whereas the VSL domain is conserved across evolution from yeast to humans and serves as the primary mode of Vps4 association, the VSE has diverged at the level of amino acid sequence. Although the VSE may represent a unique aspect of yeast Vta1 function, we speculate that an analogous mechanism contributes to Vps4 activation in higher eukaryotes.

The VSE mediates ESCRT-III enhancement of Vta1 stimulated Vps4 activity. Mutation of the VSE perturbed Vta1-mediated stimulation of Vps4 by Vps60 or Did2 and reduced the enhanced activity observed within the Vta1(275–330) truncation. These data support a model in which Vps60 or Did2 binding to the Vta1 MIT domains confers a conformational shift in the Vta1 linker region to relieve autoinhibition of the VSE. Recent structural analyses of ESCRT-III subunit interactions with the Vta1 and Lip5 MIT domains revealed that the Vta1 MIT domains themselves do not undergo significant rearrangements upon ESCRT-III binding (46, 47). These studies did not address conformational changes within the linker region, and thus the mechanisms of autoinhibition and ESCRT-III enhancement of Vta1 remain unresolved at the structural level. Deletion of the MIT domains along with the majority of the linker region resulted in the hyperactive Vta1(275–330) peptide. Restoration of the majority of the linker region (Vta1(183–330)) reduced stimulation to levels observed with full-length Vta1 or Vta1 lacking the MIT domains (Vta1(165–330)) (35, 43). These data suggest the existence of repressive elements within the Vta1 linker region. In this model, Vps60 and Did2 stimulation of Vta1 is achieved through Vta1 conformational changes that relieve the linker region repression of the VSE (Fig. 6). The position of the VSE distal to the surfaces of the VSL implicated in binding to Vps4 β-domain suggests that VSE contacts the Vps4 AAA domain to stimulate ATP hydrolysis. Whereas further structural analyses will be required to establish the mechanisms conferring ESCRT-III enhancement of Vta1 activity and VSE stimulation Vps4, the VSE appears to be the critical surface of Vta1 mediating enhanced Vps4 activation upon Did2 or Vps60 binding. These observations fit a model in which stimulation by Vta1 can be divided into three modes: (i)
induced oligomerization of Vps4 mediated by Vta1 VSL domain interactions with the Vps4 β-domain; (ii) further stimulation of Vps4 ATP hydrolysis by the Vta1 VSE; and (iii) Vps60- or Did2-enhanced Vta1 stimulation resulting from conformational changes that reorient the Vta1 VSE for maximal stimulation of Vps4.

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