Structure and Function of the ESCRT-II-III Interface in Multivesicular Body Biogenesis

Young Jun Im, Thomas Wollert, Evzen Boura, and James H. Hurley

INTRODUCTION

Newly internalized receptors and transporters, lysosomal hydrolases, and other cargo destined for the lysosome arrive through a pathway in which portions of the limiting membrane of endosomes invaginate into the lumen of the endosome (Gruenberg and Stenmark, 2004; Piper and Katzmann, 2007; Russell et al., 2006). Endosomes filled with intralumenal vesicles (ILVs) are referred to as multivesicular bodies (MVBs). The major pathway for the entry of ubiquitinated transmembrane proteins into MVBs is catalyzed by the Endosomal Sorting Complex Required for Transport (ESCRT) machinery (Hurley, 2008; Raiborg and Stenmark, 2004; Williams and Urbe, 2007). There are five ESCRT complexes: ESCRT-0, -I, -II, and -III, and the Vps4-Vta1 complex. The system is conserved from yeast to humans (throughout this report, all nontailized capitals ["VPS4"] denote human proteins while mixed case ["Vps4"] denotes either yeast proteins or all orthologs collectively). In human cells it has additional roles in cytokinesis and HIV-1 budding (Carlton and Martin-Serrano, 2009). ESCRT-0 functions to disassemble and recycle the ESCRT-III complex following vesicle scission (Babst et al., 1998; Wollert et al., 2009). The ESCRT system can be conceptually divided into machinery that binds to cargo (ESCRT-0, -I, and -II); machinery that remodels the membrane (ESCRT-III), and recycling machinery (Vps4-Vta1). The pivotal links between these sets of machines are at the ESCRT-II-ESCRT-III and ESCRT-III-Vps4-Vta1 stages. The structural basis for ESCRT-III-Vps4-Vta1 interactions has been characterized (Kieffer et al., 2008; Obita et al., 2007; Stuchell-Brereton et al., 2007; Xiao et al., 2008), while the key ESCRT-II-ESCRT-III interaction has not.

ESCRT-II in yeast and humans consists of one copy each of the Vps22 and Vps36 subunits, and two copies of the Vps25 subunit. The subunits are arranged in the shape of the letter “Y” with Vps22 and Vps36 forming one branch of the Y and the two copies of Vps25 forming the other branch (Hierro et al., 2004; Im and Hurley, 2008; Teo et al., 2004). The presence of both copies of Vps25 is essential for function in yeast (Hierro et al., 2004). ESCRT-III was first described in yeast, where it consists of the Vps20, Snf7, Vps24, and Vps2 subunits (Babst et al., 2002a). The ESCRT-III subunits assemble from cytosolic monomers into a detergent-insoluble membrane-bound array in the order listed above (Teis et al., 2008). Vps20 is the first subunit and thus is the key initiator of ESCRT-III assembly on endosomes (Teis et al., 2008).

ESCRT-II physically interacts with Vps20 in yeast (Babst et al., 2002b; Bowers et al., 2004) and humans (where it is also known as CHMP6) (Martin-Serrano et al., 2003; von Schwedler et al., 2003; Yorikawa et al., 2005). Vps20 binds to ESCRT-II via the Vps25 subunit of the latter (Teo et al., 2004). Both Vps20 and ESCRT-II interact strongly with membranes, Vps20 is myristoylated at its N terminus in both yeast and human cells (Babst et al., 2002a; Yorikawa et al., 2005). ESCRT-II binds tightly to membranes due to a phosphoinositide-specific interaction with the GLUE domain in its VPS36 (also known as EAP45) subunit (Slagsvold et al., 2005; Teo et al., 2006), and a basic N-terminal helix in its VPS22 (also known as EAP30) subunit (Im and Hurley, 2008). Progress in understanding how ESCRT-II binds to and activates Vps20 has been hampered because at concentrations used for structural studies, ESCRT-II and Vps20 form an insoluble precipitate (Teo et al., 2004).

In this study, we set out to circumvent this problem and thereby fill in the structural missing link between the ESCRT-II and ESCRT-III complexes. We were able to determine the crystal structure of a complex of the minimal interacting fragments of...
VPS20 and VPS25. The affinity of the ESCRT-II-VPS20 interaction was characterized in solution. We applied structural insights to design alleles of ESCRT-II and Vps20 selectively disabled for their interaction with one another, and characterized the activation of MVB biogenesis by ESCRT-II in yeast cells. We show that ESCRT-II and Vps20 bind to membranes with nanomolar to tens of nanomolar affinity. The activation of ESCRT-III by ESCRT-II was directly demonstrated using a recently developed assay for ESCRT function in vitro, and shown to depend on the structural interactions. Finally, docking of the VPS20-VPS25 crystal structure and previously solved structures was used to build a structural model of the ESCRT-II-VPS20 (2) supercomplex, revealing a convex curved membrane-binding face with implications for the mechanism of MVB biogenesis.

RESULTS

Structural Basis of the ESCRT-II-ESCRT-III Interaction

The N-terminal half of VPS20 binds to the C-terminal winged helix (WH2) domain of VPS25 (also known as EAP20) (Langelier et al., 2006), which is referred to hereafter as VPS25c. We screened a series of constructs to precisely map the smallest fragment of VPS20 competent to bind to VPS25. We found that residues 11–48 in helix α1 of VPS20 were necessary and sufficient for association with VPS25c (see Figure S1 available online). These fragments were coexpressed in order to overcome difficulties with the insolubility of the VPS20 fragment when expressed alone. The VPS25c (residues 102–176)–VPS20 α1 (residues 11–48) complex was crystallized and diffracted to 2.0 Å. The structure to accommodate the crystallized portion of VPS20 (residues 11–14) is a random coil, while residues 15–44 form an α helix (Figure 1A). VPS25c has a hydrophobic surface patch centered on Val-124 on the β sheet formed by β6, β7, and β8 (Figure 1D). This β sheet forms the main binding surface for VPS20. The β7–β8 loop of VPS25 wraps partway around the VPS20 α1 helix (Figure 1A). Polar interactions are extensive, with a total of eight hydrogen bonds (r<3 Å between heteroatoms) and three salt bridges. VPS20 Val-124 makes the most extensive hydrophobic interaction with VPS20 (Figure 1D). The side chain of VPS20 Leu-21 inserts into a hydrophobic pocket formed by Val-124, Ile-165, and the β7–β8 loop of VPS25. Among key polar interactions, VPS20 Thr-126 makes a hydrogen bond with VPS20 Asp-28, and VPS25 Glu-129 makes salt bridges with Lys-24 and Arg-27 of VPS20. VPS20 binding buries 713 Å² of VPS25c surface, which amounts to 14.5% of the total VPS25c surface.

ESCR-T II Binds to VPS20 with Micromolar Affinity

Biosensor binding experiments were performed with purified recombinant ESCRT-II, VPS25, and VPS20 proteins. In order to validate the crystallographic interactions, residues observed to interact structurally were mutated. VPS25 mutants V124E and T126K (hereafter referred to as VPS25<sup>AVPS20-1</sup> and VPS25<sup>AVPS20-3</sup>) completely abolished binding to VPS20. The triple mutation in VPS20 (L21R, R27A, D28A; hereafter referred to as VPS20<sup>ESCR-T II</sup>) also completely abolished the VPS25–VPS20 interaction (Figure 1E). These data confirm that the VPS25–VPS20 interaction in solution depends on the residues identified in the crystal structure.

In order to probe the role of avidity in the ESCRT-II–VPS20 interaction, the binding to monomeric VPS25c, full-length ESCRT-II containing two VPS25 subunits, or an artificially dimerized glutathione S-transferase (GST)-VPS25c construct, was compared (Figure 1E). Full-length ESCRT-II showed higher affinities than VPS25c for all VPS20 constructs. Full length ESCRT-II or GST-VPS25c dimer bound more tightly to VPS20 α1–α3 with K<sub>d</sub> values of 0.48 and 0.33 μM, respectively (Figure 1E).

In order to determine if C-terminal elements of VPS20 autoinhibited binding, as has been observed for other ESCRT-III interactors, a series of VPS20 constructs of varying length were tested (Figure 1F). VPS25 bound to full-length VPS20 with a dissociation constant of 7.0 ± 0.5 μM. VPS25 bound somewhat more tightly to VPS20 constructs lacking the C-terminal z5 helix. VPS25 bound to VPS20 α1–α3 and VPS20 α1–α4 with similar affinity, with K<sub>d</sub> values of 1.8 ± 0.2 and 2.5 ± 0.3 μM, respectively. In the context of the isolated VPS25c domain, the presence of C-terminal sequences after z5 in VPS20 slightly reduced the VPS25 binding affinity. Full-length ESCRT-II manifested less difference than VPS25c in its affinities for different VPS20 constructs (Figure 1G), which did not vary by more than a factor of two. This suggests that in the functionally relevant context of the full ESCRT-II complex, autoinhibition by the C terminus of VPS20 is not playing a major regulatory role.

Determinants for Selective Recognition of VPS20 by ESCRT-II

All ESCRT-III proteins have a common secondary structure organization with two N-terminal basic α helices and three to four C-terminal acidic α helices. The sequences of the α1 helices of Vps20 orthologs are well conserved, particularly in the residues that bind to Vps25 (Figure S2A). These include Leu-21, Arg-27, and Asp-28, which are required for binding in vitro. These residues are identical in animals. In yeast, the only variation is the conservative replacement of Arg-27 by Lys. Key residues on the β sheet of VPS25 involved in VPS20 binding are strictly conserved from yeast to humans (Figure S2B). These residues include Val-124 and Thr-126, essential for binding in vitro. Despite the common overall folding of all ESCRT-III proteins, the ten other known human ESCRT-III proteins have a much lower level of sequence identity in the α1 helix (Figure S2C). VPS25 binding involves 13 residues of VPS20, yet in other ESCRT-III proteins, no more than four of these residues are identically conserved. Leu-21 is conserved only in VPS24. Arg-27 is conservatively replaced by Lys in DID2, but is not conserved in any other ESCRT-III protein. Asp-28 is conservatively replaced by Glu in VPS60 and the N-terminal half of CHMP7, and otherwise not conserved. Thus even among the three residues analyzed mutationally, no other ESCRT-III protein sequence conserves all three positions at once. This sequence analysis indicates that the different species share a common mode of ESCRT-II–ESCRT-III interaction and explain the selectivity of ESCRT-II for VPS20 over all other ESCRT-III proteins.
Figure 1. The VPS20-VPS25 Complex

(A) Overall structure of the complex. The WH2 domain of VPS25 is colored in a blue to red gradient from the N- to C-terminal direction. Secondary structures are numbered as they occur in intact VPS25.

(B) Electron density from a final 2FO-FC map contoured at 1.0 σ in the vicinity of VPS25-VPS20 binding site. The final refined structure was shown in a ball and stick model.

(C) Superposition of apo VPS25 and VPS25-VPS20 complex structures.

(D) Interactions between VPS25 and VPS20. VPS25 is colored green and VPS20, blue. The side chains in the interface residues are shown in a ball and stick model. Selected hydrogen bonds and salt bridges are shown in dashed lines.

(E) Binding isotherms for ESCRT-II, VPS25c, and GST-VPS25c binding to wild-type VPS20 α1–α3 (residues 1–118) and, where indicated, VPS20 D ESCRT-II α1–α3.

(F) Binding of VPS25c to truncated VPS20 constructs (VPS20 α1–α4, residue 1–145; VPS20 α1–α5, residue 1–167).

(G) Binding of full-length ESCRT-II to the indicated VPS20 constructs. Binding isotherms from the equilibrium phases were fitted to a 1:1 binding model to determine the dissociation constants and statistical fitting errors shown beneath panels (E–G). All binding experiments were carried out twice with similar results.
Table 1. Statistics of Data Collection and Crystallographic Refinement

<table>
<thead>
<tr>
<th>Crystal</th>
<th>Native</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constructs</td>
<td>VPS25 (102–176) VPS20 (11–48)</td>
</tr>
<tr>
<td>Space group, unit cell</td>
<td>P2₁, a = 58.6 Å, b = 51.0 Å, c = 77.0 Å, β = 90.4</td>
</tr>
<tr>
<td>X-ray source</td>
<td>APS 22-ID</td>
</tr>
<tr>
<td>Wavelength (Å)</td>
<td>1.0000</td>
</tr>
<tr>
<td>Resolution (Å) (last shell)</td>
<td>2.0 (2.07–2.00)</td>
</tr>
<tr>
<td>No. of unique reflections</td>
<td>30553</td>
</tr>
<tr>
<td>I/σ(I) (last shell)</td>
<td>29.1 (3.0)</td>
</tr>
<tr>
<td>Rsym (%)</td>
<td>5.9 (28.1)</td>
</tr>
<tr>
<td>Data completeness (%)</td>
<td>98.4 (87.6)</td>
</tr>
<tr>
<td>Refinement</td>
<td></td>
</tr>
<tr>
<td>R factor (%)</td>
<td>23.6 (22.4)</td>
</tr>
<tr>
<td>Free R factor (%)</td>
<td>27.7 (34.8)</td>
</tr>
<tr>
<td>R.m.s. bond length (Å)</td>
<td>0.012</td>
</tr>
<tr>
<td>R.m.s. bond angle (°)</td>
<td>1.326</td>
</tr>
<tr>
<td>Average B value (Å²)</td>
<td>38.8</td>
</tr>
<tr>
<td>Number of atoms</td>
<td>protein 3661, water 213</td>
</tr>
</tbody>
</table>

The values in parentheses relate to highest-resolution shells.

ESCRT-II and VPS20 Interaction Site Is Required for Cargo Sorting

The epitopes used by VPS20 and VPS25 to bind each other are highly conserved, leading us to expect a common function from yeast to animals. Cps1 is a vacuolar hydrolase that is ubiquitinated and sorted to the lumen of the vacuole by the ESCRT pathway. Disruption of the ESCRT pathway can be diagnosed by the mislocalization of Cps1 to the limiting membrane of the vacuole and to the class E compartment. The latter appears by the mislocalization of Cps1 to the limiting membrane of the vacuole and to the class E compartment. The absence of GFP-Cps1 from the vacuolar lumen (Figure 2B)—in other words, a strong class E phenotype. The mutant alleles VPS20L18R/K24A/D25A (referred to as VPS20V148E and VPS25V148E (referred to as VPS25V148E and VPS25V148E)) were designed to cripple the interaction with Vps20, based on the equivalence of yeast Vps25 Val-148 and Thr-150 to Val-124 and Thr-126 in human VPS25 (Figure S2B). Expression of these two alleles resulted in strong class E phenotypes (Figures 2D and 2E). This indicates that the interaction of ESCRT-II with helix α1 of Vps20 is essential for the cargo-sorting function of ESCRTs.

ESCRT-II Binding Is Not Required for Membrane Recruitment of Vps20 in Yeast

The use of GFP fusions has been established as an approach to monitor ESCRT-III membrane localization in yeast. These constructs have a dominant-negative phenotype, resulting in the stabilization of their membrane binding on the class E membrane compartment (Figures 3A and 3B). Vps20-GFP is predominantly localized to the punctate class E structures (Figure 3A), as previously reported (Teis et al., 2008). When visualized with high sensitivity, a diffuse background of cytosolic Vps20-GFP was also observed (Figure 3A). Localization of Vps20ΔESCRT-II-GFP to both punctate structures and cytosol is essentially identical to wild-type (Figure 3B), indicating that abrogating ESCRT-II binding does not affect the localization of Vps20. These findings were confirmed by western blotting of the cytosol and membrane fractions, indicating that the majority of both wild-type and mutant Vps20-GFP was in the pellet fraction (data not shown).

VPS20 and ESCRT-II Bind Strongly to Membranes In Vitro

In order to probe the basis for the ESCRT-II-independent endosomal localization of Vps20, the membrane binding was assessed in vitro by monitoring FRET between rhodamine-labeled proteins and fluorescein-labeled liposomes. Recombinant unmyristoylated VPS20 was labeled on a unique engineered Cys residue at residue 119, which is outside of the ESCRT-II binding site and the membrane binding basic face. VPS20 bound with Kd = 29 nM to liposomes containing 3 mol% PI(3)P in an endosome-like background of phosphatidylcholine (PC), phosphatidylserine (PS), and cholesterol (Figure 3C). Human ESCRT-II labeled on native Cys residues bound to the same lipid mixture with Kd = 9 nM (Figure 3C). The affinity of the ESCRT-II-VPS20 supercomplex for membranes was monitored using labeled VPS20 and unlabeled ESCRT-II. ESCRT-II was preincubated with liposomes at each step in the titration in order to avoid difficulties with the insolubility of the supercomplex. The context of the supercomplex, VPS20 bound to membranes with Kd = 6 nM (Figure 3C). These results show synergism in the membrane binding of ESCRT-II and VPS20, consistent with the interaction of the proteins with each other. The binding of each protein or complex to liposomes by themselves, however, is very tight, explaining their independent targeting to endosomes in vivo.

ESCRT-II Accelerates ESCRT-III-Dependent ILV Budding In Vivo

Purified yeast ESCRT-III subunits are capable of supporting budding and scission of ILVs into giant unilamellar vesicles (GUVs) (Wollert et al., 2009), providing that superphysiological...
protein concentrations and a partially activated C-terminal deletion mutant of Vps20 are used. When full-length versions of all of the ESCRT-III subunits are used at concentrations closer to physiological levels (40:200:40:40 nM Vps20:Snf7:Vps24:Vps2), no ILVs are observed above background (Figures 4A and 4B). Addition of 40 nM ESCRT-II to this mixture led to the production of high levels of ILVs (Figure 4C), while addition of 40 nM ESCRT-II alone produced no ILVs (not shown). Addition of the doubly mutated ESCRT-II-Vps25^{A14D}Vps20^{A14D} led to essentially no ILV production above background (Figure 4D). This demonstrates that ESCRT-II potently and directly activates ESCRT-III for membrane budding and scission in the absence of cargo or other proteins, and that it does so in a manner that depends on the structural interaction between Vps25 and Vps20.

**The ESCRT-II-VPS20<sub>2</sub> Supercomplex Has a Convex Membrane-Binding Surface**

To gain insight into the interaction of ESCRT-II and full-length VPS20, we modeled a structure of ESCRT-II and VPS20 complex based on the crystal structures of human ESCRT-II (Im and Hurley, 2008) and VPS24 (Muziol et al., 2006). The conservation of the GLUE domain, VPS22, as described (Im and Hurley, 2008). The positioning of the GLUE domain and VPS22 α0 in the composite structure of full-length human ESCRT-II is approximate, but appeared reasonable in that it placed the myristoylation sites of both VPS20 molecules on the same side of the membrane, and allowed the VPS20<sup>66</sup> molecule to present its basic membrane-binding face on the same side of the overall supercomplex (Figure 5D). The membrane binding face is nonplanar, however. The WH2 domain of VPS25 is attached to the rest of the ESCRT-II core complex through a short linker that has some flexibility as judged by the difference in orientation between the domains in human and yeast, and the high B-factors for this domain in both human and yeast structures. Even allowing for linker movements, it was not possible to devise any orientation of the GLUE domain, VPS22 α0, and either VPS20 that occupied a common plane. However, a concave membrane surface can be readily modeled such that the basic faces of the GLUE domain, VPS22 α0, the myristoylation sites of both VPS20 molecules, and the basic face of VPS20<sup>66</sup> all contact the membrane without any movement of the VPS25 linker (Figure 5D). It appears that the basic face of VPS20<sup>22</sup> can be modeled onto this curved membrane provided the WH2 of

![Figure 2. Cargo Is Mislocalized in Yeast Expressing VPS20<sup>ΔESCRT-II</sup> and VPS25<sup>ΔVps20</sup> Alleles](image-url)

(A–E) The uppermost panel of each column shows the sorting of the GFP-Cps1 construct (green) in various strains, as indicated at the top of each column. The middle panels show the limiting membrane of the vacuole as labeled by FM4-64 (red), and the lower panels show the DIC image. Results presented here are characteristic of observations of >100 cells for each strain shown. One copy is bound to the VPS25 that is in turn bound to VPS36, and it will be referred to as VPS20<sup>26</sup>. The second molecule is bound to the copy of VPS25 that is in turn bound to VPS22, and we refer to it as VPS20<sup>22</sup>. VPS20<sup>22</sup> is nearly perpendicular to the plane of the Y. The N-terminal myristoylation site of VPS20 is eight amino acids upstream from the N terminus of the VPS20 model, which would allow the myristoyl groups of both VPS20 molecules in the complex to touch the lipid membrane. The ESCRT-II-VPS20<sub>2</sub> supercomplex has multiple points for membrane binding such as ubiquitinated cargo, the GLUE domain, VPS22 α0, and two N-terminal myristoyl groups of VPS20. The two VPS20 molecules are separated by 110 Å between the α1–α4 tips of VPS20 molecules. A similar orientation of VPS20 molecules was observed in a model of yeast ESCRT-II-VPS20<sub>2</sub> supercomplex. However, VPS20<sup>22</sup> is rotated 40° toward the inside of the Y, reducing the tip-to-tip distance to 90 Å in the yeast complex (Figure 5C).
VPS2522 is rotated about the WH1-WH2 connecting loop, although Figure 5D shows the geometry without domain rotations or any other adjustments.

**DISCUSSION**

The ESCRT-II-Vps20 interaction is central to MVB biogenesis, and the structure of the minimal complex explains the specificity of ESCRT-II for Vps20. The simplest model for ESCRT-II activation of ESCRT-III would invoke recruitment of Vps20 to membranes by ESCRT-II. However, we found that Vps20 had normal localization. This unexpected result was explained by the finding that both Vps20 and ESCRT-II have nanomolar affinities for endosome-like synthetic liposomes. The normal localization of Vps20 stands in sharp contrast to the
strong block in cargo sorting observed when the VPS20<sup>ESCRT-II</sup> allele was expressed. ESCRT-II coordinates ESCRT-III scission to cargo locations, which could account for the functional requirement for the direct ESCRT-II-Vps20 interaction. Using a GUV-based assay, the direct activation of ESCRT-III by ESCRT-II was visualized. The GUV assay does not contain cargo, so while the coordination of cargo to ILV biogenesis is undoubtedly important, these results show that there is also a fundamental, cargo-independent aspect to ESCRT-III activation.

One of the central questions in MVB biogenesis is how the limiting membrane of the endosome is bent into a negatively curved ILV (Barelli and Antonny, 2009). ESCRT-III subunits overexpressed in cells coat the inside of plasma membrane evaginations, promoting negative curvature (Hanson et al., 2008). Recombinant ESCRT-III subunits mixed with liposomes have similar effects. These results lead to a conundrum. The coating of the interior of the evaginations or invagination leaves no room for transmembrane cargo, and following scission, would also lead to the uptake of the ESCRT-III proteins into ILVs and thus to their degradation. This is at odds with the lack of reports of ESCRT-III localization in ILVs, and with the observation that ESCRT-III is recycled from endosomes by Vps4 (Babst et al., 1998). The source of negative curvature in MVB biogenesis has therefore remained obscure.

The ESCRT-II-VPS20<sub>2</sub> supercomplex has a convex curvature that is complementary to that of a nascent ILV, and this shape is only formed upon the assembly of the supercomplex (Figure 5D). The ESCRT system is complex, and nothing in the above is inconsistent with the concept that ESCRT-II causes an activating conformational change in Vps20 (Saksena et al., 2009). We find robust ILV formation with full-length Vps20, while the use of a partially truncated and activated Vps20 construct must be added at relatively high levels to support budding in vitro without ESCRT-II (Wollert et al., 2009). The curvature of the large in vitro ILVs that can be visualized by light microscopy is lesser than that in the physiological setting and does not match the curvature of the ESCRT-II-VPS20<sub>2</sub> supercomplex. The allosteric activation mechanism offers the most appealing explanation for the potential activation of ESCRT-III by ESCRT-II in the GUV system. Similarly, we do not discount that other factors such as ESCRT-I or ALIX could also contribute to regulating ILV size (Kostelansky et al., 2007) or promoting negative curvature (Kim et al., 2005).

ESCRT-II has a special role in MVB biogenesis, in that overexpression of ESCRT-II rescues the loss of ESCRT-I, but not the converse (Babst et al., 2002b). In contrast, ESCRT-I is critical, but ESCRT-II dispensable, for HIV-1 budding (Langelier et al., 2006) and cytokinesis (Morita et al., 2007). In HIV-1 budding, self-assembly of Gag drives membrane curvature. The membrane neck between daughter cells is also established independent of the ESCRTs. In contrast to MVB biogenesis, HIV-1 budding and cytokinesis use other mechanisms to establish membrane curvature, explaining why these processes do not require ESCRT-II. The proposed model (Movie S1) rationalizes the ESCRT-II-VPS20<sub>2</sub> supercomplex could play a key role in scaffolding negative membrane curvature by imposing its shape on the membrane. An analogous mechanism is well-established for positive curvature stabilization by the crescent-shaped BAR domains (McMahon and Gallop, 2005).
the dramatic differences in the ESCRT-II requirement in these three ESCRT-dependent pathways.

In summary, we have elucidated the determinants for the ESCRT-II-Vps20 interaction, which has multiple and vital roles in MVB biogenesis. First, the interaction coordinates cargo binding to ESCRT-II with the initiation of ESCRT-III assembly. Second, the interaction promotes an activating conformational change in Vps20 (Saksera et al., 2009). Finally, the composite structure of the ESCRT-II-VPS20 complex suggests that the interaction could directly promote the negative membrane curvature required for ILV biogenesis. The structural analysis explains how this crucial interaction is conserved from yeast to humans. The conserved specificity of ESCRT-II for Vps20, as compared to all other ESCRT-III subunits, highlights the central role for this interacting pair in MVB biogenesis.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification

The codon-optimized synthetic gene for VPS25c (residues 102–176) was subcloned into the pGSt1 vector (Sheffield et al., 1999). VPS25c was tagged with an N-terminal GST followed by a tobacco etch virus (TEV) protease cleavage site. DNA coding for GST-VPS25c was subcloned into the first cassette of the polycistronic pSt39 vector (Tan, 2001). Various Vps20 constructs with an N-terminal histidine tag followed by a TEV cleavage site were subcloned into the second cassette of pSt39. The resulting bicistronic plasmid was transformed into E. coli strain BL21(DE3) Star and expressed overnight at 30°C. Cells were resuspended in buffer (2 x PBS plus 20 mM imidazole) and lysed by sonication. The resulting VPS25c–VPS20 complexes were isolated using sequential Ni2+ and glutathione affinity chromatography. The eluate was concentrated and the GST and histidine tags were removed by cleavage with TEV protease. The VPS25c–VPS20 complexes were further purified by Superdex 200 size exclusion chromatography (SEC). The fractions containing the complex were concentrated to 10 mg/ml in a buffer of 10 mM Tris–HCl (pH 8.0) and 100 mM NaCl for crystallization.

For the biosensor binding assay, various Vps20 constructs were subcloned into a vector providing an N-terminal His6-tagged maltose binding protein (MBP) followed by a TEV cleavage site. The MBP-VPS20 constructs were expressed as described above and purified by Ni-NTA affinity chromatography. The samples were further purified by Hi-trap anion-exchange or SEC. The purified MBP-VPS20 proteins were diluted in 1 x PBS buffer for biosensor binding assay. Full-length human ESCRT-II complex was prepared as previously described (Im and Hurley, 2008).

Yeast ESCRT-II subunits Vps20, Snf7, Vps24, and Vps2 for GUV experiments were expressed and purified as described (Wollert et al., 2009). Briefly, N-terminal His6-MBP fusion proteins were expressed at 30°C for 3 hr after induction with 0.5 mM isopropyl thiogalactoside (IPTG) at an optical density (OD 600 nm) of 0.8. The proteins were affinity purified using Ni-NTA resin (QIAGEN). Elution fractions were cleaved using TEV protease and further purified by SEC (Superdex 200 column). Purified proteins were immediately flash frozen in liquid nitrogen and stored at −80°C until use. Full-length yeast ESCRT-II was purified from E. coli with an N-terminal His6 fusion-tagged to Vps22 using Ni-NTA affinity purification and SEC as described (Hierro et al., 2004).

Crystallography and Crystallographic Analysis

Crystals of VPS25c–VPS20 complex were grown by vapor-diffusion methods at 25°C over a reservoir of 100 mM Tris HCl (pH 8.0), 35% PEG 4000 for 1 week. Crystals were cryoprotected in Paratone and stored using liquid nitrogen. The samples were further purified by Hi-trapQ anion-exchange or SEC. The fractions containing the complex were concentrated to 10 mg/ml in a buffer of 0.5 mM isopropyl thiogalactoside (IPTG) at an optical density of 2500–3000 RU. Binding studies were performed in duplicate by passing the wild-type or mutant VPS25c and full-length ESCRT-II over the captured MBP-VPS20 proteins at a flow rate of 10 μl min⁻¹ in a 1 x PBS buffer. The surface was regenerated with an injection of 10 mM Glycine–HCl at pH 2.0 at a flow rate of 10 μl min⁻¹ for 30 s. The data were fit with the following equation:

\[ R = R_{\text{max}} \cdot \left[ \frac{[\text{VPS25}]}{K_d + [\text{VPS25}]} \right] + \text{offset} \]

where [VPS25] is the protein concentration of the flowing analyte, \( K_d \) is the dissociation constant, \( R_{\text{max}} \) is the maximal response, and "offset" is the background signal. The data was processed using Biacore Evaluation software and the figures were made using SigmaPlot (GraphPad Software).

Biosensor Binding Experiments

Binding of VPS25c and ESCRT-II to VPS20 was analyzed using a Biacore T100 instrument at 25°C using a CM5 sensor chip. Approximately 9000 response units (RU) of anti-MBP monoclonal antibody (Abcam) was immobilized on two flow cells using amine-coupling chemistry. The MBP-VPS20 constructs (\( 1\alpha -3\) residues 1–118, \( 1\alpha-3\) mutant L21R/R28A/D29A [ESCRT-II], \( 1\alpha-4\) residues 1–145, \( 1\alpha-5\) residues 1–167, and full-length) were captured to densities of 2500–3000 RU. Binding studies were performed in duplicate by passing the wild-type or mutant VPS25c and full-length ESCRT-II over the captured MBP-VPS20 proteins at a flow rate of 10 μl min⁻¹ in 1 x PBS buffer. The surface was regenerated with an injection of 10 mM Glycine–HCl at pH 2.0 at a flow rate of 10 μl min⁻¹ for 30 s. The data was fit with the following equation:

\[ R = R_{\text{max}} \cdot \left[ \frac{[\text{VPS25}]}{K_d + [\text{VPS25}]} \right] + \text{offset} \]

where [VPS25] is the protein concentration of the flowing analyte, \( K_d \) is the dissociation constant, \( R_{\text{max}} \) is the maximal response, and "offset" is the background signal. The data was processed using Biacore Evaluation software and the figures were made using SigmaPlot (GraphPad Software).

Plasmid Construction and Yeast Strains

DNA coding for the complete expression cassettes for VPS20 and VPS25 were amplified from yeast genomic DNA and cloned into YCplac111 (Odorizzi et al., 1998), were transformed into wild-type and mutant strains. The following yeast strains were used: BY4742 vps25Δ::KanR and BY4742 vps15Δ::KanR (Open Biosystems).

Fluorescence Microscopy

Yeast strains expressing the appropriate alleles were harvested at an A600 of 0.4–0.6, and labeled with FM4-64 during membrane staining (Vida and Emr, 1995). Uptake of FM4-64 by live cells was performed at 30°C for 1 hr, after which cells were resuspended in selection media and incubated for 30 min at 30°C. Visualization of cells was performed on an LSM510 fluorescence microscope (Carl Zeiss Microimaging) equipped with fluorescein isothiocyanate (FITC) and rhodamine filters and captured with a digital camera. GUVs were visualized in a 200 μ observation chamber (Lab-Tek chambered #1.0).
and was allowed to equilibrate for 1 min, and then a stoichiometric amount of binding in the presence of ESCRT-II, ESCRT-II was titrated first into the cuvette. In the case of VPS20 PI(3)P (3 mol%), and fluorescein-PE (1 mol%). Increasing amounts of rhodamine-oleoyl-sn-glycero-3-phosphoserin (POPS) (10 mol%), cholesterol (25 mol%), 1-Palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl). Phosphatidylinositol 3-phosphate (diC16) was purchased from Echelon.

Cell Fractionation and Western Blotting

Yeast cells grown to OD600 = 0.9 were harvested and resuspended in 50 mM Tris-HCl (pH 7.4) with the addition of yeast protease inhibitor cocktail (Sigma). The cells were disrupted by glass beads with a Beadbeater. The resulting cell lysate was centrifuged at 500 × g for 5 min to remove unlysed cells and cell walls. The resulting extracts were separated by centrifugation at 200,000 × g for 20 min into soluble and membrane-bound pellet fractions. The samples were analyzed for the presence of Vps20-GFP by western blotting using an antibody specific for the GFP tag. Quantification of the resulting blot was performed by the LabWorks 4.6 program (UVP).

Membrane Binding In Vitro

The engineered single-Cys mutant C109S/S119C of VPS20, and wild-type ESCRT-II, were incubated with tetramethylrhodamine-5-iodacetamide dihydrodilide (5-TMRIA, Molecular Probes) overnight at 4°C to allow for normal endosome function. EMBO J. 17, 2982–2993. Unreacted dye was removed by gel filtration. This procedure yielded VPS20 labeled at 100% and ESCRT-II at ~140%. Fluorescence intensity measurements were performed using a Fluorolog-3 (HORIBA Jobin Yvon) fluorescence spectrometer at room temperature with 0.1 mg/ml liposomes consisting of 1-Palmi-toyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) (61 mol%), 1-Palmi-toyl-2-oleoyl-sn-glycero-3-phosphoserin (POPS) (10 mol%), cholesterol (25 mol%), PI(3P) (3 mol%), and fluorescein-PE (1 mol%). Increasing amounts of rhodamine-labeled protein were titrated into the cuvette. In the case of VPS20 binding in the presence of ESCRT-II, ESCRT-II was titrated first into the cuvette and was allowed to equilibrate for 1 min, and then a stoichiometric amount of VPS20 was titrated into the cuvette. At each concentration, the intensity of fluorescence was recorded (excitation at 460 nm and emission at 520 nm). Percent saturation was calculated was calculated as $D = I/I_{\text{max}}$ where $D = I/I_{\text{max}}$ (I represents the measured intensity of fluorescence, $I_{\text{max}}$ is the intensity of fluorescence prior addition of any protein, $I_{\text{max}}$ represents the change of the fluorescence intensity, and $I_{\text{max}}$ represents the change of fluorescence intensity at saturation). Percent saturation was plotted against the protein concentration and fitted by Langmuir isotherm to obtain the $K_d$ values.

Preparation of GUVs

GUVs were prepared as described (Wollert et al., 2009). Briefly, lipid mixtures containing POPC (62 mol%), POPS (10 mol%), cholesterol (25 mol%), PI(3P) (3 mol%), and lissamine-rhodamine-PE (0.1 mol%) were applied to indium-tin oxide covered glass slides and placed into a custom-made Teflon chamber. An electric AC-field (1V, 10 Hz) was applied for 4 hr at 60°C (Angelova and Dimitrov, 1998). The following lipids were purchased from Avanti Polar Lipids: 1-palmityl-2-oleoyl-sn-glycero-3-phospho-L-serine, 1-palmityl-2-oleoyl-sn-glycero-3-phospho-L-serine, 1-palmityl-2-oleoyl-sn-glycero-3-phosphocholine, cholesterol, 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl). Phosphatidylinositol 3-phosphate (diC16) was purchased from Echelon.

ACCESSION NUMBERS

Crystallographic coordinates have been deposited in the Protein Data Bank with the accession code 3HTU.

SUPPLEMENTAL DATA

Supplemental data for this article include three figures and one movie and can be found at http://www.cell.com/developmental-cell/supplemental/S1534-5807(09)00266-4.

ACKNOWLEDGMENTS

We thank Boris Babakov for assistance with microscopy, Jurrien Dean and Jennifer Lippincott-Schwartz for use of their microscopes, Will Prinz for providing yeast strains and discussions, Adriana Rojas for help with crystallographic data collection, Ethan Tyler for producing Movie S1, and the SER-CAT staff for user support at the Advanced Photon Source (APS). Use of the APS was supported by the U.S. Department of Energy, Basic Energy Sciences, Office of Science, under Contract No.W-31-109-Eng-38. This research was supported by NIH intramural support, NIDDK and IATAP. T.W. is an EMBO long-term fellow.

Received: March 13, 2009
Revised: June 10, 2009
Accepted: July 7, 2009
Published: August 17, 2009

REFERENCES


