Sla1 and Rvs167 are yeast proteins required for receptor internalization and organization of the actin cytoskeleton. Here we provide evidence that Sla1 and Rvs167 are orthologues of the mammalian CIN85 and endophilin proteins, respectively, which are required for ligand-stimulated growth factor receptor internalization. Sla1 is similar in domain structure to CIN85 and binds directly to the endophilin-like Rvs167. Akin to CIN85, Sla1 interacts with synaptojanins and a ubiquitin ligase that regulates endocytosis. This ubiquitin ligase, Rsp5, binds directly to both Sla1 and Rvs167. The interaction between Rsp5 and Rvs167 is mediated through Rsp5 WW domains and PXY motifs in the central Gly-Pro-Ala-rich domain of Rvs167. Rvs167 PXY motifs are required for Rsp5-dependent monoubiquitination of Rvs167, but may control specific Rvs167 SH3 domain-protein interactions or negatively regulate SH3 domain activity.

A diverse protein network is required to sort and internalize cell surface cargo into primary endocytic vesicles that bud from the plasma membrane. Many protein-protein interactions between members of this network have been mapped, suggesting a dynamic process that must be exquisitely temporally and spatially regulated. Phosphorylation is an important regulator of interaction between endocytic proteins (1–2), particularly as an unknown site(s) by both Nedd4 and Cbl (7, 18). The specificity of ubiquitination reactions is regulated by ligand-stimulated growth factor receptor internalization (e.g. see Refs. 4, 7, 8, and 9) and modify cell surface proteins with a ubiquitin sorting signal that targets cargo for internalization and degradation in the lysosome (10, 11). Ubiquitin-dependent endocytosis controls the cell surface activity of a large variety of proteins (reviewed in Ref. 12). Ubiquitin is the principal signal for the entry of plasma membrane cargo into primary endocytic vesicles in yeast and can also function as an internalization signal in mammalian cells (13, 14). Ubiquitin-dependent internalization relies on proteins that orchestrate the selection of endocytic cargo, rearrangements of the underlying actin cytoskeleton at the plasma membrane, and deformation and scission of the plasma membrane. Of these proteins, epsin, Ep15, β-arrestin, and CIN85 are known to be ubiquitinated. Specifically, they are modified with monoubiquitin (4, 7, 15–19).

CIN85 (Cbl-interacting protein of 85 kDa) and its close homologue, CMS (Cas ligand with multiple SH3 domains), are part of the endocytic network and bind to many proteins involved in endocytosis and signaling (20). A number of observations suggest that CIN85/CMS regulates the actin cytoskeleton and may act as one link between actin polymerization and receptor internalization (e.g. see Refs. 21 and 22). CIN85 has three N-terminal SH3 domains followed by a proline-rich region and a coiled-coil region that mediates dimerization. The Cbl ubiquitin ligase was recently found to recruit a complex of CIN85 with another endocytic protein, endophilin, to facilitate the internalization of activated, ubiquitinated growth factor receptors (5, 23). Furthermore, CIN85 is monoubiquitinated at an unknown site(s) by both Nedd4 and Cbl (7, 18).

Endophilin and amphiphysin are proteins that share a number of properties and are required for the internalization step of endocytosis. Both proteins have N-terminal BAR (Bin, amphiphysin, Rvs) domains that mediate lipid interactions in vitro and a C-terminal SH3 domain that binds to dynamin and to the polyphosphoinositide phosphatases, synaptojanins (24, 25). Each protein can independently promote tubulation or vesiculation of membranes (26, 27), and both proteins are predicted to coordinate membrane deformation with actin cytoskeleton dynamics (28, 29). Endophilin and amphiphysin are localized to the highly curved necks that link a deeply invaginated vesicle to the donor membrane (30, 31), and they are thought to function at a late step in vesicle budding together with dynamin to promote vesicle scission. Like CIN85, endophilin is implicated in regulation of the actin cytoskeleton. Endophilin binds to and regulates N-WASP, an activator of actin polymerization (29). In addition, after treatment of cells

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* This work was supported by National Institutes of Health Grant DK61299. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: SH3, Src homology 3; GST, glutathione S-transferase; HA, hemagglutinin; MES, 4-morpholinethanesulfonic acid; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

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The Rsp5 Ubiquitin Ligase Binds to and Ubiquinates Members of the Yeast CIN85-Endophilin Complex, Sla1-Rvs167*
with growth factor, the CIN85-endophilin complex recruits cortactin, another regulator of actin polymerization (21, 29). The yeast endophilin/amphiphysin homologue, Rvs167 (reduced viscosity), another regulator of actin polymerization (21, 29). The growth medium of each strain was added to 2% agar-containing medium prior to autoclaving.

Here we identify endocytic proteins that bind to Rsp5, the sole yeast homologue of the Nedd4 family of ubiquitin ligases. Rsp5, like most Nedd4 family members, contains a N-terminal C2 domain, WW protein-protein interaction domains, and a C-terminal catalytic HECT (homology to E6-AP C terminus) domain. We find that Rsp5 co-precipitates with the interacting proteins Slal and Rvs167 required for receptor internalization and organization of the actin cytoskeleton. We further investigate the similarity of yeast Slal-Rvs167 to the mammalian CIN85-endophilin complex and the consequences of Rsp5 interaction with Slal and Rvs167.

**Experimental Procedures**

**Strains, Media, and Reagents**—Yeast strains used in this study are listed in Table I. Genetic manipulations were performed by standard techniques, and cell transformations were performed by the lithium acetate method (34). The *rsv167Δ::TRP1* disruption has been described (33). Cells were propagated in synthetic minimal medium (34), YPUAD medium (35), casamino acids medium (0.67% yeast nitrogen base, 0.5% vitamin assay casamino acids, 2% glucose supplemented with 50 mg/liter adenine, histidine, and tryptophan), or YNB selective medium (0.67% yeast nitrogen base, 2% glucose supplemented with commercially purchased selective amino acid mixes) as indicated in the figure legends. Sodium chloride (1 M) was added to 2% agar-containing medium prior to autoclaving. The growth medium of each strain was added to 2% agar-containing medium prior to autoclaving.

**Plasmid Construction**—RVS167 yeast expression plasmids were based on a URA3 marked centromeric plasmid, pGBK5 (a gift from Florian Bauer) (32). A triple hemagglutinin (HA) epitope was introduced at the C terminus of RVS167 by two steps. First, a NotI site was introduced at the 3′-end of RVS167 by QuikChange™ mutagenesis (Stratagene, La Jolla, CA) to

| Strain | Genotype |
|--------|----------|
| LHY10  | ste2Δ::LEU2 ura3 his3 trp1 bar1-1 |
| LHY23  | rps5-1 ura3 leu2 trp1 bar1 |
| LHY291 | end4-1 ura3 leu2 his4 |
| LHY501 | ura3 met15Δ leu2Δ his3Δ |
| LHY1850| rvs167Δ::TRP1 trp1 ura3 leu2, his3 and/or his4 bar1 |
| LHY2086| MATα rvs167Δ::TRP1 trp1 ura3 leu2 ade2 his3 and/or his4 bar1 |
| LHY2088| ade2 leu2 trp1 ura3 his3 and/or his4 bar1 |
| LHY2089| ste2Δ::LEU2::TRP1 ade2 ura3 leu2 bar1 |
| LHY2363| pRVS167-HA[URA3] rvs167Δ::TRP1 trp1 ura3 leu2 his3 and/or his4 bar1 |
| LHY2422| his4Δ::kanMX4 ura3 met15Δ leu2Δ his3Δ |
| LHY2423| his4Δ::kanMX4 ura3 met15Δ leu2Δ his3Δ |
| LHY2491| ura3 leu2 trp1 |
| LHY2492| rps5Δ::HIS3 leu2 ura3 trp1 bar1 |
| LHY2510| pRVS167PΔSTK1,PPΔ1-5-HA[URA3] rvs167Δ::TRP1 trp1 ura3 leu2 bar1 his3 or his4 |
| LHY2529| rvs167Δ::kanMX4 bar1 trp1 leu2 ura3 his3 |
| LHY2579| pRVS167[URA3] rvs167Δ::kanMX4 bar1 trp1 leu2 ura3 his3 |
| LHY2582| pRVS167-AAY[URA3] rvs167Δ::kanMX4 bar1 trp1 leu2 ura3 his3 |
| LHY2627| pRVS167-370Stop[URA3] rvs167Δ::kanMX4 bar1 trp1 leu2 ura3 his3 |
| LHY2664| pRVS167-K290R[URA3] rvs167Δ::kanMX4 bar1 trp1 leu2 ura3 his3 |
| LHY2685| pRVS167-K418R[URA3] rvs167Δ::kanMX4 bar1 trp1 leu2 ura3 his3 |
| LHY2726| pHA-Yps0[LEU2] rps5Δ::kanMX4 his3 leu2 met15 ura3 |
| LHY2769| pHA-ARC15[URA3] pyEplac112[TRP1] his3 trp1 lys2 ura3 leu2 bar1 |
| LHY2794| pHA-ARC15[URA3] pCP1-1-cmyc-UBI[TRP1] his3 trp1 lys2 ura3 leu2 bar1 |
| LHY2795| pHA-ARC15[URA3] pCP1-1-UBI[TRP1] his3 trp1 lys2 ura3 leu2 bar1 |
| LHY3111| pRVS167-P572A,AAAY[URA3] rvs167Δ::kanMX4 bar1 trp1 leu2 ura3 his3 |
| LHY3112| pRVS167-P534,372A,AAAY[URA3] rvs167Δ::kanMX4 bar1 trp1 leu2 ura3 his3 |
| LHY3113| pRVS167Δ::PPP[URA3] rvs167Δ::kanMX4 bar1 trp1 leu2 ura3 his3 |
| LHY4050| rps5Δ::HIS3 trp1 ura3 his3 leu2 |
| LHY4386| pRPS5[TRP1] rps5Δ::HIS3 trpl ura3 his3 leu2 |
| LHY4388| pRPS5-5X[:TRP1] rps5Δ::HIS3 trp1 ura3 his3 leu2 |
| LHY4370| pRPS5-5X[:TRP1] rps5Δ::HIS3 trp1 ura3 his3 leu2 |
| LHY4372| pRPS5-5X[:TRP1] rps5Δ::HIS3 trp1 ura3 his3 leu2 |
| LHY4374| pRPS5-5X[:TRP1] rps5Δ::HIS3 trp1 ura3 his3 leu2 |
| LHY4842| pSTE2-3xHA[URA3] rvs167Δ::LEU2 ura3Δ leu2Δ met15Δ0 ura3Δ0 |
| LHY4845| pSTE2-3xHA[URA3] slalΔ::kanMX4 his3Δ leu2Δ0 met15Δ0 ura3Δ0 |
| LHY4851| pHA-IF52[LEU2] his3 trp1 lys2 ura3 leu2 bar1 |

a All strains are MATα unless specified otherwise.
b These strains were obtained from EUROSCARF (Microbiology Institute, Frankfurt, Germany).
create pRVS167-Not1 (LHP1026). A triple HA epitope was ligated into the NotI site to generate pRVS167-HA (LHP1027). RVS167-HA completely resolved the growth and internalization defects of rvs167A cells.

Site-directed Pro → Ala mutations in pFBKS were generated by sequential QuikChange™ mutagenesis (pRVS167/P90A, P120A, LHP1131; pRVS167/P344A, P727A, P903A, P909A, LHP1439; pRVS167/P334A, P727A, P903A, P909A, LHP1450). The SAPP-MUTATION (LHP1436) was introduced by mutagenic QuikChange™ oligonucleotides that looped out the SAPP-HAY sequence (codons 397–401) of pFBKS. Plasmids encoding hexahistidine (His6)-tagged Rvs167 or Slal fragments were generated by ligation-independent cloning of the appropriate PCR fragments into the pET-30 bacterial expression vector (Novagen, Madison, WI) to construct the RVS167 plasmids pET30-BAR (codons 1–291; LHP1494), pET30-GPA (codons 292–427; LHP1495), pET30-SH3 (codons 428–482; LHP1496), and pET30-RVS167 (codons 1–482; LHP1497) or the SLA1 plasmids pET30-1–240 (LHP2017), pET30-420–720 (LHP2018), and pET30-720–1240 (LHP2019). Introduction of the appropriate mutations or the in-frame fusion of the His6 tag in plasmids was verified by automated sequencing.

Plasmid pGEX-6P-2 (Amersham Biosciences) was used for production of GST-Sla1. Plasmids encoding GST fused to the N terminus of Sla1 (codons 1–420; LHP2098) or to a central fragment (codons 420–720; LHP2099) were generated by PCR amplification of the appropriate DNA using BamHI site-containing oligonucleotides. The PCR products were digested and ligated into BamHI-digested pGEX-6P-2. The in-frame fusions were verified by automated sequencing.

Plasmids used for production of GST and GST-Rsp5 were pGEX-4T (Amersham Biosciences) and pGST-Rsp5 (a gift from Jon Huibregtse, University of Texas, Austin, TX). A plasmid encoding GST fused to the three WW domains of Rsp5 (pGST-3×WW, LHP703) was generated by PCR amplification of the WW domains (codons 228–430) using a 5’ EcoRI site-containing oligonucleotide and a 3’ XhoI site-containing oligonucleotide. The PCR product was digested and ligated into EcoRI- and XhoI-digested pGEX-6P-2. The in-frame fusions were verified by automated sequencing.

Plasmids used for production of GST-Sla1 and GST-Rsp5 were pGEX-4T and pGST-Rsp5 (a gift from Jon Huibregtse, University of Texas, Austin, TX). A plasmid encoding GST fused to the three WW domains of Rsp5 (pGST-3×WW, LHP703) was generated by PCR amplification of the WW domains (codons 228–430) using a 5’ EcoRI site-containing oligonucleotide and a 3’ XhoI site-containing oligonucleotide. The PCR product was digested and ligated into EcoRI- and XhoI-digested pGEX-KPT (37).

INP52 was amplified from yeast genomic DNA using 5’ PstI and 3’ BamHI site-containing oligonucleotides, digested and ligated into PstI- and BamHI-digested YEplac181 (LHP1456). The NotI site was inserted at the 5’-end of INP52 in LHP1459 via QuikChange™ mutagenesis. A triple HA epitope was ligated into the NotI site to generate pHA-INP52 (LHP1463). A PstI-SacI fragment was subcloned into a LEU2-marked multicopy plasmid (YEplac181) digested with PstI and SacI to generate LHP2077.

**Yeast Two-hybrid Analysis—RVS167** (codons 74–482) and RSP5 (codons 1–809) were cloned into the GAL4 yeast two-hybrid fusion vectors pAS2-1 and pACT2 (Clontech, Palo Alto, CA), respectively, by standard procedures. The plasmids and control vectors were transformed into Y190 cells (Clontech), and three independent cultures of representative transformants were assayed for β-galactosidase activity according to the manufacturer’s protocol.

**Preparation of Cell Lysates for Immunoblotting**—For detection of Ste2, cell extracts were prepared by glass bead lysis and SDS/urea extraction as previously described (35, 36). For detection of ubiquitinated Rvs167, cell lysates were prepared by alkaline lysis and trichloroacetic acid precipitation as described (38).

**Native Co-immunoprecipitations**—Cells were lysed in native immunoprecipitation buffer (0.2 M sorbitol, 50 mM potassium acetate, 25 mM potassium chloride, 10 mM HEPES, 1 mM EDTA, pH 7.0) containing yeast protease inhibitors by vortexing the cell suspension with acid-washed glass beads 4–6 times with intervening rests on ice. Whole cell lysates were extracted with 2 mg/ml β-mercaptoethanol for 30–60 min on ice and clarified by centrifugation at 20,000 × g for 10 min at 4°C. A fraction of each cleared lysate was reserved for analysis, and the remainder was incubated with Rsp5 antiserum followed by incubation with protein A-Sepharose beads (Amersham Biosciences). Beads were collected at 100 × g and washed four times in native immunoprecipitation buffer containing yeast protease inhibitors. Bound proteins were eluted by boiling in SDS sample buffer (39), resolved by SDS-PAGE, and analyzed by immunoblotting.

**GST Fusion Protein Precipitation Experiments**—GST pull-down experiments with yeast lysates were performed as previously described with minor modifications (35). Lysates were prepared in MES lysis buffer with the addition of 1% Triton X-100. The lysates were extracted with 2 mg/ml β-mercaptoethanol and incubated with glutathione-Sepharose beads containing equivalent amounts (~20 μg each) of GST, GST-3×WW, or GST-Rsp5 for 2 h at room temperature or overnight at 4°C. After the binding incubation, beads were washed four times in MES lysis buffer, and bound proteins were eluted by boiling in SDS sample buffer. Eluted proteins were analyzed by immunoblotting.

**E. coli** cells were induced to express His6-tagged Rvs167 proteins with 0.1 mM isopropyl-β-D-thiogalactopyranoside for 1–3 h. Cells expressing full-length His6-Rvs167 were lysed by pulsed sonication in phosphate-buffered saline containing protease inhibitors. The lysate was extracted with 1% Triton X-100 and clarified by centrifugation at 4°C. A fraction of the cleared lysate was reserved for analysis, and the remainder was split into equal aliquots and incubated with GST, GST-3×WW, or GST-Rsp5 glutathione-bound Sepharose beads for 2 h at 4°C. After several washes in phosphate-buffered saline containing protease inhibitors and 0.2% Triton X-100, bound proteins were eluted by boiling in SDS sample buffer. Cells expressing His6-tagged GPA and SH3 fragments were lysed by the same procedure in 50 mM sodium phosphate buffer, 300 mM sodium chloride with 1 mM phenylmethylsulfonyl fluoride and 1 μg/ml pepstatin. The tagged proteins were purified on TALON metal affinity resin according to the manufacturer’s recommendations for native purification (Clontech) and eluted with 200 mM imidazole in the same buffer. Purified proteins were diluted into an E. coli cell lysate (prepared as described above) to equivalent concentrations. Binding to GST and GST-3×WW was performed as described for full-length His6-Rvs167. Samples were resolved by SDS-PAGE and stained with Coomassie Brilliant Blue G-250 (Bio-Rad) or analyzed by immunoblotting.

**Immobilization of and Binding to Bacterially Expressed His6-tagged Proteins**—His6-tagged Sla1 polypeptides were immobilized on TALON metal affinity resin according to the manufacturer’s recommendations for native purification (Clontech) and eluted with 200 mM imidazole in the same buffer. Purified proteins were diluted into an E. coli cell lysate (prepared as described above) to equivalent concentrations. Binding to GST and GST-3×WW was performed as described for full-length His6-Rvs167. Samples were resolved by SDS-PAGE and stained with Coomassie Brilliant Blue G-250 (Bio-Rad) or analyzed by immunoblotting.

A yeast lysate containing HA-Inp52 was prepared as previously described (41), except that the lysis buffer contained 3 mg/ml bovine serum albumin and 1% Nonidet P-40. In addition, beads with immobilized His6-tagged polypeptides were preincubated in buffer containing 1 mg/ml bovine serum albumin in Tris-buffered saline, pH 7.5, for 1 h at 4°C. After the
Rvs167 Is Monoubiquitinated by Rsp5

binding incubation, beads were washed two times in lysis buffer, 10 mM imidazole, and 1% Nonidet P-40 and two times with lysis buffer, 20 mM imidazole, and 1% Nonidet P-40.

RESULTS

The Rsp5 Ubiquitin Ligase Binds Directly to Rvs167 and Sla1—Genetic studies suggested that Rsp5 regulates components of the receptor internalization machinery (3). To identify proteins involved in endocytosis that bind to Rsp5, we generated polyclonal rabbit antisera against full-length native Rsp5. On immunoblots, Rsp5 antisem recognized a single protein of the expected size in wild type cells but not in rps5Δ cells (Fig. 1A). We then performed native immunoprecipitations with anti-Rsp5 on lysates prepared from wild type yeast strains, some of which expressed HA-tagged versions of different endocytic proteins. Although interactions with a number of endocytic proteins were observed inconsistently, Rvs167-HA and Sla1 reproducibly co-precipitated with Rsp5 (Fig. 1B). The Rsp5-Rvs167 interaction was further confirmed by yeast two-hybrid experiments (Table II). During the course of this study, a systematic analysis of interactions in the yeast proteome identified a complex containing both Rsp5 and Rvs167, providing a third independent confirmation of this protein-protein interaction (42).

Rvs167 and Sla1 are proteins required for receptor internalization and regulation of the actin cytoskeleton organization (32, 33, 43, 44), and both bind to many other proteins involved in actin polymerization and endocytosis. Sla1 carries three N-terminal SH3 domains, central SHD1 ([Sla1-homology domain 1) and coiled-coil domains, and a C-terminal repeat domain (Fig. 1C). Sla1 associates with other endocytic proteins at the plasma membrane early in the formation of an endocytic vesicle (45), but the molecular mechanisms by which Sla1 acts are unknown. Rvs167 is an endophilin/amphiphysin-like protein that is one of two yeast proteins containing a BAR domain. The Rvs167 BAR domain is at the N terminus, followed by a Gly-Pro-Ala-rich (GPA) region of 176 amino acids and a C-terminal SH3 domain (Fig. 1C). Rvs167 forms a BAR domain-mediated heterodimer with the other yeast BAR protein, Rvs161 (46, 47).

To test whether the observed physical interaction between Rsp5 and Rvs167 was direct, we expressed recombinant Hisa-tagged Rvs167 in bacteria and tested for interaction with recombinant GST-Rsp5 fusion proteins. Rvs167 bound specifically to immobilized GST fusion proteins containing full-length Rsp5 or a fragment containing the WW domains alone (Fig. 2A). To localize the Rsp5 interaction domain in Rvs167, we expressed Hisa-tagged Rvs167 fragments comprising each of the defined domains (BAR, GPA, and SH3; see Fig. 1C). Whereas the SH3 domain showed no interaction with either GST or the WW domains, the GPA domain bound specifically to immobilized WW domains (Fig. 2B). It was not possible to definitively test interaction of the BAR domain by this assay, because the recombinant BAR domain bound to GST alone. However, binding of BAR to GST-3×WW domains was similar to its interaction with GST, suggesting that the BAR domain does not contribute significantly to the interaction of Rvs167 with WW domains. These data demonstrate that the GPA domain of Rvs167 interacts directly with Rsp5 WW domains.

Rsp5 contains type I WW domains, defined by affinity for PPIX motif-containing ligands (48). The Rvs167 GPA domain carries the sequence P398P399AY and two imperfect PPIX motifs, P394SY and P372QY (Fig. 3A). To analyze the role of these motifs in Rsp5 interaction, we generated a series of mutations in the Rvs167 PPIX and PXY sequences. All of the mutant proteins were expressed at normal levels in cells carrying a chromosomal disruption of RVS167 (Fig. 3A). In addition, the mutant alleles restored growth of rvs167Δ cells on high salt medium at all temperatures and rescued temperature-sensitive growth of rvs167Δ cells on medium containing caffeine,2 suggesting that the mutant proteins fold normally and complement at least some aspects of Rvs167 function. The P398A,P399A mutation significantly diminished but did not abolish interaction with Rsp5 in vitro (Fig. 3B) and inhibited the ability of Rvs167 to co-precipitate with Rsp5 in yeast lysates (Fig. 3C). Complete deletion of this motif (APPPAY) also did not abolish Rsp5 binding, suggesting that the two imperfect PXY motifs upstream in the GPA domain contribute to the

2 R. Dunn and L. Hicke, unpublished results.
interaction. Mutations of one (3P3A) or two (4P3A) of the upstream PXY motifs in combination with the P398A,P399A mutation further reduced binding, indicating that the GPA domain contains redundant proline-based interaction motifs for Rsp5.

Rvs167 and Sla1 have been reported to interact in the yeast two-hybrid system (49); thus, it is possible that our observed co-precipitation of Sla1 with Rsp5 (Fig. 1B) was due to an indirect interaction via Rvs167. To test this possibility, we performed native Rsp5 immunoprecipitations in wild type and rvs167/H9004 cells. Sla1 precipitates with Rsp5 regardless of the presence of Rvs167 (Fig. 4A). To determine whether the Sla1-Rsp5 interaction is direct, we expressed three His6-tagged fragments of Sla1 in E. coli: Sla1 1–420, which includes the three SH3 domains, Sla1 420–720, which harbors 26 repeats of the approximate consensus sequence TGGX2–6PQ and a Gln-rich region. Sla1 420–720 bound specifically to recombinant GST-Rsp5 purified from bacterial lysates (Fig. 4B), indi-

TABLE II

| DNA-binding domain plasmid       | Activation domain plasmid        | β-Galactosidase activitya |
|----------------------------------|----------------------------------|--------------------------|
| GAL4 DBD-RVS167 (amino acids 74–482) | GAL4-Act                         | 0.036 ± 0.003            |
| GAL4 DBD-RVS167 (amino acids 74–482) | GAL4-Act-RSP5                    | 8.6 ± 0.2                |
| GAL4 DBD                         | GAL4-Act-RSP5                    | 0.06 ± 0.01              |

a β-Galactosidase activity is represented as the mean activity and the S.D. for three independent cultures.

Fig. 2. The Rvs167 GPA domain binds directly to the WW domains of Rsp5. A, binding of recombinant full-length Rvs167 to Rsp5 WW domains and full-length Rsp5. An E. coli lysate containing recombinant His6-tagged Rvs167 was incubated with GST, GST-3×WW, or GST-Rsp5 immobilized on beads for 2 h at 4 °C. Bound proteins and a fraction of the input lysate were analyzed by SDS-PAGE and Coomassie Blue staining. The identity of the Rvs167 species was confirmed by its reactivity with both anti-His6 and anti-Rvs167 antibodies.3 B, interaction of recombinant fragments of Rvs167 with Rsp5 WW domains. Bacterially expressed His6-tagged GPA and SH3 domains were purified on and eluted from a metal affinity resin. To prevent nonspecific binding, the purified domains were diluted into E. coli lysates at equivalent concentrations and incubated with immobilized GST and GST-3×WW as in A. Bound proteins and a fraction of the input lysate were resolved on a 16.5% Tris-Tricine-SDS gel and analyzed by Coomassie Blue staining (top panel) and immunoblotting with Rvs167 antiserum (bottom panel).

Fig. 3. Rsp5 binds to PXXY sequences in the Rvs167 GPA domain. A, mutations in Rvs167 PXXY motifs. The sequence of the Rvs167 GPA domain is shown with the PXXY and PPXXY motifs highlighted in boldface type. The expression of Rvs167 mutant proteins was analyzed by preparing lysates of rvs167Δ::kanMX4 cells (LHY2529) carrying plasmid copies of the indicated RVS167 alleles (3P3A is P372A,P398A,P399A, and 4P3A is P334A,P372A,P398A,P399A) or a vector control. Lysates of cells grown in selective minimal medium were prepared, and samples were analyzed by immunoblotting with Rvs167 antiserum (upper panel). The blot was stripped and reprobed with hexokinase antiserum (lower panel) to control for equivalent protein loading. B, analysis of Rsp5 interaction with Rvs1673P3A mutant proteins. Cell lysates prepared from strains identical to those in A were incubated with equivalent amounts of immobilized GST and GST-Rsp5 overnight at 4 °C. Lysates (left panel) and bound proteins (right panel) were analyzed by immunoblotting with Rvs167 antiserum. C, interaction of Rvs1673P3A,3P99A with Rsp5 in vivo. Native precipitations with Rsp5 antiserum were performed from lysates of rvs167Δ cells expressing Rvs167-HA (LHY2363) or Rvs1673P3A,3P99A-HA (LHY2510). Total lysates and immunoprecipitates (IP) were analyzed by immunoblotting (IB) with anti-HA and anti-Rsp5 antibodies.
Sla1 and Rvs167 Bind Directly to Each Other and Are Similar to the Mammalian CIN85-Endophilin Complex—Above, we described physical interactions between the Rsp5 ubiquitin ligase and two yeast proteins required for receptor internalization. In mammalian cells, the Cbl ubiquitin ligase binds to the CIN85-endophilin complex, a component of the endocytic machinery important for growth factor receptor internalization. Sla1 is the yeast protein most like CIN85 in domain structure (Fig. 1C), sharing 34% similarity over its first 600 amino acids. Like CIN85, Sla1 is a multivalent adaptor that binds a large number of other proteins involved in endocytosis, actin organization, including Pan1, End3, Las17, and Sla2 (50–52).

Rvs167 is similar in structural organization to mammalian endophilins, although Rvs167 has a central GPA domain not found in endophilins (Fig. 1C). In addition, Rvs167 and Sla1 have been observed to interact in a high throughput yeast two-hybrid analysis (49). These observations suggest that Sla1 and Rvs167 may be functionally similar to CIN85 and endophilin, respectively. To test this idea, we first determined whether the N-terminal SH3 domain fragment of Sla1 binds to Sla1 1–420 immobilized on metal affinity beads and incubated with yeast lysates. We tested whether the N-terminal SH3 domain fragment of Sla1 (amino acids 1–420) bound to epitope-tagged versions of the yeast synaptojanins Inp51 and Inp52. We incubated immobilized Sla1 1–420 with lysates from yeast cells expressing HA-Inp52 (LHY4651) and expressed Sla1 1–420 immobilized on metal affinity beads. A fraction of the lysate and eluted proteins were analyzed by SDSPAGE followed by immunoblotting with anti-HA antibodies. We detected specific binding of HA-Inp52 to Sla1 1–420, indicating that Rvs167 binds to Sla1 directly, perhaps via multiple sites. The observation that rs16Δ and sla1Δ mutations are synthetically lethal provides evidence for a functional relationship between Rvs167 and Sla1 (53). Thus, Sla1, like CIN85, interacts physically and functionally with an endophilin-like protein.

Because CIN85 SH3 domains bind to mammalian synaptojanins, proteins that dephosphorylate phosphoinositides, we tested whether the N-terminal SH3 domain fragment of Sla1 (amino acids 1–420) bound to epitope-tagged versions of the yeast synaptojanins Inp51 and Inp52. We incubated immobilized Sla1 1–420 with lysates from yeast cells expressing HA-Inp51 or HA-Inp52. We detected specific binding of HA-Inp52 to Sla1 1–420 (Fig. 5C) and weaker binding of HA-Inp51 to Sla1 1–420. Thus, Sla1 can bind to yeast synaptojanins in vitro.

Rvs167 Is Monoubiquitinated by Rsp5—Rsp5 is required to modify plasma membrane proteins with a ubiquitin internalization signal (reviewed in Ref. 10), although it is not known how Rsp5 recognizes its membrane substrates. We considered the possibility that Rvs167 or Sla1 may link Rsp5 to plasma membrane endocytic cargo to facilitate cargo ubiquitination. To test this idea, we analyzed ligand-stimulated ubiquitination of
or revealed a higher molecular mass, 66-kDa species of Rvs167 that and cells deficient in Rsp5 or the related ubiquitin ligases Hul4 ubquitinated forms of both proteins. We first examined these strates of Rsp5, we performed a variety of experiments to detect detected in the same lysates.2 These data indicate that Rvs167 is for actin regulation and receptor internalization, was not de-
taged Arc15, another PP domain are required for Rsp5 interaction, we tested whether the site of ubiquitination. To determine the site of Rvs167 monoubiquitination, we mutated the single lysine in the C-terminal SH3 domain (LHY501), sla1Δ (LHY4845), and ste2Δ (LHY10) cells expressing Ste2-HA. The cells were grown at 30 °C and lysed before or 8 min after treatment with α-factor. Ubiquitination of Ste2 was analyzed as described for A except that Ste2 was detected by immunoblotting with anti-HA antibodies.

To investigate the possibility that Sla1 and Rvs167 are substrates of Rsp5, we performed a variety of experiments to detect ubiquitinated forms of both proteins. We first examined these proteins in concentrated lysates prepared from wild type cells and cells deficient in Rsp5 or the related ubiquitin ligases Hul4 and Hul5. Immunoblotting with anti-Rvs167 antibodies revealed a higher molecular mass, 66-kDa species of Rvs167 that was absent in rps5Δ cells but was unaffected by deletions of HUL4 or HUL5 (Fig. 7A). The 66-kDa form migrated ~8.5 kDa above the primary Rvs167 species, consistent with the molecular weight of a single ubiquitin moiety. To confirm that the Rsp5-dependent modification of Rvs167 was a ubiquitin conjugate, Rvs167 species were analyzed in cells expressing plasmid-borne ubiquitin, Myc epitope-tagged ubiquitin, or a vector control. Ubiquitin overexpression induced the higher molecular mass form of Rvs167, and the mobility of this form shifted when ubiquitin carried a Myc epitope (Fig. 7B). Induced ubiquitination was highly specific, because modification of HA-tagged Arc15, another PPXY motif-containing protein required for actin regulation and receptor internalization, was not detected in the same lysates.2 These data indicate that Rvs167 is modified by monoubiquitin in vivo in an Rsp5-dependent reaction. We have not been able to detect ubiquitination of Sla1 using similar assays.

Because the PPXY and PPXY sequences in the Rvs167 GPA domain are required for Rsp5 interaction, we tested whether point mutations in these sequences reduce its ubiquitination. Disruption of the PPXY motif (P398A,P399A) significantly re-
duced the ubiquitinated Rvs167 species, and additional loss of upstream PPXY motifs (3P→A and 4P→A) further diminished the modification (Fig. 7C). Furthermore, mutations in the Rsp5 WW domains, which bind to Rvs167, also reduced Rvs167 ubiquitination. Deletion of the entire C2 domain or point mutation of each WW domain individually had little effect on Rvs167 ubiquitination; however, mutation of all three WW domains simultaneously severely diminished ubiquitination (Fig. 7D).2 These observations together indicate that there is strong correlation between the requirements for Rvs167-Rsp5 binding and Rvs167 monoubiquitination.

Site and Consequences of Rvs167 Monoubiquitination—Rsp5 binds to Rvs167 through PPXY motifs in the GPA domain. However, because the GPA domain contains no lysines, it cannot be the site of ubiquitination. To determine the site of Rvs167 monoubiquitination, we mutated the single lysine in the C-terminal SH3 domain (Lys416) or the 32 lysine residues at the N terminus individually or in pairs to arginine. Mutation of lysines in the N terminus (such as K290R immediately up-
stream of the GPA domain) did not affect the level of the ubiquitinated Rvs167 species, but monoubiquitinated Rvs167 was reduced by the K481R mutation and was not detectable upon deletion of the entire SH3 domain (Fig. 8A and unpublished data). These results are consistent with recently published mass spectrometry data (54, 55), indicating that the site of Rvs167 ubiquitination is Lys416, the penultimate amino acid in the protein.

The SH3 domain of Rvs167 binds to proteins important for endocytosis and/or regulation of the actin cytoskeleton, includ-
Rvs167 is similar to the mammalian amphiphysins and endophilins, and we propose that Rvs167 is an orthologue of both proteins. Rvs167, amphiphysins, and endophilins all regulate endocytosis and the actin cytoskeleton. Rvs167 binds to the other yeast BAR domain protein, Rvs161, analogous to amphiphysins isoforms that dimerize through their BAR domains (1, 58, 59). Like endophilin, Rvs167 binds to the actin assembly regulator N-WASP (Las17) through its C-terminal SH3 domain (29, 47, 59, 60). Unlike the amphiphysins and endophilins, Rvs167 does not carry clathrin and clathrin adapter (AP)-binding motifs and does not appear to bind either of these proteins. This difference may exist because clathrin adaptors are not required for receptor internalization in yeast (61, 62), and clathrin itself is not strictly required (63).

The interaction of Rsp5 with Rvs167 and Sla1 is not required for cargo ubiquitination, but an Rsp5-Rvs167 interaction is important for monoubiquitination of Rvs167. PXY and PPXY motifs in Rvs167 are necessary to bind to Rsp5 WW domains in vitro and for Rvs167 monoubiquitination in vivo, indicating that the Rvs167-Rsp5 interaction is physiologically relevant. Rsp5 binds to the central region of Sla1 (amino acids 420–720). This domain does not have PXY motifs but does carry an SHD1 domain that interacts with NPF motifs (56). Rsp5 carries an NPF sequence in the HECT domain (amino acids 513–515); however, mutation of this sequence to APA does not inhibit Sla1 interaction or receptor internalization, and the Sla1 SHD1 domain is not required for co-precipitation of Sla1 with Rsp5. Therefore, it is unlikely that the Sla1 SHD1 and Rsp5 NPF sequences mediate interaction between the proteins, and at this time it is not known how Sla1-Rsp5 binding occurs.

CIN85 appears to be constitutively monoubiquitinated by Nedd4 and undergoes ligand-induced ubiquitination by Cbl (7). Ubiquitination of endophilin has also been reported (64). We have observed Rvs167 monoubiquitination that is dependent on the Nedd4 homologue Rsp5, and this event is not stimulated by the addition of α-factor ligand. Although we have not detected Sla1 ubiquitination in the absence or presence of α-factor, this event may occur at low levels or only in response to specific stimuli. Mutations in Rvs167 PXXY motifs (Pro → Ala mutations) severely inhibit binding to Rsp5 in vitro and Rvs167 ubiquitination in vivo; however, we have not observed internalization, growth, or actin cytoskeleton phenotypes associated with these mutations.

Ubiquitination occurs on the penultimate amino acid of Rvs167, Lys481, in the SH3 domain. Lys481 is not required for normal α-factor internalization kinetics but is important for growth on high salt medium. Because these defects are not observed with rvs167P → A mutations that inhibit Lys481 ubiquitination, this residue is likely to be important for protein-protein interactions in addition to being a ubiquitination site. It is unlikely that Lys481 is important structurally, because it is in a location that is surface-exposed on known SH3 structures. Ubiquitination at Lys481 may negatively regulate Rvs167 functions or its ability to interact with an SH3-binding partner. Alternatively, Lys481 may be a site for other Lys-dependent modifications, such as acetylation, sumoylation, or methylation.

In sum, we have identified a system of protein interactions between the Rsp5 ubiquitin ligase and the yeast CIN85-endophilin homologues, Sla1-Rvs167. Rsp5 is required to monoubi-
Rvs167 Is Monoubiquitinated by Rsp5

biquitinate Rvs167. Ubiquitination occurs on the SH3 domain of Rvs167 and may regulate SH3 binding to proteins that control actin polymerization.

Acknowledgments—We are grateful to Florian Bauer, Jon Hrubegste, Bob Lamb, Greg Payne, Howard Riezman, and Jeff Schatz for reagents.

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