Control of Bro1-Domain Protein Rim20 Localization by External pH, ESCRT Machinery, and the *Saccharomyces cerevisiae* Rim101 Pathway

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Bro1-domain proteins such as yeast Bro1 and mammalian AIP1/Alix are well-established participants in endosome metabolism. The Bro1-domain interacts with endosomal surface protein Snf7/Vps32 in yeast, a subunit of the ESCRT complex. Yeast Bro1-domain protein Rim20 has no role in endosome function, but is required for alkaline pH-stimulated cleavage of transcription factor Rim101. Rim20-GFP is cytoplasmic under acidic conditions but concentrated in punctate foci under alkaline conditions. Bro1-GFP also accumulates in foci, but they are more numerous under acidic than alkaline conditions. Colocalization experiments indicate that some Rim20-GFP foci correspond to Bro1-RFP foci, whereas others do not. Rim8, Rim9, Rim21, Dfg16, Snf7, Vps20, Vps23, and Vps25, which are required for Rim101 cleavage, are required for appearance of Rim20-GFP foci. ESCRT complexes accumulate on endosome-derived compartments in cells that lack the AAA-ATPase Vps4. We find that Rim20-GFP foci accumulate in a *vps4* mutant background independently of external pH, Rim101 pathway-specific genes, and most ESCRT subunit genes except for *SNF7*. Rim20-GFP foci seem to represent endosomes, because they colocalize with Snf7-RFP and because they correspond to a perivacuolar compartment in the *vps4* strain. We propose that alkaline growth conditions alter the endosomal surface to favor Rim20-Snf7 interaction and Rim101 cleavage. Our findings raise the possibility that Bro1-domain proteins may be differentially regulated in the same cell, thereby coupling endosome metabolism to signaling.

INTRODUCTION

All cells recognize and respond to external signals. For microorganisms, the responses are vital for adaptation to a changeable environment. For multicellular organisms, the responses are critical for coordination of developmental and physiological processes. The plasma membrane is well recognized as the chief sensory organelle of the cell, where receptors undergo environmentally directed changes that relay information to the cytoplasm and nucleus. The endocytic machinery is routinely used for down-regulation of receptors and metabolism or destruction of ligands. However, there is growing evidence that endocytosis plays diverse roles in signal transduction and cell physiology (Di Fiore and De Camilli, 2001; Conner and Schmid, 2003). Recent analysis of the fungal Rim101 pH-response pathway suggests that the endocytic machinery may participate directly in signaling (Kullas *et al.*, 2004; Xu *et al.*, 2004; Barwell *et al.*, 2005; Rothfels *et al.*, 2005). Our focus here is the interaction of endosomal multivesicular body (MVB) formation components with Rim20, a key participant in pH-dependent signaling.

Rim20 is required for proteolytic activation of the transcription factor Rim101 (Xu and Mitchell, 2001). Rim101 cleavage removes a ~100-residue C-terminal PEST-like region, allowing the N-terminal zinc finger region to effect

transcriptional changes (Li and Mitchell, 1997; Lamb *et al.*, 2001). Ultimately, cleaved Rim101 is required for expression of many alkaline pH-inducible genes (Lamb *et al.*, 2001; Lamb and Mitchell, 2003). Rim20 belongs to the family of Bro1-domain proteins, which includes *Saccharomyces cerevisiae* Bro1 and mammalian AIP1/Alix. Most well-studied Bro1-domain proteins function in endosome metabolism (Odorizzi *et al.*, 2003; Babst, 2005) to promote packaging of endocytic cargo into an MVB, a specialized vesicle that contains additional vesicles within its lumen (Lemmon and Traub, 2000; Raiborg *et al.*, 2003; Babst, 2005). AIP1/Alix is also required for HIV budding, in which the virus seems to have coopted the MVB formation pathway (Strack *et al.*, 2003; von Schwedler *et al.*, 2003). The Bro1-domain is the site of interaction with a class of vesicle surface proteins such as *S. cerevisiae* Snf7 or mammalian CHMP4b (Katoh *et al.*, 2003; Kim *et al.*, 2005). Recent studies reveal that Bro1 functions as an adaptor, binding Snf7 to bring the ubiquitin hydrolase Doa4 (Amerik *et al.*, 2000) into proximity with endocytic cargo proteins (Luhtala and Odorizzi, 2004), whose cytoplasmic domains are ubiquitinated (Raiborg *et al.*, 2003; Babst, 2005). Rim20 is also thought to function as an adaptor, binding Snf7 to bring the protease Rim13 into proximity with Rim101, thus promoting Rim101 cleavage and activation (Xu and Mitchell, 2001; Vincent *et al.*, 2003).

Snf7 is one component of the ESCRT machinery (Kranz *et al.*, 2001; Babst *et al.*, 2002), a group of three protein complexes that sort endocytic cargo into MVBs (Babst, 2005). Once the cargo is sorted into presumed subdomains of the vesicle surface, it is de-ubiquitinated through the action of Bro1-Doa4, and MVBs are produced by invagination of the surface subdomain. The ESCRT machinery is subsequently

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dissociated through the action of the AAA-ATPase Vps4 (Babst *et al.*, 1997; Babst *et al.*, 1998; Raiborg *et al.*, 2003; Babst, 2005), ultimately permitting fusion of the MVB with the vacuole or lysosome and renewing ESCRT complex formation at early endosomes.

Prior studies indicate that subunits of the ESCRT machinery are also required for Rim101 cleavage (Xu *et al.*, 2004). This idea traces back to a large-scale two-hybrid survey (Ito *et al.*, 2001) that revealed interactions between Snf7 and Rim20. Functional significance of the interaction was underscored by the observation that mutants defective in any of eight ESCRT-I, -II, and -III subunits failed to produce cleaved Rim101 (Xu *et al.*, 2004). On the other hand, mutants defective in Vps4 or in Vps2 and Vps24, the ESCRT-III subunits that recruit Vps4, were capable of producing cleaved Rim101 (Xu *et al.*, 2004). Largely consistent results have been obtained independently for *S. cerevisiae* (Barwell *et al.*, 2005; Hayashi *et al.*, 2005; Rothfels *et al.*, 2005) and other fungi (Kullas *et al.*, 2004; Xu *et al.*, 2004; Blanchin-Roland *et al.*, 2005; Cornet *et al.*, 2005). These findings led to the proposal that vesicle-associated Snf7 is required for productive interaction with Rim20, leading to Rim101 cleavage (Xu *et al.*, 2004).

Accumulation of the two Rim101 forms, full-length or cleaved, is governed by external pH. In acidic conditions, cells accumulate uncleaved Rim101; in alkaline conditions, cells accumulate mainly cleaved Rim101 (Li and Mitchell, 1997). These observations were predicated upon extensive analysis of *Aspergillus nidulans* PacC, a homolog of Rim101 and the first known eukaryotic pH-response regulator (Penalva and Arst, 2002). The PacC processing regulators have proven to be broadly conserved among fungi (Penalva and Arst, 2002). The *S. cerevisiae* homologues, which are required along with ESCRT subunits for Rim101 cleavage, include the arrestin homolog Rim8, the transmembrane proteins Dfg16, Rim21, and Rim9, as well as the scaffold Rim20 and the protease Rim13 (Futai *et al.*, 1999; Penalva and Arst, 2002; Barwell *et al.*, 2005; Rothfels *et al.*, 2005). The transmembrane proteins have generally been thought to serve as sensors of external pH (Penalva and Arst, 2002). This view is particularly inviting because Dfg16 and Rim21, like their sole *A. nidulans* homolog PalH, have substantial similarity to 7-transmembrane domain (7-TMD) receptors (Penalva and Arst, 2002; Barwell *et al.*, 2005). We have suggested that external pH activates the Rim101 pathway through Rim8-directed endocytosis of Dfg16 or Rim21 (Barwell *et al.*, 2005). The Rim8 homolog PalF indeed behaves as a pH-responsive arrestin: it undergoes phosphorylation and ubiquitination only under alkaline growth conditions (Herranz *et al.*, 2005). This recent analysis of PalF is the only demonstration thus far that an upstream component of the Rim101 or PacC processing pathways responds to the regulatory signal, external pH.

In this study, we have explored the relationship between Rim20, the ESCRT pathway, and external pH. We find that Rim20 undergoes pH-responsive interaction with the ESCRT pathway. This association depends on upstream Rim101 pathway components, and the genetic control of Rim20-ESCRT association provides support for its functional significance. We provide a model for Rim101 pathway signal transduction that organizes current observations. Aspects of this model might be operative in higher organisms, which have numerous Bro1-domain proteins of unknown function.

MATERIALS AND METHODS

Strains, Media, and Growth Conditions

S. cerevisiae strains are listed in Table 1. To assay colocalization, diploids were created by crossing JBY46 (*MATa RIM20-GFP*) to JBY120 (*MATa SNF7-RFP*). Strain JBY120 was provided by James Falvo and Erin O'Shea (Huh *et al.*, 2003). Strain JBY224 (*MATa RIM20-GFP BRO1-RFP*) was constructed as follows: the carboxy-terminal *BRO1-RFP* construct was generated by a PCR-directed gene disruption using primers Bro1-pFA6 F (5'-acgtcttttacaatagaccctctgttttgatgaaaatgtactccaataacagcagtggtcgacggatccccgggtt-3') and Bro1-pFA6 R (5'-taattatcatattagttgaaaaaaaagctacaataaaatgaagaataagaatgcactcgatgaattc-gagctcggtt-3') against genomic DNA from strain JBY120. Parent strain JBY46 was then transformed with selection for the presence of the *KanMX4* gene.

The *RIM20-GFP* and *SNF7-RFP* constructs were functional, as determined by Ura3-V5-Rim101p processing assays (Barwell *et al.*, 2005). The *BRO1-GFP* construct was functional, as determined by FM4-64 pulse-chase analysis (Odorizzi *et al.*, 2003).

All mutations except the *vps4Δ::URA3* mutation were introduced into the parent GFP strain by a PCR-directed gene disruption using genomic DNA from the appropriate *KanMX4* deletion strain as a template, as previously described (Xu *et al.*, 2004). The *vps4Δ::URA3* mutation was introduced by a PCR-directed gene disruption using primers VPS4.URA3 F (5'-ttgagacatg-gaagacaaaaataaagcagcatagatgctctatagtagatgggtacaaatgtcgaaagctacataag-3') and VPS4.URA3 R (5'-ttttttatttttttttttcatgtacacagaatctacattag-cagtttaactaattgattagtttctgctggcgcactc-3') against plasmid pRS306 as template. Yeast cells were then transformed with selection for the presence of the *URA3* gene. All genotypes were confirmed by PCR analysis of genomic DNA using outside primers.

Yeast growth media (YPD and SC) were of standard composition (Kaiser *et al.*, 1994). For pH exposure assays, SC media containing 0.1 M HEPES was freshly titrated to pH 4.0 with HCl or to pH 8.3 with NaOH and used immediately. All cultures and plates were incubated at 30°C.

Localization and Focus Quantification Assays

To observe localization changes in response to external pH, strains were grown to saturation in YPD (unbuffered, pH ~ 6.6), inoculated into YPD at an OD₆₀₀ of 0.1, and grown to an OD₆₀₀ of 0.6–0.8 (equivalent to ~10⁷ cells/ml). Cells were washed once in unbuffered SC and then resuspended in an equal volume of pH 4.0- or pH 8.3-buffered SC medium, placed directly on the slide and immediately subjected to microscopic analysis at room temperature. Equivalent localization results were obtained using buffered YPD media, with SC media yielding lower background fluorescence than YPD media. Cells were viewed using a wide-field Nikon Eclipse E800 fluorescence microscope (Melville, NY) and a 100 × NA 1.4 objective. Fluorescent images were acquired with an exposure time of 1.5 s on an Orca100 (Hamamatsu, Bridgewater, NJ) camera utilizing Openlab (Improvision, Lexington, MA) software. Images were processed using Adobe Photoshop 6.0 software (San Jose, CA).

To quantify foci, strains were subjected to pH changes as described above. After image acquisition, foci quantification was carried out as follows: all GFP images were processed to enhance contrast, and a subsection of the image field was defined. Images were arranged sequentially, and cells falling entirely within the defined subsection of the image field were assayed for presence or absence of foci. This process was repeated (n = 100) with each strain under acidic and alkaline conditions. Statistical significance was assessed with the Steel test or Steel-Dwass test (Steel, 1959) for multiple non-parametric comparisons.

Membrane Staining

Staining with *N*-(3-triethylammoniumpropyl)-4-(*p*-diethylaminophenyl)-hexatrienyl-pyridinium dibromide (FM4-64, purchased from Molecular Diagnostics, Chicago, IL) was performed as described previously (Barwell *et al.*, 2005). Briefly, cells were grown to midlog phase in YPD medium at 30°C, harvested, and resuspended in 166 μl YPD to which 0.4 μl 16 mM FM4-64 in dimethyl sulfoxide was added. The tubes were wrapped in foil and incubated at 30°C for 20 min on a shaker. The cells were harvested, washed once with 200 μl YPD medium, resuspended in 200 μl YPD medium, and incubated at 30°C for 60 min on a shaker. To reduce background, cells were harvested, washed once in unbuffered SC medium, and resuspended in 200 μl SC medium. Cells were then placed directly onto slides and analyzed by fluorescence microscopy as above.

Immunoblots

Cells carrying the plasmid pKJB11 (Barwell *et al.*, 2005), specifying the *URA3-V5-RIM101* gene under the control of the *RIM101* native 5' region were grown to saturation in selective SC-Leu media. Fresh 10-ml SC-Leu cultures were inoculated at a consistent OD₆₀₀ and grown for two doublings, collected, resuspended in 100 μl 3 × Laemmli Buffer, vortexed with glass beads, and boiled for 5 min. After centrifugation, 15–25 μl of the supernatant was fractionated on a 9% SDS-PAGE gel and transferred to nitrocellulose. The filter was probed with anti-V5-HRP antibody (Invitrogen, Carlsbad, CA; 1:3000 dilution in TBST) and was developed with ECL detection reagents

Table 1. Yeast strains

Strain	Genotype	Source
EY0986	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i>	ATCC 201388
JB46	EY0986; <i>RIM20-GFP-HIS3MX6</i>	Invitrogen
JB224	JB46; <i>BRO1-RFP-KanMX4</i>	This study
JB197	JB46; <i>vps23Δ::KanMX4</i>	This study
JB198	JB46; <i>vps28Δ::KanMX4</i>	This study
JB199	JB46; <i>vps37Δ::KanMX4</i>	This study
JB200	JB46; <i>vps25Δ::KanMX4</i>	This study
JB201	JB46; <i>vps36Δ::KanMX4</i>	This study
JB202	JB46; <i>snf8Δ::KanMX4</i>	This study
JB203	JB46; <i>vps20Δ::KanMX4</i>	This study
JB204	JB46; <i>snf7Δ::KanMX4</i>	This study
JB205	JB46; <i>vps2Δ::KanMX4</i>	This study
JB206	JB46; <i>vps24Δ::KanMX4</i>	This study
JB207	JB46; <i>rim8Δ::KanMX4</i>	This study
JB208	JB46; <i>rim9Δ::KanMX4</i>	This study
JB209	JB46; <i>rim13Δ::KanMX4</i>	This study
JB210	JB46; <i>rim20Δ::KanMX4</i>	This study
JB211	JB46; <i>rim21Δ::KanMX4</i>	This study
JB212	JB46; <i>rim101Δ::KanMX4</i>	This study
JB213	JB46; <i>dfg16Δ::KanMX4</i>	This study
JB115	JB46; <i>vps4Δ::URA3</i>	This study
JB133	JB115; <i>vps23Δ::KanMX4</i>	This study
JB176	JB115; <i>vps28Δ::KanMX4</i>	This study
JB179	JB115; <i>vps37Δ::KanMX4</i>	This study
JB136	JB115; <i>vps25Δ::KanMX4</i>	This study
JB182	JB115; <i>vps36Δ::KanMX4</i>	This study
JB185	JB115; <i>snf8Δ::KanMX4</i>	This study
JB139	JB115; <i>vps20Δ::KanMX4</i>	This study
JB142	JB115; <i>snf7Δ::KanMX4</i>	This study
JB145	JB115; <i>vps2Δ::KanMX4</i>	This study
JB148	JB115; <i>vps24Δ::KanMX4</i>	This study
JB169	JB115; <i>rim8Δ::KanMX4</i>	This study
JB154	JB115; <i>rim9Δ::KanMX4</i>	This study
JB172	JB115; <i>rim13Δ::KanMX4</i>	This study
JB175	JB115; <i>rim20Δ::KanMX4</i>	This study
JB157	JB115; <i>rim21Δ::KanMX4</i>	This study
JB161	JB115; <i>rim101Δ::KanMX4</i>	This study
JB163	JB115; <i>dfg16Δ::KanMX4</i>	This study
JB47	EY0986; <i>BRO1-GFP-HIS3MX6</i>	Invitrogen
JB214	JB47; <i>vps4Δ::URA3</i>	This study
JB216	JB214; <i>vps23Δ::KanMX4</i>	This study
JB217	JB214; <i>vps25Δ::KanMX4</i>	This study
JB218	JB214; <i>vps20Δ::KanMX4</i>	This study
JB219	JB214; <i>snf7Δ::KanMX4</i>	This study
JB120	<i>MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 SNF7-RFP-KanMX4</i>	Gift of J. Falvo and E. O'Shea
JB166	JB120; <i>vps4Δ::URA3</i>	This study

(Amersham, Indianapolis, IN). Every gel included control lanes of wild-type and *rim20Δ* mutant strains carrying the *URA3-V5-RIM101* processing reporter gene.

RESULTS

pH-responsive Localization of Rim20 and Bro1

Rim20 has a pivotal role in the response to external pH changes. To determine whether Rim20 itself may respond to external pH, we examined the localization of a functional Rim20-GFP fusion, expressed from the *RIM20* genomic locus. Rim20-GFP was predominantly cytoplasmic under acidic conditions (Figures 1A and 2A); fewer than 10% of cells had punctate GFP foci. This observation agrees with the large-scale protein localization analysis (Huh *et al.*, 2003), which was carried out in acidic SD medium. Under alkaline conditions, the majority of cells had one or more punctate GFP foci (Figures 1A and 2A). Therefore, the subcellular localization of Rim20-GFP reflects the external pH.

Rim20 is homologous to Bro1, an endosome-associated protein that promotes MVB formation. It seemed possible that Bro1 localization might also be affected by external pH. We found that a functional Bro1-GFP fusion was cytoplasmic with some punctate foci under acidic and alkaline conditions (Figures 1A and 2A). However, Bro1-GFP foci were more numerous under acidic conditions than alkaline conditions ($p < 0.05$). Therefore, the subcellular localization of Bro1-GFP also reflects external pH, with a pattern that is opposite that of Rim20-GFP.

To determine whether Rim20 and Bro1 foci represented the same sites in the cell, we examined the distribution of coexpressed Rim20-GFP and Bro1-RFP. Under acidic conditions, only Bro1-RFP foci were apparent, as expected (unpublished data). Under alkaline conditions, some Rim20-GFP and Bro1-RFP foci coincided (Figure 1B, white arrows) and others did not (Figure 1B, open arrows). Therefore, Rim20 and Bro1 localize to both distinct and common compartments.

Figure 1. Effect of external pH on Rim20 and Bro1 localization. (A) Isogenic *RIM20-GFP* and *BRO1-GFP* strains were analyzed by fluorescence microscopy. Cells grown to midlogarithmic phase in rich medium were washed and resuspended in SC medium buffered to pH 4.0 (acidic) or pH 8.3 (alkaline). (B) A strain expressing both *RIM20-GFP* and *BRO1-RFP* was grown to midlogarithmic phase in rich medium, washed, and resuspended in SC medium buffered to pH 8.3 (alkaline). A representative field is shown. White arrows, coincident Rim20-GFP and Bro1-RFP foci; open arrows, unique Rim20-GFP and Bro1-RFP foci.

Effect of pH-Response Defects on Rim20 Localization

If Rim20 localization is related to its function, then mutations that impair Rim20 function may alter its localization. Thus we examined Rim20-GFP localization in a panel of mutants defective in Rim101 pathway function. The mutants fell into two classes. One class, which included *rim8*, *rim9*, *rim21*, *dfg16*, *vps20*, *vps23*, and *vps25* mutants, had a reduced frequency of Rim20-GFP foci under alkaline conditions compared with the wild type (Figure 2B; $p < 0.05$). Rim9, Rim21, and Dfg16 are the three membrane proteins in the Rim101 pathway, and Rim8 probably associates with Dfg16 or Rim21, based on analysis of its *Aspergillus* homolog (Penalva and Arst, 2002; Barwell *et al.*, 2005; Herranz *et al.*, 2005). Vps20, Vps23, and Vps25 are ESCRT complex subunits that are required for Rim101 cleavage (Xu *et al.*, 2004; Hayashi *et al.*, 2005; Rothfels *et al.*, 2005). The other class, which included *rim13* and *rim101* mutants, had a slightly increased frequency of foci under alkaline conditions ($p < 0.05$), an issue taken up in the Discussion. We conclude that several proteins that are required for Rim20 function are required for accumulation of Rim20 foci.

Relationship between Rim20 Localization and Snf7

Rim20 function is dependent on Snf7, and Rim20-Snf7 interaction has been detected by two-hybrid analysis. Snf7 is an endosome-associated protein (Kranz *et al.*, 2001; Babst *et al.*, 2002). To determine whether Rim20 and Snf7 foci coincide, we examined cells expressing functional Rim20-GFP and Snf7-RFP fusions under alkaline conditions. We observed that most Rim20-GFP foci coincided with Snf7-RFP foci. A

Figure 2. Control of Rim20-GFP localization. (A) Quantification of pH effect on accumulation of Rim20-GFP and Bro1-GFP foci. Each strain was subjected to acidic or alkaline pH conditions and the number of foci per cell ($n = 100$) was tabulated. For both Rim20-GFP and Bro1-GFP, the distribution of foci under alkaline conditions was significantly different from under acidic conditions ($p < 0.05$). Across strains, the distribution of Rim20-GFP foci was significantly less than Bro1-GFP foci under acidic conditions ($p < 0.05$), but not under alkaline conditions. (B) The number of Rim20-GFP foci per cell was tabulated in wild-type, *rim8*, *rim9*, *rim21*, *dfg16*, *vps20*, *vps23*, *vps25*, *rim13*, and *rim101* strains under alkaline conditions. All mutant distributions were significantly different from the wild type ($p < 0.05$).

Figure 3. Comparison of Rim20-GFP and Snf7-RFP localization. (A) A diploid strain coexpressing *RIM20-GFP* and *SNF7-RFP* was analyzed by fluorescence microscopy under alkaline conditions. Nomarski and fluorescent images for Rim20-GFP and Snf7-RFP were obtained, and the GFP and RFP images were merged. (B) A diploid *vps4/vps4* strain coexpressing *RIM20-GFP* and *SNF7-RFP* was analyzed by fluorescence microscopy under acidic conditions. (C) Quantification of Rim20-GFP foci in wild-type and *snf7* backgrounds under acidic and alkaline conditions.

typical image is shown in Figure 3A, which illustrates two points. First, each Rim20-GFP focus corresponds to a Snf7-RFP focus. Second, Snf7-RFP foci are more numerous than Rim20-GFP foci, so that some Snf7-RFP foci that do not yield a clear Rim20-GFP signal. Presence of Snf7 is critical for focus formation, because a *snf7* mutation abolished detectable Rim20-GFP foci (Figure 3C). These findings support the model that Snf7 and Rim20 interact in vivo and indicate that Snf7 is required for pH-responsive distribution of Rim20. In addition, these results suggest that association of Snf7 and Rim20 is regulated by external pH, because Snf7 foci appear under both acidic and alkaline conditions (unpublished data), whereas Rim20 foci appear primarily under alkaline conditions.

Relationship of Rim20 Localization and Vps4 Function

Like Rim20, the endosome-associated proteins of the ESCRT-I, -II, and -III complexes colocalize with Snf7. One additional feature of ESCRT proteins is that they hyperaccumulate on endosomes in the absence of the recycling

factor Vps4 (Babst *et al.*, 1998; Kranz *et al.*, 2001). Defects in Vps4 ultimately result in ESCRT accumulation on a large, endosome-derived "class E" compartment found at the periphery of the vacuole. Indeed, Rim20-GFP foci were much more numerous in a *vps4* strain than in the wild type, and their presence was independent of external pH (Figure 3B, and unpublished data). Most Rim20-GFP foci colocalized with Snf7-RFP foci in the *vps4* mutant background, and some additional Snf7-RFP foci were apparent (Figure 3B). Many cells had larger regions of Rim20-GFP accumulation, and these regions were near the periphery of the vacuole, as visualized with FM4-64 staining (Figure 4). This is the property expected for the class E compartment that appears in *vps4* and other MVB pathway mutants (Vida and Emr, 1995). These results indicate that Vps4 is formally a negative regulator of accumulation of Rim20 foci. In addition, the hyperaccumulation of Rim20 foci in a *vps4* mutant is a parallel between Rim20 and ESCRT subunit behavior.

Recruitment of Vps4 to the endosome depends on Vps2 and Vps24 (Babst *et al.*, 2002). We observed abundant Rim20-

Figure 4. Perivacuolar accumulation of Rim20-GFP. A *RIM20-GFP vps4* strain was stained with the vacuolar membrane staining dye FM4-64. GFP (left panel) and FM4-64 (middle panel) images were obtained and merged (right panel).

GFP foci in both *vps2Δ* and *vps24Δ* mutant strains, independent of the external pH (unpublished data). These observations support the idea that Vps4-endosome association is critical for regulation of Rim20 focus accumulation.

Effects of MVB Pathway Defects on Rim20-GFP Focus Accumulation

Our observations suggest that Rim20 may be associated with one or more ESCRT subunits. We tested this hypothesis in a *vps4* mutant background, where Rim20-GFP foci are abundant and stable. Mutations that eliminate any of the three ESCRT-I subunits (Vps23, Vps28, Vps37) caused no change in Rim20-GFP localization (Figure 5A and unpublished data). In contrast, mutations affecting any of the three ESCRT-II subunits (Vps25, Vps36, Snf7) caused accumulation of Rim20-GFP in a few large foci (Figure 5A and unpublished data). These large foci were perivacuolar, as indicated by costaining with FM4-64 (unpublished data). Vps20, Snf7, Vps2, and Vps24 comprise the ESCRT-III complex. Previous analysis has shown that Vps20 and Snf7 form a membrane-proximal subcomplex of ESCRT-III, with Vps2 and Vps24, forming a second ESCRT-III subcomplex. A defect in Snf7 abolished accumulation of Rim20-GFP foci (Figure 5A), as we noted above for the *VPS4* background. In contrast, a defect in Vps20 caused Rim20-GFP to accumulate in a few large foci (Figure 5A), much as seen with ESCRT-II mutants. This finding is consistent with previous studies showing that Snf7 can associate with endosomes in *vps20 vps4* mutants (Babst *et al.*, 2002). Defects in Vps2 or Vps24 did not alter the accumulation of Rim20-GFP foci observed in the *vps4* background (unpublished data). These results indicate that Snf7 is absolutely required for Rim20 focus formation, whereas ESCRT-II subunits and Vps20 have a modulatory role.

We also examined the localization of the previously characterized Rim20 homolog Bro1. In agreement with previous observations (Huh *et al.*, 2003; Odorizzi *et al.*, 2003), Bro1-GFP localized to faint foci in wild-type cells, and loss of Vps4 resulted in an increase in focus number and intensity (Figure 5B). In the *vps4* background, defects in ESCRT-I subunits caused little change in Bro1-GFP localization, and defects in ESCRT-II subunits or Vps20 caused Bro1-GFP to accumulate in a few perivacuolar compartments (Figure 5B and unpublished data). A Snf7 defect in the *vps4* background caused cytosolic Bro1-GFP localization and eliminated accumulation of foci (Figure 5B), as previously reported (Odorizzi *et al.*, 2003). Thus, in a *vps4* background, the behavior of Bro1-GFP parallels that of Rim20-GFP.

Relationship of the Rim101 Pathway to Vps4

We found above that all Rim101 pathway proteins are required for proper pH-dependent regulation of Rim20-GFP focus accumulation. Because a *vps4* mutation overrides the pH dependence of focus formation, it seemed possible that it would also override the need for Rim101 pathway proteins. We observed that a *vps4* mutation promoted accumulation of Rim20-GFP foci in all Rim101 pathway mutants (Figure 5C). Therefore, Vps4 is required to establish both pH-dependence and Rim101 pathway dependence of Rim20-GFP focus formation.

If Rim20 focus formation is required for function, then a *vps4* mutation may alter the genetic requirements for Rim20 function. To test this hypothesis, we monitored processing of a Ura3-V5-Rim101 fusion protein in a panel of *VPS4* and *vps4* strains. In the *VPS4* strains, we verified that Ura3-V5-Rim101 has the same requirements described previously for native Rim101 (Figure 6A; Xu *et al.*, 2004; Barwell *et al.*, 2005; Rothfels *et al.*, 2005). These requirements include most ESCRT-I, -II, and -III subunits and all Rim101 pathway proteins except Rim101 itself. However, in the *vps4* background, only Snf7, Rim13, and Rim20 were required for processing (Figure 6B). These requirements for processing include the only two gene products necessary for Rim20 focus formation in the *vps4* background—Snf7 and Rim20 itself—and the protease that is thought to catalyze Rim101 cleavage.

DISCUSSION

Our results are consistent with a model in which pH signaling governs Rim101 processing through formation of Rim20 foci. Our findings argue that these foci represent the association of Rim20 with an endosomelike compartment. Comparison of Rim20 and Bro1 behavior indicates that two Bro1-domain proteins may undergo environmentally regulated endosome association in the same cell. The unifying feature of Bro1-domain proteins is that they are adaptors coupling Snf7 homologues to effector proteins. Thus our results may reflect a broadly utilized mechanism for coupling signal transduction to endosome function and the extracellular environment.

Rim20 Foci and Rim101 Processing Activity

We argue that Rim20 foci represent the form of Rim20 that directs Rim101 cleavage, based on three lines of evidence. First, in a *VPS4* background, several genes required specifically for Rim101 processing are also required for focus formation. Second, again in a *VPS4* background, the environmental signal that promotes Rim101 processing—alkalinity—also promotes focus formation. Third, a defect in Vps4 relieves the dependence of both Rim101 processing and Rim20 focus accumulation on the numerous Rim101

Figure 5. Rim20-GFP and Bro1-GFP localization in ESCRT subunit mutants. (A) RIM20-GFP localization was examined by fluorescence microscopy in wild-type, *vps4*, *vps23 vps4* (ESCRTI), *vps25 vps4* (ESCRTII), *vps20 vps4* (ESCRTIII), and *snf7 vps4* (ESCRTIII) backgrounds. (B) BRO1-GFP localization was examined as in A. (C) RIM20-GFP localization was examined by fluorescence microscopy in *rim8 vps4*, *rim9 vps4*, *rim13 vps4*, *rim21 vps4*, *rim101 vps4*, and *dfg16 vps4* backgrounds.

pathway proteins and ESCRT subunits. Therefore, the correlation between Rim20 focus accumulation and Rim101 processing includes not only circumstances that block, but also those that augment, both phenomena.

Our results provide a clear distinction between the functions of Rim101 pathway proteins. Dfg16, Rim8, Rim9, and Rim21 are required for focus formation; Rim13 and Rim101 itself are not. This distinction is consistent with four other lines of evidence. First, Dfg16, Rim9, and Rim21 have sequence features of transmembrane proteins, so it is reasonable that they may act together, perhaps as receptor subunits or as participants in one another's biogenesis (Penalva and Arst, 2002; Barwell *et al.*, 2005). Second, *A. nidulans* PalF binds to PalH (Herranz *et al.*, 2005), which suggests that the PalF homolog Rim8 may function through interaction with the PalH homologues Dfg16 or Rim21. Third, we show here that the functions of Dfg16, Rim8, Rim9, and Rim21 may be bypassed by a *vps4* defect, whereas the function of Rim13 cannot. A recent independent study reported completely convergent findings (Hayashi *et al.*, 2005). Finally, the fact that Rim13 and Rim101 are not required for Rim20 focus formation is consistent with the proposal that Rim20 functions by recruiting both Rim13 and Rim101, if one assumes

that a Rim20 focus serves as a recruitment site. Thus the grouping of these proteins based on Rim20 focus formation fits well with their structural features, functional properties, and our current understanding of the Rim101 and PacC pathways.

One unexpected observation is the finding that Rim20 focus formation in an otherwise wild-type background is augmented in the absence of Rim13 or Rim101. We suggest that the feedback circuit in which RIM8 is repressed by processed Rim101 (Lamb and Mitchell, 2003) can explain this finding. RIM8 is overexpressed in *rim101* and *rim13* mutants (Lamb and Mitchell, 2003; Barwell *et al.*, 2005). Given that Rim8 activity may be rate-limiting for focus formation, as suggested by the induced modification of the Rim8 homolog PalF (Herranz *et al.*, 2005), it seems reasonable that increased Rim8 accumulation may cause increased focus accumulation.

RIM8 is overexpressed in *dfg16* and *rim21* mutants (Barwell *et al.*, 2005) and presumably in *rim9* mutants as well. However, in these cases, there is no Rim20-GFP focus formation. This finding is consistent with the idea that Rim8 action is dependent on Rim9, Rim21, and Dfg16.

Figure 6. Processing of Ura3-V5-Rim101. (A) Protein extracts from *RIM20-GFP VPS4+* strains carrying the Ura3-V5-Rim101 processing reporter were analyzed on anti-V5 immunoblots. Loaded protein amounts were approximately equal, as determined by Ponceau S staining. (B) *vps4* strains, derived from the strains in A, were tested for Ura3-V5-Rim101 processing on anti-V5 immunoblots.

Nature of Rim20 Foci

Our results extend the model that Rim20 is associated with Snf7 (Ito *et al.*, 2001; Xu and Mitchell, 2001; Xu *et al.*, 2004) in two ways. First, this interaction was demonstrated previously only through two-hybrid assays (Ito *et al.*, 2001; Xu *et al.*, 2004); our colocalization data argue that the interaction occurs *in vivo* at natural protein expression levels. Second, our findings suggest that both genetic and environmental signals influence the extent or site of Rim20-Snf7 association, a relationship previously undetected.

It seems likely that the site of Rim20-Snf7 association that we visualize as a focus is an endosome for two reasons. First, Snf7 and its homologues are endosome-associated proteins in numerous species (Raiborg *et al.*, 2003; Babst, 2005), so colocalization of Rim20 and Snf7 argues strongly that Rim20 is also associated with endosomes. Second, such vesicles accumulate in *vps4* mutants and are associated with Snf7, which corresponds to the behavior of Rim20-GFP foci. The finding that Rim20-GFP is often associated with a large perivacuolar compartment in *vps4* mutants is also characteristic of endosome-associated proteins. Finally, we note that the abundance and appearance of Bro1-GFP and Rim20-GFP foci are under similar genetic control in the *vps4* background. The inference that Rim20 is associated with endosomes thus accounts for several observations.

Bro1-Rim20-ESCRT Relationship

Although one might expect Rim20 to respond to changes in external pH, we have made the surprising observation that Bro1-GFP focus accumulation is also pH-dependent. Given that the abundance of Bro1 and Rim20 foci show a reciprocal pH dependence, a simple possibility is that one protein displaces the other from interaction with Snf7. pH-depen-

dent signaling might then be generated by a pH-dependent change that affects the affinity of Bro1 or Rim20 for endosomal Snf7. One model is that Bro1 or Rim20 may be modified under alkaline growth conditions to alter their relative affinities for Snf7. A second model is that endosomes or the associated ESCRT complexes are different at acidic and alkaline pH, effecting different relative affinities for Bro1 and Rim20. For example, the nature of endocytic cargo or lipid composition may reflect the external pH, as has been suggested previously (Matsuo *et al.*, 2004; Barwell *et al.*, 2005). Our finding that some Rim20 foci and Bro1 foci are distinct at alkaline pH would be expected if some endosomes have cargo or composition that recruits Rim20, whereas others have cargo or composition that recruits Bro1. Therefore, we favor the second model.

Model for pH-responsive Signal Transduction

Our findings provide a new element in the understanding of pH-responsive signaling through fungal Rim101/PacC pathways: that the state of Rim20 is modulated by external pH and that this state is critical to relay a pH-dependent signal. In addition, our findings suggest that association of Rim20 with an endocytic compartment is critical for it to enter an active state and thus fit well with the ESCRT requirement for pH-responsive signaling. We have combined these findings with recent insights into PalF and Dfg16 (Barwell *et al.*, 2005; Herranz *et al.*, 2005; Rothfels *et al.*, 2005) to arrive at a model for pH-responsive signaling (Figure 7). Under acidic and alkaline conditions, many plasma membrane proteins undergo endocytosis, which delivers them to ESCRT-containing compartments. These compartments recruit Bro1 and ultimately form MVBs. Under acidic conditions, we suggest that Rim8 is inactive and that Dfg16 re-

Figure 7. Model for pH regulation of Rim20 localization. (A) Under acidic and alkaline conditions, many plasma membrane proteins undergo endocytosis, which delivers them to ESCRT-containing compartments. Subsequent recruitment of Bro1 results in incorporation of the cargo proteins into MVBs. (B) Under alkaline conditions, Rim8 is modified and stimulates endocytosis of Dfg16. Endosomal Dfg16 stimulates interaction of Rim20 with Snf7 and other ESCRT subunits. The Rim20-Snf7 complex recruits Rim13 and Rim101, resulting in Rim101 cleavage and activation.

sides primarily at the cell surface. Rim9 and Rim21 may be in a complex with Dfg16 or may be required for Dfg16 biogenesis. Under alkaline conditions, we suggest that Rim8 is modified, by analogy with *A. nidulans* PalF (Herranz *et al.*, 2005), resulting in association with and endocytosis of Dfg16, as proposed previously (Barwell *et al.*, 2005). We suggest that endosomal Dfg16 then favors interaction of Rim20 with Snf7 and other ESCRT subunits. Increased accumulation of endosome-associated Snf7 in the *vps4* mutant (Babst *et al.*, 2002) bypasses the regulatory system, permitting Rim20-Snf7 complex formation in the absence of Dfg16. In either situation, the Rim20-Snf7 complex recruits Rim13 and Rim101, resulting in Rim101 cleavage and activation.

Although complete Rim101/PacC pathways have been found only in fungi (Penalva and Arst, 2002), multiple Bro1-domain proteins are specified in eukaryotic genomes. The recent analysis of Bro1-domain structure makes it clear that all Bro1-domain proteins may interact with Snf7 or its or-

thologues (Kim *et al.*, 2005). Like Snf7 itself (Kranz *et al.*, 2001; Babst *et al.*, 2002), the characterized mammalian Snf7 orthologues have been localized to the endosomal surface and implicated in endosome trafficking (Katoh *et al.*, 2003; Peck *et al.*, 2004; Yorikawa *et al.*, 2005). With that as a backdrop, our finding that Rim20 is localized to Snf7-containing endosomes is entirely expected. What is novel is that the association of Rim20 with these endosomes is regulated by an external signal and that this regulation ultimately governs Rim20 activity. There are some interesting extrapolations of this finding that might be explored with other eukaryotes. We suggest that some of the numerous Bro1-domain proteins in a higher eukaryotic cell may undergo regulated endosome association, that this association may govern their activities, and that their endosomal association may not reflect a direct role in endosome trafficking but, rather, that endosome properties feature a signal that ultimately alters activity of a Bro1-domain protein's downstream effector.

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