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Sla1p serves as the targeting signal recognition factor for NPFX$_{(1,2)}$D-mediated endocytosis

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Efficient endocytosis requires cytoplasmic domain targeting signals that specify incorporation of cargo into endocytic vesicles. Adaptor proteins play a central role in cargo collection by linking targeting signals to the endocytic machinery. We have characterized NPFX$_{(1,2)}$ (NPFX$_{[1,2]}$D) targeting signals and identified the actin-associated protein Sla1p as the adaptor for NPFX$_{(1,2)}$D-mediated endocytosis in *Saccharomyces cerevisiae*. 11 amino acids encompassing an NPFX$_{(1,2)}$D sequence were sufficient to direct uptake of a truncated form of the pheromone receptor Ste2p. In this context, endocytic targeting activity was not sustained by conservative substitutions of the phenylalanine or aspartate. An NPFX$_{1,2}$D-related sequence was identified in native Ste2p that functions redundantly with ubiquitin-based endocytic signals. A two-hybrid interaction screen for NPFX$_{(1,2)}$D-interacting proteins yielded *SLA1*, but no genes encoding Eps15 homology (EH) domains, protein modules known to recognize NPF peptides. Furthermore, EH domains did not recognize an NPFX$_{(1,2)}$D signal when directly tested by two-hybrid analysis. *SLA1* disruption severely inhibited NPFX$_{(1,2)}$D-mediated endocytosis, but only marginally affected ubiquitin-directed uptake. NPFX$_{(1,2)}$D-dependent internalization required a conserved domain of Sla1p, *SLA1* homology domain, which selectively bound an NPFX$_{(1,2)}$D-containing fusion protein in vitro. Thus, through a novel NPF-binding domain, Sla1p serves as an endocytic targeting signal adaptor, providing a means to couple cargo with clathrin- and actin-based endocytic machineries.

Introduction

Internalization of cell surface components by endocytosis mediates important processes such as nutrient uptake, cell surface remodeling, and downregulation of signal transduction events. Rapid internalization of cell surface proteins relies largely on endocytic targeting determinants within their cytoplasmic domains (Marks et al., 1997). In general, such targeting signals are recognized by adaptors that also interact with core components of the endocytic machinery, thereby guiding bound cargo into nascent endocytic vesicles (Kirchhausen, 1999). Thus, defining endocytic targeting signals and identifying the corresponding adaptors is critical to understanding the endocytic process.

Common examples of mammalian endocytic targeting signals include tyrosine-based motifs, YXXΦ and NPXY (where X is any amino acid [aa]* and Φ is a bulky hydrophobic aa), and di-leucine motifs, all of which act as signals for packaging into endocytic clathrin-coated vesicles (Kirchhausen et al., 1997; Marks et al., 1997). In the best-characterized case, YXXΦ motifs interact with the μ subunit of the heterotetrameric AP-2 adaptor complex (Ohno et al., 1995). AP-2 also binds to clathrin, promoting assembly of individual clathrin molecules into a polyhedral scaffold (Kirchhausen, 1999). By serving as an adaptor linking receptors to the clathrin coat, AP-2 is thought to couple cargo recruitment with vesicle formation. A variety of other endocytic targeting sequences likely functions in a similar fashion by direct or indirect interaction with AP-2 (Kirchhausen et al., 1997; Kirchhausen, 1999). In a possible exception, the NPXY motif has been reported to interact directly with clathrin (Kibbey et al., 1998).

In the yeast *Saccharomyces cerevisiae*, two distinct classes of clathrin-facilitated endocytic targeting signals have been uncovered, one containing critical lysine residues, and the other consisting of the sequence NPFX$_{(1,2)}$ (NPFX$_{[1,2]}$D) (Rohrer et al., 1993; Tan et al., 1996). The lysine-based signal relies on ubiquitylation subsequent to local serine and threonine phosphorylation (Hicke and Riezman, 1996; Hicke et al., 1998). Mono-ubiquitylation is sufficient to trigger endocytosis, in contrast to the polyubiquitin chains of four or more residues required for proteasome-mediated degradation (Chau et al., 1989; Galan and Haguenauer-Tsapis,

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*Abbreviations used in this paper: aa, amino acid; EH, Eps15 homology; GST, glutathione-*S*-transferase; SHD, *SLA1* homology domain.

Key words: endocytosis; SLA1; NPF; adaptor; clathrin

1997; Roth et al., 1998; Terrell et al., 1998; Thrower et al., 2000). Expanding evidence suggests that ubiquitin acts as an endocytic targeting signal in mammals as well as yeast, although the mechanism by which this signal directs cargo to the endocytic machinery is currently unclear (Hicke, 2001).

The NPFX$_{(1,2)}$D signal was originally discovered in the cytoplasmic domain of the furin-like protease Kex2p (Tan et al., 1996). Kex2p cycles between the Golgi complex and endosomes without normally encountering the cell surface (Wilcox et al., 1992; Brickner and Fuller, 1997). Despite this itinerary, the Kex2p cytoplasmic domain directs rapid endocytosis when fused to an endocytically inactive version of a cell surface protein, the α-factor mating pheromone receptor, Ste2p (Tan et al., 1996). Mutational analysis of the Ste2p/Kex2p chimeric protein defined the sequence NPFSD as the endocytic targeting signal. Characterization of a similar sequence, NPFSTD, naturally present in Ste3p (the cell surface receptor for α-factor mating pheromone), established NPFX$_{(1,2)}$D as a motif that can direct rapid internalization of an integral membrane protein that normally transits the plasma membrane.

The NPFX$_{(1,2)}$D motif shares sequence features with the mammalian NPXY endocytic targeting signal. Furthering this resemblance, phenylalanine can function in place of tyrosine in the NPXY signal (Davis et al., 1987). In addition, both motifs can direct clathrin-mediated uptake, raising the possibility that the two signals might function by similar mechanisms (Davis et al., 1986; Tan et al., 1996). An alternative model derives from identification of NPF as a motif recognized by proteins bearing Eps15 homology (EH) domains (Salcini et al., 1997). Involvement in endocytosis is a common characteristic of EH domain proteins, including Eps15 and the yeast proteins Pan1p, End3p, and Ede1p (Benedetti et al., 1994; Wendland et al., 1996; Carbone et al., 1997; Benmerah et al., 1998; Gagny et al., 2000). Accordingly, EH domain proteins are candidate endocytic adaptors for the NPFX$_{(1,2)}$D signal.

Here we present in-depth mutational analysis of the NPFX$_{(1,2)}$D signal, indicating a closer relationship with EH domain ligands than with the NPXY endocytic targeting signal. However, the NPFX$_{(1,2)}$D motif is not efficiently recognized by yeast EH domain proteins. Instead, we identified the actin-associated protein Sla1p as the component of the endocytic machinery that recognizes NPFX$_{(1,2)}$D. Sla1p binds NPFX$_{(1,2)}$D through a domain unrelated in sequence to EH domains. Our results suggest that Sla1p represents a novel type of endocytic adaptor that can link NPFX$_{(1,2)}$D-containing cargo to the actin and clathrin-based endocytic machinery in yeast.

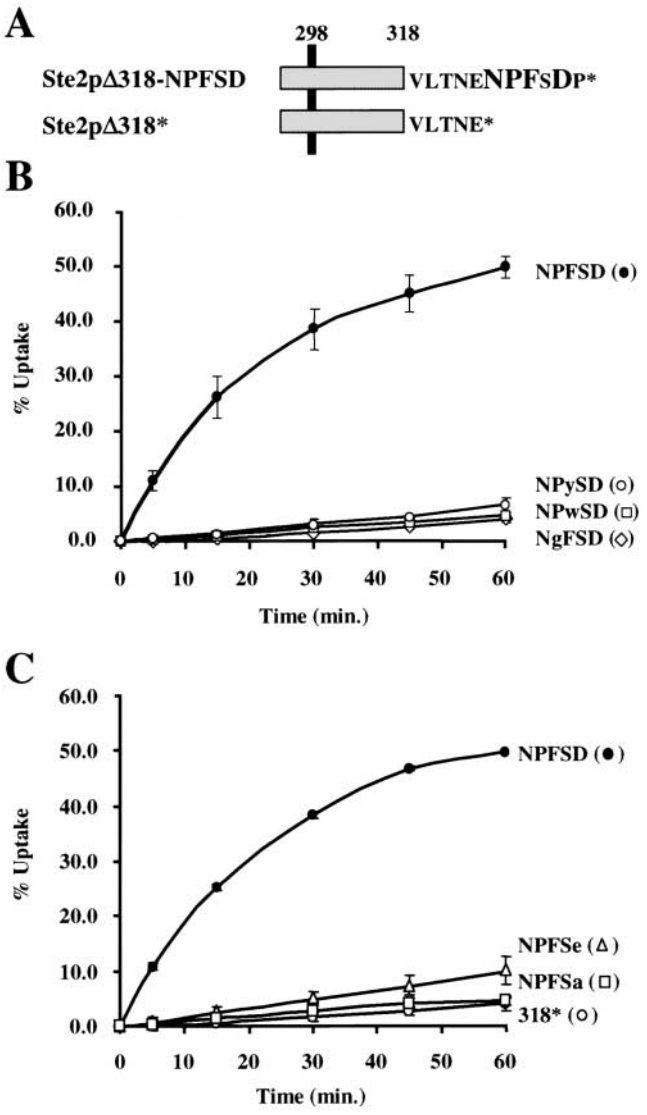

Figure 1. **Sequence requirements for NPFX$_{(1,2)}$D-mediated internalization.** (A) Diagrams of Ste2pΔ318-NPFSD and Ste2pΔ318*. The indicated sequences from Kex2p were fused to Ste2p at aa 318. Shaded rectangles represent the COOH-terminal region of Ste2p to aa 318 and the vertical black line represents the plasma membrane. The cytosolic tail domain of Ste2p is predicted to begin at aa 298. Residues previously defined as important for endocytic targeting by this signal are indicated by larger font. (B and C) Uptake of prebound, radiolabeled α-factor was measured in GPY779 expressing wild-type and indicated mutant forms of Ste2pΔ318-NPFSD at 30°C. Mutant residues are indicated in lowercase font. Error bars represent the standard deviation in three separate experiments.

Results

Conservative mutations in the NPFX$_{(1,2)}$D motif inhibit endocytosis

NPFX$_{(1,2)}$D was originally defined as an endocytic targeting signal by introducing alanine substitution mutations into a Ste2p/Kex2p chimera and a truncated form of Ste3p (Tan et al., 1996). However, in neither case was the targeting signal demonstrated to function completely independently of other Kex2p or Ste3p sequences. Consequently, an 11-aa sequence containing NPFSD from Kex2p was appended to residue 318 of Ste2p, replacing most of the Ste2p COOH-terminal cytoplasmic domain (Fig. 1 A, Ste2pΔ318-NPFSD). Truncation of Ste2p at position 318 leaves pheromone binding intact but eliminates the native endocytic targeting determinants (Konopka et al., 1988; Reneke et al., 1988). For comparison, a second construct was generated that carries the first five aa of the Kex2p sequence, but lacks the NPFSD signal (Fig. 1 A, Ste2pΔ318*). Ste2pΔ318-NPFSD and Ste2pΔ318* were introduced into cells lacking endogenous *STE2,* and endocytosis was assessed by measur-

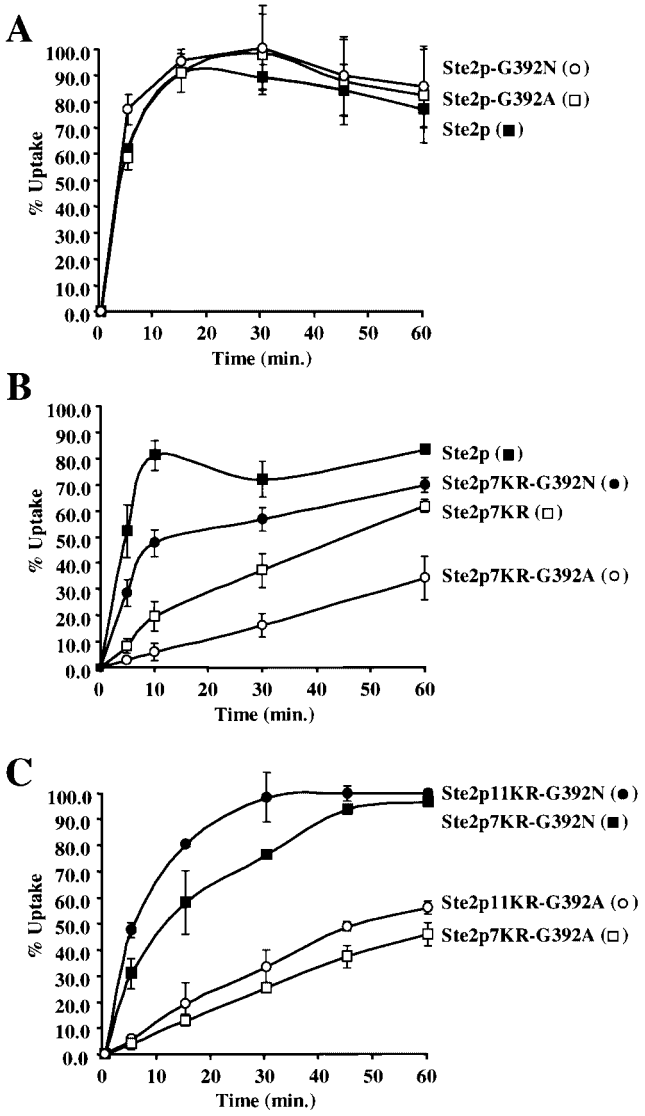

Figure 2. **NPFX$_{(1,2)}$D directs rapid endocytosis in the absence of ubiquitin acceptor sites.** Endocytosis of wild-type and mutant forms of Ste2p expressed in GPY779 were determined by measuring α-factor uptake as described in the legend to Fig. 1.

ing uptake of radiolabelled α-factor (Fig. 1, B and C). Ste2pΔ318-NPFSD was rapidly internalized (Fig. 1 C), albeit not as efficiently as wild-type Ste2p (compare Figs. 1 B and 2 A). In contrast, Ste2pΔ318* was not internalized (Fig. 1 C). These results indicate that the 11-aa peptide encompassing NPFSD functions autonomously to direct endocytosis in the absence of other Kex2p sequences.

The NPFX$_{(1,2)}$D motif resembles two signals involved in the endocytic process, the mammalian NPXY endocytic targeting signal and the NPF ligand of EH domains. To examine this relationship in more detail, we introduced mutations at selected sites in Ste2pΔ318-NPFSD and measured endocytosis of the resulting constructs. Both NPXY and NPF peptides adopt type I β turns in vitro and lose activity when proline is replaced with aa such as alanine or glycine that disfavor the turn conformation (Chen et al., 1990; Bansal and Gierasch, 1991; Salcini et al., 1997; de Beer et al., 2000). Mutation of NPFSD to NPASD in the original Ste2p/Kex2p chimera eliminated internalization (Tan et al., 1996). To extend this finding, we replaced proline with glycine in Ste2pΔ318-NPFSD. The resulting mutant was internalization-defective (Fig. 1 B, NgFSD), consistent with a possible role for a β turn conformation in endocytic targeting by NPFX$_{(1,2)}$D. NPXY and NPF signals differ in dependence on aromatic residues; tyrosine in NPXY can be functionally replaced by other aromatic aa, whereas phenylalanine in NPF cannot (Davis et al., 1987; Salcini et al., 1997). Mutation of phenylalanine to tyrosine or tryptophan in Ste2pΔ318-NPFSD abolished endocytosis (Fig. 1 B, NPySD and NPwSD). These results suggest that the NPFX$_{(1,2)}$D motif more closely resembles an EH domain ligand.

In the context of the original Ste2p/Kex2p chimera, the aspartate in NPFSD is an important component of the signal, although an aspartate to alanine mutant displayed a residual level of endocytosis (Tan et al., 1996). We tested the role of aspartate by substituting either alanine or glutamate in Ste2pΔ318-NPFSD. In this receptor, the alanine mutation reduced endocytosis to background levels (Fig. 1 C). More importantly, the glutamate mutation was also disruptive (Fig. 1 C), establishing that aspartate, not simply a negatively charged side chain, is required for full endocytic targeting activity.

Serine phosphorylation is required for ubiquitylation of the yeast lysine-based endocytic targeting signal and several serine and/or threonine kinases have been implicated in endocytosis (Hicke et al., 1998; Cope et al., 1999; Zeng and Cai, 1999). Serine and/or threonine residues are located within and surrounding the NPFX$_{(1,2)}$D signals derived from Kex2p and Ste3p. To determine whether phosphorylation might influence efficacy of the NPFX$_{(1,2)}$D motif, serine and threonine residues were converted to alanine in Ste2pΔ318-NPFSD. In the same construct, the glutamate preceding the NPFSD motif was also replaced with alanine. Alternatively, serine and threonine residues were changed to aspartate to mimic phosphoserine. Endocytosis of both mutants was normal (unpublished data), arguing that local phosphorylation/negative charge is not important for NPFX$_{(1,2)}$D targeting activity.

Native Ste2p contains a functional NPFX$_{(1,2)}$D-like motif

Ste2p contains a sequence related to the NPFX$_{(1,2)}$D signal, GPFAD, beginning at residue 392 in the COOH-terminal cytoplasmic domain. We examined the endocytic targeting potential of this sequence in the context of the native receptor by introducing alanine (inactivating) or asparagine (possibly activating) mutations at *G392*. The alanine mutation did not affect Ste2p endocytosis (Fig. 2 A). However, *G392N* resulted in a reproducible enhancement in uptake kinetics at the 5-min time point (Fig. 2 A), suggesting that the sequence contributes to endocytosis.

Ste2p utilizes ubiquitin as an endocytic targeting determinant (Hicke and Riezman, 1996). Multiple Ste2p lysines can serve as ubiquitin acceptors, providing redundant endocytic targeting information (Terrell et al., 1998). Also, there are examples of proteins with more than one type of endocytic tar-

geting signal (Kirchhausen et al., 1997). Therefore, it was possible that ubiquitylation masks a role for the GFPAD sequence in endocytosis. Accordingly, *G392A* and *G392N* were introduced into a version of Ste2p in which seven of eight COOH-terminal domain lysines were converted to arginine (Ste2p7KR) to prevent ubiquitin conjugation while maintaining overall charge. The remaining lysine, *K304*, does not suffice to direct internalization (Fig. 1 C, Ste2pΔ318*), and does not appear to be ubiquitylated (Dunn and Hicke, 2001). Ste2p7KR was internalized at initial rates four- to fivefold slower than wild-type (Fig. 2 B). The *G392A* mutation further reduced the initial internalization rate about fourfold, whereas *G392N* increased internalization to a rate approaching that of wild-type Ste2p (Fig. 2 B).

Although Ste2p7KR does not appear to be ubiquitylated, it was formally possible that the remaining cytosolic lysines could act as low-level or transient ubiquitin acceptors. To assess this possibility, the *G392A* and *G392N* mutations were analyzed in a form of Ste2p in which arginines replaced all 11 cytoplasmic lysines (Ste2p11KR).

Ste2p11KR and Ste2p11KR-G392N were internalized ~1.5-fold more efficiently than the cognate 7KR versions (Fig. 2 C; unpublished data), for reasons that are currently unclear. However, internalization remained sensitive to the *G392A* mutation (Fig. 2 C). Thus, NPFAD and GPFAD function in the absence of cytoplasmic lysines, indicating that $NPFX_{(1,2)}D$-mediated endocytosis does not require cargo ubiquitylation.

Collectively, these data provide evidence that an $NPFX_{(1,2)}D$-related motif participates redundantly with ubiquitin in directing endocytosis of Ste2p. Although GPFAD directs endocytosis, glycine is less optimal than asparagine at the first position. Consequently, Ste2p11KR-G392N and Ste2p-G392A represent sensitive reporters to distinguish $NPFX_{(1,2)}D$- and ubiquitin-dependent modes of endocytosis. Henceforth, we will refer to these reporters as Ste2p-NPFXD and Ste2p-Ub, respectively.

NPFSD interacts with Sla1p

To isolate an endocytic adaptor for the $NPFX_{(1,2)}D$ signal, we carried out a yeast two-hybrid screen. We generated a bait that carried the cytoplasmic domain sequences from Ste2pΔ318 fused to three tandem copies of the 11-residue Kex2p-derived NPFSD sequence (Fig. 3 A). To eliminate activation of reporter genes by the NPFSD bait alone, glutamates in the Kex2p derived sequences were altered to alanine, a mutation that does not affect $NPFX_{(1,2)}D$-mediated endocytosis (Tan et al., 1996). We reasoned that the triple repeat might improve chances of detecting interactions, as the initial internalization rate of Ste2pΔ318-NPFSD was enhanced ~2.5-fold by adding two additional copies of the Kex2p NPFSD-containing sequence (unpublished data). Using the triple-repeat bait we identified a plasmid carrying a fragment of the *SLA1* gene, but no plasmids expressing EH domain proteins. *SLA1* encodes a 1,244-aa protein implicated in actin dynamics (Holtzman et al., 1993; Ayscough et al., 1997, 1999). Furthermore, Sla1p interacts with Pan1p and End3p, two proteins that function in actin organization and endocytosis, suggesting a role for Sla1p in the endocytic process (Tang et al., 2000). Sla1p contains three NH_2-terminal SH3 domains, a central region with a proline rich sequence flanked by two domains unrelated to each other but

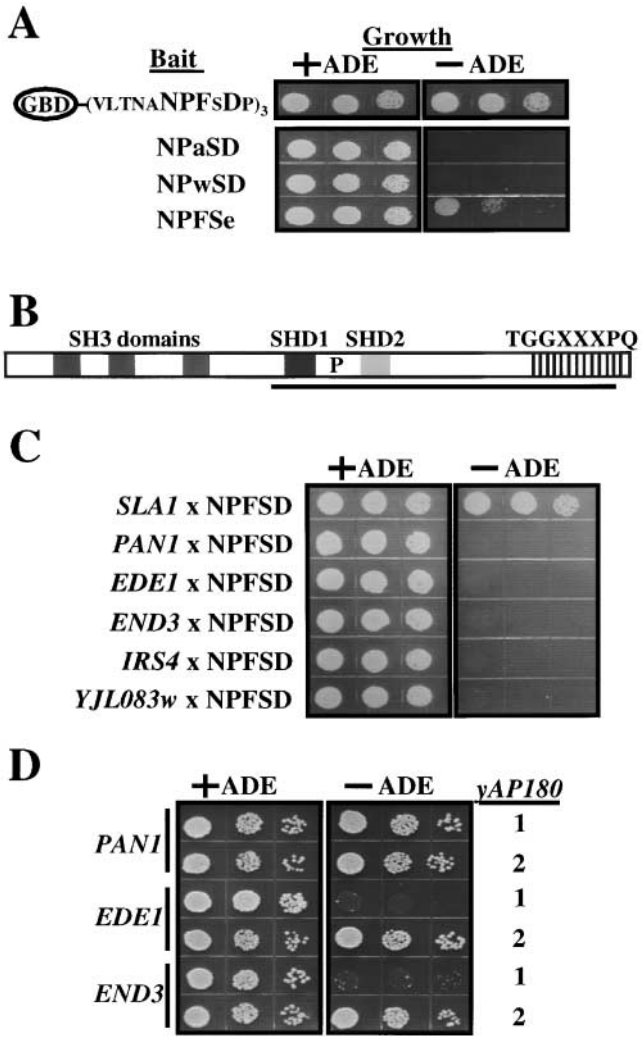

Figure 3. **NPFSD specifically interacts with the COOH-terminal region of Sla1p.** (A) Bait constructs used in the two-hybrid analysis. Three repeats of the NPFSD signal linked by Ste2p aa298–318 to the Gal4p DNA binding domain (GBD), and mutant versions shown below, were tested for interaction with the *SLA1* fragment isolated in the two-hybrid screen. Interaction was assessed in PJ69–4A by growth of serial diluted cells at 30°C on synthetic media containing (+ADE) or lacking (−ADE) adenine. (B) Domain architecture of Sla1p. Indicated are SH3 domains, SLA1 homology domain 1 and 2 (SHD1 and SHD2), a predicted proline-rich SH3 binding site (P), and multiple repeats of TGGXXXPQ (vertical lines). The Sla1p fragment isolated from the two-hybrid screen (K471-T1185) is underlined. (C) The Sla1p fragment and indicated EH-domain proteins were tested for interaction with NPFSD as in A. (D) Yeast EH-domain encoding genes were tested for interaction with *YAP180*s as above.

conserved in other fungal and plant species (*SLA1* homology domains [SHDs]), and multiple repeats of the sequence TGGXXXPQ in the COOH-terminal domain that potentially serve as phosphorylation sites (Fig. 3 B) (Ayscough et al., 1999; Zeng and Cai, 1999). The fragment isolated in the two-hybrid screen spans aa 471–1185, lacking the SH3 domains.

Because an endocytic adaptor for $NPFX_{(1,2)}D$ would be expected to interact only with an endocytically functional version of the motif, we compared interaction of the Sla1p fragment with NPFSD, or with endocytically defective vari-

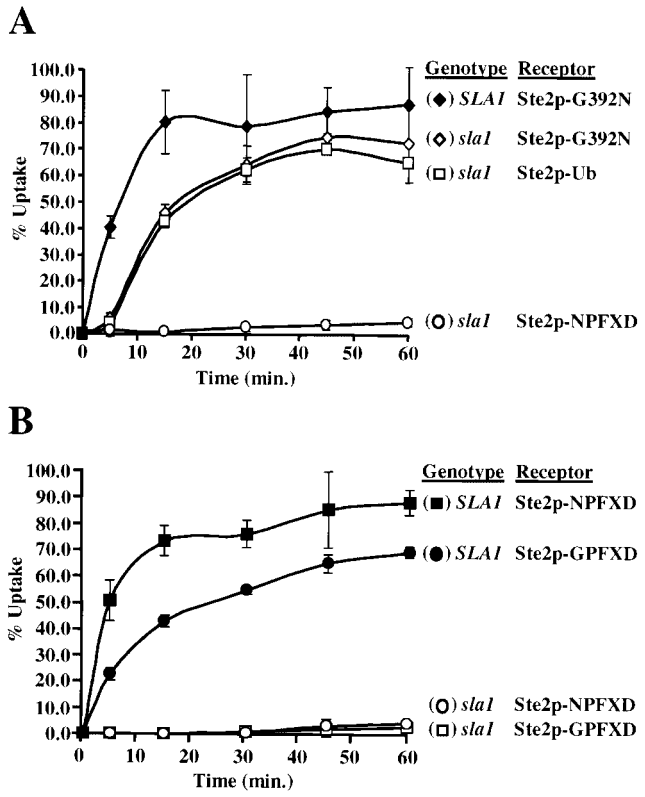

Figure 4. **NPFX$_{(1,2)}$D-mediated endocytosis requires *SLA1*.** (A and B) Centromere plasmids encoding Ste2p-G392N, Ste2p-NPFXD (Ste2p11KR-G392N), and Ste2p-GPFXD (Ste2p11KR) were introduced into *SLA1* cells (GPY2490, GPY2454, and GPY2665), and the same plasmids plus Ste2p-Ub (Ste2p-G392A) were introduced into *sla1Δ* cells (GPY2487, GPY 2488, GPY2489, GPY2451, and GPY2666). Endocytosis was monitored by α-factor uptake as described in the legend to Fig. 1.

ants, by two-hybrid analysis. As monitored by growth on selective media, two-hybrid interactions paralleled endocytic activity of the different sequences; the Sla1p fragment interacted strongly with NPFSD, very weakly with NPFSE, and not at all with NPASD or NPWSD (Fig. 3 A).

We also tested NPFSD for interaction with each of the five EH domain-containing proteins encoded in *S. cerevisiae*. These proteins were of particular interest because of sequence similarities between the NPFX$_{(1,2)}$D signal and EH domain ligands, and because three of these proteins (Pan1p, End3p, and Ede1p), are involved in endocytosis (Benedetti et al., 1994; Wendland et al., 1996; Gagny et al., 2000). Using the two-hybrid assay, only the *SLA1* fragment displayed interaction with NPFSD (Fig. 3 C). In contrast, Pan1p interacted with Yap1801p and Yap1802p, two proteins with five NPF motifs each, none containing an aspartate at position +5 and only one containing an aspartate at +6 (Fig. 3 D) (Wendland and Emr, 1998). Ede1p and End3p interacted with Yap1802p but not Yap1801p (Fig. 3 D). These results imply that Sla1p uniquely recognizes the NPFX$_{(1,2)}$D motif.

NPFX$_{(1,2)}$D-mediated endocytosis requires *SLA1*

The role of Sla1p in NPFX$_{(1,2)}$D-mediated endocytosis was investigated by evaluating the effects of disrupting *SLA1* (*sla1Δ*) on internalization of Ste2p-G392N, Ste2p-NPFXD (Ste2p11KR-G392N), and Ste2p-Ub (Ste2p-G392A). Uptake of α-factor mediated by Ste2p-NPFXD was abolished in *sla1Δ* cells (Fig. 4 A). The effects of *sla1Δ* on Ste2p-G392N and Ste2p-Ub were far more subtle; after a 5-min lag, internalization of both receptors was essentially normal. Sla1p was also required for uptake directed by the native GPFAD sequence in Ste2p (Fig. 4 B). The selectivity of *sla1Δ* effects on Ste2p-NPFXD and Ste2p-GPFXD supports the hypothesis that Sla1p acts as a specific endocytic adaptor for the NPFX$_{(1,2)}$D signal. However, the lag in ubiquitin-mediated endocytosis suggests Sla1p also participates more generally in the initiation of cargo internalization.

A conserved domain of Sla1p binds NPFX$_{(1,2)}$D

To define the region of Sla1p that interacts with NPFSD, deletion mutants of the *SLA1* fragment were tested for NPFSD binding by two-hybrid analysis. The fragment isolated in the original two-hybrid screen extended from K471 to T1185, encompassing the two SHD domains, the proline-rich sequence, and a majority of the COOH-terminal TGGXXXPQ repeats (Fig. 5 A). Using β-galactosidase activity as a measure of interaction, COOH-terminal excision of most of the fragment, leaving only K471-E555, produced little effect on NPFSD interaction (Fig. 5 A). In contrast, an NH_2-terminal truncation of 39 aa reduced β-galactosidase activity to ~10% of the original fragment (Fig. 5 A). These results suggest that the 84 aa between K471 and E555 contain an NPFX$_{(1,2)}$D interaction site. This fragment includes SHD1 (P492-G551), first recognized as a domain of unknown function that is conserved in an orthologue of *SLA1* from *Schizosaccharomyces pombe* (Ayscough et al., 1999). (It should be noted that SHD1 is distinct from a region in Pan1p containing TGGXXXPQ repeats that has been referred to as a Sla1p homology domain [Tang and Cai, 1996].)

Affinity chromatography was used as an independent assessment of NPFSD binding by SHD1. Glutathione-*S*-transferase (GST) fusion inserts to triple repeats of either NPFSD or NPASD were expressed in bacteria and purified by binding to glutathione-Sepharose. Equivalent amounts of the purified fusions were tested for binding to hexahistidine-tagged SHD1 (S486-E555) in *Escherichia coli* extracts (Fig. 5 B), or purified SHD1 (Fig. 5 C). SHD1 present in bacterial extract (Fig. 5 B, lanes 1 and 3, Immunoblot) was enriched in the bound fraction from GST-NPFSD but not GST-NPASD beads, as detected by Coomassie blue staining or immunoblotting with antibody to a hexahistidine tag (Fig. 5 B, lanes 2 and 4). Similarly, purified SHD1 bound preferentially to GST-NPFSD beads (Fig. 5 C, lanes 2 and 3). These results demonstrate that SHD1 can directly associate with a NPFX$_{(1,2)}$D motif.

Sla1p SHD1 is required for NPFX$_{(1,2)}$D-mediated endocytosis

We designed a mutant of *SLA1* lacking sequences encoding SHD1 (P492-G551, *sla1-Δshd1*) to examine the role of this domain in endocytosis. Centromere-based plasmids expressing either *SLA1* or *sla1-Δshd1* were introduced into *sla1Δ* cells and Sla1p expression was monitored by immunoblot-

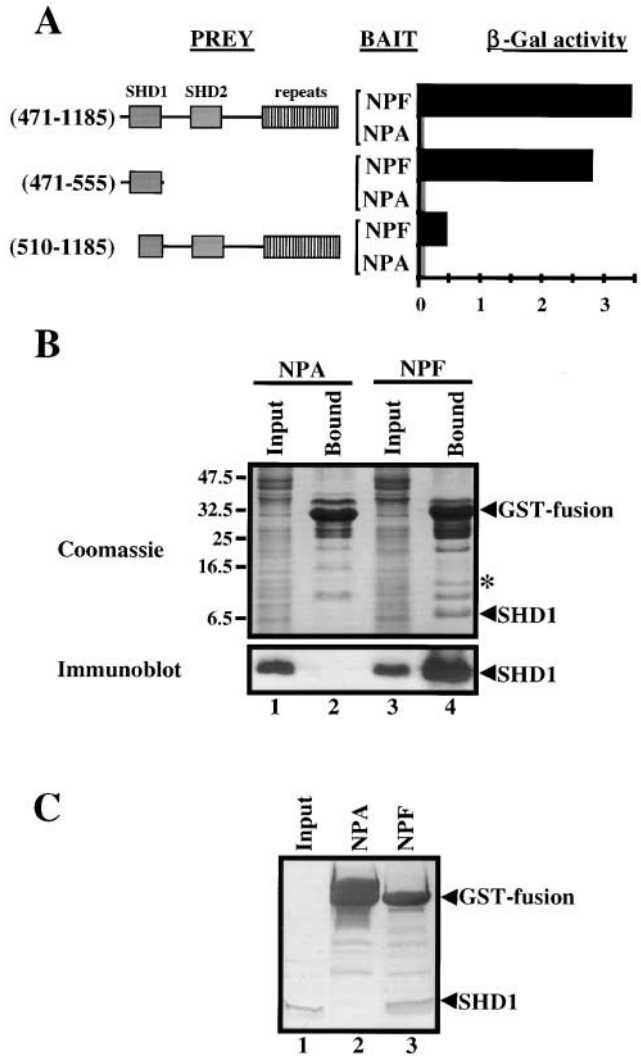

Figure 5. **NPFSD interacts specifically with the SHD1 region of Sla1p.** (A) Two-hybrid analysis of the NPFSD-interacting region of Sla1p. Fragments encoded by the original *SLA1* clone isolated in the two-hybrid screen (471–1,185) and two deletion mutants are diagrammed. Numbers indicate the amino acid boundaries of Sla1p fused to the Gal4p activation domain. Each construct was tested for interaction with active (NPF) and inactive (NPA) versions of the Kex2p-derived signal by measuring β-galactosidase activity (units × 10^3) in cell extracts. (B) GST-fusion protein affinity chromatography of SHD1 from cell extracts. Triple repeats of active (NPF) and inactive (NPA) forms of the 11 aa Kex2p-derived signal joined to Ste2p residues 298–318 were fused to GST and expressed in *E. coli*. Fusion proteins bound to glutathione-Sepharose were incubated with extracts from *E. coli* cells expressing hexahistidine-tagged SHD1. Bound proteins were eluted with reduced glutathione, separated by SDS-PAGE, and visualized by staining with Coomassie blue or by immunoblotting with antibodies against hexahistidine epitope. Positions of molecular mass standards (in kD), GST fusions, SHD1, and an apparently dimeric form of SHD1 (*) are indicated. Input represents 0.5% of the extract used for the incubations with GST fusions. (C) SHD1 purified as described in Materials and methods was tested for interactions with GST-NPASD (NPA) or GST-NPFSD (NPF) as described in B. Eluted proteins were subjected to SDS-PAGE and detected by Coomassie blue staining. Input represents 30% of purified SHD1 incubated with GST fusions.

ting. The results indicated that deletion of SHD1 does not destabilize Sla1p (unpublished data). We then compared endocytosis of Ste2p-G392N, Ste2p-NPFXD, and Ste2p-Ub in *sla1-Δshd1* cells to Ste2p-G392N endocytosis in wild-type cells. As shown in Fig. 6 A, internalization of Ste2p-G392N and Ste2p-Ub in *sla1-Δshd1* cells lagged only at the 5-min time point compared to the wild-type control. The delay was less pronounced than that observed for the same receptors in *sla1Δ* cells (compare Figs. 4 and 6 A). However, uptake of Ste2p-NPFXD was essentially eliminated (Fig. 6 A), as was uptake of Ste2p-GPFXD (unpublished data), equivalent to the effects of deleting the entire *SLA1* gene (Fig. 4).

Other Sla1p-related phenotypes were monitored to probe the specificity of *sla1-Δshd1* effects on $NPFX_{(1,2)}D$-mediated endocytosis. Deletion of *SLA1* leads to temperature-sensitive growth at 37°C and defects in organization of the actin cytoskeleton (Holtzman et al., 1993). As shown in Fig. 6 B, *SLA1* or *sla1-Δshd1* expressed from centromere-based plasmids fully restored growth at 37°C, whereas vector alone did not. Similarly, *sla1Δ-shd1* was comparable to *SLA1* in suppressing the loss in number and intensity of cortical actin patches in *sla1Δ* cells (Fig. 6 C). These results provide evidence that deletion of SHD1 specifically compromises $NPFX_{(1,2)}D$-mediated endocytosis, whereas other Sla1p activities remain intact.

Discussion

We have characterized sequence requirements for endocytic targeting by the motif $NPFX_{(1,2)}D$ and uncovered a novel role for Sla1p as an adaptor for $NPFX_{(1,2)}D$-mediated internalization in yeast. A role for Sla1p in endocytosis is further supported by previous studies documenting physical interactions of Sla1p with End3p and Pan1p, two components of the general actin-based endocytic machinery in yeast (Tang et al., 1997, 2000). We propose that Sla1p can act as an endocytic adaptor, coupling $NPFX_{(1,2)}D$-containing cargo to the endocytic machinery by its ability to bind the $NPFX_{(1,2)}D$ targeting signal, End3p, and Pan1p. To our knowledge, Sla1p represents the first example of a protein involved in recognition of endocytic targeting signals in yeast.

SHD1: a novel NPF-binding domain

Our results identify the Sla1p SHD1 as a novel NPF-binding domain, unrelated in primary structure to NPF-binding EH domains. Some basic characteristics of NPF recognition by SHD1 can be inferred from analysis of endocytic targeting by the $NPFX_{(1,2)}D$ signal and from two-hybrid interaction studies. Recognition can occur relatively independent of $NPFX_{(1,2)}D$ position within the cytoplasmic domain of the receptor constructs tested, as close as 20 aa from the membrane spanning domain in Ste2pΔ318-NPFSD, or as far as 96 aa from the membrane spanning domain of Ste2p-NPFXD. Several sequence features of $NPFX_{(1,2)}D$ appear to be especially important for SHD1 binding. Previous alanine mutagenesis identified key residues at positions 1–3 and 5 in NPFSD (Tan et al., 1996). Our current results indicate a preference for asparagine over glycine at position 1, and

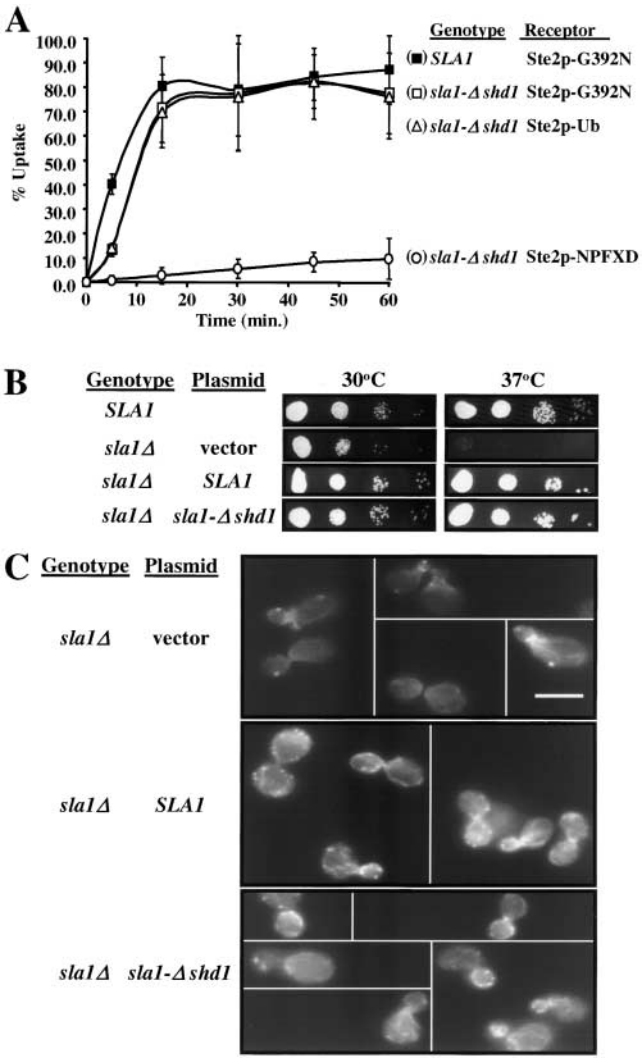

Figure 6. **NPFX$_{(1,2)}$D mediated endocytosis requires SHD1.** (A) Effects of deleting Sla1p SHD1 on receptor mediated endocytosis. Uptake of Ste2p-G392N, Ste2p-Ub (Ste2p-G392A), or Ste2p-NPFXD (Ste2p11KR-G392N) was measured in *sla1-Δshd1* cells (GPY 2493, GPY2494, and GPY 2495) and Ste2p-G392N in *SLA1* cells (GPY2490) as in Fig. 1. (B) Effects of deleting Sla1p SHD1 on growth. GPY2448 (*sla1Δ*) was transformed with centromeric plasmids containing no insert (vector), wild-type *SLA1*, or *sla1-Δshd1* and the resulting strains (GPY2487, GPY 2490, and GPY 2493, respectively) plus wild-type strain GPY1805 were serially diluted onto synthetic media and examined for growth after 3 d at the indicated temperatures. (C) Effects of deleting Sla1p SHD1 on actin organization. Gallery of cells from strains GPY2487 (*sla1Δ*, vector), GPY2490 (*sla1Δ*, *SLA1*), and GPY2493 (*sla1Δ*, *sla1-Δshd1*) grown at 30°C and then fixed and stained with rhodamine phalloidin. Bar, 5 microns.

strict requirements for phenylalanine rather than tryptophan and tyrosine at position 3, and aspartate instead of glutamate at position 5. The sequence requirements at positions 1–3 are similar to EH domain ligands and consistent with the type I–like β turn conformation observed in the solution structure of an NPF peptide bound to the second EH domain of Eps15 (de Beer et al., 2000). Accordingly, we suggest that the NPFX$_{(1,2)}$D signal binds to SHD1 in a conformation similar to a type I β turn with intimate hydrophilic contacts through the aspartate at position 5 or 6.

The NPFSD signal interacted with SHD1 but not EH domain–containing proteins. For EH domains, residues flanking NPF can influence specificity for different domains (Paoluzi et al., 1998). Of the EH domains from yeast proteins implicated in endocytosis (Pan1p, End3p, and Ede1p), only the second EH domain of Pan1p preferentially selected NPFXD peptides from a phage display library (Paoluzi et al., 1998). However, this binding was reportedly weaker than other EH domain–NPF interactions. Thus, aspartate may play a key role in determining the preference of NPFX$_{(1,2)}$D for SHD1 rather than EH domains, although other surrounding residues may also contribute to binding specificity.

After discovery of the NPFSD endocytic targeting signal in Kex2p, we sought the motif in proteins that normally undergo endocytosis. Two such proteins, Ste2p and Ste3p, contain NPFX$_{(1,2)}$D and ubiquitin-based signals, although the apparent contributions differ in the two receptors. In Ste2p, these two signals appear to function in both pheromone-stimulated and constitutive uptake (Hicke et al., 1998; unpublished data). In Ste3p, the NPFX$_{(1,2)}$D signal is particularly important for pheromone-stimulated internalization (Tan et al., 1996). A search of the *Saccharomyces* Genome Database revealed seven established or putative plasma membrane proteins with the sequence [G,N]PFX$_{(1-2)}$D, suggesting that the NPFX$_{(1,2)}$D motif, either alone or with ubiquitin-based signals, potentially directs endocytosis of a subset of cell surface proteins. Diversification provided by NPFX$_{(1,2)}$D signals could allow special regulatory inputs, or increase overall endocytic efficiency by contributing contacts to the endocytic machinery distinct from those of other signals. Furthermore, the targeting function of NPFX$_{(1,2)}$D may not be limited to endocytosis, as the signal was first identified in Kex2p, a protein that cycles between the Golgi and endosomes without normally transiting the cell surface. Therefore, it is possible that Sla1p SHD1 interactions with NPFX$_{(1,2)}$D signals play roles at multiple protein sorting steps.

Sla1p: an endocytic adaptor

SLA1 was originally uncovered in a screen for mutations that result in lethality when combined with a deletion of the gene encoding the actin binding protein Abp1p (Holtzman et al., 1993). Characterization of *sla1* mutants and Sla1p interaction partners indicate a role for Sla1p in actin cytoskeleton dynamics (Ayscough et al., 1997, 1999; Li, 1997; Madania et al., 1999; Drees et al., 2001). Importantly, Tang et al. (2000) have provided evidence that Sla1p forms a complex with Pan1p and End3p, two proteins essential for endocytosis. Our results extend the connection of Sla1p to endocytosis by demonstrating the role of the SHD1 domain in NPFX$_{(1,2)}$D-mediated internalization. Considering these findings, we propose that the Sla1p/Pan1p/End3p complex serves as an endocytic adaptor complex linking cargo to the actin and clathrin-based endocytic machinery in yeast (Fig. 7).

The Sla1p component of the Sla1p/Pan1p/End3p complex has the capacity to bridge NPFX$_{(1,2)}$D-containing cargo with the actin cytoskeleton. Deletion of *SLA1* disrupts actin organization, a phenotype primarily attributable to the region encompassing the most COOH-terminal SH3 domain (Holtzman et al., 1993; Ayscough et al., 1999). This region may be particularly important in mediating interactions

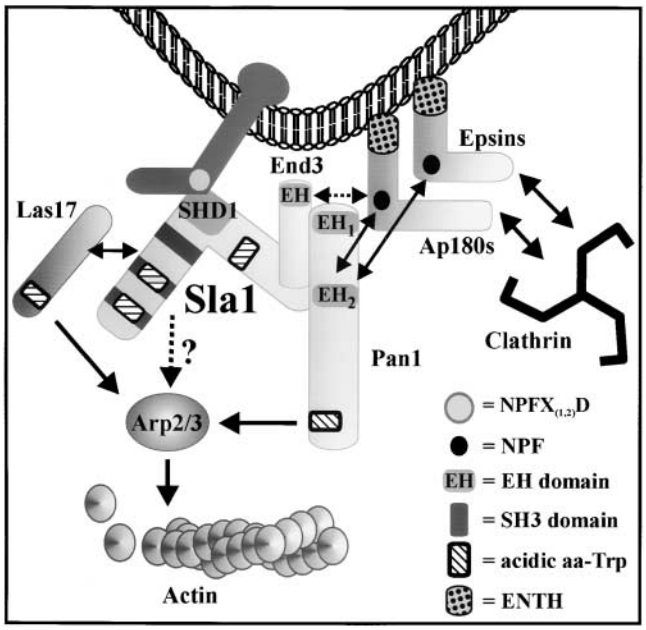

Figure 7. **Model for Sla1p/Pan1p/End3p endocytic adaptor complex.** Solid arrows indicate established physical interactions, dotted arrows indicate possible interactions. See text for details.

with the actin cytoskeleton. Additionally, Pan1p functions as an activator of the ARP2/3 complex, a key factor in actin polymerization (Duncan et al., 2001). Like other ARP2/3 activators, Pan1p contains a motif consisting of an acidic aa stretch followed by a tryptophan. Mutation of this sequence affected Pan1p activity in vivo, and ARP2/3 activation in vitro (Duncan et al., 2001). Interestingly, Sla1p also harbors three versions of the acidic aa tryptophan motif, two within SH3 domains and another COOH-terminal to SHD1 (Fig. 7). Therefore, the Sla1p/Pan1p/End3p complex could stimulate actin polymerization by Pan1p- and/or Sla1p-mediated ARP2/3 complex activation (Fig. 7). Sla1p also interacts, directly or indirectly with Las17p, and genetically with Abp1p, two additional proteins that stimulate the ARP2/3 complex (Fig. 7) (Holtzman et al., 1993; Li, 1997; Winter et al., 1999; Goode et al., 2001). Perhaps formation of an endocytic vesicle requires a high level of ARP2/3 stimulatory activity, provided through presentation of multiple activators by the Sla1p/Pan1p/End3 complex. In this scenario, the unusual lag observed in uptake of Ste2p-G392N and Ste2p-Ub in Sla1p-deficient cells could result from a delay in effective ARP2/3 activation.

Members of the Sla1p/Pan1p/End3p complex also interact with clathrin-associated proteins (Fig. 7). Through its second EH domain, Pan1p binds yeast epsins (Ent1p and Ent2p) and AP180s (Yap1801p and Yap1802p), presumably by recognizing NPF motifs within these proteins (Wendland and Emr, 1998; Wendland et al., 1999). Our two-hybrid results suggest that End3p can also associate with Ap1802p. In turn, yeast epsins and AP180s interact with clathrin, likely through COOH-terminal clathrin-binding motifs (Wendland and Emr, 1998; Wendland et al., 1999). Epsins and AP180s contain NH_2-terminal ENTH domains, which in mammalian proteins have been reported to mediate binding to phosphatidyl 4,5 bisphosphate (Ford et al., 2001; Itoh et al., 2001; Mao et al., 2001). Considering these interactions in sum, the Sla1p/Pan1p/End3p complex can coordinate a network linking the vesicle coat protein clathrin to the plasma membrane and endocytic cargo. Mutations of clathrin generally have less severe effects on endocytosis than mutations affecting actin cytoskeleton components. However, clathrin inactivation decreases internalization rates of different receptors (including $NPFX_{[1,2]}D$-dependent proteins) between two- and fivefold (Tan et al., 1993, 1996; Payne et al., 1988). These results, and the well-established role of clathrin in mammalian cell endocytosis, argue that clathrin coat formation at least facilitates endocytosis in yeast. Through the interactions shown in Fig. 7, the Sla1p/Pan1p/End3p complex has the potential to couple cargo collection to clathrin coat formation, similar to the mammalian AP-2 clathrin adaptor complex.

Database searches for homologues of Sla1p or the SHD1 domain identify significant matches in several fungal species and *Arabidopsis*, but not other organisms for which sequence information is available. However, there are provocative parallels between the Sla1p/Pan1p/End3p complex and a protein network coordinated by Eps15, raising the possibility of an analogous complex in mammals. Pan1p and Eps15 similarly consist of multiple NH_2-terminal EH domains, central predicted coiled-coil regions, and proline-rich COOH termini, and both participate in endocytosis (Wendland et al., 1996; Carbone et al., 1997; Tang et al., 1997; Benmerah et al., 1998). Eps15 interacts with intersectin, a protein with two NH_2-terminal EH domains, and five COOH-terminal SH3 domains that has also been implicated in endocytosis (Yamabhai et al., 1998; Sengar et al., 1999; Simpson et al., 1999). Like Sla1p, intersectin combines NPF recognition modules with SH3 domains. Furthermore, through its EH domains, intersectin recognizes NPFXD sequences in a cytoplasmic domain of the integral membrane protein, SCAMP1 (Fernandez-Chacon et al., 2000). Importantly, the NPFXD motifs in SCAMP1 can interact with intersectin EH domains, but not Eps15 EH domains, a selectivity mirroring preferential association of $NPFX_{(1,2)}D$ with SHD1 over Pan1p EH domains. Therefore, the intersectin EH domain and the Sla1p SHD1 domain may exemplify convergent evolution for ligand binding specificity, suggesting that Sla1p and intersectin may be, at least in part, functionally homologous.

As we have proposed for the Sla1p/Pan1p/End3p complex, the Eps15/intersectin complex has the potential to couple NPFXD-containing cargo to the actin cytoskeleton and clathrin-coats. One such cargo could be SCAMP1, as it cycles through the plasma membrane and interacts with intersectin through NPFXD sequences (Brand et al., 1991; Brand and Castle, 1993). Eps15 and intersectin each contain candidate acidic aa tryptophan ARP2/3 activation sequences. Additionally, a splice variant of intersectin contains a DBL homology domain that stimulates actin polymerization through Cdc42 nucleotide exchange activity (Hussain et al., 2001). Interactions with clathrin coats could be mediated by EH domains in both Eps15 and intersectin that bind epsins (Chen et al., 1998; Yamabhai et al., 1998; Sengar et al., 1999), and SH3 domains in intersectin that bind proline-rich domains of

Table I. **Strains used in this study**

Strain	Genotype	Reference
GPY779	*MATa leu2-3, 112 his4 or 6 trp1-289 ura3-52 sst1-3 ste2::Leu2*	Tan et al., 1996
GPY1563	GPY779 + pJH108	This study
GPY1564	GPY779 + pJH121	This study
GPY1565	GPY779 + pJH122	This study
GPY1570	GPY779 + pJH106	This study
GPY1571	GPY779 + pJH106-G392A	This study
GPY1572	GPY779 + pJH106-G392N	This study
GPY1574	GPY779 + pJH108-G392A	This study
GPY1575	GPY779 + pJH108-G392N	This study
GPY1810	GPY779 + pJH121-NPySD	This study
GPY1811	GPY779 + pJH121-NPwSD	This study
GPY1812	GPY779 + pJH121-NgFSD	This study
GPY1923	GPY779 + pJH121-NPFSa	This study
GPY1924	GPY779 + pJH121-NPFSe	This study
GPY1995	GPY779 + pJH108-11KR-G392N	This study
GPY1996	GPY779 + pJH108-11KR-G392A	This study
GPY1805	*MATa ura3-52, leu2-3, 112 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 sst1::LYS2 ste2::LEU2*	This study
GPY2448	GPY1805 *sla1::HIS3*	This study
GPY2487	GPY2448 + pRS313 + pJH106-G392N	This study
GPY2488	GPY2448 + pRS313 + pJH106-G392A	This study
GPY2489	GPY2448 + pRS313 + pJH108-11KR-G392N	This study
GPY2490	GPY2448 + pKA51 + pJH106-G392N	This study
GPY2493	GPY2448 + pKA51Δshd1 + pJH106-G392N	This study
GPY2494	GPY2448 + pKA51Δshd1 + pJH106-G392A	This study
GPY2495	GPY2448 + pKA51Δshd1 + pJH108-11KR-G392N	This study
PJ69-4A	*MATa trp1-901 leu2-3, 112 ura3-52 his3-200 gal4Δ gal80Δ LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ*	James et al., 1996

clathrin coat accessory factors (Roos and Kelly, 1998; Yamabhai et al., 1998; Sengar et al., 1999). Also, Eps15 binds AP-2 (Benmerah et al., 1995). Thus, the interaction networks radiating from the Sla1p/Pan1p/End3p and Eps15/intersectin complexes incorporate common functional targets. Based on the models derived from our studies, it should now be possible to test the extent of functional analogy between these components of the endocytic machinery in yeast and mammals.

Materials and methods

General methods and media

General molecular biology methods were performed as described (Sambrook et al., 1989). PCR amplifications were performed with Elongase (Invitrogen), and automated sequencing carried out with ABI Prism® Big Dye™ Terminators Cycle Sequencing Kit (Applied Biosystems). All PCR products were sequenced to confirm accurate amplification. Yeast extract peptone dextrose media (YPD) and synthetic media (SD and SD$_{CAA}$) have been described previously (Tan et al., 1996).

Strains and plasmids

Table 1 describes the strains used in this study. Plasmids pEK3 (*sst1::LYS2*), a gift from Howard Riezman (University of Basel, Basel, Switzerland) and pAB506 (*ste2::LEU2*), a gift from James Konopka (University of Oregon, Eugene, OR) were used for single-step gene replacements (Rothstein, 1991) after cleavage with *Eco*RI and *Bam*HI, respectively. *SLA1* was disrupted by generating two overlapping PCR fragments and cotransformation. DNA was introduced into yeast strains by a lithium acetate protocol (Woods and Gietz, 2001).

Plasmids used in this study were constructed as follows. pJH106 (pRS314-*STE2*): a 1.3-kb *Hpa*I/*Sac*I fragment from pJR3 (Rohrer et al., 1993) was inserted into pRS314-*STEX22* (Tan et al., 1996), resulting in replacement of the Kex2p cytoplasmic domain with the native Ste2p cytoplasmic domain. pJH108 (pRS314-*STE2-7KR*): the analogous *Hpa*I/*Sac*I fragment from a plasmid-encoding mutant *ste2* (*ste2-7xR*) (Terrell et al., 1998) was inserted into pJH106, introducing lysine to arginine mutations in the last seven lysine residues of Ste2p. *G392A* and *G392N* mutations were generated by the megaprimer method (Sarkar and Sommer, 1990) and introduced into pJH106 and pJH108. pJH108-11KR: a 0.94-kb *Hpa*I/*Pst*I fragment from LHP409, a gift from Linda Hicke (Northwestern University, Evanston, IL), was inserted into pJH108 plasmids, altering the remaining cytosolic lysines to arginines. pJH121 (pRS314-STE2Δ318-NPFSD): a PCR product encoding the sequence VLTNENPFSDP* fused to aa 318 of Ste2p was introduced into the *Cla*I/*Hind*III sites of pRS314-*STEX2h* (Tan et al., 1996). This insertion replaces the Kex2p cytoplasmic tail in a Ste2p/Kex2p fusion with VLTNENPFSDP*. In addition, a PCR product containing ~500 bp of the *KEX2* 3′ untranslated region was introduced into the *Hind*III site. Point mutations in Ste2pΔ318-NPFSD were similarly engineered by PCR using mutant primers. pJH122 (pRS314-STE2Δ318*) was derived by a similar strategy with a PCR product encoding VLTNE* fused to aa 318 of Ste2p. pKA51 (pRS313-*SLA1*) is described in Ayscough et al. (1999). pKA51Δshd1: deletion of Sla1p aa 492–551 was generated using the megaprimer method and insertion into the *Apa*I and *Aat*II sites of pKA51. pGBD-NPFSDx3: a PCR product encoding aa 298–318 of Ste2p fused to the sequence VLTNANPFSDP repeated three times in tandem was inserted into pGBD-C1 (James et al., 1996). Plasmids containing mutant versions of the targeting signal were constructed similarly. Complementary plasmids in pGAD-C1 (James et al., 1996) were generated using fragments from pGBD plasmids. Yeast two-hybrid plasmids encoding NH$_2$-terminal regions of Pan1p and Ede1p, and full-length Yap1801p and Yap1802p, are described in Wendland and Emr (1998). Yeast two-hybrid plasmids containing *END3*, *IRS4*, and *YJL083w* were generated by PCR amplification of genomic DNA and insertion into pGBD-C1. pGEX-KG-NP(F/A)SD: the corresponding sequences from pGBD-NP(F/A)SDx3 were amplified by PCR and inserted into pGEX-KG (Amersham Pharmacia Biotech), to generate GST fused NH$_2$-terminal to NP(F/A)SDx3 (as above). pET21-SHD1: the region of *SLA1* encoding K471 to E555 was amplified by PCR and inserted into pET21b (Novagen), introducing a COOH-terminal hexahistidine tag. p926-SHD1: the region of *SLA1* encoding K482 to E555 was amplified by PCR and inserted into p926, a gift from Jean L. Perry (University of California, Los Angeles, Los Angeles, CA). This plasmid encodes a TEV protease cleavage site separating an NH$_2$-terminal hexahistidine tag from SHD1.

Yeast two-hybrid screen

Strain PJ69-4A was cotransformed with genomic DNA libraries in pGAD yeast two-hybrid vectors (James et al., 1996) and plasmid pGBD-NPFSDx3, and incubated on SD agar plates lacking adenine for up to 2 wk at 30°C. More than 2×10^6 transformants were plated for each reading frame, covering the entire library at a >99% confidence level. Bait plas-

mids from Ade$^+$ His$^+$ colonies were cured by growth in nonselective media, and prey plasmids isolated and sequenced by standard methods. Cells cured of bait vector were retransformed with either pGBD-NPFSDx3, or versions carrying mutations of the signal, and examined for growth on SD plates lacking adenine or histidine. No prey genes were isolated more than once, suggesting that the screen was not carried out to saturation. Other than *SLA1*, there were no other isolates likely to be involved in endocytosis or other protein trafficking steps.

Liquid β-galactosidase assays were performed as described (Breeden and Nasmyth, 1987). Units were calculated as $(1{,}000 \times OD_{420})/([\text{ml extract}] \times [\text{incubation time \{min\}}] \times [\text{protein concentration extract \{mg/ml\}}])$. To assess relative growth on agar plates, freshly transformed PJ69-4A cells were grown overnight to stationary phase in SD_{CAA}–trp-leu media, diluted to 1×10^7 cells/ml, and then tenfold serially diluted. Dilutions were spotted onto SD plates with or without adenine and incubated at 30°C for 5 d. Images were collected on a SPEEDLIGHT PLATINUM gel documentation system (Lightools Research) and adjusted using standard settings in Adobe Photoshop (Adobe Systems).

Preparation of ^{35}S-labeled α-factor

Metabolically labeled α-factor was prepared and purified from filtered culture supernatant over a cellulose-phosphate column essentially as described (Tan et al., 1993), using strain RK537-3B, a gift from Kendall Blumer (Washington University School of Medicine, St. Louis, MO).

^{35}S-labeled α-factor internalization assay

Metabolically labeled α-factor was prepared as described in (Tan et al., 1993). The assay for binding and internalization of ^{35}S-labelled α-factor was performed essentially as that described (Tan et al., 1996), except that internalization was carried out in 1% Bacto-yeast extract (Difco), 2% Bacto-peptone (Difco), 50 mM PO_4, pH 6, 1% BSA, 20 μg/ml histidine, adenine and uracil) and dextrose was added to 5% to initiate endocytosis. Percent uptake is 100× the ratio of ($internal_{CPM}$-$background_{CPM}$/$bound_{CPM}$-$background_{CPM}$). All samples were measured in duplicate, and each experiment performed at least three times.

GST fusion affinity binding

pGEX-KG-NPFSDx3 and pGEX-KG-NPASDx3 were expressed in bacterial strain BL21 (DE3), and GST fusion proteins were affinity purified with glutathione-Sepharose (Amersham Pharmacia Biotech) as described in Smith and Johnson (1988). pET21b-SHD1 was induced by 0.1 M IPTG for 16 h at 18°C and cells were lysed by sonication. Lysates were incubated with GST-NPFSD or GST-NPASD beads overnight at 4°C. Bound proteins were resolved by SDS-PAGE on a 15% gel, and visualized by Coomassie blue stain or immunoblotting (Burnette, 1981) with anti-pentaHis antibody (QIAGEN) and secondary antibodies coupled to HRP (Bio-Rad Laboratories). HRP was visualized using enhanced chemilumenescence (Amersham Pharmacia Biotech). p926-SHD1 was expressed and purified as described (Pettit et al., 1998), except that protein expression was induced for 12 h at 18°C. To remove the NH_2-terminal tag, purified protein was dialyzed into Tris buffer (20 mM Tris, 10% [vol/vol] glycerol, pH 8.0) using Spectra/Por dialysis membrane 2 (Spectrum), and then treated with rTEV protease (Invitrogen) for 1 h at 30°C in TEV cleavage buffer (50 mM Tris, 0.5 mM EDTA, and 1 mM DTT. pH 8.0). KCl was added to a final concentration of 100 mM, and then cleaved tag and recombinant protease were removed by incubation with Ni-NTA beads (QIAGEN) for 20 min at 4°C. Binding experiments with GST fusion proteins were carried out as above, except that the incubation step was performed for 20 min at 4°C. Samples were resolved by SDS-PAGE analysis on a 20% gel (Giulian et al., 1983) and visualized by Coomassie blue staining. Images were collected by laser densitometry and adjusted using standard settings in Adobe Photoshop.

Temperature-sensitive growth assay

Cells grown to stationary phase in SD_{CAA} media at 30°C were diluted to 1×10^6 cells/ml, and then tenfold serially diluted. Dilutions were spotted onto SD plates and incubated at 30°C or 37°C for 3 d.

Phalloidin staining of yeast cells

Phalloidin staining of actin was carried out as described (Pringle et al., 1989). Staining was visualized using a 100× oil immersion lens on a Nikon FXA fluorescence microscope. Images were collected using a Photometrics cooled charge-coupled device camera and Isee software from Inovision and adjusted using standard settings in Adobe Photoshop.

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