Cleavage of the signaling mucin Msb2 by the aspartyl protease Yps1 is required for MAPK activation in yeast

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Signaling mucins are cell adhesion molecules that activate RAS/RHO guanosine triphosphatases and their effector mitogen-activated protein kinase (MAPK) pathways. We found that the Saccharomyces cerevisiae mucin Msb2p, which functions at the head of the Cdc42p-dependent MAPK pathway that controls filamentous growth, is processed into secreted and cell-associated forms. Cleavage of the extracellular inhibitory domain of Msb2p by the aspartyl protease Yps1p generated the active form of the protein by a mechanism incorporating cellular nutritional status. Activated Msb2p functioned through the tetraspan protein Sho1p to induce MAPK activation as well as cell polarization, which involved the Cdc42p guanine nucleotide exchange factor Cdc24p. We postulate that cleavage-dependent activation is a general feature of signaling mucins, which brings to light a novel regulatory aspect of this class of signaling adhesion molecule.

Introduction

Signal transduction pathways control multiple aspects of cellular behavior, including regulating the connectivity between cells and their adhesion to external surfaces. Among the signaling proteins that regulate cell adhesion are members of the mucin family of proteins. Signaling mucins are cell-surface proteins composed of a heavily glycosylated extracellular domain that contains a Ser/Thr/Pro-rich mucin homology domain (MHD; Silverman et al., 2003), which is connected to a cytoplasmic domain that regulates RAS/RHO GTPases and their effector MAPK pathways (Carraway et al., 2007). Proteolytic processing separates the domains and results in the shedding of the extracellular glycodomain from the cell (Singh and Hollingsworth, 2006). Signaling mucins also contribute to the regulation of other pathways including the Wnt, nuclear factor-κB, and estrogen receptor pathways (Huang et al., 2003; Wei et al., 2006).

In Saccharomyces cerevisiae, the signaling mucin Msb2p regulates MAPK activity by interaction with the RHO GTPase Cdc42p (Cullen et al., 2004), a global regulator of cell signaling and polarity (Johnson, 1999). Cdc42p and its effector kinase Ste20p regulate multiple MAPK pathways including the filamentous growth (Peter et al., 1996; Leberer et al., 1997), pheromone response (Simon et al., 1995), and high osmolarity glycerol response (HOG) pathways (Tatebayashi et al., 2006). Differential MAPK activation is regulated in part by pathway-specific MAPKs (Madhani et al., 1997) and recruitment of core signaling modules to pathway-dedicated receptor complexes by scaffolding proteins (Butty et al., 1998; Harris et al., 2001). Msb2p is required for activation of the filamentous growth pathway (Cullen et al., 2004) and may function in a minor way in the HOG pathway (O’Rourke and Herskowitz, 2002). Indeed, Msb2p associates with the tetraspan protein Sho1p (Cullen et al., 2004), which operates in both the filamentous growth and HOG pathways (Maeda et al., 1995; Cullen et al., 2004; Zarrinpar et al., 2004). Although it is unclear how Msb2p becomes activated, loss of the MHD induces the signaling function of the protein, which suggests an inhibitory role for this domain in MAPK signaling (Cullen et al., 2004).

In the present study, we show that Msb2p, like its mammalian counterparts, is processed into secreted and cell-associated forms. This discovery, coupled with the fact the secreted domain...
of Msb2p is inhibitory, suggests a mechanism where processing and release of the extracellular domain activates the protein. We confirmed this hypothesis and identified an aspartyl protease, Yps1p, that is required for Msb2p processing. Intriguingly, the gene encoding Yps1p is induced in response to nutrient limitation, which provides a straightforward mechanism for regulated processing of Msb2p. We speculate that cleavage-dependent activation may be a general feature of mucin receptors.

Results and discussion

Msb2p is processed into distinct polypeptides

The extracellular portion of the Msb2p protein was detected in supernatants by Western blotting, which indicates that it is secreted from the cell. To determine if secretion results from processing in the extracellular domain, a version of the protein was examined in which the extracellular (HA, at 500 residues) and cytoplasmic (GFP, at 1,306 residues) domains were differentially tagged. Conditioned media derived from cells expressing HA-Msb2p-GFP showed that the extracellular but not cytoplasmic domain was secreted (Fig. 1 A). This result confirms topological and subcellular fractionation data that indicate that Msb2p is a membrane-spanning protein (Cullen et al., 2004). Although the extracellular domain of Msb2p migrated as multiple intermediate forms (Fig. 1 A), the majority of the secretion defect of HA-Msb2p in the 5ypsΔ strain (Fig. S1 A), confirming that Yps1p is the primary Yps protease required for Msb2p processing.

Yps1p has been shown to process heterologously expressed glycoprotein substrates by cleavage in the extracellular domain at monobasic residues (Bourbonnais et al., 2000). By these criteria, Msb2p is a likely substrate of Yps1p and the first physiological target to be identified. Several lines of evidence support this possibility. First, yps mutants are not generally defective in protein secretion, and several proteins have been examined that are secreted in yps mutants to wild type (WT) or higher levels (unpublished data). The Msb2p protein was delivered to the cell surface in yps mutants based on the fact that HA-Msb2p lacking its TM domain was secreted in the 5ypsΔ mutant (Fig. 2 A). Intriguingly, Msb2p-GFP was absent from vacuoles in yps mutants (Fig. 2 B). Several other cell-surface proteins showed a similar localization pattern (unpublished data), which suggests a general requirement for Yps proteins in delivery of a subset of cell-surface proteins to vacuoles. Second, although yps mutants have defects in cell integrity and cell wall biosynthesis (Krysan et al., 2005), secretion of HA-Msb2p was not restored by conditional rescue of the cell integrity defect of the 5ypsΔ mutant grown in 1 M sorbitol (Fig. 2 A). Likewise, cell-wall mutant combinations defective for β-1,3 and β-1,6-glucan synthesis that mimic yps phenotypes showed elevated secretion of HA-Msb2p (Fig. S1 B). Third, the ypsΔ mutant was defective in processing Msb2p-TAP, resulting in accumulation of the full-length protein and loss of a processing intermediate (Fig. S1 C).

Cleavage of Msb2p is required for MAPK activation and is stimulated by nutrient limitation-dependent induction of YPS1 expression

Msb2p lacking the MHD hyperactivates the filamentous growth pathway (Cullen et al., 2004). Versions of Msb2p lacking larger portions of the extracellular domain were also hyperactive, more so than Msb2pΔMHD (Fig. 3 A), which confirms that the extracellular domain (from 100 to 950 residues) functions in an inhibitory capacity. Cleavage of the inhibitory domain may provide an explanation for how the protein becomes activated. In support of this possibility, the noncleavable version of Msb2p, Msb2pΔCD, was defective for MAPK activity (Fig. 3 A) and filamentous growth (not depicted). Versions of
Msb2p lacking the TM and CYT domains were similarly defective for signaling (Fig. 3 A).

To determine if Msb2p processing is regulated, secretion of Msb2p was examined in cells incubated under different conditions, particularly nutrient limitation, a known inducer of filamentous growth (Cullen and Sprague, 2000). Secretion of HA-Msb2p was induced >10-fold by nutrient limitation, and the enhanced secretion was Yps1p dependent (Fig. 3 B). Unprocessed Msb2p accumulated in the \( \text{yps} \Delta \) mutant under this condition (Fig. 3 B). To determine how Yps1p regulates Msb2p processing in a nutrient-dependent manner, we turned to the expression of the \( \text{YPSI} \) gene, which is highly regulated (Krysan et al., 2005). Expression of \( \text{YPSI} \) was induced under nutrient-limiting conditions that promote filamentous growth (Fig. 3 C). Overexpression of \( \text{YPSI} \) or \( \text{MKC7/YPS2} \) from a high-copy plasmid or inducible promoter stimulated Msb2p-dependent MAPK activity (Fig. 3 D) and polarized growth (Fig. 3 E and not depicted). Cells lacking Yps1p were partially defective for MAPK activity, and this effect was exacerbated in cells lacking Mkc7p and Yps3p (Fig. 3 D). Therefore, Msb2p processing is regulated by starvation-dependent induction of its cognate protease. Cleavage by Yps1p may trigger Msb2p activation or be a prerequisite for activation.

Activated Msb2p functions through Sho1p

Msb2p interacts with the tetraspan protein Sho1p (Cullen et al., 2004). The C-terminal domain of Msb2p was sufficient to recapitulate the Msb2p–Sho1p interaction (Fig. 4 A). To better understand the role of Sho1p in Msb2p-dependent signaling, alleles of \( \text{SHO1} \) were isolated that hyperactivate the filamentous growth pathway. A dominant gain-of-function allele was identified by random mutagenesis that encodes a P120L change in the short DGKK linker of the second extracellular loop of the protein (Fig. 4 B). The mutation at P120L did not affect Sho1p protein levels (Fig. 4 B) but stimulated the filamentous growth pathway based on hyperpolarized growth of haploid and diploid cells (Fig. 4 C), agar invasion phenotypes (Fig. S2 A, available at http://www.jcb.org/cgi/content/full/jcb.200704079/DC1), and induction of filamentous growth pathway reporters (Fig. S2 B). Sho1p\( \text{P120L} \) functioned normally in the HOG pathway (Fig. S2, C–F).
Gain-of-function alleles of MSB2 and SHO1 were used to explore the functional relationships between the cell-surface proteins. Activated versions of Msb2p were dependent on Sho1p for MAPK signaling (Fig. 4 D) and hyperpolarized growth (Fig. 4 E). In contrast, Sho1p P120L partially induced MAPK signaling (Fig. 4 D) and hyperpolarized growth (Fig. 4 E) in the absence of Msb2p. Sho1p P120L also restored MAPK activity to the Msb2pACD strain and theyps1Δ mkc2Δyps3Δmutant (not depicted). This property

Other alleles of SHO1 that differentially influence its function between pathways map to the cytoplasmic SH3 domain and affect the Sho1p-Pbs2p interaction (Marles et al., 2004; Zarrinpar et al., 2004; Tatebayashi et al., 2006). The P120L mutation maps to the tetraspan domain that is thought to be dispensable for Sho1p function in the HOG pathway (Raitt et al., 2000) and may identify a region of the protein that specifically regulates Sho1p function in the filamentous growth pathway.

Figure 2. The role of yapsin proteases in Msb2p processing. (A) Western blot analysis of the secretion defect of HA-Msb2p in yps mutants. Molecular mass standards (kD) are indicated on the left of the gel blots. P, pellet; S, supernatant. (B) Msb2p-GFP localization in WT, yps1Δ, and 5ypsΔ strains. Bar, 10 μm.
Cdc24p interaction is mediated by other proteins. We also tested for an interaction between Sho1p and the positional marker Bud8p (Harkins et al., 2001), which is required for unipolar budding during filamentous growth (Taheri et al., 2000; Cullen and Sprague, 2002) and for GEF recruitment during vegetative growth by the bud-site selection GTPase Rsr1p (Shimada et al., 2004). Sho1p and Bud8p were found to associate (Fig. 5B). These results are consistent with the idea that Sho1p contributes to Bud8p-dependent recruitment of Cdc24p by a mechanism that requires MAPK activation. Sho1p and Cdc24p might interact by affiliation with bud-site selection proteins; however, the Sho1p–Cdc24p interaction was independent of Rsr1p (Fig. 5A) and Bud8p (Fig. 5C).

Hyperactive alleles of \( SHO1 \), or its overexpression, induced hyperpolarized growth in a manner that was partially independent of bud-site selection cues and the MAPK pathway (Fig. 5D). These results indicate that Sho1p may also have a function in stabilization of Cdc24p at polarized sites.

**Summary**

We have identified a novel MAPK activation mechanism in yeast by processing and release of the inhibitory domain of Msb2p. Cleavage-dependent activation has been found for Notch (Schweisguth, 2004) and PAR receptors (Barry et al., 2006) and reflects a change in the activation state of Sho1p, as another gain-of-function allele that was present at higher levels (S220F; Fig. 4B), or overexpression of \( SHO1 \), failed to bypass \( msb2\Delta \) (Fig. 4D, shown for S220F). These results indicate that MAPK signaling is initiated by Msb2p and propagated through Sho1p. This conclusion may oversimplify the relationship between the proteins as they show some degree of interdependence (Fig. 4D, \( SHO1^{S220F} \) in WT compared with \( msb2\Delta \)) but is nevertheless consistent with the idea that cleavage of Msb2p is an early or initial event in MAPK activation.

**Sho1p contributes to guanine nucleotide exchange factor (GEF) polarization by multiple mechanisms**

Msb2p interacts with the GTPase Cdc42p (Cullen et al., 2004) and has genetic interactions with the GEF Cdc24p (Bender and Pringle, 1992). \( CDC24 \) showed genetic interactions with \( SHO1 \) and other filamentous growth pathway components (Fig. S3A, available at http://www.jcb.org/cgi/content/full/jcb.200704079/DC1). The Cdc24p protein also associated with Sho1p (Fig. 5A); an interaction with Msb2p was not detected (not depicted). Given the complex associations between receptors and GEFs in other contexts (Nern and Arkowitz, 1999), it is possible that the Sho1p–Cdc24p interaction is mediated by other proteins. We also tested for an interaction between Sho1p and the positional marker Bud8p (Harkins et al., 2001), which is required for unipolar budding during filamentous growth (Taheri et al., 2000; Cullen and Sprague, 2002) and for GEF recruitment during vegetative growth by the bud-site selection GTPase Rsr1p (Shimada et al., 2004). Sho1p and Bud8p were found to associate (Fig. 5B). These results are consistent with the idea that Sho1p contributes to Bud8p-dependent recruitment of Cdc24p by a mechanism that requires MAPK activation. Sho1p and Cdc24p might interact by affiliation with bud-site selection proteins; however, the Sho1p–Cdc24p interaction was independent of Rsr1p (Fig. 5A) and Bud8p (Fig. 5C). Hyperactive alleles of \( SHO1 \), or its overexpression, induced hyperpolarized growth in a manner that was partially independent of bud-site selection cues and the MAPK pathway (Fig. 5D). These results indicate that Sho1p may also have a function in stabilization of Cdc24p at polarized sites.
may extend to other members of the signaling mucin family. We have also identified a glycosylphosphatidylinositol (GPI)-anchored aspartyl protease that regulates Msb2p function. In mammalian cells, secreted aspartyl proteases are coregulated with signaling mucins (Liaudet-Coopman et al., 2006), which suggests the possibility that aspartyl proteases may be general regulators of signaling mucin receptors.

Materials and methods

Strains, plasmids, and microbiological techniques

Yeast strains are listed in Table S1 (available at http://www.jcb.org/cgi/content/full/jcb.200704079/DC1) and plasmids in Table S2. β-galactosidase assays were performed as described and represent at least two independent trials (Cullen et al., 2000). FUS1-HIS5 expression was used to confirm FUS1-lacZ reporter data and was measured by spotting equal amounts of cells onto synthetic medium lacking histidine and containing 4-amino-1,2,4-triazole. Unless otherwise indicated, the locations of the epitope tags are at 500 residues for Msb2p and at 367 residues for Sho1p. HA-Msb2p-GFP contains a GFP epitope at position 1,306 residues. All fusion proteins were functional with respect to filamentation phenotypes and MAPK reporter activity. Other functional C-terminal Msb2p fusions show similar processing intermediates as Msb2p-GFP.

Yeast and bacterial strains were manipulated by standard methods (Sambrook et al., 1989; Rose et al., 1990). The budding pattern was based on established methodology (Chant and Pringle, 1995) and by visual inspection as described previously (Cullen and Sprague, 2002) using the single cell invasive growth assay (Cullen and Sprague, 2000). The plate-washing assay (Roberts and Fink, 1994) and pseudohyphal growth assay (Gimeno et al., 1992) were performed as described previously.

The plasmid containing the MSB2 gene (pMSB2; PC1287) was identified in a genetic screen for high-copy suppressors of the signaling defect of an msb2Δ mutant (PC948) using a galactose-inducible...
To examine the interaction between Sho1p and Cdc24p, co-immunoprecipitation (co-IPT) analysis was performed on extracts derived from PC1652. To measure the interaction in a strain lacking Rsr1p, co-IPT analysis was performed from extracts derived from PC2281. For the Sho1p–Bud8p interaction, PC2249 was transformed with pBUD8-GFP (PC1883) and a control plasmid. Co-IPT analysis was based on previous methodology (Kemp and Sprague, 2003). 100 ml of exponentially grown cells were pelleted at room temperature for 5 min at 3,000 g. Cells were resuspended in 4 ml of DTT buffer (50 mM Tris-HCl, pH 7.5, and 10 mM DTT) and incubated at room temperature for 15 min. The cells were pelleted at room temperature for 5 min at 15,800 g, and pellets were resuspended in 3.75 ml of sphero-plast buffer (1.2 M sorbitol, 50 mM potassium phosphate, pH 7.4, 1 mM MgCl, and 250 μg/ml of zymolyase) and incubated in 30 °C on an end-to-end rotator for 1 h. Spheroplasts were centrifuged for 2 min at 2,300 g. Cell pellets were washed in 1 ml of 1.2 M sorbitol and centrifuged for another 1 h. IPT complexes were pelleted at 15,800 g for 1 min. Beads were washed in IPT buffer three times for 1 min each time. The protein concentration of precleared supernatants was determined by the Bradford Coomassie blue assay. 5–7 mg of proteins were immunoprecipitated with a polyclonal antibody in 1:75 dilution for 1 h, and complexes were pulled down by adding 75 μl of protein G and incubating for another 1 h. Beads were washed in IPT buffer three times for 1 min each time. The standard deviation of the values derived from at least two separate experiments.

Protein analysis
Secreted proteins were analyzed from conditioned media that was passed through a 2-μm filter to remove cells. Western blots were performed as described previously (Cullen et al., 2004). Monoclonal mouse anti-HA antibodies (12CA5.16.4), monoclonal goat anti-GFP antibodies (Rockland Scientific International), and monoclonal antibodies to Dpm1p were used.

Co-immunoprecipitation (co-IPT) analysis
To examine the interaction between Sho1p and Cdc24p, co-IPT analysis was performed on extracts derived from PC1652. To measure the interaction in a strain lacking Rsr1p, co-IPT analysis was performed from extracts derived from PC2281. For the Sho1p–Bud8p interaction, PC2249 was transformed with pBUD8-GFP (PC1883) and a control plasmid. Co-IPT analysis was based on previous methodology (Kemp and Sprague, 2003). 100 ml of exponentially grown cells were pelleted at room temperature for 5 min at 3,000 g. Cells were resuspended in 4 ml of DTT buffer (50 mM Tris-HCl, pH 7.5, and 10 mM DTT) and incubated at room temperature for 15 min. The cells were pelleted at room temperature for 5 min at 15,800 g, and pellets were resuspended in 3.75 ml of sphero-plast buffer (1.2 M sorbitol, 50 mM potassium phosphate, pH 7.4, 1 mM MgCl, and 250 μg/ml of zymolyase) and incubated in 30 °C on an end-to-end rotator for 1 h. Spheroplasts were centrifuged for 2 min at 2,300 g. Cell pellets were washed in 1 ml of 1.2 M sorbitol and centrifuged for 4 min at low speed as before. Spheroplasts were lysed in 1 ml of ice-cold IPT buffer (50 mM Tris-HCl, pH 8, 1 mM EDTA, 50 mM NaCl, and 1.5% (w/v) Brij-35) supplemented with 1× protease inhibitor cocktail, EDTA free, and 1 mM PMSF before use. Lysates were centrifuged at 4 °C for 20 min at 15,800 g. Supernatants were precleared by incubating with 75 μl of immobilized Protein G plus (Thermo Fisher Scientific) on an end-to-end rotator for 30 min. Beads were resuspended at 4 °C for 10 min at 15,800 g. The protein concentration of precleared supernatants was determined by the Bradford Coomassie blue assay. 5–7 mg of proteins were immunoprecipitated with a polyclonal antibody in 1:75 dilution for 1 h, and complexes were pulled down by adding 75 μl of protein G and incubating for another 1 h. Beads were washed in IPT buffer three times for 1 min each time. 50 μl of Thorner buffer (8 M urea, 5% SDS, 40 mM Tris-HCl, pH 6.8, 0.1 M EDTA, 0.4 mg/ml...
Bromophenol blue, and 1% β-mercaptoethanol) was added to beads, and IPT complexes were released from beads by boiling for 10 min. Beads were removed by centrifuging for 1 min at 15,800 g, after which the pellet was discarded. Supernatants (protein complexes) were either immediately processed by SDS-PAGE or frozen at −80°C.

Microscopy

Differential interference contrast and fluorescence microscopy of the GFP protein using FITC filter sets were performed using an fluorescent microscope (Axioplan 2; Carl Zeiss, Inc.) with a Plan-Apochromat 100x/1.4 (oil) objective (NA 0.17; Carl Zeiss, Inc.). For most experiments, cells were visualized by resuspending in water at 25°C. Digital images were obtained with a camera (AxioCam MRm; Carl Zeiss, Inc.). Axiosvision 4.4 software (Carl Zeiss, Inc.) was used for image acquisition and analysis. Images were further analyzed in Photoshop (Adobe), where adjustments of brightness and contrast were made.

SHO1 mutagenesis

Mutagenesis of the SHO1 gene was performed by plasmid mutagenesis based on a protocol for direct transformation into yeast (Rose and Fink, 1987). Efficiency was 5% as determined by the frequency of sho1 null mutations. An allele of SHO1 was used (S171C and NF300GS; Marles et al., 2004) that fully complemented the sho1 mutant. Hyperactive alleles were selected on media lacking histidine and containing 4-amino-1,2,4-triazole by resuspending in water at 25°C. Digital images were obtained with a camera (AxioCam MRm; Carl Zeiss, Inc.). Axiosvision 4.4 software (Carl Zeiss, Inc.) was used for image acquisition and analysis. Images were further analyzed in Photoshop (Adobe), where adjustments of brightness and contrast were made.

Western blots and protein analysis

Proteins were separated by SDS-PAGE on 10% precast gels (Bio-Rad Laboratories) and transferred to nitrocellulose membranes (protran BA85, Pharmacia). Proteins were separated by SDS-PAGE on 10% precast gels (Bio-Rad Laboratories) and transferred to nitrocellulose membranes (protran BA85, Pharmacia). The secondary antibodies were O-GLYCBASE version 2.0 (Center for Biological Sequence Analysis, Technical University of Denmark; Hansen et al., 1997). The secondary antibody was O-GLYCBASE version 2.0: a revised database of O-glycosylated proteins. Western blotting reagents were selected on media lacking histidine and containing 4-amino-1,2,4-triazole using the FUS1-HIS3 reporter (PC1531) integrated into ste4Δ 1Δ2766 strains (Cullen et al., 2004). 253,580 URA3+ colonies were screened and 100 AIA resistant isolates were identified, of which six were plasmid-dependent; five of the six resulted in the P120L change and were identified in separate pools. Plasmid-dependent mutations were analyzed by sequence identification in the Roswell Park sequencing facility.

Online supplemental material

Figure S1 shows the secretion defect of Msb2p in yeast and in cell wall mutant combinations. Figure S2 shows analysis of the SHO1Δ256 allele. Figure S3 shows genetic data between filamentous growth pathway components and Cdc24p. Table S1 lists yeast strains. Table S2 lists plasmids used in this study. Table S3 shows analysis of secretion of the Msb2p-HA protein in protease mutants. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200704079/DC1.

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