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Aspergillus nidulans Pmts form heterodimers in all pairwise combinations

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ABSTRACT

Eukaryotic protein *O*-mannosyltransferases (Pmts) are divided into three subfamilies (Pmt1, Pmt2, and Pmt4) and activity of Pmts in yeasts and animals requires assembly into complexes. In *Saccharomyces cerevisiae*, Pmt1 and Pmt2 form a heteromeric complex and Pmt 4 forms a homomeric complex. The filamentous fungus *Aspergillus nidulans* has three Pmts: PmtA (subfamily 2), PmtB (subfamily 1), and PmtC (subfamily 4). In this study we show that *A. nidulans* Pmts form heteromeric complexes in all possible pairwise combinations and that PmtC forms homomeric complexes. We also show that MsbA, an ortholog of a Pmt4-modified protein, is not modified by PmtC.

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1. Introduction

Protein *O*-mannosylation is a type of protein glycosylation found in prokaryotes and eukaryotes [1–3]. In eukaryotes, protein *O*-mannosyltransferases (Pmts) are integral membrane proteins localized in the endoplasmic reticulum (ER) [4,5]. Pmts transfer a mannose residue from dolichyl phosphate mannose to the hydroxyl residue of serine or threonine in secreted proteins facing the luminal side of the ER [6,7]. Further elongation of *O*-mannosyl glycans takes place in the Golgi using a different set of enzymes and GDP-sugar [1]. *O*-mannosylation is important for stability, localization and function of secreted proteins [8–10] and a total lack of *O*-mannosylation is lethal in eukaryotes. Pmts in eukaryotes are grouped into 3 subfamilies with names based on the *Saccharomyces cerevisiae* enzymes Pmt1, Pmt2 and Pmt4 [11]. *S. cerevisiae* has 7 Pmts; *Candida albicans* has 5 [12]; and *Aspergillus nidulans* and other filamentous fungi have 3, one from each subfamily [2,13].

In *S. cerevisiae* Ser/Thr-rich domains of secreted proteins are likely to be mannosylated and membrane-associated proteins are mannosylated by Pmt4 [14,15]. But beyond these generalizations, the consensus sequence directing *O*-mannosylation is not known [3]. Nonetheless, some targets of Pmts have been found empirically and these exhibit specificity toward individual target proteins [8,16]. However, there is evidence of limited substrate overlap. For example, the *S. cerevisiae* Pmt4 and Pmt1/Pmt2 complex mannosylate different domains of Ccw5p [17].

O-mannosyltransferase activity requires a Pmt complex [18–20]. In *S. cerevisiae* and *S. pombe*, members of the Pmt1 subfamily form heteromeric complexes with members of the Pmt2 subfamily [13,21]. The Pmt4 subfamily of *S. cerevisiae* forms a homomeric complex [13].

In previous work, both our group and another group independently showed that the filamentous fungus *A. nidulans* has three *pmts* each representing a different subfamily: PmtA from subfamily 2, PmtB from subfamily 1, and PmtC from subfamily 4. Both groups also showed that *ΔpmtA*, *ΔpmtB*, *ΔpmtC* and the double *ΔpmtA ΔpmtB* were viable and that each null mutant had a distinctive phenotype [22,23]. These results strongly suggested that either PmtA and Pmt B do not form complexes in *A. nidulans* as the orthologous Pmt2 and Pmt1 do in *S. cerevisiae*, or that such subfamily 1/subfamily 2 complexes are not required for viability in *A. nidulans* as they are in *S. cerevisiae*. In this study we tested the three *A. nidulans* Pmts for the ability to form heteromeric and homomeric complexes. We also examined modifications of the *A. nidulans* ortholog of *S. cerevisiae* Msb2, a HOG pathway osmosensor modified by Pmt4 [24,25].

Abbreviations: HA, hemagglutinin; HOG, high osmolarity glycerol; Pmt/PMT, protein *O*-mannosyltransferase

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2. Materials and methods

2.1. Aspergillus strains and media

The *A. nidulans* strains used in this study (Table 1) were incubated in complete and minimal media (CM and MM) with supplements as previously described [22]. Genetic manipulations were carried out using standard *A. nidulans* protocols as previously described [22].

2.2. Construction of tagged Pmts

Strains bearing single copy epitope tagged Pmts were constructed by fusion PCR [26] using primers listed in Table 2. Primer names in Table 2 indicate tag identity and whether primers are upstream or downstream of the designated *pmt* gene. Amplicons were purified and transformed into ATK45. Homologous integration resulting in strains bearing a single tagged Pmt replacing the original Pmt was verified by PCR and Southern. All strains constructed along with detailed genotypes are shown in Table 1.

2.3. Construction of S-tagged Pmt target proteins

The GA4 S-tag fragment with stop codon was amplified from pAO81 and the gene of interest was amplified from the start codon to one codon before the stop codon. Amplicons were fused by PCR, ligated into the pENTR/D-TOPO vector using the pENTR/D-TOPO Cloning Kit (Invitrogen Co., CA) and transferred into the pMT-DV2 destination vector using Gateway LR Clonase II (Invitrogen Corp., CA) and transformed into A850 and Δ*pmt* strains. All strains constructed along with detailed genotypes are shown in Table 1. All primer sequences are shown in Table 2. Primer names in Table 2 indicate tag identity and whether primers are upstream or downstream of the designated *pmt* gene.

2.4. Membrane fraction preparation

1×10^8 conidia/ml of the specified tagged-PMT strain were inoculated to CM (50 mL for target protein extraction and 1 L for immunoprecipitation) and shaken at 200 rpm and 30°C for 8 h. Mycelia were filtered, washed with cold stop buffer (0.9% NaCl, 1 mM NaN_3, 10 mM EDTA, 50 mM NaF, pH 7.0), and ground in liquid nitrogen. Two milliliters of cold extraction buffer (50 mM Tris–HCl, pH 7.5, 0.3 mM $MgCl_2$ plus protease Inhibitors (Complete, Mini, EDTA-free; Protease Inhibitor Cocktail Tablets, Roche) were added to 1 g ground mycelia and vortexed for 10 min at 4 °C. The cell suspension was centrifuged at 500×*g* for 10 min at 4°C. The supernatant was collected and centrifuged for 30 min at 20,000 rpm at 4 °C (Sorvall SS34 rotor). One mL buffer containing 50 mM Tris–HCl, pH 7.5, 7.5 mM $MgCl_2$, and 15% glycerol was added per 1 mL of pellet and stored at −80°C. Protein was quantified with RC DC Protein Assay Kit (Bio-Rad Laboratories, CA) using bovine serum albumin as a standard.

2.5. Immunoprecipitation

Immunoprecipitation methods were adapted from Girrbach and colleagues [7]. Twenty milligrams of membrane fraction was solubilized in 4 mL of lysis buffer (20 mM Tris–HCl, pH 7.5, 140 mM NaCl, 0.3 mM $MgCl_2$, 10% glycerol, 0.35% sodium deoxycholate, 0.5% Triton X-100 plus Protease Inhibitor (Complete, Mini, EDTA-free; Protease Inhibitor Cocktail Tablets, Roche). One hundred μL of agarose immobilized anti-epitope tag antibody slurry was added per 20 mg of protein. Agarose immobilized rabbit anti-S tag or

Table 1
A. nidulans strains and plasmids.

Strain	Genotype/phenotype	Source or reference
A850	*argB2::trpC_B methG*	FGSC*
ATK08	*pyrG89 argB2::trpC_B pyroA4 ΔpmtA::AfpyrG*	[22]
ATK16	*pyrG89 ΔpmtB::AfpyrG argB2 pyroA4*	[22]
ATK38	*pyrG89 wA3 argB2 pyroA4 ΔpmtC::AfpyrG*	[22]
ATK42	*pyrG89 wA3 argB::trpC_B pyroA4*	This study
ATK45	*pyrG89 argB::trpC_B nkuA::Afpyro pyroA4*	[22]
ATK89	*pyrG89 argB::trpC_B nkuA::Afpyro pyroA4 pmtA::*S-tag-*AfpyrG*	This study
ATK95	*pyrG89 argB2::trpC_B pyroA4 ΔpmtA::Afpyro*	This study
ATK103	ATK38::*AfargB-gpd(P)*- ANID_01359-S-tag	This study
ATK104	*pyrG89 wA3 ΔpmtB::Afpyro argB2 pyroA4*	[22]
ATK154	*pyrG89 argB::trpC_B nkuA::Afpyro pyroA4 pmtC::*HA-tag-*AfargB*	This study
ATK165	*pyrG89 argB::trpC_B pyroA4 pmtA::*S-tag-*AfpyrG pmtC::*HA-tag-*AfargB*	This study
ATK168	*pyrG89 ΔpmtB::Afpyro argB::trpC_B pyroA4 pmtA::*S-tag-*AfpyrG pmtC::*HA-tag-*AfargB*	This study
ATK172	A850::*AfargB-gpd(P)*-ANID_07041(MsbA)-S-tag	This study
ATK177	ATK16::*AfargB-gpd(P)*-AN ID_07041(MsbA)-S-tag	This study
ATK179	ATK08::*AfargB-gpd(P)*-AN ID_07041(MsbA)-S-tag	This study
ATK184	ATK38::*AfargB-gpd(P)*-AN ID_07041(MsbA)-S-tag	This study
ATK187	*pyrG89 pmtB::*HA-tag-*AfargB argB::trpC_B nkuA::Afpyro pyroA4*	This study
ATK192	*pyrG89 pmtB::*HA-tag-*AfargB argB::trpC_B pyroA4 pmtA::*S-tag-*AfpyrG*	This study
ATK193	*pyrG89 pmtB::*HA-tag-*AfargB argB::trpC_B pyroA4 pmtC::*S-tag-*AfpyrG*	This study
ATK195	ATK149 X ATK104	This study
ATK200	*pyrG89 pmtB::*HA-tag-*AfargB argB::trpC_B pyroA4ΔpmtA::Afpyro pmtC::*S-tag-*AfpyrG*	This study
ATK208	*pyrG89 argB::trpC_B pyroA4 pmtC::*S-tag-*AfpyrG*	This study
ATK211	ATK177 X ATK95	This study
ATK217	*pyrG89/pyrG89 argB::trpC_B/argB::trpC_B pyroA4/pyroA4 pmtC::*S-tag-*AfpyrG/pmtC::*HA-tag-*AfargB*	This study
Plasmids		
pAfargB2	Ampr *argB2*	G. S. May
pAO81	GA4-S-Tag *AfpyrG*	[26]
pDV2	Ampr *argB-gpd(p)-ccdB-sgfp*	[28]
pMT-3xHA	Ampr *argB-alcA(p)-ccdB-3xHA*	[28]
pFNO3	Kanr GA5-GFP *AfpyrG*	[26]
pTK74	pDV2::ANID_07041-S-tag	This study

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Table 2
Primers.

Primer name	Sequence
Pmt2FWent	CACCATGGCTGAAATTGGCTTTG
Pmt2RVnostop	GTTAGCGATTCGCCAACCG
pmt2-GA4Fw	GAGCGGTTGGCGAATCGCTAACGGAGCTGGTGCAGGCGC
Stag-pmt2RV	GAACTGTAACGCTCTATCACGGTTCCTGTCTGAGAGGAGGCACTGATGC
pmt2DnFW	GAACCGTGATAGAGCGTTACAGTTCCCGTTG
pmt2DNRV	GCGATCCCAATCCTTCCTATCTCTGTCATC
PMT2F301	CCGAATCGGCCGCTCAAACATCGTG
PMT2R6311	TGCGACTGGGCGAGAAAGCGTGAGG
up177PMT2F	TCCGCAGTCGCCCGCCAGATATGAG
StagR	GCGCCAATTGCTGTTGCCAGGTGAGG
PMT11093F	GTGCTTGGTCAATCCGGGAGAGGG
Pmt1RVnostop	ACGGGTCGCCTCGTTGCCTTC
PMT1-GA5F	GCAACGAGGCGACCCGTGGAGCTGGTGCAGGCGC
argBAf-PMT1DnR	CAACAGACCGACATATTATCTCATAGGATTTTCCCCTTG
Pmt1dn-FW	GATAATATGTCGGTCTGTTGAACTACCTGCC
PmtBdnR2	CTGAGAGGGAGTTCGGAAACGTGC
463Pmt4F	GTCCCCTATGTCGCCCTCCG
Pmt4RVnostop	TTTCGCGAAGTGCAAGTCATAGC
Pmt4GA4F	CTATGACTTGCACTTCGCGAAAGGAGCTGGTGCAGGCGCTGGAG
pyrGAFRV-Pmt4SPRV	AGAACGAGATGAGATCAGATGCTCCTGTCTGAGAGGAGGCACTGATGCG
Pmt4dnFW	GAGCATCTGATCTCATCTCGTTCTCTCCC
Pmt4dnRV	CTATCCACGGTATGAGCTGAGCGAGTAATG
argBpmt4RV	AGAACGAGATGAGATCAGATGCTCCGATTTTCATAGGATTTTCCCCTTG
StagR	GCGCCAATTGCTGTTGCCAGGTGAGG
MSB2entF	CACCATGGTTTCCCAGACGGCTC
MSB2-StagR	GCGCCTGCACCAGCTCCGTTCCATCCCAGAGAGTTC
StagF	GGAGCTGGTGCAGGCGCTGGAGC
StagR	GCGCCAATTGCTGTTGCCAGGTGAGG

rabbit anti-HA antibody was used for immunoprecipitation (Immunology Consultants Laboratory, Inc., Newberg, OR). Incubation with the solubilized membrane fraction was carried out at 4°C on a rocker for 2 h followed by 5 washes at 4°C with equal volume of cold lysis buffer and one wash with 1 mL cold Tris-buffered saline. Agarose beads were resuspended in 50 μL cold Tris-buffered saline. The bound proteins were eluted with 3 × SDS loading dye by 5 min at 95°C.

2.6. Western blot analyses

20 μL per lane of epitope-tagged Pmt eluent was loaded into 2 gels to be further probed with anti-S tag or anti-HA antibody. The proteins were resolved on 4–20% gradient Tris–HEPES SDS gels (Pierce Protein Gels, Thermo Fisher Scientific Inc., IL) and transferred to a nylon membrane. S-tagged Pmts were detected with mouse anti-S-tag antibody (1:5000) (Abcam Inc., MA), followed by Sheep anti-Mouse IgG ECL antibody coupled to horseradish peroxidase (1:5000) (GE Healthcare, NJ). HA-tagged proteins were detected with mouse anti-HA-tag antibody (1:5000) (Invitrogen Co., CA), followed by Sheep anti-Mouse IgG ECL antibody coupled to horseradish peroxidase (1:5000) (GE Healthcare, NJ).

For Pmt target protein analyses, an equal volume of 2 × SDS–PAGE sample buffer [27] was added to membrane fractions and heated for 5 min at 95°C. Forty μg solubilized protein were loaded per lane and detected as described above.

3. Results and discussion

To investigate Pmt complex formation in *A. nidulans*, we constructed the following strains with C-terminal purification tags: ATK89 ($PmtA^S$), ATK187 ($PmtB^{HA}$) and ATK154 ($PmtC^{HA}$). Because we were concerned that over-expression of individual Pmts might alter interactions within complexes, we constructed these strains by replacing native Pmts with the corresponding tagged protein using homologous integration. In these strains, the tagged Pmt fusion constructs were integrated at native Pmt loci behind native Pmt promoters resulting in expression levels as close to wildtype as possible. Correct integrations were confirmed by PCR and Southern hybridization. All tagged Pmt strains showed wildtype phenotypes at 30°C and 42°C (data not shown). The S and HA tags are both small, with deduced molecular mass of 2.26 kDa and 3.98 kDa, respectively. The predicted molecular mass of $PmtA^S$ is 86.86 kDa, of $PmtB^{HA}$ is 108.98 kDa, and of $PmtC^{HA}$ is 92.28 kDa.

Attempts to detect tagged Pmts by western blot of total membrane fractions were unsuccessful, suggesting low levels of fusion protein. Therefore, immunoprecipitation of solubilized membrane fractions with agarose-immobilized anti-HA or anti-S tag antibodies was used, followed by SDS–PAGE and immunoblot (Fig. 1). The $PmtA^S$ strain yielded two bands with apparent molecular mass of approximately of 68 kDa and 165 kDa. The $PmtB^{HA}$ strain yielded two bands with apparent molecular mass of approximately 120 kDa and 180 kDa. The $PmtC^{HA}$ strain yielded two bands with apparent molecular mass of approximately 80 kDa and 160 kDa. In all cases, the lower band is likely the Pmt based on predicted molecular mass, strength of the signal and reproducibility. Pmts isolated from yeasts and animals frequently show apparent molecular masses that are smaller or larger than predicted [13,18]. Girrbach and Strahl reported similar double band patterns in *S. cerevisiae* blue native PAGE experiments with the higher bands resulting from heteromeric PMT complex formation [13]. However, our experiments included SDS so that proteins are expected to be denatured. Further these bands always appear at the same molecular mass for specific Pmts and their presence in strains carrying a Pmt deletion along with a tagged Pmt suggests that they are not heterodimers. A similar double band pattern was reported when the human PMT ortholog Pomt1 was expressed in HEK293T cells. In this case the authors speculated that the upper band resulted from protein aggregation due to Pomt1 over-expression [18]. Though the Pmts in our study were all expressed from endogenous promoters rather than over-expressed, it is possible that the higher bands seen in our immunoblots resulted from aggregation of these hydrophobic proteins during processing. It is also possible that the upper bands represent homodimers of tagged Pmts.

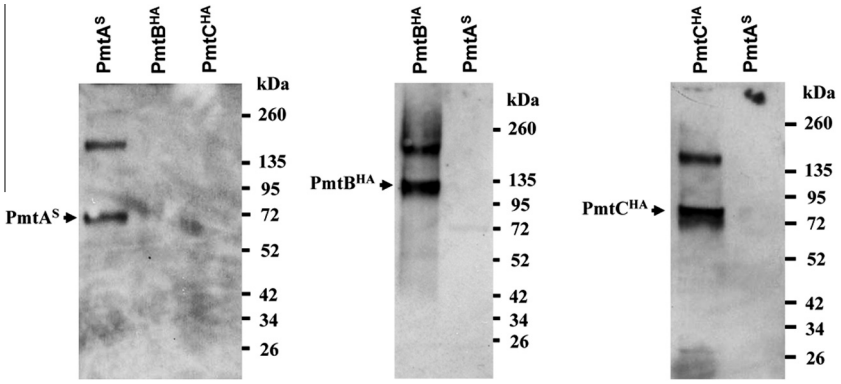

Fig. 1. Immunoprecipitation of Epitope-tagged Pmts. Western blot analyses of immunoprecipitates from ATK89 (PmtA^S), ATK187 (PmtB^{HA}) and ATK154 (PmtC^{HA}). Proteins were expressed from their native loci under the control of their endogenous promoters. Solubilized membrane-enriched fractions were immunoprecipitated with the corresponding agarose immobilized antibody against the epitope tag. Immunoprecipitates were resuspended in SDS loading dye and resolved on 4–20% gradient Tris–HEPES–SDS–polyacrylamide gels, transferred to membranes, and probed with anti-S tag antibody (left) or anti-HA antibody (two blots on right). Arrows indicate Pmts.

3.1. PmtA (subfamily 2) forms heteromeric complexes with PmtB (subfamily 1)

To investigate whether *A. nidulans* PmtA and PmtB form a heteromeric complex like their *S. cerevisiae* orthologs, Pmt2 and Pmt1 [13], we derived a PmtA^S PmtB^{HA} strain (ATK192) from sexual crosses. Immunoprecipitation was performed with agarose-immobilized anti-HA antibodies and agarose-immobilized anti-S tag antibody. The immunoprecipitate was divided into two aliquots. One aliquot was probed with anti-S tag antibody and the other was probed with anti-HA antibody. When probed with anti-S tag antibody, the same bands that were seen in the PmtA^S strain were visible (approximately 68 kDa and 165 kDa) (Fig. 2). When probed with anti-HA antibody, the same bands that were seen in the PmtB^{HA} strain were visible (approximately 120 kDa and 180 kDa) (Fig. 2). Reciprocal experiments performed using anti-HA antibodies for immunoprecipitation before immunoblotting showed very high background, though the results were consistent with heteromeric complex formation by PmtA and PmB (data not shown). Our results show that PmtA and PmtB form heteromeric complexes in *A. nidulans*.

3.2. PmtA (subfamily 2) forms heteromeric complexes with PmtC (subfamily 4)

In *S. cerevisiae*, Pmt2 and Pmt4 do not form complexes [13]. To investigate the interactions between the *A. nidulans* orthologs, strains carrying PmtA^S and PmtC^{HA} in the presence of PmtB (*pmtA*::S tag, *pmtC*::HA, ATK165) and absence of PmtB (*pmtA*::S tag, *pmtC*::HA, Δ*pmtB*; ATK168) were derived from sexual crosses. Immunoprecipitations were performed with agarose-immobilized anti-S tag antibody as described above. When probed with anti-HA antibody, Western blots of immunoprecipitates from both strains showed a band of approximately 80 kDa whether or not PmtB was present (Fig. 3). Reciprocal experiments performed using anti-HA antibodies for immunoprecipitation before immunoblotting were consistent with heteromeric complex formation by PmtA and PmtC (Fig. S1). Our results show that *A. nidulans* PmtA forms a heteromeric complex with PmtC in the presence and absence of PmtB.

3.3. PmtB (subfamily 1) forms heteromeric complexes with PmtC (subfamily 4)

To investigate the interactions between PmtB and PmtC, a strain bearing PmtB^{HA} and PmtC^S in the presence of PmtA (*pmtB*::HA, *pmtC*::S tag; ATK193) and absence of PmtA (*pmtB*::HA, *pmtC*::S tag, Δ*pmtA*; ATK200) were derived from sexual crosses. Immunoprecipitation and Western blots were performed as described above. Immunoblots probed with anti-HA antibody showed a band of approximately 120 kDa whether or not PmtA was present (Fig. 4). Reciprocal experiments performed using anti-HA antibodies for immunoprecipitation before immunoblotting showed very high background, though the results were consistent with heteromeric complex formation by PmtB and PmtC (data not shown). Our

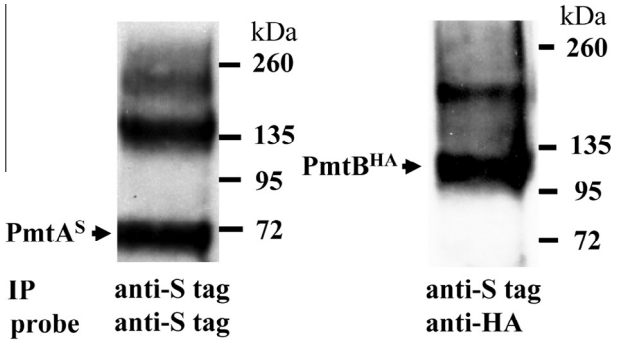

Fig. 2. Immunoprecipitation of PmtA-PmtB heteromeric complexes. Solubilized membrane-enriched fractions from ATK192 (PmtA^S PmtB^{HA}) were immunoprecipitated with agarose immobilized anti-S tag antibody. Co-immunoprecipitates were resuspended in SDS loading dye and resolved on 4–20% gradient Tris–HEPES–SDS–polyacrylamide gels, transferred to membranes, and probed with anti-S tag antibody (left) or anti-HA antibody (right). Arrows indicate Pmts.

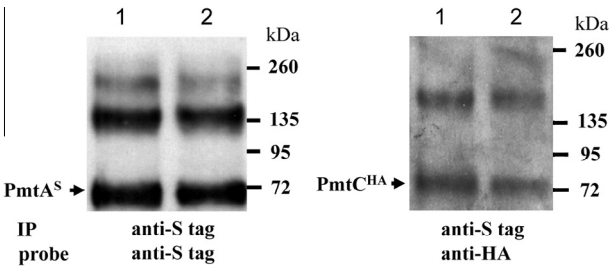

Fig. 3. Immunoprecipitation of PmtA-PmtC heteromeric complexes. Solubilized membrane-enriched fractions from ATK168 (PmtA^S, PmtC^{HA}, Δ*pmtB;* lane 1) and ATK165 (PmtA^S, PmtC^{HA}; lane 2) were immunoprecipitated with agarose immobilized anti-S-tag antibody. Co-immunoprecipitates were resuspended in SDS loading dye and resolved on 4–20% gradient Tris–HEPES–SDS–polyacrylamide gels, transferred to membranes, and probed with anti-S tag antibody (left) or anti-HA antibody (right). Arrows indicate Pmts.

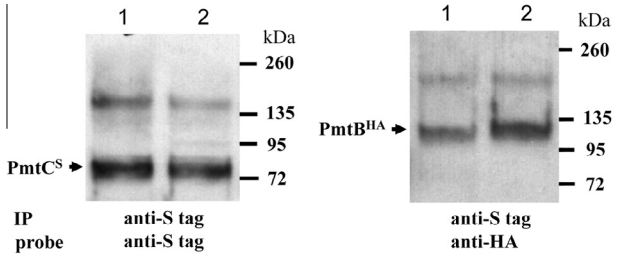

Fig. 4. Immunoprecipitation of PmtB-PmtC heteromeric complexes. Solubilized membrane-enriched fractions were immunoprecipitated with agarose immobilized anti-S-tag antibody. Lane 1 is co-immunoprecipitate from ATK200 ($PmtB^{HA}$, $PmtC^{S}$, Δ*pmtA*), lane 2 is co-immunoprecipitate from ATK193 ($PmtB^{HA}$, $PmtC^{S}$). Co-immunoprecipitates were resuspended in SDS loading dye and resolved on 4–20% gradient Tris–HEPES–SDS–polyacrylamide gels, transferred to membranes, and probed with anti-S tag antibody (left) or anti-HA antibody (right).

results show that *A. nidulans* PmtB forms a heteromeric complex with PmtC in the presence and absence of PmtA.

3.4. PmtC (subfamily 4) forms homomeric complexes

In *S. cerevisiae*, Pmt4 functions as a dimer, not a monomer [13]. To investigate whether the *A. nidulans* ortholog, PmtC, is a monomer or forms homomeric complexes in *A. nidulans*, a stable diploid $PmtC^{S}$ $PmtC^{HA}$ strain (*pmtC*::S tag/*pmtC*::HA; ATK217) was constructed. Immunoprecipitation and Westerns were performed as described above and probed with anti-S tag antibody or anti-HA antibody. In both cases the same bands that were seen in the $PmtC^{HA}$ strain were visible (approximately 80 kDa and 160 kDa) (Fig. 5). Reciprocal experiments performed using anti-HA antibodies for immunoprecipitation before immunoblotting were consistent with homomeric complex formation by PmtC (Fig. S1). Our results show that *A. nidulans* forms a PmtC homomeric complex as is true for the orthologous *S. cerevisiae* Pmt4 [13].

Genetic evidence from our previous work suggests that this PmtC–PmtC homomeric complex is sufficient to support growth because the Δ*pmtA* Δ*pmtB* strain is viable [22]. Despite repeated attempts, we were unable to recover appropriately marked strains to investigate PmtA–PmtA and PmtB–PmtB homomeric complex formation. However, if PmtA–PmtA and PmtB–PmtB homomeric complexes exist, neither appears to be sufficient to support *A. nidulans* growth in the absence of the other Pmts since both the Δ*pmtA* Δ*pmtC* and Δ*pmtB* Δ*pmtC* double mutants are inviable.

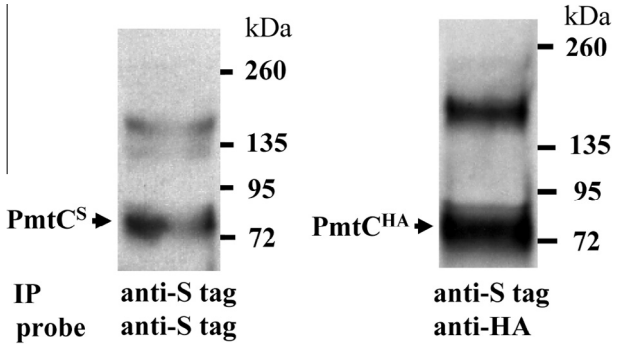

Fig. 5. Immunoprecipitation of PmtC-PmtC homomeric complexes. A solubilized membrane enriched fraction from diploid strain ATK217 ($PmtC^{S}$ coexpressed with $PmtC^{HA}$) was immunoprecipitated with agarose immobilized anti-S tag antibody. Co-immunoprecipitate was resuspended in SDS loading dye and resolved on 4–20% gradient Tris–HEPES–SDS–polyacrylamide gels, transferred to membranes, and probed with anti-S tag antibody (left) or anti-HA antibody (right).

3.5. Pmt complexes are formed in vivo

To eliminate the possibility that the *A. nidulans* Pmt complexes we detected might result from nonspecific aggregation of Pmts in solubilized membrane fractions, we performed the following control experiment. Membrane fractions were isolated from strains in which S tagged and HA tagged Pmts were co-expressed, and solubilized. Membrane fractions were also isolated separately from strains in which S tagged Pmts were expressed, and from strains in which HA tagged Pmts were expressed. Isolated membrane fractions were combined and solubilized. Agarose-immobilized anti-S tag antibody was used to immunoprecipitate protein from solubilized membranes of the strains co-expressing S tagged and HA tagged Pmts or from the combined and solubilized membranes of the strains separately expressing tagged Pmts. Identical aliquots were probed with anti-S tag antibody or anti-HA antibody. The co-expressed Pmts co-precipitated, but the separately expressed and combined Pmts did not, suggesting that the Pmt complexes we detected in earlier experiments were formed *in vivo* and not the result of nonspecific protein aggregation (Fig. 6).

3.6. MsbA, an ortholog of a Pmt4-modified protein, is not modified by PmtC

In *S. cerevisiae*, Msb2, an osmosensor in the HOG pathway, is modified by Pmt4 [24,25]. Using the *S. cerevisiae* Msb2 protein sequence to query the *A. nidulans* genome database at the Broad Institute (http://www.broad.mit.edu) yielded ANID_07041.1, which we named "MsbA". $MsbA^{S}$ was expressed from the *gpd* promoter in wildtype and Δ*pmt* mutants, membrane fractions were isolated and probed with anti-S tag antibodies in Westerns (Fig. 7). The apparent molecular mass of MsbA in the Δ*pmtA* and Δ*pmtB* mutants was 160 and 170 kDa respectively, while the molecular mass of $MsbA^{S}$ in Δ*pmtA*Δ*pmtB* mutant appeared lower at 150 kDa. The apparent molecular mass of MsbA in the Δ*pmtC* mutant was approximately 240 kDa, which was the same as that in the wildtype. MsbA possesses a putative *N*-glycosylation site. The molecular mass of PNGase F treated $MSbA^{S}$ in Δ*pmtA*, Δ*pmtB* and Δ*pmtC* was unchanged (data not shown). Our results suggest that MsbA is modified by PmtA and PmtB, but not by PmtC, and that PmtA and PmtB make separate modifications to MsbA.

In *S. cerevisiae* both Pmt subfamily 1 and Pmt subfamily 2 have two members (Fig. 8). In immunoprecipitation experiments, the predominant interaction partner for Pmt1 is Pmt2, though low levels of Pmt3 (from subfamily 2) are also reproducibly precipitated. Similarly, the predominant interaction partner for Pmt 5 (from subfamily 1) is Pmt3, though low levels of Pmt2 are also precipitated [13]. The Pmt 4 subfamily has only one member and it only forms homodimers. Thus in *S. cerevisiae* five Pmt complexes exist and low level complexes can substitute if the predominant complexes are lost.

In contrast *A. nidulans* contains only 3 Pmts, one from each subfamily (Fig. 8). Not surprisingly, we found that PmtA (subfamily 2) interacts with Pmt B (subfamily 1) and PmtC (subfamily 4) interacts with itself. More surprisingly, we also found that both PmtA and Pmt B interact with Pmt C (subfamily 4). Because we were unable to generate appropriately marked diploid strains, we do not know if PmtA and Pmt B can also form homodimers and because single copy Pmts driven by native promoters were not produced in large quantities, we were unable to address quantitative issues such as which complexes predominate. However, it is clear that in *A. nidulans* at least four Pmt complexes exist, though it is not clear which complexes predominate. *A. nidulans* is the first fungus reported to form heteromeric complexes involving the Pmt4 subfamily, though such complexes have been reported in animals where POMT1 (subfamily 4) forms a heteromeric complex with

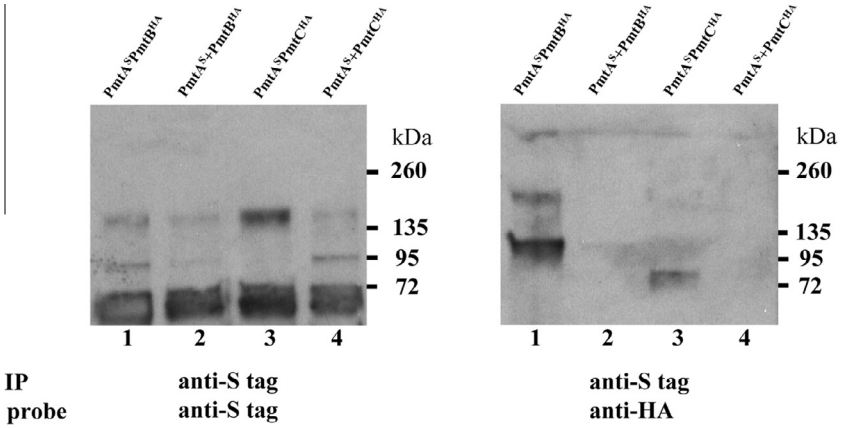

Fig. 6. Pmt complexes form *in vivo*. Solubilized membrane enriched fractions or combined solubilized membrane fractions were immunoprecipitated with agarose immobilized anti-S tag antibody. Lane 1 is co-immunoprecipitate from ATK192 ($PmtA^S$, $PmtB^{HA}$). Lane 2 is co-immunoprecipitate from membrane fractions of ATK89 ($PmtA^S$) combined with membrane fractions of ATK187 ($PmtB^{HA}$). Lane 3 is co-immunoprecipitate from ATK165 ($PmtA^S$, $PmtC^{HA}$). Lane 4 is co-immunoprecipitate from membrane fractions of ATK89 ($PmtA^S$) combined with membrane fractions of ATK154 ($PmtC^{HA}$). Co-immunoprecipitates were resuspended in SDS loading dye and resolved on 4–20% gradient Tris–HEPES–SDS–polyacrylamide gels, transferred to membranes, and probed with anti-S tag antibody (left) or anti-HA antibody (right).

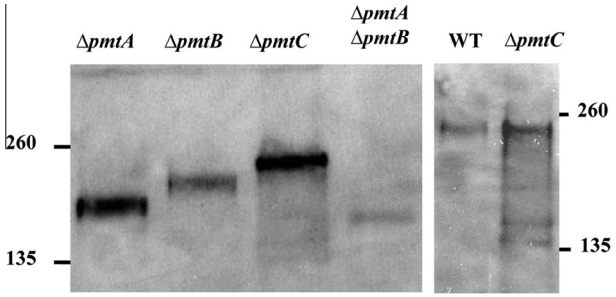

Fig. 7. MsbA (AN7041) is modified by PmtA and PmtB, but not by PmtC. Crude membrane fractions containing $MsbA^S$ from Δ*pmt* mutants and wildtype were treated with 2 × SDS loading dye and separated on 7% SDS–polyacrylamide gel, transferred to a membrane, and probed with anti-S tag antibody.

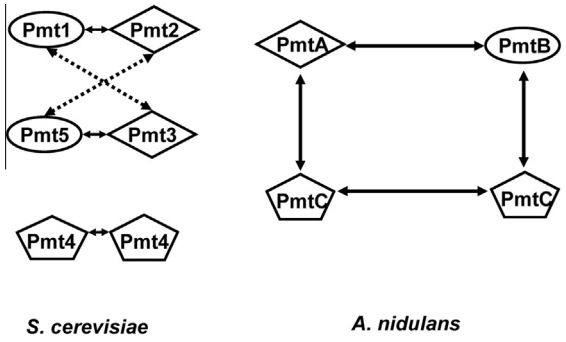

Fig. 8. Diagram comparing Pmt complex formation in *A. nidulans* and *S. cerevisiae* (adapted from Girrbach and Strahl) [13]. In *S. cerevisiae* Pmt1 forms heteromeric complexes with Pmt2 and Pmt4 forms homomeric complexes. Pmt1 forms heteromeric complexes with Pmt3 (subfamily 2) in the absence of Pmt2, while Pmt2 forms heteromeric complexes with Pmt5 (subfamily 1) in the absence of Pmt1. In *A. nidulans* PmtB (subfamily 1) forms heteromeric complexes with PmtA (subfamily 2) and with PmtC (subfamily 4). PmtA (subfamily 2) forms heteromeric complexes with PmtC (subfamily 4). PmtC (subfamily 4) forms homomeric complexes. Ovals indicate Pmt1 subfamily. Diamonds indicate Pmt2 subfamily. Pentagons indicate Pmt4 subfamily.

POMT2 (subfamily 2) [18,19]. It is possible that the novel heterodimers we observed with Pmt C might be low level forms that can substitute when other complexes are lost allowing *A. nidulans* to survive with its reduced complement of single Pmts from each subfamily [22].

4. Conclusions

In this study we show that the three *A. nidulans* Pmts (PmtA from subfamily 2, PmtB from subfamily 1, and PmtC from subfamily 4) form heteromeric complexes in all pairwise combinations and that PmtC forms a homomeric complex. We also show that MsbA, the *A. nidulans* ortholog of *S. cerevisiae* Msb2, is modified by PmtA and PmtB, but not by PmtC.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fob.2014.03.006.

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