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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.

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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