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FUNGAL EXTRACELLULAR POLYSACCHARIDES INVOLVED IN RECYCLING OF METABOLITES AND OSMOTOLERANCE OF *PENICILLIUM FELLUTANUM* : APPLICATION OF ^{13}C-NMR SPECTROSCOPY FOR THE STUDY ON FUNGAL PHYSIOLOGY AND METABOLISM

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Abstract

Penicillium fellutanum produces a phosphorylated, choline-containing extracellular peptido-polysaccharide, peptidophosphogalactomannan (pPxGM) (8). The ^{13}C-methyl labeled pPxGM ([*methyl*-^{13}C]pPxGM) was prepared from the cultures supplemented with L-[*methyl*-^{13}C]methionine or [2-^{13}C]glycine and was used as a probe to monitor the fate of phosphocholine in this polymer. Addition of purified [*methyl*-^{13}C]pPxGM to growing cultures in low phosphate medium resulted in the disappearance of [*methyl*-^{13}C]phosphocholine and -*N,N*-dimethyl-phosphoethanolamine from the added [*methyl*-^{13}C]pPxGM. Two ^{13}C-methyl-enriched cytoplasmic solutes, choline-*O*-sulfate and glycine betaine, were found in mycelial extracts, suggesting that phosphocholine-containing extracellular pPxGM of *P. fellutanum* is a precursor of intracellular choline-*O*-sulfate and glycine betaine and thus of phosphatydilcholine (10). ^{13}C-Methyl-labeled cells grown in 3 M NaCl-containing medium showed 2.6- and 22-fold more accumulation of ^{13}C-methyl labeled choline-*O*-sulfate and glycine betaine, respectively, originated from the extracellular [^{13}C-methyl]pPxGM than those grown without added NaCl. The results suggest that, in addition to glycerol and erythritol, glycine betaine and choline-*O*-sulfate and thus choline are also osmoprotectants and hence that pPxGM is involved in osmotolerance of this fungus (11). Taken collectively, the ^{13}C- and ^{31}P-NMR analyses of cytosolic solute pools and structural modulation of extracellular pPxGM corresponding to environmental stimuli in *P. fellutanum*, provided evidence that pPxGM is involved in cellular choline metabolism, osmotolerance, and recycling of metabolites.

Introduction

Fungi produce complex phosphorylated polysaccharides and glycopeptides which are membrane-bound, cell wall-associated, or extracellular (1, 8, 15). Although the structural chemistry of these polymers has been studied (2, 8), their role(s) in fungal physiology are mostly unknown. *Penicillium fellutanum* (formerly *P. charlesii* G. Smith) produces two classes of complex exocellular polysaccharides, i) soluble peptidophosphogalactomannan (pPxGM), and ii) membrane-bound lipo-$pP_{30}GM$, where "x" and "30" refer to the number of phosphodiester residues (6, 7, 8, 9, 12). The mannan backbone of this polymer is attached to a 3-kDa peptide through O-glycosidic linkage and to which eight to 10 galactofuran chains are attached. Approximately 10 phosphocholine and phosphoethanolamine residues are attached to the C-6 position of mannopyranosyl residues (16); *N*-peptidyl phosphoethanolamine phosphodiester residues are attached to the C-6 position of galactofuranosyl residues (Fig. 1) (3, 5, 12, 16, 17).

Using mainly ^{13}C- and ^{31}P-NMR analysis, sets of experiments were performed to elucidate physiological role(s) of the phosphodiester residues in extracellular pPxGM. We found that phosphocholine in extracellular pPxGM is a precursor of intracellular choline-*O*-sulfate and glycine betaine (10) and that, under low-phosphate and high osmolarity conditions, these intracellular choline derivatives and thus extracellular pPxGM are involved in osmotolerance of *P. fellutanum* (11). These results provided some insight into the interrelationships between the apparently unrelated physiological functions of extracellular polysaccharides, choline metabolism, phospholipid metabolism, medium osmolarity, phosphate storage, and sulfate storage in filamentous fungi.

Experimental

Isolation and fractionation of extracellular soluble pPxGMs For the production of pP_XGM, *P. fellutanum* was grown in either the standard growth (SG) medium containing 20 mM phosphate or low phosphate standard growth (LPSG) medium containing 2 mM phosphate (13). Medium compositions and growth conditions were described previously (14). Soluble pPxGMs were obtained by established methods (5) from 2 liters of day-8 cultures in SG or LPSG medium. Crude preparations of pPxGMs were fractionated on DEAE-cellulose pre-equilibrated with 50 mM $K_2B_4O_7$. Fractions were assayed for total carbohydrate at 490 nm (4). Carbohydrate-containing fractions were pooled, dialyzed against dH_2O, and freeze-dried.

Preparation of [methyl-^{13}C]pPxGM by metabolic labeling *P. fellutanum* was cultured for 8 days in 4 liters of SG medium enriched with L-[*methyl*-^{13}C]methionine (50 mg/200 ml); ^{13}C-methyl labeled pPxGM was isolated from the culture filtrate as described above. Thirty hours after inoculation, 50 mg of L-[*methyl*-^{13}C]methionine dissolved in dH_2O was filter sterilized

(0.22 μm, Millipore) and added to each culture flask. The [*methyl*-^{13}C]pPxGM obtained was analyzed by proton-decoupled ^{13}C-NMR spectroscopy.

Release of phosphocholine from extracellular pPxGM The ^{13}C-methyl labeled pPxGM obtained was used to determine the release of phosphocholine from extracellular pPxGM. Two hundred milligrams of purified [*methyl*-^{13}C]pPxGM were dissolved in 4 ml dH_2O, and added 72 h after inoculation, by filtration through 0.22 μm membrane filter (Millipore), to 200 ml cultures in LPSG medium. The same quantity of unlabeled pPxGM was used as control. At day 8, the added [*methyl*-^{13}C]pPxGM was recovered from the culture filtrates through a modified isolation procedure. Compositional changes in the recovered [*methyl*-^{13}C]pPxGM were analyzed by proton-decoupled ^{13}C-NMR spectroscopy. The intracellular solute pools of mycelia obtained from the same cultures were examined for the existence of any intracellular ^{13}C-methyl labeled substance(s) (see below).

Preparation of mycelial extracts Intracellular solute pools of mycelia were analyzed by proton decoupled ^{13}C-NMR spectroscopy after extraction with 80% ethanol. Fifty milligrams of L-[*methyl*-^{13}C]methionine was added to each 200-ml culture at 30 h which is the approximate time of germination. Mycelia were obtained by filtration *in vacuo*, washed with water and then were treated with 80% ethanol for 24 h at room temperature and centrifuged at 8,000 x *g* for 30 min at 4°C. Insoluble debris were removed by vacuum filtration through M-type sintered glass filters. The filtrates were evaporated *in vacuo* to near dryness and only water-soluble substances were analyzed by ^{13}C-NMR spectroscopy.

^{13}C-NMR spectroscopy of mycelial extracts and pPxGMs Natural-abundance proton-decoupled ^{13}C-NMR spectra were recorded at 75.45 MHz using a NT-300 spectrometer with a 7 Tesla Oxford magnet operating in the pulsed Fourier transform mode. Unless otherwise stated, all samples were examined at 22°C, with broad-band decoupling of protons, with full nuclear Overhauser enhancement (NOE), and without sample spinning. Four milliliters of each mycelial extract were analyzed by proton decoupled ^{13}C-NMR spectroscopy with addition of 0.5 ml D_2O containing sodium (trimethylsilane)-1-propanesulfonate (TSP) as internal reference.

Identification of intracellular choline-O-sulfate and glycine betaine Four authentic choline derivatives (COS, glycine betaine, choline chloride, and phosphocholine; 30 mM each) were sequentially dissolved directly in mycelial extracts and analyzed by ^{13}C-NMR spectroscopy.

Osmotic up- and downshift To monitor the fate of intracellular choline derivatives, cells were grown for 8 days in L-[*methyl*-^{13}C]methionine-containing (50 mg/200 ml medium) LPSG medium stressed with 3 M NaCl and then these NaCl-stressed cells were transferred aseptically to a fresh LPSG medium without added NaCl and L-[*methyl*-^{13}C]methionine (11). L-[*methyl*-^{13}C]Methionine was used to increase sensitivity. As a control, mycelium was transferred

separately to a fresh medium containing 3 M NaCl. Each mycelium was harvested 3 days after transfer, extracted with 80% ethanol, and the water-soluble portions of extracts were analyzed with ^{13}C NMR spectrometry.

Results and Discussion

Preparation of [methyl-^{13}C]pPxGM by metabolic labeling The ^{13}C-NMR spectrum of Fig. 2A represents normal pPxGM obtained from SG medium; that of Fig. 2B represents the [*methyl*-^{13}C]pPxGM from the same medium but enriched with L-[*methyl*-^{13}C]methionine. In Fig. 2A, the signal at 56.83 ppm (designated as 'a') represents methyl carbons of phosphocholine attached to C-6 of mannopyranosyl residues. The methyl carbon signal at 47.24 ppm (designated as 'b') was tentatively identified as that of *N,N'*-dimethyl-phosphoethanolamine (17) attached to C-6 of other mannopyranosyl residues in pPxGM. In Fig. 2B, the signal intensities of methyl groups of phosphocholine and *N,N'*-dimethyl-phosphoethanolamine increased about 25-fold compared with those in Fig. 2A, indicating that the methyl carbons of both choline and dimethyl ethanolamine of extracellular pPxGM were metabolically enriched with ^{13}C. The purified [*methyl*-^{13}C]pPxGM was then used as a probe for the initial survey of ^{13}C-methyl labeled substances in intracellular solute pools by ^{13}C-NMR spectrometry.

Release of phosphocholine from extracellular pPxGM The fate of phosphocholine in extracellular pPxGM was determined during the growth of *P. fellutanum* in low phosphate medium. Purified [*methyl*-^{13}C]pPxGM was added to the low phosphate (LPSG) cultures and recovered from the culture filtrates 5 days later and then analyzed by ^{13}C-NMR for any compositional changes. The two signals, representing ^{13}C-labeled phosphocholine (56.83 ppm) and *N,N'*-dimethyl-phosphoethanolamine (47.24 ppm), completely disappeared from the added [*methyl*-^{13}C]pPxGM (Fig. 2C). This interpretation of the data was confirmed by ^{31}P-NMR spectroscopy, which showed that phosphocholine signal resonating at 0.22 ppm significantly decreased (data not shown). It was also observed that signals at ~ 110 and 84 ppm, which represent galactofuranosyl residues, were no longer present in the polymer (Fig. 2C).

Evidence for the cytosolic localization of methyl carbons of phosphocholine from extracellular pPxGM Intracellular solute pools of mycelia obtained from cultures initially containing 2 mM phosphate and either unsupplemented (Fig. 3A), supplemented with [*methyl*-^{13}C]pPxGM (Fig. 3B) or supplemented with unlabeled pPxGM (Fig. 3C), were surveyed for the presence of any ^{13}C-labeled substance(s) (Fig. 3B). Each extract contained the same concentration of TSP (0.22%) as a reference for integration of peak intensities of individual signals. Integration was performed using Felix for Windows 1.02 Software (Molecular Simulations Inc.). With the TSP signal as a reference, comparison of relative intensities of

signal *P* and *Q* of Fig. 3B with those in Fig. 3A and 3C showed that the relative intensities of signals *P* and *Q* were about 4.3-fold and 2.3-fold larger than those in the extract from the culture supplemented with unlabeled pPxGM (Fig. 3C). These data show that *P* (56.77 ppm) and *Q* (56.23 ppm) signals in Fig. 3B are the ^{13}C-enriched signals and that they originated from the extracellular ^{13}C-methyl labeled phosphocholine of the [*methyl*-^{13}C]pPxGM added to the culture. This suggests that at least the methyl groups of phosphocholine from extracellular pPxGM are relocalized as a component of intracellular methyl carbon-containing solutes. Addition of unlabeled pPxGM to the culture (Fig. 3C) resulted in a small increase in signals *P* and *Q*, compared with those in Fig. 3A.

Identification of two intracellular choline derivatives of P. fellutanum as choline-O-sulfate and glycine betaine Identification of the two methyl-containing substances that accumulated inside of the mycelia was performed by proton-decoupled ^{13}C-NMR spectroscopy. Dissolving authentic commercial glycine betaine (30 mM) directly into a mycelial extract resulted in a significant increase in signals at 56.23, 69.04, and 172.04 ppm, showing that the mycelial extract contained glycine betaine derived from extracellular pPxGM. Subsequently, solid authentic choline-*O*-sulfate (COS) (30 mM) was dissolved in the mycelial extract and this resulted in an increase of the three signals resonating at 56.77, 64.90, and 67.68 ppm, indicating that the 56.77 ppm-substance in the original extract was COS. There was no detectable choline in the original mycelial extract. These results showed that phosphocholine (and possibly *N*,*N*'-dimethyl-phosphoethanolamine) attached to extracellular pPxGM, accumulates primarily as COS and glycine betaine in *P. fellutanum* cultured for 8 days in low phosphate medium.

Effect of osmotic upshock and downshift on intracellular choline derivatives in P. fellutanum The peak at 56.76 ppm represents $-N^{+}-(CH_3)_3$ of COS and that at 56.23 ppm represents $-N^{+}-(CH_3)_3$ of GB. There were no significant differences in the pattern and intensities of spectral ^{13}C-NMR signals from intracellular solute pools of the mycelium grown in control medium lacking added NaCl (data not shown), with added 0.5 M or 1.0 M NaCl. In contrast, the levels of GB and COS increased significantly in the mycelium grown with 2 M and especially with 3 M NaCl. However, upon osmotic downshift, their intracellular levels significantly decreased (data not shown), suggesting that these two choline derivatives are involved in osmoprotection of this fungus. This was confirmed by the fact that 2 mM each of GB, COS, and choline added to cultures stressed with 3 M NaCl stimulated the growth of *P. fellutanum* (data not shown).

During the study to determine possible role(s) of phosphocholine attached to the C-6 position of mannopyranosyl residues of extracellular pPxGM, we found that, under low phosphate condition, *P. fellutanum* has a mechanism for recycling phosphate and for conserving

excess choline not immediately required for synthesis of new membranes (10). It was also shown that, under low phosphate and high osmolarity conditions, more phosphocholine is released from extracellular pPxGM and then its derivatives, GB and COS, function as osmoprotectants in this fungus (11).

The overall process of reutilization of specific nutrients from complex polysaccharides is likely associated with the induction of corresponding enzymes with varying environmental stimuli (13). Coupling the release of choline with that of phosphate from a storage polymer, pPxGM, is consistent with synthesis of membranes which are major sinks for both phosphate and choline derivatives. This type of recycling of nutrients back into the organism provides a means of conserving important nutrients or metabolites until they are needed physiologically and metabolically. This may be a general mechanism for providing for continued survival and growth of organisms in a nutritionally unbalanced environment.

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