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Lipid and Cell Wall Changes in an Inositol-Requiring Mutant of *Neurospora crassa*

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An inositol deficiency in the inositol-requiring (*inl*) mutant of *Neurospora crassa* led to changes in the composition of the inositol-containing lipids and the cell wall. On deficient levels of inositol, phosphatidyl inositol decreased by 23-fold, di(inositolphosphoryl) ceramide decreased by 4-fold, and monoinositolphosphoryl ceramide increased slightly. The inositol deficiency also led to an aberrant hyphal morphology and changes in both the amount of cell wall and the amino sugar content of the cell wall. The glucosamine content of the cell wall decreased by 50%, the galactosamine increased by 50%, but no significant changes were found in the content of the cell wall amino sugar precursors, or in the amino acid, glucose, or total hexose content of the cell wall. Inositol-containing compounds were found associated with purified cell wall material. These compounds were bound tightly to the cell wall but could be removed by treatment with alkali, a treatment which disrupts the cell wall integrity. Possible mechanisms of how changes in lipid composition can affect cell wall biosynthesis are discussed.

Mutationally induced aberrations in hyphal formation of many fungi are considered to be the result of associated changes in cell wall structure (2, 6, 29). Most of these mutations affect some aspect of carbohydrate metabolism in these organisms (2, 6, 7, 10, 21, 22, 29). However, in *Neurospora crassa*, there is a unique group of mutations which specifically affect lipid biosynthesis and which also result in aberrant hyphal formation. These mutants are choline (*chol*) and inositol (*inl*) auxotrophs (3, 4, 12-18, 25-28, 30, 32, 35, 36, 39).

Of the two mutant classes, the *inl* strain has been more extensively studied. When the *inl* mutant is grown on less than 5.6 μM inositol, changes occur in the intracellular levels of three inositol-containing lipid classes: phosphatidyl inositol (PI), di(inositolphosphoryl) ceramide (IP_2C), and monoinositolphosphoryl ceramide (IPC) (13, 14, 17, 20, 30). These changes in the inositol metabolism of the *inl* mutant are the result of a defective inositol synthetase system (34). The mycelium of the *inl* strain grown under these conditions becomes very highly branched.

This mutant strain can be used to address two questions about the relationships of lipid biosynthesis to aberrant hyphal growth: first, whether changes in lipid composition can be correlated with changes in hyphal morphology and cell wall structure; and if so, by what mechanisms these changes in lipid composition affect hyphal growth and cell wall structure.

Two lines of investigation were followed in this present study. First, changes in the content and location of inositol-containing lipids and low-molecular-weight compounds were examined in the *inl* mutant grown on different levels of inositol. Second, several parameters of cell wall structure, i.e., hexose, amino sugar, glucose, and amino acid content were measured in the mutant grown on different levels of inositol to correlate these changes with the corresponding changes in inositol metabolism. (This work was submitted in partial fulfillment of the requirements for the Ph.D. degree in biology.)

MATERIALS AND METHODS

Organism. The Rockefeller-Lindegren inositolless (*inl* 89601) strain of *N. crassa* was obtained from the Fungal Genetics Stock Center, California State University, Humboldt, Calif.

Growth and harvest of mycelia. The *inl* strain was grown on Vogel medium (37) plus 2% glucose, supplemented with inositol (3.3 to 55.5 μM) and 0.06 mCi of *myo*-[2-^{3}H]inositol (New England Nuclear; specific activity, 1 to 5 Ci/μmol). The inositol incorporated by the mutant from the medium was not further metabolized in the cell (20). Cultures were grown in 1,000- to 1,500-ml shake flasks for 48 to 66 h at 22°C. These early stationary-phase cultures, which showed no cell lysis, were harvested by filtration through Whatman no. 1 paper and washed twice with cold Vogel medium without inositol. A portion of the my-

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celia was used for lipid extractions and the remainder was frozen immediately, lyophilized for 24 h, and then stored at −20°C. Lyophilized mycelia were used for all cell wall experiments.

Cell wall isolation and fractionation. Cell walls were isolated and fractionated by the Edson modification (10) of the Mahadevan and Tatum procedure (23, 24). One of the modifications was that the cell walls were treated with hot 80% ethanol during their isolation to remove loosely associated inositol-containing lipids. In this procedure, cell walls were treated with 2 N NaOH for 16 h at 23°C and then centrifuged. Material in the supernatant solution was precipitated with ethanol to yield fraction I (FI). This fraction is relatively uncharacterized and contains galactosamine and amino acids (23). The residue was hydrolyzed with 1 N H_2SO_4 for 16 h at 90°C, and the material extracted after neutralization with $Ba(OH)_2$ was designated fraction II (FII). This fraction consists primarily of glucose liberated from the cell wall (23). Further treatment of the cell wall pellet with 2 N NaOH for 30 min at 23°C yielded fraction III (FIII). This fraction was primarily β-1, 3-glucan (23). After these three extractions, the remaining material, primarily chitin (23), was designated fraction IV (FIV).

Total inositol content of mycelia. Samples of approximately 50 mg of lyophilized mycelia were hydrolyzed in 6 N HCl under vacuum for 42 to 48 h at 100°C. Cell wall samples, 10 to 15 mg each, were hydrolyzed under identical conditions but for only 16 h. These conditions gave optimal yields of inositol from the hydrolysates. Longer periods for higher temperatures resulted in a decrease in the inositol content of the hydrolysates (B. A. Hanson, Ph.D. thesis, University of California, San Diego, La Jolla, 1975). The inositol released by hydrolysis co-chromatographed in three solvent systems with a *myo*-inositol standard (11, 33).

The hydrolysates were filtered through Whatman no. 1 filter paper and activated charcoal. The mycelial residue was rinsed thoroughly with distilled water. Each filtrate residue was suspended in 5 ml of water. Duplicate 0.1-ml samples of the mycelial hydrolysates were analyzed for radioactivity as described previously (10). The inositol content of the hydrolysates was determined by bioassay (3) or calculated from the specific activity of [^{3}H]inositol in the medium.

Inositol-containing lipids of mycelia. The procedure for the extraction of PI, IP_2C, and IPC involved soaking damp mycelia in boiling 80% ethanol for 20 min and then rapidly filtering them through Whatman no. 1 paper (14). This process was repeated twice. The filtered solutions were combined and evaporated to a volume of 0.5 to 1.0 ml and analyzed for ^{3}H content. The inositol-containing lipids were resolved by two-dimensional chromatography on Whatman SO81 paper dipped in EDTA (20, 38). Compounds were located by comparing with standard compounds. These regions were eluted in 80% ethanol overnight and the eluates were analyzed for ^{3}H content. The inositol content of the different lipid compounds were calculated from the [^{3}H]inositol specific activity.

Galactosamine and glucosamine content of the cell wall and mycelia. Samples of lyophilized mycelia, cell wall, and cell wall fractions (10 to 15 mg) were hydrolyzed in 6 N HCl for 16 h at 100°C in sealed, evacuated hydrolysis tubes. This time and temperature for hydrolysis gave the maximal release of amino sugars and inositol from the cell wall (10). The HCl was removed from each filtrate by flash evaporation. The residues were suspended twice in distilled water and evaporated to dryness. The nucleotide sugars were extracted from the mycelia and chemically analyzed by the method of Schmit et al. (31).

Total hexose and glucose content of the cell wall. Samples of mycelia and cell wall (4 to 7 mg) were hydrolyzed in 2 N trifluoroacetic acid by the method of Albersheim et al. (1). The optimal hydrolysis time at 120°C was determined to be 1 to 1.5 h. After 2 h of hydrolysis the hexose and glucose content of the samples decreased significantly. After hydrolysis, any remaining residue in the samples was removed by filtering the samples through Whatman no. 5 filter paper. Trifluoroacetic acid was removed from each sample by flash evaporation. The remaining residues were suspended twice in distilled water and evaporated to dryness.

After the samples were resuspended in water, the total glucose and hexose content were determined. Total hexose was measured by the phenolsulfuric acid method by using glucose as a standard (8). Total glucose was determined by the Glucostat enzyme assay (Worthington Biochemicals, Inc.)

Total amino acid content of cell wall. Samples of cell wall were hydrolyzed for 24 h at 100°C in 6 N HCl in evacuated, sealed hydrolysis tubes. The HCl was removed by flash evaporation, and the sample residue was resuspended in distilled water. This was repeated twice. The total amino acid content was determined by the method of Schmit et al. (31) on a Beckman 120 C amino acid analyzer.

Chemicals. The sugar, amino acid, and lipid standards for chromatography were obtained from Sigma Chemical Co., St. Louis, Mo. Phosphatidyl inositol was obtained from Pierce Chemical Co., Rockford, Ill.

RESULTS

Inositol and inositol-containing lipids of the *inl* mycelium. Three classes of phosphoinositides exist in *N. crassa*: PI, IP_2C, and IPC. These classes of lipids were resolved by two-dimensional paper chromatography by the method of Lester et al. (20, 38). This chromatography system will resolve the different classes of phosphoinositides but will not resolve the lipids within a class of phosphoinositides which contain different fatty acids. Therefore, each general class of lipids discussed here may contain more than one type of lipid.

In the *inl* mutant, the level of those lipids showed a dependence upon the inositol concentration in the medium. On deficient levels of inositol (< 3.3 μM) both the total inositol of the *inl* mycelia and the total inositol-containing lipid content decreased by fivefold (Table 1). There was a 23-fold decrease in the PI content from 3.2 μmol/g (dry weight) to 0.14, whereas only a 4-

TABLE 1. *Distribution of inositol-containing compounds in the mycelium of the inl mutant grown on varying levels of inositol in the medium*[a]

Compound	Inositol in medium (μM)			
	3.3		55.4	
	μmol/g (dry wt)	%	μmol/g (dry wt)	%
IP_2C[b]	0.8	47.3	3.3	41.2
IPC[b]	0.4	23.7	0.3	3.8
PI[b]	0.14	8.3	3.2	40.0
Soluble compounds[c]	0.05	3.0	0.5	6.2
Residue[d]	0.3	17.7	0.7	8.8

[a] Isolated according to the hot-ethanol extraction procedure in Materials and Methods.

[b] Compounds were resolved by two-dimensional paper chromatography by the method of Lester et al. (20, 38).

[c] Inositol-containing material, primarily inositol and inositol phosphate.

[d] [^{3}H]inositol remaining in the residue after lipid extraction was released by hydrolysis of the residues in 6 N HCl at 110°C for 40 h by the method of Lester et al. (34). Residue consists of cell wall and any nonextractable cell components.

fold decrease in the IP_2C content of the inositol-deficient *inl* mycelia occurred. The IPC content increased slightly under these conditions.

These three lipids also changed in proportion to each other in the inositol-deficient *inl* mycelia. The proportion of total cell inositol found in IP_2C remained constant. However, the proportion of IPC increased by about sixfold in the inositol-deficient *inl* mutant. The proportion of total cell inositol found in PI decreased by fivefold under the same growth conditions (Table 1). After lipid extraction, the amount of inositol remaining in the residue was ~5 to 8% of the total cell inositol. This amount was reduced further to less than 0.5% by multiple extractions with water-ethanol-diethylether-pyridine (15:15:5:1[vol/vol]) according to the method of Lester et al. (20). The inositol-containing material extracted from the residue included all three classes of phosphoinositides.

Cell wall components affected by the inositol deficiency in the *inl* mutant. The inositol-deficient *inl* mycelium was analyzed for changes in cell wall content (the proportion of mycelia that is cell wall). The cell wall content of *inl* mycelium grown on sufficient levels of inositol in the medium was 12%. This value increased in the inositol-deficient *inl* mutant to 22.4%.

The galactosamine and glucosamine contents were measured in hydrolysates of the mycelia and cell wall of the *inl* mutant grown on varying levels of inositol. In addition, measurements were made of the intracellular pools of uridine diphosphate-*N*-acetylglucosamine (UDP-*N*-acetyl glucosamine) and uridine diphosphate-*N*-acetylgalactosamine (UDP-*N*-acetyl galactosamine), the compounds shown by Edson and Brody to be the precursors of the glucosamine and galactosamine polymers found in the cell wall in *Neurospora* (10, 31).

Both amino sugars, galactosamine and glucosamine, showed changes in their content in the inositol-deficient *inl* mutant. The galactosamine content increased by 50% on deficient levels of inositol (3.3 μM), whereas the glucosamine content decreased by 50% (Table 2).

The intracellular nucleotide sugar pools were also measured in the mycelia of the *inl* mutant grown on varying levels of inositol. The UDP-*N*-acetylglucosamine pools remained relatively constant at about 5 μmol/g of mycelia. The soluble pools of UDP-*N*-acetylgalactosamine were found only in trace amounts. Thus, the changes in total content of amino sugars were mainly in the cell wall fraction and not in the soluble nucleotide sugar pools. For each of the amino sugars, the sum of the amino sugar content of the cell wall plus the amino sugar content of the soluble nucleotide sugars approximately equaled the total amino sugar content of the mycelia.

Inositol content of the cell wall. When the cell wall of the *inl* mutant was extracted from *Neurospora* mycelium grown on [^{3}H]inositol-containing medium, [^{3}H]inositol was found associated with the cell wall. This amount of inositol varied depending on the molarity of inositol in the medium. On deficient levels of inositol in

TABLE 2. *Levels of inositol and the amino sugars in the cell walls of the mycelium of the inl mutant grown on varying concentrations of inositol*

Inositol in medium (μM)	Cell wall content (mg of cell wall/g [dry wt] mycelia)[a]	Concn (μmol/100 mg of cell wall)		
		Glucosamine[b]	Galactosamine[b]	Inositol[c]
3.3	22.4	14.9	31.0	0.15
8.3	15.5	28.2	16.6	0.18
27.7	15.8	24.6	17.0	0.25
55.4	12.8	29.5	19.0	0.22

[a] Cell wall was isolated by the method of Edson (10).

[b] Glucosamine values for cell wall and mycelia are the average of three determinations. Soluble glucosamine values were determined in one experiment.

[c] [^{3}H]inositol remaining in cell wall after isolation from *inl* mycelia was released by acid hydrolysis as in Materials and Methods. Micromoles of inositol were calculated from the specific activity of the [^{3}H]inositol.

the growth medium the cell wall inositol was 1.5-fold lower than cell wall inositol from an inositol-sufficiently grown *inl* mycelia (Table 2).

Attempts were made to release [^{3}H]inositol from the cell wall by various extraction procedures at 23°C. Treatment with 2:1 chloroform-methanol in 1 N HCl released only 1 to 2% of the inositol, as did treatment with 3:1 ethanol-ether. Alkali treatment of the cell wall was the most effective method of releasing [^{3}H]inositol in that 25% NH_4OH released ~25% of the inositol, and 2 N KOH released 35 to 42% of the [^{3}H]inositol.

Treatment with 2 N KOH or 2 N NaOH released inositol and inositol phosphate from the cell wall. These compounds were identified by co-chromatography with standards in three different solvent systems. The ratio of inositol phosphate to inositol in the alkali extracts was 1.6.

Galactosamine, glucosamine, and inositol content of the *inl* cell wall fractions. Most of the galactosamine, glucosamine, and inositol of the cell wall was found only in a few specific fractions. The galactosamine and inositol were found mainly in FI, whereas most of the glucosamine was localized in FIV (Table 3). The inositol deficiency in the *inl* mutant was associated with a change in the amino sugar contents of the fractions; the glucosamine in the chitin-containing fractions FIV decreased from 16.6 to 8.5 μmol/100 mg of cell wall.

Cell wall components not affected by the inositol deficiency in the *inl* mutant. The total amino acid content and the content of individual amino acids was measured in cell walls extracted from *inl* mycelia grown with different inositol concentrations in the medium.

TABLE 3. *Glucosamine and galactosamine content of cell wall fractions of the inl mutant grown on different concentrations of inositol in the growth medium*

Inositol (μM) in medium	Amino sugar	Amino sugar content				
		Unfractionated cell wall (μmol/100 mg of cell wall)	Fractionated cell wall (μmol/100 mg of cell wall[a])			
			FI	FII	FIII	FIV
3.3	Galactosamine	30.9	18.9	0.6	1.2	1.4
110.8		16.5	13.7	0.1	0.8	1.2
3.3	Glucosamine	16.4	0.05	0.1	Trace	8.4
110.8		31.0	0.04	0.2	Trace	16.4

[a] See Materials and Methods for a brief description of these fractions and reference 24 for a fuller description.

These values remained relatively constant regardless of the inositol concentration of the medium. The total amino acid content averaged 21.5 μmol/100 mg of cell wall.

The total hexose and glucose content was measured in both the mycelia and the cell wall of the *inl* mutant. Both values remained relatively constant regardless of the inositol concentration in the medium. The average glucose content was 220 μmol/100 mg of cell wall. The average hexose content was 315 μmol/100 mg of cell wall.

DISCUSSION

Since these studies have shown that changes in cell wall structure, specifically the content of amino sugars, correlated with changes in three classes of phosphoinositides, it suggested a possible role for these lipids in cell wall biosynthesis in *Neurospora*. However, it is not evident why these changes in lipid composition affect the amino sugar content of the cell wall.

Initially, the localization of inositol on the cell wall, even after treatment with hot 80% ethanol and sodium dodecyl sulfate, appeared as an important hint about how changes in inositol metabolism affect the cell wall in *Neurospora*. However, repeated lipid extractions of whole mycelia removed all but 0.5% of the total cell inositol. Thus, inositol on the cell wall may be trapped lipid which is released when the wall is partially hydrolyzed with base. In addition, inositol may be cleaved from any one of the three classes of phosphoinositides by base treatment.

The amino sugar perturbations in the cell wall caused by inositol starvation may be a fruitful area for future studies of the role of inositol in cell wall biosynthesis. These amino sugar perturbations, especially in glucosamine, are important to cell wall biosynthesis in *Neurospora*. Glucosamine, in the form of UDP-*N*-acetylglucosamine, is the precursor of a major structural polymer forming the innermost cell wall layer, chitin. In the *inl* mutant starved for inositol, this structural component is reduced by 50%, whereas there is no compensatory increase in the other structural polymer, β-1, 3-glucan (Table 3). This probably results in a greatly weakened cell wall, as suggested by the work of Katz and Rosenberger (21, 22). They have also shown, using temperature-sensitive chitin synthetase mutants of *Aspergillus*, that chitin helps to stabilize and strengthen the cell wall. At nonpermissive temperatures, chitin synthetase mutants lyse when they are not osmotically stabilized.

By what mechanisms could inositol starvation cause changes in the cell wall biosynthesis of the chitin? Several possibilities exist. First, the

membrane and, as a secondary consequence, the cell wall structure, may be physically disrupted by a deficiency in the membrane phosphoinositides. Matile suggested inositol deprivation in *Neurospora* caused membrane deterioration (26). In an analogous manner, inositol deprivation in the *inl* mutant may cause a deterioration in the membrane organelles responsible for cell wall polysaccharide formation and transport, therefore preventing proper cell wall formation. A disappearance or loss of the membrane organelles which incorporate cell wall precursors or contain cell wall biosynthetic enzymes from an *inl* mutant grown on limiting levels of inositol would be strongly indicative of a degenerating cell membrane.

There is, however, a problem with the idea that membranes are deteriorating in the inositol-deficient *inl* strains. Most of the work of Matile and others on the membrane deterioration of the inositol deficient *inl* mutant have been done on cells in the stationary phase of growth (13, 26, 28, 32, 36). In these types of cells one may be seeing the results of inositol starvation rather than inositol-limiting growth. Studies on the phospholipid composition of choline and inositol-requiring mutants of *Neurospora* (19) actually suggested that in logarithmic cells the membranes remain intact and that there exist compensatory mechanisms for conserving phospholipid membrane charge and content. These studies indicated that a decrease in the content of the acidic phosphatidyl inositol is compensated for by an increase in another acidic phospholipid, phosphatidyl serine. As a result, the total phospholipid content and charge were conserved. Similar findings were subsequently reported (5) for inositol starvation in *Saccharomyces cerevisiae*.

A second possible explanation for aberrant cell wall synthesis in the *inl* mutant is that at the growing hyphal tips the membrane lipid pattern is so altered that the cell wall polysaccharides and biosynthetic enzymes cannot be extruded from the cell or properly aligned on the surface of the membrane. A possible experimental approach, based on the observed changes in the cell wall amino sugars of the *inl* mutant, would be to examine more closely the transport and biosynthesis of these two sugars and their polymers in the cell wall. The change in the amount of *N*-acetylglucosamine of the chitin layer in the *inl* mutant is significant because the enzyme which synthesizes its polymer, chitin synthetase, is a membrane-bound enzyme (9) and, therefore, susceptible to changes in the membrane. It must be remembered that attempts to understand the role of the membrane in cell wall synthesis are hindered by the complexity of and the lack of knowledge about the biosynthesis and structure of the cell wall and membrane in *Neurospora*. The *inl* and *chol* mutants have the additional problem of causing pleiotropic effects, so it is difficult to decide which effects to study. By focusing on the appropriate specific phenomena in the mutants, one may be able to unravel the relationship of cell membrane to cell wall synthesis in *N. crassa*.

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