Characterization of *un-17*, *chol-3* and *chol-4*, three phospholipid biosynthetic mutants of *Neurospora crassa*

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Four choline-requiring mutants of *Neurospora crassa* have been identified genetically. The *chol-1* and *chol-2* mutants are impaired in phosphatidylcholine biosynthesis. Phospholipid synthesis is also impaired in the *inl* and *un-17* mutants; the *inl* mutant cannot synthesize inositol phospholipids. Defects in phospholipid biosynthesis are reported here for three previously uncharacterized mutants, *un-17*, *chol-3* and *chol-4*.

Phospholipid biosynthesis in fungi is best characterized in *Saccharomyces cerevisiae* and *Neurospora crassa*. Much of the early work on phospholipid synthesis relied on the biochemical and genetic characterization of the choline-requiring mutants *chol-1* and *chol-2*, and the inositol-requiring mutants at the *inl* (*inos*) locus of Neurospora. These mutants have defects at the following points in the biosynthetic pathway (Figure 1): *chol-1*, the enzyme that carries out the first of the three methylation steps converting the phospholipid phosphatidylethanolamine to phosphatidylcholine; *chol-2*, the bifunctional enzyme that carries out the second and third methylation steps of phosphatidylethanolamine to phosphatidylcholine (Crocken and Nyc, J. Biol. Chem. 239: 1727-1730, 1964); and *inl*, the enzyme that converts glucose-6-phosphate to inositol-1-phosphate (Zsindely et al., Acta Biol. Acad. Sci. Hung. 28: 381-290, 1977).** The *inl* defect results in the inability to synthesize inositol phospholipids, including phosphatidylinositol (Shatkin and Tatum, Am. J. Bot. 48: 760-771, 1961). More recently, several laboratories have advanced the biochemical, genetic and molecular understanding of phospholipid biosynthesis in Saccharomyces, for which a large number of mutants have now been characterized. Among the genes identified are the apparent homologs of the Neurospora *chol-1*, *chol-2*, and *inl* genes, several genes affecting their regulation, and genes affecting earlier steps of the phosphatidylcholine biosynthetic pathway (see Carman and Zeimetz, J. Biol. Chem. 271: 13293-13296, 1996, for a brief review).

In Neurospora, one potential regulatory mutant has been described (a suppressor of *inl*; Giles and Partridge, Proc. Natl. Acad. Sci. USA 39: 479-488, 1953) and three additional mutants that may affect the phospholipid biosynthetic pathway are available, but have not been characterized. One of these is the temperature-sensitive *un-17* mutant. This mutant is a potential mutant in phospholipid biosynthesis because its growth cessation at the restrictive temperature (34°C) correlates wi a dramatic reduction in phosphorus incorporation into phospholipids (Inoue and Ishikawa, Arch. Microbiol. 104: 1-6, 1975). The choline-requiring mutants *chol-3* and *chol-4* are also potential mutants in phospholipid biosynthesis; supplementation with choline allows biosynthesis of phosphatidylcholine by a minor pathway that utilizes CDP-choline. In Saccharomyces, mutations earlier in the phosphatidylcholine biosynthetic pathway can also lead to a choline requirement. Aside from phospholipid biosynthetic or regulatory mutants, no other choline-requiring mutants are known. These two Neurospora mutants originated from the collection of Tatum, who identified them as choline requiring (Perkins and Pollard, Fungal Genet. Newsl. 34: 52-53, 1986). The *chol-4* mutant, because of its choline requirement and close linkage to *chol-1*, initially was suspected to be an allele of *chol-1*; however, *chol-1* and *chol-4* have recently been shown to be nonallelic (D. Perkins, pers. comm.). Phospholipid biosynthesis in *un-17* and in the *chol-3* and *chol-4* mutants was therefore compared to wild type and to the previously characterized Neurospora phospholipid biosynthetic mutants.

Wild-type *Neurospora crassa* strain 74-OR23-1*A* (FGSC #987), the *chol-1* (FGSC #2982), *chol-2* (FGSC #4095), *inl* (FGSC #497) and *un-17* (FGSC #2356) mutants were obtained from the Fungal Genetics Stock Center. The *chol-3* (FGSC #4646) and *chol-4* (FGSC #4648) mutants were obtained from David Perkins, as was a temperature-sensitive *inl* allele (FGSC #2258). The *chol-3* and *chol-4* mutants grow much more slowly than wild type, *chol-1*, or *chol-2*, even in the presence of choline. Only trace amounts of phospholipids and other lipids are produced in *chol-3* and *chol-4* cultures (similar to unsupplemented *inl* or *chol-1* or *chol-2*). Other water-soluble supplements (for example inositol, ethanolamine, monomethylethanolamine, dimethylethanolamine, and fatty acids in the form of Tween detergents) were tested for ability to better support growth of *chol-3* and *chol-4*, and of *un-17* above its restrictive temperature, without success. The *chol-3* and *chol-4* mutants are leaky, and *chol-4* showed a tendency toward reversion to a wild-type phenotype.

To determine profiles of phospholipid synthesis relative to wild type, cultures were radiolabelled for 1 hr with 0.5 μCi of the phospholipid precursor ^{14}C-glycerol (Moravek) in the presence of suboptimal (20 μM) or no supplement at 35°C. This temperature is a restrictive temperature for *chol-2*, for *un-17*, and for the temperature-sensitive *inl* mutant. To increase mass, cultures were grown for 1 day prior to labelling (30°C), then shifted to 35°C, with label added 30 min after the temperature shift. For some experiments, ^{14}C-acetate (Amersham) was instead used as a precursor.

After radiolabelling, lipids were extracted and phospholipids separated by TLC in the solvent system 1-propanol/chloroform/propionic acid/0.1% KCl, 3/2/2/1. Lipids were stained with iodine, and label incorporation was determined by radiodensitometry. For all of the mutants, ^{14}C-glycerol incorporation into phospholipids differed more from the wild-type profile than did ^{14}C-acetate incorporation, most likely because fatty acid groups (derived from acetate) are exchanged relatively rapidly among different lipids, which obscures effects of a mutation at a specific point in the pathway.

The profiles of labelled precursor incorporation into phospholipids of the *chol-1*, the *chol-2*, and the *inl* mutant showed the expected differences relative to wild type (Figure 2). For example, the *chol-2* mutant accumulated the intermediate phosphatidylmonomethylethanolamine. (The inhibition of phosphatidylinositol accumulation was greater for the temperature-sensitive inl allele than for the other *inl* strain grown with suboptimal inositol.) Such clear-cut results supported the use of a similar radiotracer approach to identify any biochemical defects in phospholipid synthesis of the uncharacterized mutants. Radiotracer results with the three mutants *chol-3*, *chol-4* and *un-17* are consistent with phospholipid biosynthetic defects. Consistent with their slow growth, incorporation of labelled precursors into phospholipids of both *chol-3* and *chol-4* was reduced (over 1 hr, about 10% that of wild type for ^{14}C-acetate, and 30% for ^{14}C-glycerol). A similar overall reduction in incorporation was observed in the absence of supplementation for *chol-1* and *chol-2* mutants and for *inl* mutants, consistent with earlier reports. Slow growth may be an indirect effect of the mutations, due to grossly altered membrane phospholipid composition (Hubbard and Brody, J. Biol. Chem. 250: 7173-7181, 1975), although in *Saccharomyces*, inhibition of phosphatidylinositol synthesis results in negative regulation of phosphatidylcholine synthesis, and vice versa.

Based on ^{14}C-glycerol incorporation results, *un-17* appears to be defective in the phosphatidylethanolamine methylation pathway. Intensely staining bands corresponding to the methylation intermediates are visible following TLC separation of lipids from cultures shifted to the restrictive temperature. The Neurospora *chol-1* and *chol-2* mutants have been well characterized as having defects in the required methyltransferases. Despite extensive genetic and biochemical studies in Saccharomyces, a second subunit of the phosphatidylmonomethylethanolamine methyltransferase has not been postulated. Also, in contrast to *chol-2*, supplementation of *un-17* with dimethylethanolamine does not restore growth. Therefore, *un-17* is unlikely to be a methyltransferase mutant, unless growth at high (>34°C) temperatures requires a distinct methyltransferase from that encoded by the *chol-2* gene. Furthermore, to explain the inability of either dimethylethanolamine or choline to restore wild-type growth, the alternative CDP-choline biosynthetic pathway for phosphatidylcholine would also have to be impaired at high temperatures. The mutation may alternatively impair all three of the methylation steps, each of which requires S-adenosylmethionine. However, the mutant maps to a distinct locus from *eth-1*, the gene for S-adenosylmethionine synthase. In *Saccharomyces*, phospholipid biosynthetic genes are coordinately regulated, so a regulatory mutation is also a possibility, provided it has an especially pronounced effect on synthesis of phosphatidylcholine.

^{14}C-glycerol incorporation also shows a distinct labelling pattern for *chol-3*, indicating that the mutant is blocked in the formation of phosphatidylinositol. In heterokaryons, *chol-3* complemented *inl* mutants, so *chol-3* is not allelic to *inl*. In addition, inositol supplementation did not restore wild-type growth of *chol-3*. These results suggest that *chol-3* affects the formation of phosphatidylinositol from CDP-diacylglycerol. An alternative possibility, that a higher activity of phosphatidylinositol lipase in *chol-3* accounts for the results, is unlikely given that both *chol-3* and *inl* incorporated significant label from ^{14}C-acetate into phosphatidylinositol, presumably by exchange of fatty acid groups from other phospholipids.

The *chol-4* mutant appears to have either a partial block in the synthesis of phosphatidylserine, or a block earlier in the pathway. A regulatory mutation cannot be ruled out. Further work is thus needed to identify the nature of the *chol-4* defect.

**Some biochemical evidence conflicts, suggesting an effect on the following biochemical step. However, a partial sequence of the cloned *inl* gene is homologous to the *S. cerevisiae* and other cloned *myo*-inositol-1-synthases, which catalyze this step (M. G.-T., unpublished data).

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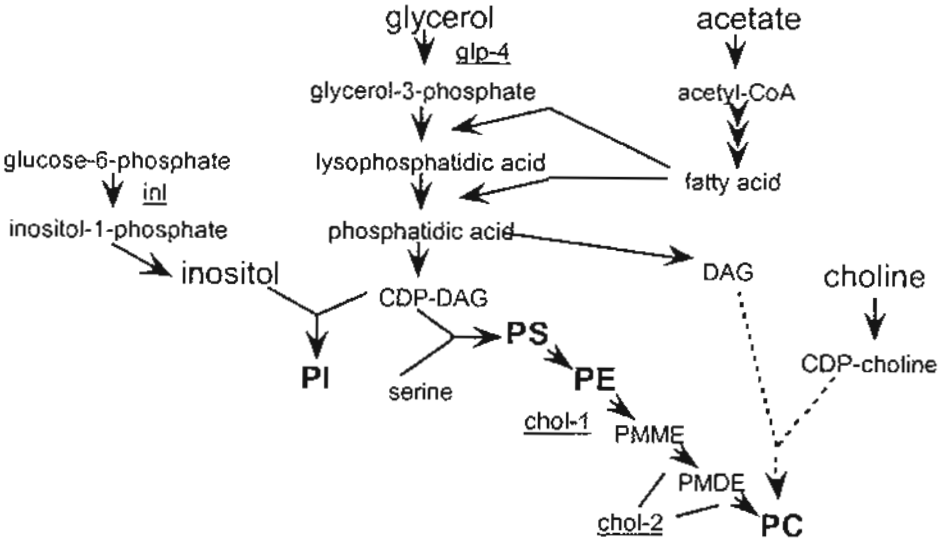

Figure 1. Outline of phospholipid biosynthesis in Neurospora. The major phospholipids (PI, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine) are shown in bold. Also shown are the points of entry into the pathway of the soluble phospholipid precursors inositol, choline, glycerol and acetate. PMME (phosphatidylmonomethylethanolamine) and PMDE (phosphatidyl-dimethylethanolamine) are minor phospholipids that accumulate in the *chol-2* mutant. Choline-supplemented *chol-1* and *chol-2* mutants are able to synthesize PC by the minor CDP-choline pathway, indicated by dotted arrows.

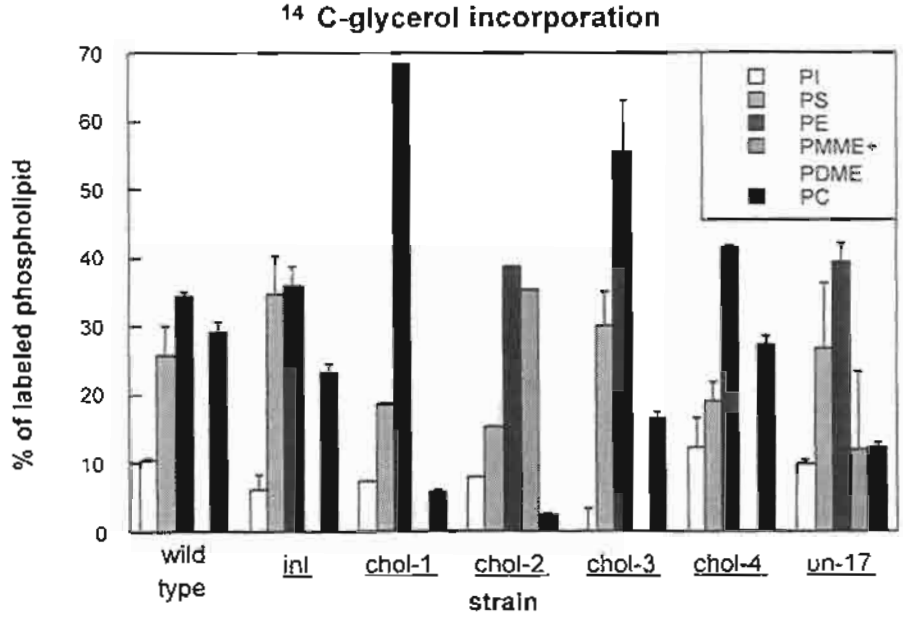

Figure 2. Incorporation of ^{14}C-glycerol into phospholipids. Cultures were labeled for 1 hr, beginning 30 min after transfer of cultures to 35°C. Data are averages ± s.d. for 3 or 4 cultures (1 each for *chol-1* and *chol-2* under these labeling conditions) In this experiment, the temperature-sensitive *inl* strain was used.