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Grant Title: *How do chitin synthases start chitin chains and at which end do chains grow?*

(a) Abstract:

1. Objectives. Hypotheses for how the enzymatic synthesis of chitin is initiated by Glycosyltransferase Family 2 chitin synthases (CSs) were tested. These included the possibility that CSs generate UDP-di-*N*-acetyl-chitobiose (UDP-$GlcNAc_2$) and that UDP-linked chitin oligosaccharides (COs) generated by CSs could be trapped using a chain-terminating UDP-GlcNAc analogue. The nature of the products of incubations was predicted to provide evidence for (1) a discrete chitin initiation reaction between two UDP-GlcNAcs and (2) for reducing- or non-reducing end chain extension. Additional analyses of priming of CO synthesis by a zymogenic CS (*S. cerevisiae* Chs1), and of the effects of a 2-acylamido analogue of GlcNAc on fungal morphogenesis, were conducted.

2. Methods used. *Saccharomyces cerevisiae* chitin synthases 1 and 2 (Chs1 and Chs2) were used as model CSs. They were overexpressed using a high copy vector under the control of a strong inducible promoter in a yeast host strain lacking detectable background CS activity. Membranes from these CS-overexpressing strains, which served as "*in vivo* proteoliposomes", were used as source of CS. Formation of both low- and high molecular weight reaction products (COs and insoluble chitin, respectively) was monitored using two types of assay. In the first, UDP-[^{14}C]GlcNAc was used a substrate, and the effects of primers and the UDP-GlcNAc analogues UDP-$GlcNAc_2$ and UDP-4-O-MeGlcNAc were tested. In the second, CO and chitin synthesis was assessed using a novel assay in which [^{14}C]GlcNAc is used as priming substrate in the presence of unlabeled UDP-GlcNAc, UDP-$GlcNAc_2$ or UDP-4-O-MeGlcNAc. Proteolytically treated and untreated zymogenic Chs1 was compared with Chs2 with respect to priming of CO formation by GlcNAc, $GlcNAc_2$ and the 2-acylamido derivatives of GlcN, GlcNPr, GlcNBu, and GlcNGc.

3. Results. Major findings are as follows.

i) We devised a [^{14}C]GlcNAc priming assay that represents a new way to characterize CSs that is distinct from UDP-GlcNAc-dependent chitin extension assays.

ii) UDP-$GlcNAc_2$ is not a substrate for Chs1 or Chs2, so reducing end extension is unlikely.

iii) UDP-4-O-MeGlcNAc is neither used as substrate by Chs2, nor does it inhibit chitin or CO synthesis by Chs1 or Chs2. The UDP-GlcNAc-binding site of CSs therefore cannot accommodate a CH_3 group at the 4-OH of GlcNAc.

iv) Chs1 and Chs2 differ from one another in their oligosaccharide product profiles and in their ability to use $GlcNAc_2$ as primer. Such differences may impact *in vivo* CS function.

v) Partial proteolysis of Chs1 does not alter the processivity of this CS by converting a CO synthase into a polysaccharide synthase, but does alter the response of this CS to GlcNAc.

vi) The 2-acylamido analogue of GlcNAc, GlcNBu, which primes CO synthesis by Chs1 and Chs2, is taken up by the human pathogenic fungus *Candida albicans* and dramatically affects cellular morphology in a way distinct from GlcNAc itself, potentially due to its conversion to UDP-GlcNBu, and formation of GlcNBu-containing chitin analogue.