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Carbohydrates as structural constituents of yeast cell wall and septum

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Abstract - The cell walls and septa of fungi are useful models for morphogenesis and potential targets for antifungal agents. In the yeast, Saccharomyces cerevisiae, the main structural components of the primary septum and of the cell wall are chitin and $\beta(1\rightarrow3)$glucan, respectively. Two chitin synthetases have been identified in yeast, one of which is essential for septum formation, whereas the other one has a repair function. Both synthetases are bound to the plasma membrane and are found in extracts in a zymogenic form that can be activated by proteases. $\beta(1\rightarrow3)$Glucan synthetase is also attached to the plasma membrane. Its activity is strongly stimulated by GTP and its analogs. By sequential extraction of the membranes, two components have been obtained in soluble form. One contains a GTP-binding protein; the other probably includes the substrate-binding site. Both components, in addition to GTP, are required for glucan synthesis.

INTRODUCTION

Fungal cell walls and septa have essential roles in the life of the fungal cell. They protect mechanically the cell from the environment, prevent osmotic bursting of the cell by the turgor pressure and act as a sieve for large molecules that might harm the cell membrane. Growth of the cell wall goes hand in hand with that of the other cellular elements; septum formation is an essential step in cell division. The shape of the fungal cell is determined by the cell wall: protoplasts that have lost the cell wall assume the spherical form. Because of this shaping role and of their relatively simple composition, fungal cell walls and septa are good models for morphogenesis in eucaryotic cells.

Cell walls and septa have also recently awakened the interest of researchers intent on finding or designing drugs against fungal infections. Because of the vital function of cell walls, interference with their biosynthesis may result in arrest of fungal growth. Furthermore, some of the main components of the cell wall are not found in animal tissues. There is hope, therefore, of finding specific agents against the cell wall that will not harm the host.

We have studied the biosynthesis of yeast (Saccharomyces cerevisiae) cell wall and septum (Fig. 1), because much is known about the biochemistry and cell biology of this organism; yeast is also admirably suited to the manipulations of genetics and molecular biology.

The major components of the yeast cell wall are carbohydrates. About half of the dry weight of the cell wall is accounted for by two beta-glucans (Table 1); the more abundant one is composed of $\beta(1\rightarrow3)$ linked glucose, with some $\beta(1\rightarrow6)$ branches; the other of $\beta(1\rightarrow6)$ linear

TABLE 1. Composition of cell wall and septum of Saccharomyces cerevisiae

	$\beta(1\rightarrow3)$glucan	$\beta(1\rightarrow6)$glucan	mannoprotein	chitin
Cell wall	+	+	+	±
Primary septum	-	?	-	+
Secondary septa	+	+	+	-

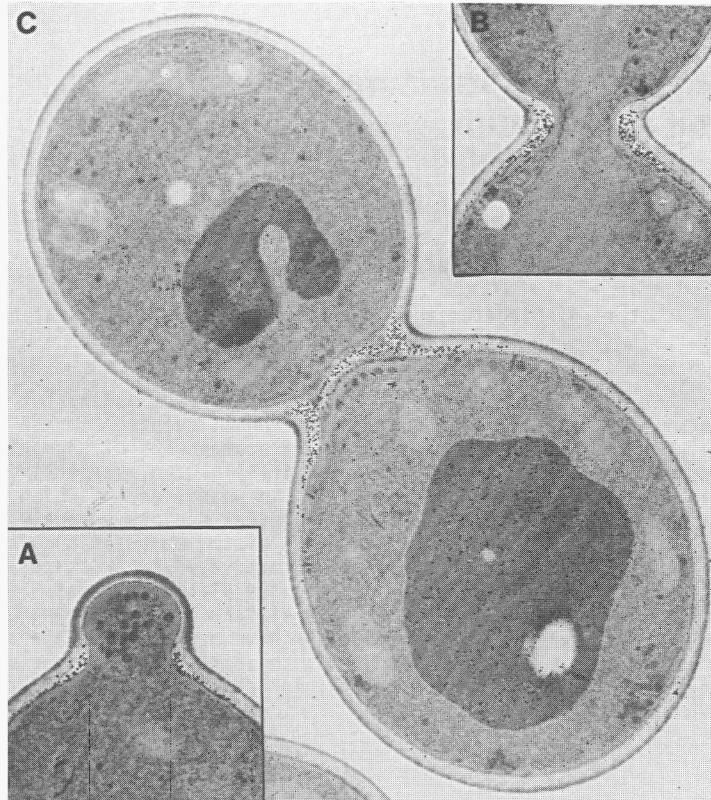

Fig. 1. Cell wall and septum of _S. cerevisiae_. In the cell wall, the inner, more electron-lucent layer is rich in glucans, whereas the outer, darker layer has a high content of mannoprotein (ref 3). The localization of chitin is indicated by the colloidal gold granules (dark dots) attached to wheat germ agglutinin, a lectin with a high affinity for chitin. _A_, emerging bud: the wall is thinner than in the mature cell; notice the ring of chitin (here seen in cross-section) at the base of the bud. _B_, channel between mother and daughter cell before septum closure. _C_, the two cells after completion of the septum. The chitin primary septum is sandwiched between the secondary septa (from ref 4). The gold grains on the vacuole areas (dark regions in the middle of each cell) do not result from attachment to chitin. They are still present in the same areas when gold without wheat germ agglutinin is used.

chain with some $\beta(1\rightarrow3)$ linkages (ref. 1). Most of the remainder of the wall consists of a mannoprotein in which carbohydrate accounts for almost 90% of the weight (ref. 2). The $\beta(1\rightarrow3)$ glucose linkage appears to be essential for maintenance of the wall structure. Treatment of isolated cell walls with purified $\beta(1\rightarrow3)$ glucanase results in solubilization of the structure, including the mannoprotein component (ref. 3). The latter, which is more concentrated in an exterior layer of the wall, appears to act as a sieve for compounds of high molecular weight. It can be solubilized without destroying the integrity of the wall (ref. 3). There is also in the wall a very small amount of chitin, too low to measure accurately, but possibly important in stabilizing in some way the β-glucan (ref. 5, 6).

The septum has a complex, stratified structure, that is best discussed in terms of its ontogenesis. The primary septum, composed of chitin, begins to be laid down at the onset of budding, as a ring around the base of the bud (Fig. 1). Towards the end of the cell division cycle, this ring grows centripetally into a disc, the primary septum, that effectively separates the two dividing cells. Secondary septa, containing the same components as the lateral wall, are then laid down from both the mother and the daughter cell side, resulting in a tri-layered structure (Fig. 1). Cell separation occurs along the chitin boundary, leaving one of the secondary septa and most of the primary septum in the bud scar on the surface of the mother cell (ref. 1).

From the foregoing it is clear that in yeast $\beta(1\rightarrow3)$glucan and chitin are the main structural polysaccharides of cell wall and primary septum, respectively. In many other fungi, chitin has a less specific distribution and is also a major component of the cell wall. Again, in those organisms, chitin and $\beta(1\rightarrow3)$glucan are the most important structural components of both cell walls and septa (ref. 7). As a consequence, it is possible to study the formation

of a biological structure, the cell wall, by investigating the biosynthesis of certain polysaccharides. This approach may lead to an understanding at the molecular level of morphogenetic processes in the intact cell: it will be used here in a discussion of the biosynthesis of chitin and the primary septum, followed by that of β(1→3)glucan, the main structural component of the cell wall.

THE BIOSYNTHESIS OF CHITIN

Two chitin synthetases in yeast

Up to the present time, two chitin synthetases have been identified in S. cerevisiae, chitin synthetase 1 (Chs1) (ref. 8) and chitin synthetase 2 (Chs2) (ref. 9). They catalyze the same reaction, a transfer of N-acetylglucosamine from UDP-N-acetylglucosamine to a growing chain of β(1→4)-linked N-acetylglucosamine residues. Chs1 and Chs2 share some properties, but differ in others (Table 2). One important difference is the cation preference: the best stimulator for Chs1 is magnesium, for Chs2 cobalt. Also, Chs2 has a higher pH optimum than Chs1. The bulk of both enzymes is attached to the plasma membrane (ref. 9, 10). Chs1 has been shown to catalyze a simultaneous synthesis and extrusion of chitin towards the external face of the membrane (ref. 11). It is probable that Chs2 functions in the same way, but no data are available as yet.

TABLE 2. Comparison of properties of chitin synthetases

Chitin synthetase 1	Chitin synthetase 2
Bound to plasma membrane	Bound to plasma membrane
Zymogen, activated by proteases	Zymogen, activated by proteases (different from Chs1 in specificity)
Stimulated by GlcNAc	Stimulated by GlcNAc
Inhibited by polyoxin D and nikkomycin	Inhibited by polyoxin D and nikkomycin less than Chs1
Mg^{2+} best stimulatory cation (Co^{2+} inactive or inhibitory)	Co^{2+} best stimulatory cation
pH optimum 6-6.5	pH optimum 7-8

Another property in common between the two synthetases is their presence in the cell in a zymogen form that can be converted into an active form by partial proteolysis (ref. 9, 12). It is not known how the zymogen is activated in vivo, but it is clear that an activation step is required: it is probably at this step that the enzymatic activity is controlled.

Function of chitin synthetase 1 and 2

Information about the function of the chitin synthetases has come for the most part from a genetic approach. The first gene to be cloned was that coding for Chs1, at that time the only chitin synthetase known in yeast (ref. 13). After a study of its structure, the gene was disrupted and substituted for a normal gene in a wild-type strain. Unexpectedly, cell division and chitin content were not affected in the cells containing the disrupted gene. Obviously, Chs1 was not essential for chitin synthesis and some other synthetase (later identified as Chs2) was present in the cell. However, further studies revealed that cells with a disrupted CHS1 gene were not completely normal. When such cells were grown in a medium insufficiently buffered to prevent the acidification that accompanies yeast growth, many buds lysed during separation from the mother cell (ref. 13, 14). The lysis is apparently due to a small perforation in the center of the birth scar (Fig. 2).

Analysis of the conditions that lead to lysis indicated the possible participation of a chitinase that normally facilitates separation between mother and daughter cell by degrading some of the chitin of the primary septum. In fact, when allosamidin, a chitinase inhibitor, was added to the medium, bud lysis was drastically reduced (ref. 14). Lysis can be totally abolished by introducing in a cell devoid of Chs1 a disrupted chitinase gene (kindly provided by M. Kuranda and P.W. Robbins). The last result shows unambiguously that chitinase is required for bud lysis in the absence of Chs1 (S.J. Silverman, J.A. Shaw and E. Cabib, in preparation). It also indicates that Chs1 must repair the damage caused by excessive activity of the chitinase by replacing some of the lost chitin. Thus, although Chs1 is not absolutely required for viability, it has an auxiliary function that may be important for survival under certain conditions.

With Chs2 the results were quite different. The structural gene was cloned by transforming chs1 strains with high-copy plasmids carrying a yeast DNA library and looking for cells that would overproduce chitin synthetase (ref. 15). Later, the gene was disrupted and

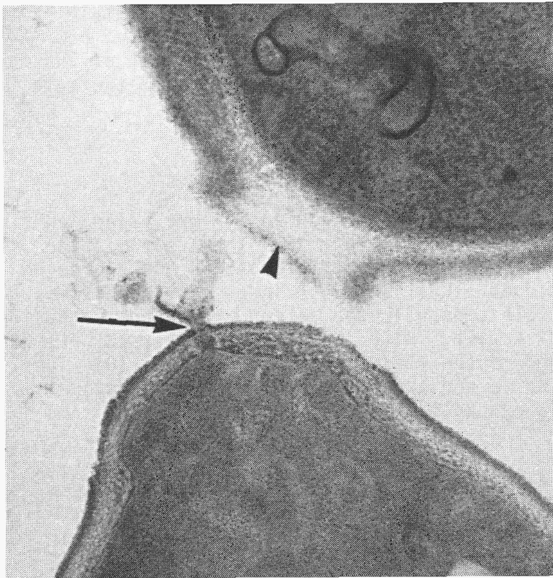

Fig. 2. Lysis of daughter cell during growth in unbuffered minimal medium. A small perforation can be seen in the birth scar (arrow) through which some membranous material has escaped. The mother cell bud scar is indicated by an arrowhead (from ref. 14).

substituted for one of the two copies of <u>CHS2</u> in a diploid strain. Sporulation and tetrad dissection showed that in each tetrad only the two spores containing the normal <u>CHS2</u> gene were able to give rise to colonies. The other two yielded small clumps of abnormal, usually elongated cells without septa. It was concluded that Chs2 is the enzyme normally involved in the construction of the primary septum (ref. 15). Whether the chitin ring that forms at bud emergence is also synthesized with the help of Chs2 or with another synthetase, is not yet clear.

In view of the different functions of the two chitin synthetases, it was of interest to compare their amino acid sequences, as predicted from the coding sequence of the respective genes. Such a comparison revealed a large region, comprising about two-thirds of each gene, in which more than 40% of the amino acids were identical in both sequences (Fig. 3). The

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1  NLTANRALKRSGTEIRKFKLWNGNFVFDSPISKTLLDQYATTTENANTLPNEFKFMRYQA
2  RRANSESKRRMVSDLPPPSKKKALLKLDNPIPKGLLD------TLPRRNSPEFTEMRYTA

1  VTCEPNQLAEKNFTVRQLKYLTPRETELMLVVTMYNEDHILLGRTLKGIMDNVKYMVKKK
2  CTVEPDDFLREGYTLR-FAEMN-RECQIAICITMYNEDKYSLARTIHSIMKNVAHLCKRE

1  NSSTWGPDAWKKIVVCIISDGRSKINERSLALLSSLGCYQDGFAKDEINEKKVAMHVYEH
2  KSHVWGPNGWKKVSVILISDGRAKVNQGSLDYLAALGVYQEDMAKASVNGDPVKAHIFEL

1  TTMINITNISESEVSLECNQGTVPIQLLFCLKEQNQKKINSHRWAFEGFAELLRPNIVTL
2  TTQVSI----NADLDY-VSKDIVPVQLVFCLKEENKKKINSHRWLFNAFCPVLQPTVVTL

1  LDAGTMPGKDSIYQLWREF-RNPNVGGACGEIRTDLGKRFVKLLNPLVASQNFEYKMSNI
2  VDVGTRLNNTAIYRLWKVFDMDSNVAGAAGQIKTMKGKWGLKLFNPLVASQNFEYKISNI

1  LDKTTESNFGFITVLPGAFSAYRFEAVR-----GQPLQKYFYGEIMENEGFHFFSSNMYL
2  LDKPLESVFGYISVLPGALSAYRYRALKNHEDGTGPLRSYFLGETQEGRDHDVFTANMYL

1  AEDRILCFEVVTKKNCNWILKYCRSSYASTDVPERVPEFILQRRRWLNGSFFASVYSFCH
2  AEDRILCWELVAKRDAKWVLKYVKEATGETDVPEDVSEFISQRRRWLNGAMFAAIYAQLH

1  FYRVWSSGHNIGRKLLLTVEFFYLFFNTLISWFSLSSFFLFFRILTVSIALAYHSAFNVL
2  FYQIWKTKHSVVRKFFLHVEFLYQFIQMLFSWFSIANFVLTFYYLAGSMNLVIKHG-EAL

1  SVIFLWLYGICTLST-FILSLGNKPKSTEKFYVLTCVIFAVMMIYMIFCSIFMSVKSFQN
2  FIFFKYLI-FCDLASLFIISMGNRPQGAKHLFITSMVILSICATYSLICGFVFAFKSLAS

1  ILKNDTISFEGLITTEAFRDIVISLGSTYCLYLISSIIYLQPWHMLTSFIQYILLSPSYI
2  GTESHKI----------FVDIVISLLSTYGLYFFSSLMYLDPWHMFTSSIQYFLTLPAFT

1  NVLNIYAFCNVHDLSWGTKGAMANP--LGKINTTEDGTFKMEVLVSSSEIQANYDKYLKV
2  CTLQIFAFCNTHDVSWGTKGSTQESKQLSKAIVVQGPDGK-QIVETDWPQEVD-KKFLEI

1  LNDFDPKSESRPTEPSYDEKKTGYYANVRSLVIIFWVITNFIIVAVVLETGGIADYIAMK
2  KSRLK-EPEFEESSGNEKQSKNDYYRDIRTRIVMIWMLSNLILIMSIIQVFTPQDTDNGY
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Fig. 3. Comparison of Chs1 (1) and Chs2 (2) sequences in the homologous region (amino acids 361-1017 for Chs1 and 213-904 for Chs2). Bars (▪) indicate identical amino acids, asterisks (*) conservative substitutions. The total length of the sequence is 1131 amino acids for Chs1 and 963 for Chs2 (from ref.16).

small remaining segment at the carboxyl terminus and the much larger one at the amino terminus were quite unrelated. These similarities and differences are conserved in the hydropathic profiles, which show a few possible membrane-spanning domains near the carboxyl end (ref. 16).

Deletion of the non-homologous amino terminal region of CHS2 in a high-copy plasmid resulted in expression of a gene product exhibiting enzymatic activity and able to complement a disruption of the chromosomal CHS2 (J.A. Shaw, S.J. Silverman and E. Cabib, unpublished results). This rather surprising result suggests that the divergent amino terminus in Chs2 and possibly also in Chs1 may be dispensable. If this is so, the sequence(s) that somehow determine the different functions of Chs1 and Chs2 must occur either in the homologous region or at the carboxyl terminus.

THE BIOSYNTHESIS OF β (1→3)GLUCAN

As in the case of chitin, synthesis of β(1→3)glucan proceeds by a transfer of a glycosyl residue from a nucleotide sugar to a growing chain of polysaccharide: the sugar here is glucose rather than N-acetylglucosamine and the product is β(1→3) rather than β(1→4)-linked (ref. 17). As shown for chitin synthetase and by the same methodology, β(1→3)glucan synthetase has been found to be attached to the plasma membrane. No activity has been detected in other fractions. No primer appears to be required for the reaction.

There is no indication that glucan synthetase is in a proteolytically activatable zymogen form. In fact, treatment with several different proteases led to inactivation of the enzyme (E. Cabib, unpublished results). On the other hand, the activity is strongly stimulated by guanosine triphosphate and its analogs, at concentrations in the 10^{-6}-10^{-7} M range (ref. 18). A study of the structure-activity relationship of many compounds revealed that several phosphorylated substances show some stimulation, but the guanine nucleotides are by far the best, probably because of a much tighter binding to the enzyme (ref. 19).

The stimulation by guanosine nucleotides is a property shared by β(1→3)glucan synthetases of several fungi (ref. 20). A study of these glucan synthetases culminated in the finding that by extraction with detergent and salts, the membrane preparations could be dissociated into two components, one soluble, one still membrane bound; both components are necessary for the reaction to occur, in addition to GTP (ref. 21). Several criteria indicated that a proteinaceous component in the soluble fraction was responsible for interaction with GTP: the soluble component was protected by GTP against thermal denaturation, inactivation by EDTA at 30°C and inactivation by salts at high concentration. On the other hand, the component that remained membrane-bound after extraction was protected by the substrate, UDP-glucose, but not by GTP, against thermal denaturation (ref. 21). It seems probable that this component contains the catalytically active site of the reaction, whereas the GTP-binding protein would act as a regulatory component.

Recently, these results have been extended to S. cerevisiae (H.-M. Park, S. Das Gupta, J.T. Mullins and E. Cabib, unpublished results). In addition to solubilizing the putative GTP-binding component, we have succeeded in obtaining in soluble form the other component as well. Here also, both components plus GTP or a GTP analog are necessary for full activity (Fig. 4). The GTP-binding component has been partially purified. From gel-filtration experiments, its molecular weight under non-denaturing conditions appears to be in the 65,000-70,000 range.

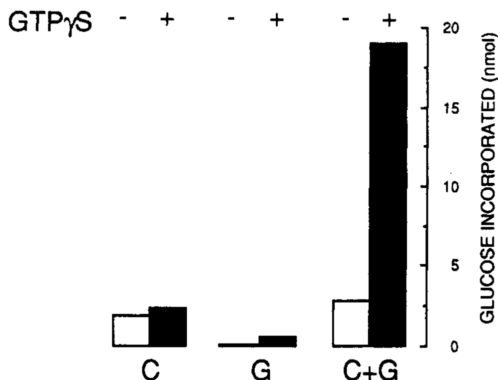

Fig. 4. Reconstitution of β(1→3)glucan synthetase activity from its components. G, GTP-binding component, obtained by extracting yeast membranes with 2M NaCl and 2% Tergitol NP40; C, presumed catalytic component, solubilized by further extraction with 1.1% octylglucoside and 0.2% (3-[(3-Cholamidopropyl)-dimethylammonio]-1-propane sulfonate (CHAPS). It can be seen that each component separately yields little activity in the presence or absence of guanosine 5'-O-(3-thiotriphosphate) (GTPγS). A mixture of both components with the addition of the nucleotide is required for high incorporation of glucose from UDP-glucose into glucan.

THE CONSTRUCTION OF CELL WALL AND SEPTUM

Because of the localization of chitin synthetases and glucan synthetase in the plasma membrane, it seems probable that the formation of both polysaccharides takes place, as has been shown for Chs1, by simultaneous synthesis and extrusion through the membrane. On the other hand, the mannoprotein of the cell wall and secondary septa appears to be synthesized inside the cell, starting at the endoplasmic reticulum and following the general secretory pathway for glycoproteins (ref. 22). To understand how the growth of septum and cell wall takes place, it will be necessary to find out how the synthesis and/or excretion of their components is localized in time and space. For instance, how is it determined that at budding chitin will be laid down in an annular space at the base of the bud? or how is glucan synthesis initiated at the budding site, regulated spatially so as to obtain an oval-shaped cell and shut off when the cell attains maturity? For chitin synthesis, the zymogenic character of the enzyme may offer a partial answer: if, as it appears to happen with Chs1, the chitin synthetases are distributed rather uniformly on the plasma membrane, their temporal and spatial regulation may be controlled by delivering an activator, such as a protease, at a specific site and a specific time, perhaps by using a vesicle or a component of the cytoskeleton as carrier. We know nothing at this point about the activator or the delivering apparatus.

The morphogenetic system must also include signals to alert the cell that chitin synthesis is required at certain locations, such as at the septum, either for construction of the primary septum, in the case of Chs2, or for repair, in the case of Chs1. Finally, the activity of each enzyme will have to be modulated according to the requirements of each situation, and somehow stopped when there is no longer a need for it.

Many of these concepts also apply to the problem of regulation of synthesis of $\beta(1\rightarrow3)$glucan. Here, however, the means by which the enzymatic activity is modulated appear to be different. Interaction with GTP is probably the principal mechanism by which the activity of glucan synthetase is regulated.

It is unlikely that the regulation is attained by changes in the level of GTP, because its average concentration in the cell is probably always higher than that necessary to saturate the synthetase. In other GTP-binding regulatory proteins (G-proteins), found in yeast or elsewhere, regulation can be conserved independently of the concentration of GTP, by modulating the ability of the G-protein to act as a GTP-ase as well as by influencing the exchange between GTP and GDP on the protein (ref. 23). This control may be exerted by interaction with other regulatory proteins, phosphorylation-dephosphorylation, and perhaps other modifications.

From the above discussion, it is clear that for the regulation of both chitin and $\beta(1\rightarrow3)$glucan biosynthesis, i.e. for septum and cell wall formation, an array of proteins, organelles and possibly elements of the cytoskeleton are necessary. To unravel this complex system is going to be a difficult task. A start could perhaps be achieved by a genetic approach. Mutants whose phenotype shows a defect or abnormality in the biosynthesis of one or the other of the cell wall polysaccharides may be helpful. One can also seek conditional-lethal mutants in the already known genes of the synthetases and then look for extragenic suppressors that may define other components of the system.

CELL WALL AND SEPTUM AS TARGETS FOR ANTIFUNGAL AGENTS

As mentioned in the Introduction, inhibition of the synthesis of cell wall components may be used as a way of stopping growth or even causing death of a pathogenic fungus. Both chitin and $\beta(1\rightarrow3)$glucan seem to constitute good targets for this type of attack, first, because they appear to be essential structural components of cell wall and/or septum; second, although $\beta(1\rightarrow3)$glucan is present in plants, neither polysaccharide is found in animals. This enhances the possibility of finding specific inhibitors. Some such inhibitors are available: among the most powerful for chitin synthetase are the polyoxins (ref. 24) and nikkomycins (ref. 25), which compete with the substrate, UDP-N-acetylglucosamine. The polyoxins have been used against plant pathogens, but neither they nor the nikkomycins have been found effective against animal or human infections. There are problems of cell permeability and probably also enzymatic breakdown of the antibiotics in the fungal cell. In _S. cerevisiae_, polyoxin D is effective _in vivo_ but at concentrations several orders of magnitude greater than those that inhibit the chitin synthetases _in vitro_ (ref. 26). It is of interest, however, that Chs2, the essential synthetase, is inhibited much less than Chs1 _in vitro_ (ref. 9 and E. Cabib, in preparation). This invites caution in judging the effect of such inhibitors on other fungi, where the properties and functions of chitin synthetases have not been established.

Inhibitors of glucan synthesis _in vivo_ have also been described. One inhibitor, papulacandin B (ref. 27), has the basic structure of a disaccharide, to which two fatty acids and an aromatic residue are attached. Another group of 3 compounds, aculeacin (ref. 28), echinocandin (ref. 29) and cilofungin (ref. 30), share a cyclic hexapeptide backbone

and differ in a side chain. We have tested papulacandin on β(1→3)glucan synthetases of different fungi (ref. 31) and aculeacin and cilofungin on yeast β(1→3)glucan synthetase (E. Cabib, unpublished results) and found them very poor as inhibitors. However, papulacandin has been found to be fairly effective against the glucan synthetase of Schizosaccharomyces pombe (ref. 32) and cilofungin against that of Candida albicans (ref. 33). At this point it is not clear whether their effect is exerted directly on the enzyme or indirectly, through some other component necessary for glucan biosynthesis and extrusion through the membrane.

The very existence of inhibitors of chitin and glucan synthetase that have at least some and in certain case considerable effects in vivo suggests that this approach towards better and more specific antifungal agents merits further efforts.

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