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Regulation of fungal cell wall growth: A guanine nucleotide-binding, proteinaceous component required for activity of (1→3)-β-D-glucan synthase

(GTP/polysaccharides)

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ABSTRACT **By treatment with detergent and NaCl, particulate (1→3)-β-D-glucan synthase (EC 2.4.1.34) from *Hansenula anomala* or *Neurospora crassa* was dissociated into a "soluble fraction" and a "membrane fraction." Each fraction alone was almost inactive, but enzymatic activity could be reconstituted by mixing the two fractions and adding GTP or one of its analogs. Based on their lability to heat and to incubation with trypsin, the activity in both fractions is proteinaceous. The active component in the soluble fraction appears to bind guanosine 5′-[γ-thio]triphosphate (GTP[γS]), since it was specifically protected by this nucleotide against heat inactivation and against inactivation in the presence of EDTA. Furthermore, precipitation of the soluble component with ammonium sulfate in the presence of GTP[γS] gave rise to a fraction that was highly active in the absence of added nucleotide, indicating either tight binding or covalent interaction between GTP[γS] and the soluble component. The membrane fraction probably contains the catalytic moiety, because it was partially protected against heat inactivation by the substrate, UDP-glucose. Soluble fractions that stimulated membrane fractions from *H. anomala* and *N. crassa* were obtained from several other fungi, including *Saccharomyces cerevisiae*. We propose that the soluble fraction contains a GTP-binding protein that modulates the biosynthesis of (1→3)-β-D-glucan of fungal cell walls and probably has a major role in the regulation of cell wall morphogenesis.**

In many fungi, a predominantly β(1→3)-linked glucan is a major component of the cell wall (1). In one species, *Saccharomyces cerevisiae*, it has been shown that (1→3)-β-D-glucan is the polysaccharide that supports and maintains the wall structure (2, 3). During cell growth and division, cell wall morphogenesis must keep pace with the other anabolic processes that take place during the cell cycle and with their changes in rate. In consequence, one may expect the synthesis of the major cell wall components to be subject to strict regulation (4). Studies with *S. cerevisiae* (5, 6) and, more recently, with several other fungi (7) showed that the activity of (1→3)-β-D-glucan synthase (UDP-glucose: 1,3-β-D-glucan 3-β-D-glucosyltransferase, EC 2.4.1.34) is stimulated by nucleoside triphosphates, guanosine derivatives being by far the most efficient. Further study of this potential regulatory system was hampered by the failure in solubilizing and purifying its constituents. We report now the successful dissociation of fungal (1→3)-β-D-glucan synthase into two proteinaceous components, one soluble and the other particulate. Both components, as well as a guanine nucleotide, are required for activity. The guanine nucleotide-binding component appears to be in the soluble fraction.

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MATERIALS AND METHODS

Organisms, materials, and experimental procedures were the same as previously reported (7) except for those described below. *Neurospora crassa* had to be subcultured four to five times successively in Vogel's minimal medium (8) in order to obtain maximal enzymatic activity.

Materials. Trypsin, soybean trypsin inhibitor, and Tergitol NP-40 were obtained from Sigma; Sephacryl S-300 was from Pharmacia.

Preparation of Soluble and Membrane Fractions. Particulate enzyme was obtained after cell breakage with glass beads as described (7) and stored at −80°C in buffer A [50 mM Tris Cl (pH 7.5) containing 1 mM EDTA, 1 mM 2-mercaptoethanol, and 33% (vol/vol) glycerol]. In initial experiments, a soluble fraction was obtained by centrifugation (165,000 × *g* for 1 hr) of preparations that had been stored for weeks or months at −80°C. In most cases, however, the following procedure was adopted: to the particulate preparation in buffer A, sufficient volume of a 20% solution of Tergitol NP-40 was added to make the detergent concentration 2%. After vigorous Vortex mixing, the suspension was centrifuged for 1 hr at 165,000 × *g*. The supernatant fluid was the "soluble fraction, first extract." The pellet was suspended, in a volume equal to the original volume of the preparation, in buffer A containing 2% Tergitol NP-40, 2 M NaCl and 20 μM guanosine 5′-[γ-thio]triphosphate (GTP[γS]). After Vortex mixing and centrifugation as above, the supernatant fluid was recovered as "soluble fraction, second extract" and dialyzed against buffer A containing 2% Tergitol NP-40 and 20 μM GTP[γS]. The pellet was washed with buffer A lacking glycerol and suspended in buffer A to one-half the original volume of the preparation. This suspension was designated the "membrane fraction." The residual glucan synthase activity in the membrane fraction could be further lowered by omitting GTP[γS] in the NaCl wash. The first extract, containing one-third of the recovered activity, provided soluble fraction free from guanine nucleotides, as needed for certain experiments. The second extraction resulted in additional (two-thirds of the total) soluble activity when performed in the presence of GTP[γS]. The specific activity was about the same in the two soluble fractions. When the nucleotide was omitted in the second extraction, the high salt concentration resulted in total loss of the soluble fraction activity but did not affect the insoluble fraction; therefore, those conditions were used to obtain insoluble fraction free from soluble-component activity. The total recovery of activity in a reconstituted system was about 60% of the original particulate enzyme. The activity of both soluble and membrane fractions was stable for several months, when the fractions were stored at −80°C.

Abbreviation: GTP[γS], guanosine 5′-[γ-thio]triphosphate.

Enzyme Assays. Activity of (1→3)-β-D-glucan synthase was measured either with original particulate preparation or with mixtures of soluble and membrane fraction, as outlined for each experiment. Unless otherwise indicated, all assay mixtures contained 20 μM GTP[γS]. Incorporation of radioactivity from UDP-[^{14}C]glucose into trichloroacetic acid-insoluble material was determined as described (7).

RESULTS

Two Proteinaceous Components of (1→3)-β-D-Glucan Synthase. High speed centrifugation of particulate preparations of (1→3)-β-D-glucan synthase that had been stored at −80°C for at least several weeks in glycerol-containing buffer (7) resulted in substantial loss of activity. The activity, however, could be regained (data not shown) by mixing pellet (designated as "the membrane fraction") and supernatant fluid (designated as "the soluble fraction"). Further studies, directed to improve the efficiency of the separation, showed that part of the "soluble" component could be extracted with the help of a detergent, Tergitol NP-40, and most of the remainder with the further addition of 2 M NaCl (see *Materials and Methods*). The resulting extracted membrane fraction, as well as either one of the two soluble fractions, had little or no enzymatic activity when tested individually, either in the absence (data not shown) or in the presence of GTP[γS] (Table 1). Mixing of soluble and membrane fractions in the absence of nucleotides was also ineffective, but further addition of GTP[γS] led to a large increase in (1→3)-β-D-glucan synthase activity (Table 1). As determined with the *Hansenula anomala* preparation, out of 12 nucleotides assayed only GTP[γS], GTP, and guanosine 5′-[β,γ-imido]triphosphate strongly stimulated the reaction. UTP, ITP, and GDP, which were somewhat effective with the unfractionated enzyme, enhanced the rate only marginally. ATP, CTP, dTTP, guanosine 5′-[β-thio]diphosphate, adenosine 5′[γ-thio]triphosphate, and adenosine 5′-[β,γ-imido]triphosphate were practically without effect (data not shown).

Heating at 100°C for 2 min or trypsin treatment of either fraction resulted in an almost complete loss of activity, an indication that both fractions contain proteins as essential components (Table 1).

Addition of variable amounts of one component to a fixed amount of the other resulted in a saturation curve (Fig. 1). Furthermore, soluble fractions from either *H. anomala* or *N. crassa* gave rise to similar maximal stimulations when added to the membrane fraction from the other organism (Fig. 2). Thus, it was possible to measure the activity of one of the components by adding a fixed amount of the other one, either from the same or a different organism, within the limits of the approximately linear portion of the saturation curve.

Table 1. Reconstitution of (1→3)-β-D-glucan synthase activity with soluble and membrane fractions

Fraction(s)	GTP[γS] (20 μM)	Glucose incorporation, nmol	
		H. anomala	*N. crassa*
Soluble	+	0.1	0.9
Membrane	+	0.8	3.9
Membrane + soluble	−	0.75	6.3
Membrane + soluble	+	19	24
Boiled soluble + membrane	+	2.0	9.6
Boiled membrane + soluble	+	0.1	1.2
Trypsin-treated soluble + membrane	+	1.4	7.8
Trypsin-treated membrane + soluble	+	0.2	1.5

Membrane and soluble fractions were prepared as described in *Materials and Methods*. In the incubation mixtures, 5 μl of membrane fraction suspension and 10 μl of soluble fraction were used. Incubation was for 1 hr at 30°C. "Boiled" samples were placed for 2 min in a boiling water bath. Trypsin treatment was for 15 min at 30°C with 10 μg of trypsin and was stopped by addition of 15 μg of soybean trypsin inhibitor. During this treatment, 40 μM GTP[γS] was present.

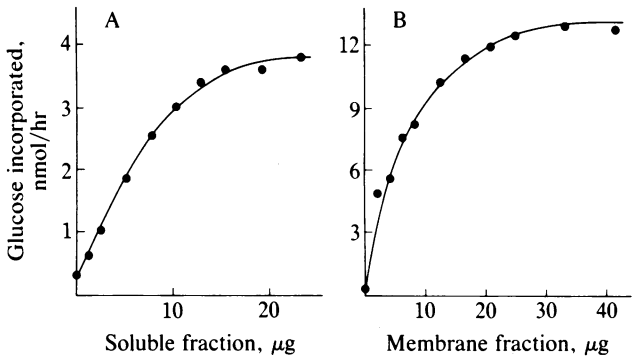

FIG. 1. Effect of varying either the soluble fraction or the membrane fraction, while the other fraction was maintained constant. In *A*, 2.1 μg of membrane fraction (as protein) was used in each tube. In *B*, 13 μg of soluble fraction was present in each tube. Both fractions were from *H. anomala*.

The Soluble Component Interacts with Guanosine Triphosphates. We previously reported (7) that the GTP[γS]-stimulatable activity of the *Neurospora* synthase was lost upon incubation at 30°C. Similar results were later obtained with the *Hansenula* enzyme, and it was also found that an increase in EDTA concentration enhanced the effect (data not shown). It was now possible to investigate which component was responsible for the loss of activity. Incubation of the soluble component at the EDTA concentration contained in the preparation (resulting in 0.5 mM EDTA in the reaction mixture) gave rise to a 50% loss in activity; further addition of EDTA resulted in almost total disappearance of activity. Almost 90% of this disappearance could be prevented by the presence of GTP[γS] during the incubation. In contrast, neither EDTA nor GTP[γS] had any effect on the loss of activity sustained after incubation of the membrane fraction (Table 2). The nucleotide specificity of the protective effect was similar to that described in the preceding section for stimulation of the activity. GTP[γS] also afforded partial protection of soluble-fraction activity from inactivation by heating (Fig. 3). These results were interpreted to mean that a factor in the soluble fraction was able to bind guanine nucleotides and was thereby protected from inactivation by either EDTA or heating. Further evidence on this point was obtained by precipitating the soluble component with ammonium sulfate in the presence of 20 μM GTP[γS]. When the

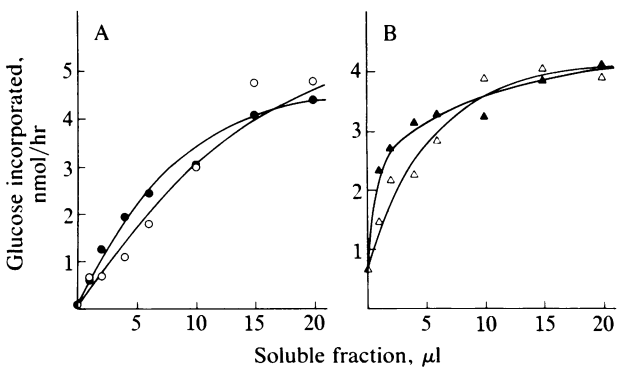

FIG. 2. Effect of soluble fraction from either *H. anomala* (filled symbols) or *N. crassa* (open symbols) on the membrane fraction from either *H. anomala* (*A*) or *N. crassa* (*B*). In each experiment, 0.5 μl of membrane fraction was used per assay.

Table 2. Effect of preincubation of soluble and membrane fractions in the presence of various additions on subsequent activity of (1→3)-β-D-glucan synthase

Fraction preincubated	Additions during preincubation	Glucose incorporated, nmol/hr
None*	—	19
	—	17†
Soluble	None‡	9.6
	2 mM EDTA	3.5
	2 mM EDTA + 40 μM GTP[γS]	15
Membrane	None§	10.6
	2 mM EDTA	12
	2 mM EDTA + 40 μM GTP[γS]	11.7

Fractions were preincubated at 30 min at 30°C with the indicated additions, in a total volume of 20 μl; enzyme activity was measured in a final volume of 40 μl, after adding the missing fraction and the other components of the assay mixture, including in all cases 20 μM GTP[γS].

*Fractions were mixed and immediately assayed, without preincubation.

†With 1 mM EDTA in the assay. Because of a dilution factor of 2, this was the same EDTA concentration present in the assay of mixtures that had received 2 mM EDTA during preincubation.

‡Because of the EDTA in the soluble fraction, this mixture contained 0.5 mM EDTA.

§Because of the EDTA in the membrane fraction, this mixture contained 0.25 mM EDTA.

system was reconstituted with the use of the precipitated fraction, not only was most of the activity recovered, but the enzyme was almost fully active without addition of guanine nucleotides (Table 3). This result cannot be attributed to carryover of GTP[γS] through the precipitation step and subsequent washing. In a parallel experiment with GTP[γ^{35}S], only 1.4% of the radioactivity was recovered in the dissolved pellet. This would correspond to 36 nM nucleotide in the assay mixture, a concentration that would result in negligible stimulation of activity, as separately determined (data not shown). To obtain the effect shown in Table 1, the concentration of GTP[γS] would have to be almost two orders of magnitude greater than that found. It is concluded

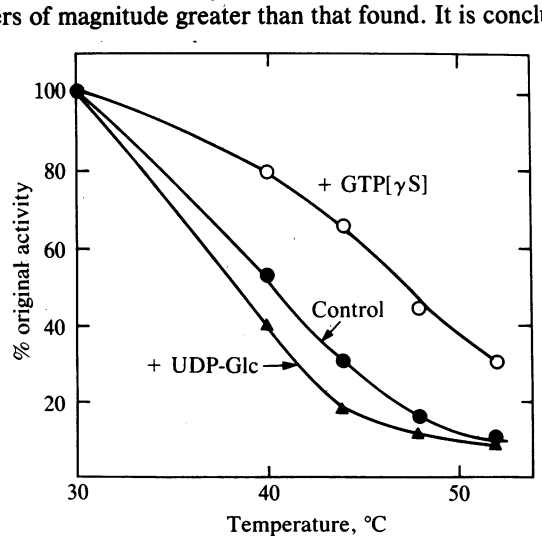

FIG. 3. Protection by GTP[γS] of soluble component of glucan synthase from *H. anomala* against heat inactivation. Each mixture contained 10 μl of *H. anomala* soluble fraction in a total volume of 20 μl. Where indicated, either 40 μM GTP[γS] or 10 mM UDP-glucose were also present. The mixtures were incubated for 5 min at the indicated temperatures and then cooled in ice. The other components of the standard assay were added, including 5 μl of membrane fraction from *H. anomala*, and the activity of glucan synthase was determined.

Table 3. Precipitation of soluble fraction from *N. crassa* with ammonium sulfate in the presence of GTP[γS]

	Total activity, μmol of glucose incorporated per hr	
	No GTP[γS]	+ 10 μM GTP[γS]
Before ammonium sulfate	0.16	1.7
After ammonium sulfate	1.36	1.56

The soluble fraction was obtained by centrifugation of a *N. crassa* preparation that had been stored at −80°C. Tergitol NP-40 was not used in this case because it precipitates with ammonium sulfate. To 0.5 ml of the preparation, 10 μl of 1 mM GTP[γS] was added, followed by 212 mg of solid ammonium sulfate (saturating amount in the presence of buffer A). After 30 min on ice, the suspension was centrifuged for 10 min at 27,000 × *g* and the pellet was washed once with 0.5 ml of buffer A saturated with ammonium sulfate. The washed pellet was dissolved in buffer A containing 2% Tergitol NP-40, to a final volume of 0.5 ml. Activity of glucan synthase was assayed after addition of an insoluble fraction from *H. anomala*, in the absence or presence of 10 μM GTP[γS].

that the activity determined in the absence of added nucleotide is due to either GTP[γS] tightly bound to the soluble component or to a covalent modification of that component by GTP[γS].

Dialysis of the redissolved ammonium sulfate precipitate did not result in loss of activity of the soluble component. Indeed, some increase in activity was detected that could quantitatively be accounted for by the removal of residual ammonium sulfate, an inhibitor of glucan synthase. On the other hand, precipitation of the soluble fraction with ammonium sulfate in the absence of nucleotide resulted in almost total loss of activity (data not shown). Thus GTP[γS] interaction with the soluble component also protects it against inactivation by high salt concentration. Advantage was taken of this fact in the preparation of soluble component (see *Materials and Methods*) by including GTP[γS] in the extraction mixture together with NaCl when activity of the factor was to be preserved.

Finally, it should be noted that the experiment with GTP[γ^{35}S] can be used to estimate the maximum carryover of the nucleotide through the procedure but not to determine the amount of GTP[γS] bound to the soluble component, because of the crude character of the fraction and of the possibility of binding to other proteins.

Is the Catalytic Center of the (1→3)-β-D-Glucan Synthase Reaction in the Soluble or in the Membrane Fraction? In contrast to GTP[γS], UDP-glucose had no protective effect on the soluble component against thermal inactivation (Fig. 3). The reciprocal situation applied to the membrane fraction; i.e., UDP-glucose protected against heat inactivation (Fig. 4), whereas GTP[γS] was ineffective. It may be observed that UDP-*N*-acetylglucosamine, which acts as a competitive inhibitor of glucan synthase (result not shown), also showed protective ability. Under the same conditions, 20 μM GTP[γS] was without effect (result not shown).

These results suggest that a UDP-glucose binding site functionally related to the activity of glucan synthase is present in the membrane fraction.

Behavior of Soluble Fraction on Gel Filtration Columns. During preparation, the soluble factor remained in the supernatant fluid after centrifugation for 1 hr at 165,000 × *g* in the presence of 33% glycerol. Because of the relatively high density of the solution, it seemed possible that the "soluble" factor may be particulate. Therefore, its behavior on Sephacryl S-300 columns was studied. The factor obtained from *H. anomala* in the first extract gave rise to three peaks of activity, the first and largest emerging at the void volume. With the material from the second extract, however, the

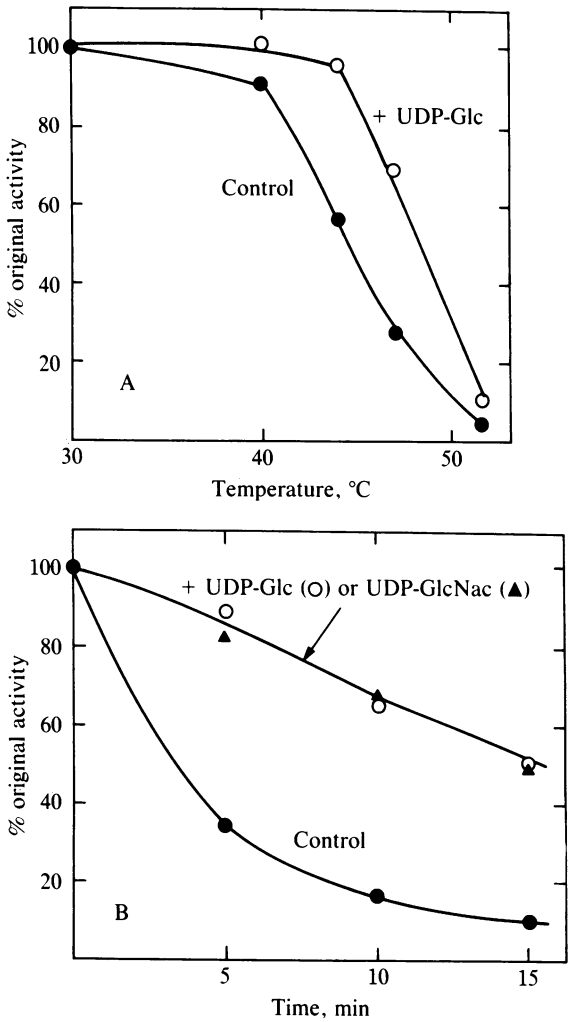

FIG. 4. Protection, by UDP-glucose or UDP-*N*-acetylglucosamine, of insoluble component of glucan synthase from *H. anomala* against heat inactivation. (*A*) Inactivation at different temperatures. Each mixture contained 5 μl of membrane fraction from *H. anomala* in a total volume of 17 μl. Where indicated, 12 mM UDP-glucose was also present. The mixtures were incubated for 5 min at the indicated temperatures and then cooled in ice. The other components of the standard assay, including 10 μl of soluble fraction from *H. anomala*, were added and glucan synthase activity was measured. (*B*) Effect of time on inactivation. The same conditions as in *A* were used, but the heating was carried out at 47°C only, and the time of heating was varied as indicated. UDP-*N*-acetylglucosamine was also used as a protector, at the same concentration as UDP-glucose. Since UDP-*N*-acetylglucosamine inhibits glucan synthase, the results were corrected for the inhibition.

second peak was the largest. The latter result was also obtained with a first extract from *N. crassa* (results not shown). The position of the major peak from the second extract from *H. anomala* or the first from *N. crassa* corresponds to that of a protein of molecular weight 350,000.

Generality of Glucan Synthase Dissociation. Addition of soluble fractions from glucan synthase preparations obtained from different fungi, including *S. cerevisiae*, to membrane fractions from *H. anomala* or *N. crassa* resulted in considerable stimulation of activity (Table 4), indicating that the synthase from the different organisms can be dissociated and that the soluble factors are, to a degree, interchangeable.

DISCUSSION

Our results clearly show that the particulate (1→3)-β-D-glucan synthase from *H. anomala*, *N. crassa*, and other fungi can be dissociated into two proteinaceous fractions, one

Table 4. Effect of soluble fractions from different fungi on membrane fractions from *H. anomala* and *N. crassa*

Origin of soluble fraction	Glucan synthase activity, cpm		
	No membrane fraction	*H. anomala*	*N. crassa*
No soluble fraction added	—	270	660
Wangiella dermatitidis	19	2027	4082
Neurospora crassa	14	1168	3199
Cryptococcus laurentii	56	1180	2283
*Cryptococcus albidus**	21	802	1591
Schizophyllum commune	52	609	2802
Saccharomyces cerevisiae	20	1855	ND†

Soluble fractions were obtained by centrifugation of enzymes prepared as described (7) and stored at −80°C, except for *S. cerevisiae*, where membranes were extracted with 2 M NaCl containing 2% Tergitol NP-40 and 20 μM GTP[γS]. Standard assay conditions were used for measurement of glucan synthase.
*Strain NIH 280e, kindly provided by K. J. Kwon-Chung. *C. albidus* was grown under the same conditions as *C. laurentii* (7).
†Not determined.

soluble and the other insoluble. Reconstitution of enzymatic activity requires both fractions and GTP or one of its analogs. In most of our experiments, GTP[γS] was used because it was the most efficient compound in both stimulating and protecting the enzymatic activity. The saturation kinetics observed when one of the fractions was varied while the other was maintained constant suggest that soluble and insoluble components combine in some stoichiometric relationship to reconstitute an active unit. The function of the guanine nucleotide may be to promote a productive association by binding to one of the components. We could find no experimental support for another possible explanation—i.e., that each one of the two components catalyzes a sequential, discrete step in the synthesis of (1→3)-β-D-glucan, as recently described for the formation of cellulose in *Acetobacter xylinum* (9, 10). Incubation of an assay mixture with one of the components, to allow for accumulation of a putative intermediate, followed by heating at 100°C and addition of the other component, did not result in glucan formation. Similarly, when one of the components was incubated in the assay mixture and the other was added later, there was no enhancement in initial reaction rate over the case in which both fractions were added simultaneously. Furthermore, from the experiment of Table 3 it appears that GTP[γS] bound to the soluble component was sufficient for near maximal stimulation of the glucan synthase reaction, a result that puts a severe limitation on the amount of substrate available for the synthesis of an active guanine nucleotide of the type found by Ross *et al.* (10).

The nature of the association between soluble component and insoluble component in the untreated enzyme preparation is not clear. The partial dissociation obtained by freezing during long periods of time in the presence of glycerol suggests that the soluble component is a peripheral rather than an intrinsic protein of the membrane, a concept supported by the effectiveness of NaCl in promoting dissociation. Nevertheless, hydrophobic interactions also seem to occur, based on the contributory effect of detergent to the solubilization. The tightness of binding between the two components varies greatly depending on the organism. Based on both the release of soluble component upon freezing and the concentration of NaCl required for immediate extraction, the enzyme of *Neurospora* is the easiest to dissociate, followed, in order, by that of *Hansenula* and that of *Saccharomyces* (unpublished results). The parallelism in extractability by freezing and by detergent plus NaCl among the

different species suggests that the same component(s) is solubilized by the two procedures. In agreement with this notion, soluble component prepared in either way supplements inactive insoluble fraction, interacts with GTP[γS] (see, for instance, Fig. 3 and Table 3), and is inactivated by incubation with EDTA at 30°C in the absence but not in the presence of GTP[γS].

The evidence indicates that the guanine nucleotide binding site is in the soluble fraction. This fraction, whether from *H. anomala* or from *N. crassa*, was protected by guanine nucleotides from inactivation at 30°C in the presence of EDTA. The specificity for guanine nucleotides as protectors or stimulators was even more stringent for the reconstituted system than for the unfractionated enzyme. GTP[γS] also showed protection of the soluble component against thermal inactivation (Fig. 3). Further evidence on tight binding of GTP[γS] to the soluble component was provided by experiments in which soluble fraction was precipitated with ammonium sulfate in the presence of the nucleotide. Enzyme that was reconstituted with this soluble fraction was highly active in the absence of added guanine nucleotides, a result that could not be explained by carryover of free GTP[γS] during the procedure. All these results, however, could be explained by an interaction between soluble component and guanine nucleotide followed by the formation of new covalent bonds. It appears unlikely that the putative reaction would be a phosphorylation of the protein, because guanosine 5′-[β,γ-imido]triphosphate, which is presumably unable to transfer its terminal phosphate, functions both as a stimulator and as a protector against inactivation. Even if a covalent interaction occurs, it seems safe to assume that it would be preceded by binding between protein and nucleotide. Attempts to adsorb the soluble component onto GTP-linked agarose gels has resulted so far in only partial binding and elution. In the crude preparations available at this time, the soluble component appeared to be polydisperse, although a major peak with an apparent molecular weight of 350,000 corresponded to most of the activity in extracts from *Neurospora*. Even this preparation, when centrifuged in a glycerol gradient, gave rise to a diffuse distribution of activity over most of the gradient. These results indicate that the factor may easily assemble into aggregates.

Several lines of evidence point to the soluble fraction as responsible for GTP binding. The localization of the glucan synthase catalytic site posed a more difficult question, because formation of the reaction product requires the simultaneous presence of both fractions. Therefore, only somewhat indirect procedures could be used to identify the catalytic site. Attempts to protect the activity with UDP-glucose against inhibition by UDP-pyridoxal (11) and thiol reagents were unsuccessful. UDP-glucose did show some protection against trypsin inactivation of the membrane fraction, but not of the soluble one (data not shown). More clear-cut results were obtained when the different fractions were subjected to heat inactivation. Here, both UDP-glucose and a competitive inhibitor of glucan synthase, UDP-*N*-acetylglucosamine, showed clear protection of the membrane fraction (Fig. 4), whereas GTP[γS] was without effect. Symmetrical results were obtained for the soluble fraction, where GTP[γS] but not UDP-glucose had a protective action (Fig. 3). We conclude that the catalytic site for glucan synthase is probably in the membrane fraction.

The interchangeability of soluble and membrane fractions from different fungi indicates that the mode of regulation of glucan synthase has been highly conserved, at least in the functional sense, during fungal evolution. We propose, therefore, that a GTP-binding or, more generally, GTP-interacting protein is a major regulator of (1→3)-β-D-glucan synthesis and, ultimately, of the morphogenesis of fungal cell walls. This concept defines a growth-related function for GTP-binding regulatory proteins, different from that found in the adenylate cyclase and cyclic GMP diesterase systems (12).

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