Stimulation of exoprotein secretion by choline and Tween 80 in *Trichoderma reesei* QM 9414 correlates with increased activities of dolichol phosphate mannose synthase

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Addition of choline (20 mM) or Tween 80 (0.06%) to the culture medium of *Trichoderma reesei* QM 9414 increased (a) the secretion of protein under both carbon-catabolite-repressed and -derepressed conditions, and (b) cellulase secretion under carbon-catabolite-derepressed conditions. In contrast, no stimulation by choline or Tween 80 was observed with the hypersecretory strain *T. reesei* RUT C-30. In view of the obligatory role of *O*-glycosylation in protein secretion by this fungus, an investigation was made into the effects on this process of choline and Tween 80. A membrane preparation was isolated from both strains of *T. reesei* and used to assay enzymes involved in *O*-glycosylation. Significant differences were observed with respect to the activity of dolichol phosphate mannose (Dol-P-Man) synthase only. Strain QM 9414, grown on media supplemented with choline or Tween 80 exhibited a two- to threefold higher activity of Dol-P-Man synthase compared to a control lacking these supplements. This stimulatory effect was observed during growth under both carbon-catabolite-repressed and -derepressed conditions. In contrast, strain RUT C-30 exhibited decreased activities of Dol-P-Man synthase when grown in media supplemented with choline. Choline had no effect on Dol-P-Man synthase *in vitro*, whereas Tween 80 decreased the activity. Thus the effect of Tween 80 or choline on protein secretion by *T. reesei* may be due to a stimulation of formation and/or activity of Dol-P-Man synthase, thereby elevating the level of *O*-glycosylation and protein secretion.

Introduction

Exoprotein secretion by micro-organisms is known to be stimulated by addition of non-ionic surfactants or phospholipid precursors (Reese & Maguire, 1969, 1971). The mechanism of this stimulation is not clear: in bacteria, these agents appear to cause alterations in membrane fluidity (Umesaki *et al.*, 1977; Wittenberger *et al.*, 1978), but this effect has not yet been demonstrated in filamentous fungi. For *Trichoderma reesei*, a fungus producing high amounts of cellulases (Enari & Niku-Paavola, 1987), we have recently shown that addition of phospholipid precursors (Schreiber *et al.*, 1986) or Tween-surfactants (Panda *et al.*, 1987) has little effect on membrane fluidity, but appears to stimulate formation of intracellular membranous structures. *T. reesei* exoproteins are usually *O*- and *N*-glycosylated (Merivuori *et*

al., 1984), and *O*-glycosylation appears to be obligatory for their secretion (Merivuori *et al.*, 1984; Messner & Kubicek, 1988; Kubicek *et al.*, 1987). We have recently shown that *O*-glycosylation in *T. reesei* (Kruszewska *et al.*, 1989) proceeds in a similar way to that in yeasts (Tanner & Lehle, 1987), and may be controlled at the level of Dol-P or GDP-mannose supply.

In the present communication we provide evidence that stimulation of enzyme secretion in *T. reesei* by exogenous choline (a phospholipid precursor) and Tween 80 (an oleic acid derivative of sorbitol) coincides with increased activities of an enzyme of *O*-glycosylation, dolichol phosphate mannose synthase (EC 2.4.1.83; 'Dol-P-Man-synthase').

Methods

Materials. GDP-[U-¹⁴C]Mannose [302 mCi mmol⁻¹ (370 kBq mmol⁻¹)] was obtained from Amersham. C₉₅-Dol and C₉₅-Dol-P were obtained from the Collection of Polyprenols of the Institute

Abbreviations: Dol-P, dolichol phosphate; Dol-P-Man, dolichol phosphate mannose.

Fig. 1. Effect of choline (20 mM) (▲) and Tween 80 (0.06%, w/v) (△) on secretion of protein by *T. reesei* QM 9414 during growth on glycerol (A) or lactose (B) as carbon source. □, Control without supplement. Both choline and Tween 80 were added at the start of cultivation.

of Biochemistry and Biophysics, Warsaw, Poland. Silica Gel G TLC plates were from Merck. All other chemicals were of analytical grade and were obtained from commercial sources.

Organism and growth conditions. *Trichoderma reesei* strains QM 9414 (ATCC 26921) and RUT C-30 (ATCC 56765) were obtained from the American Type Culture Collection. The fungi were cultivated at 30 °C in wide-mouthed 1 litre Erlenmeyer shake flasks containing 200 ml of the medium described by Mandels & Andreotti (1978), except that the carbon source (glycerol or lactose, as indicated) was used at 0.5% (w/v). All other conditions were as described previously (Kammel & Kubicek, 1985).

Quantification of fungal dry weight. Fungal dry weight was quantified by filtering culture samples through G1 sintered funnels, washing the biomass with a threefold volume of tap water and then distilled water, and drying the washed mat to constant weight at 110 °C.

Cell fractionation. A membrane fraction, capable of carrying out O-glycosylation, was isolated from the fungus by homogenization of mycelia with glass beads and centrifugation as described previously (Kruszewska *et al.*, 1989).

Determination of cellulase activity. Cellulase activity was determined by assaying 'avicelase', using Avicel cellulose as a substrate as described previously (Kubicek, 1981). One unit (1 U) is the amount of enzyme forming 1 µmol reducing sugar min⁻¹ at 50 °C under these assay conditions.

Determination of protein. Protein was determined by the dye-binding procedure (Bradford, 1976) using BSA as a standard. Intracellular protein was extracted with alkali as described by Kubicek (1981) and neutralized with 1 M-HCl before determination.

Determination of glycoprotein. The presence of glycoproteins in the culture filtrate was assessed by subjecting samples of the culture filtrate to SDS-PAGE (Laemmli, 1970) and staining by the periodate procedure as described by Kammel & Kubicek (1985).

Incorporation of [¹⁴C]mannose into Dol-P and into endogenous proteins. This was determined as described previously (Kruszewska *et al.*, 1989).

Reproducibility of results. All experiments presented were done at least in triplicate, which yielded essentially consistent results.

Results

Stimulation of *T. reesei* cellulase and protein secretion by exogenous addition of choline or Tween 80

The stimulation of protein secretion in *T. reesei* by addition of choline or Tween 80 has been reported recently, using cellulose as a carbon source (Schreiber *et al.*, 1986; Panda *et al.*, 1987). However, during growth on this carbon source, mycelia cannot be separated from the insoluble cellulose, and hence preparation of mycelial subcellular fractions is difficult. In the present study we have therefore made use of a soluble carbon source (lactose) that is metabolized only slowly by *T. reesei*; this relieves the fungus from carbon catabolite repression and allows secretion of cellulases (Merivuori *et al.*, 1984). Controls using glycerol (which does cause catabolite repression) as carbon source were included. Figs 1–3 show the formation of cellulase as well as extracellular and mycelial protein by *T. reesei* QM 9414 under these conditions. Protein secretion was observed during cultivation under both conditions, albeit at a lower rate in glycerol medium, and was stimulated by both choline and Tween 80 under both cultural conditions. Cellulase secretion, on the other hand, was significant only during growth on lactose, but was stimulated by both choline and Tween 80 in a similar way to formation of total extracellular protein. When the secreted proteins were

Fig. 2. Effect of choline (20 mM) (▽, ▼) and Tween 80 (0.06%, w/v) (△, ▲) on secretion of cellulase by *T. reesei* QM 9414 during growth on glycerol (open symbols) or lactose (filled symbols) as carbon source. □, ■ Control without supplement. Both choline and Tween 80 were added at the start of cultivation.

subjected to SDS-PAGE and staining for glycoprotein, most of the secreted proteins were shown to be glycosylated, irrespective of the presence of Tween 80 or choline in the medium (data not shown). No significant influence on the formation of mycelial protein (i.e. growth) by either choline or Tween 80 was detected. These results indicate that the differences in protein secretion mainly concern glycoprotein secretion and are not the result of different growth rates of the fungus. This lack of effect of choline on the fungal growth rate is

supported by recent similar findings with *Fusarium graminearum* (Wiebe *et al.*, 1989).

The hyperproducing mutant strain *T. reesei* RUT C-30 develops a greatly increased amount of endoplasmic reticulum under conditions of cellulase formation. We were therefore interested whether this mutant would also be affected by choline or Tween 80. As shown in Figs 4 and 5, stimulation of both protein and cellulase secretion by either supplement was low to negligible. No effect of choline or Tween 80 on the formation of mycelial protein was seen (results not shown). This indicates that the effect brought about by choline or Tween 80 may be complementary to that accomplished by the mutation in RUT C-30.

Mannosyl transfer from GDP-[U-¹⁴C]mannose to endogenous and exogenous Dol-P

In order to find out whether the observed stimulation of glycoprotein synthesis was due to a change in protein glycosylation activity, a microsomal fraction was isolated from *T. reesei* QM 9414 that was able to transfer [U-¹⁴C]mannose from GDP-[U-¹⁴C]mannose to two endogenous acceptors, Dol-P and protein. Addition of 'cold' exogenous Dol-P strongly increased the rate of transfer of labelled mannose to the lipid acceptor (Table 1), indicating that the endogenous concentration of Dol-P was low. In the absence of added Dol-P, higher activities were observed under carbon-catabolite-derepressed conditions (Table 1). This increase was less pronounced with mycelia grown on glycerol as a carbon source, which might indicate elevated Dol-P levels in *T. reesei* microsomes under conditions of carbon catabolite derepression. Also, higher activities of Dol-P-Man synthase were found in catabolite-derepressed mycelia,

Fig. 3. Effect of choline (20 mM) (□, ■) and Tween 80 (0.06%, w/v) (○, ●) on intracellular protein in *T. reesei* QM 9414 during growth on glycerol (B) or lactose (A) as carbon source. △, ▲ Without supplement. Both choline and Tween 80 were added at the start of cultivation. The intracellular protein content is given as mg protein per amount of mycelium present in 1 ml of cultivation broth (mg ml⁻¹).

Fig. 4

Fig. 4. Effect of choline (20 mM) (○) and Tween 80 (0.06%, w/v) (▲) on secretion of total extracellular protein by *T. reesei* RUT C-30. ■, Control without supplement. Lactose was used as carbon source.

Fig. 5

Fig. 5. Effect of choline (20 mM) (○) and Tween 80 (0.06%, w/v) (▼) on secretion of cellulase by *T. reesei* RUT C-30. ■, Control without supplement. Lactose was used as carbon source.

Table 1. Mannosyl transfer from GDP-[U-¹⁴C]mannose to lipid and protein

Conditions for mannosyl transfer are described in Methods. The terms 'lactose', 'glycerol', 'choline' and 'Tween 80' indicate that the mycelia, used for preparation of the membranes, had been grown on media containing either lactose or glycerol as the carbon source in the presence of 20 mM-choline or 0.06% (w/v) Tween 80. Data given are from a single experiment, but replicate experiments yielded essentially the same result.

Growth conditions	[U- ¹⁴ C]Mannose transfer [pmol (mg protein) ⁻¹ per 5 min]	
	+ Unlabelled Dol-P	No unlabelled Dol-P
Glycerol	65.0	7.4
+ Choline	169.7	12.1
+ Tween 80	154.6	8.0
Lactose	84.0	17.6
+ Choline	298.4	34.9
+ Tween 80	173.3	26.6

which coincides with previous findings (Kruszewska *et al.*, 1989).

When membrane fractions isolated from mycelia of QM 9414 and RUT C-30 grown in the absence or presence of choline or Tween 80, were analysed for Dol-P-Man synthase activity, significant differences were observed. RUT C-30 exhibited higher activities than

Table 2. Activities of Dol-P-Man synthase in cell-free extracts of *T. reesei* QM 9414 and RUT C-30 grown on media with and without choline (20 mM)

Conditions for the experiment are given in Methods.

Carbon source	Choline	Dol-P-Man synthase activity [pmol (mg protein) ⁻¹ per 5 min]	
		QM 9414	RUT C-30
Glycerol	—	65.0	278.3
	+	169.7	135.8
Lactose	—	84.0	243.4
	+	287.1	74.8

QM 9414, but the latter had comparable levels in the presence of choline. RUT C-30, in contrast, had lower Dol-P-Man synthase activities in membranes isolated from mycelium grown in choline-supplemented media (Table 2).

It should be noted that these differences in activities were observed throughout the whole growth phase.

Mannosyl transfer from GDP-[U-¹⁴C]mannose to endogenous protein

In order to find out whether other enzymes involved in O-glycosylation might exhibit increased activities in

Table 3. *Effect of GDP-mannose addition and divalent cations on mannosyl transfer from GDP-[U-¹⁴C]mannose to lipid and protein by membranes from T. reesei grown in media supplemented with choline or Tween 80*

Conditions for mannosyl transfer are described in Methods. Unlabelled GDP-mannose (4 μ mol) or MnCl₂ (to give a final concentration of 125 mM) was added after 60 min and the reaction continued for a further 60 min.

Growth conditions	[U- ¹⁴ C]Mannose transfer [pmol (mg protein) ⁻¹ per 120 min]					
	–		+ GDP-Mannose		+ Mn ²⁺	
	Dol-P-Man	Protein	Dol-P-Man	Protein	Dol-P-Man	Protein
Glycerol	12.7	40.2	9.9	48.2	13.1	104.5
+ Choline	26.8	61.7	16.6	71.4	20.5	145.6
+ Tween 80	19.7	61.3	12.9	68.8	14.3	132.4
Lactose	36.6	25.4	27.6	35.2	29.9	65.9
+ Choline	62.3	63.7	37.8	71.0	50.2	99.9
+ Tween 80	42.9	45.1	31.0	48.2	36.0	79.4

mycelia after supplementation by choline or Tween 80, the incubation system for the assay of Dol-P-Man synthase was 'chased' with cold GDP-mannose and the reaction was allowed to proceed in the presence of manganese ions (Kruszewska *et al.*, 1989). The results are shown in Table 3: pulsing with GDP-mannose led to a moderate increase in labelling of protein, which occurred at a comparable rate with membrane preparations isolated under various conditions. This indicates that none of the mannosyl transferases is limiting under any of the conditions applied. Hence Dol-P-Man synthase is the only enzyme influenced by the presence of choline or Tween 80, or by carbon catabolite repression.

Effect of choline and Tween 80 on Dol-P-Man synthase in vitro

The results described above are consistent with increased activities of Dol-P-Man synthase in *T. reesei* cultured in media containing either choline or Tween 80. The effect of choline and Tween 80 on the activity of this enzyme *in vitro* was also investigated. Evidence was obtained (not shown) that choline has little if any effect on Dol-P-Man synthase activity, whereas Tween 80 is actually inhibitory. Both agents produced the same effect with membranes isolated from glycerol- or lactose-grown mycelia. These findings indicate that the stimulation of Dol-P-Man synthase by Tween 80 and choline is not due to a direct effect of these compounds on the activity of the enzyme.

Discussion

On the basis of the present results, at least two different interpretations may be discussed with respect to how

exogenously applied lipids or lipid precursors may lead to enhanced activities of Dol-P-Man synthase. One possibility is that they might stimulate formation of the endoplasmic reticulum by relieving the cell from a shortage of lipid sources, as originally suggested by Schreiber *et al.* (1986); this may simultaneously increase the total cellular activity of associated enzymes (Dol-P-Man synthase has been recommended as an endoplasmic reticulum marker enzyme; cf. Braell, 1988). This explanation is strongly supported by the findings that the hypersecretory *T. reesei* mutant RUT C-30 – which contains an increased cellular amount of endoplasmic reticulum (Ghosh *et al.*, 1982, 1984) – exhibited almost no stimulation of exoprotein secretion by choline or Tween 80. It is also supported by the fact that this mutant already contains elevated activities of Dol-P-Man synthase. That addition of choline to *T. reesei* RUT C-30 even led to decreased activities of this enzyme is puzzling; however, if phospholipid metabolism in this strain is different, this effect can be due to various reasons. The activity of another enzyme from this compartment, UDP-NAcGlc:Dol-P-GlcNAc-1-P-transferase, was not influenced by Tween 80 or choline supplementation (results not shown); hence, not all of the enzymes associated with the endoplasmic reticulum display the observed stimulation in response to choline or Tween. This would indicate that the effect brought about by choline or Tween 80 is more complex than simply a relief from a shortage in membrane biogenesis.

An alternative view on the mechanism of stimulation by choline or Tween 80 is that exogenous lipids directly stimulate the activity of Dol-P-Man synthase. It has been reported that the enzyme from rat liver microsomes displays optimal activity in a phospholipid matrix with preferred non-bilayer organization (Jensen & Schutzbach, 1985). A high proportion of mycelial choline has

been reported to occur on the endoplasmic reticulum of *Neurospora crassa* (Bowman *et al.*, 1987). Hence it is possible that the exogenous addition of choline or Tween 80 selectively enriches this membrane system with phosphatidylcholine or oleic acid, respectively, and thereby enhances the activity of Dol-P-Man synthase. We have shown in this paper that choline and Tween 80 do not stimulate Dol-P-Man synthase activity *in vitro*, but we cannot exclude the possibility that these lipids need to become metabolized or incorporated into the membranes to exert their effect. Isolation of Dol-P-Man synthase and its inclusion in liposomes may be a useful tool to elucidate this point further.

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