Localization by Autoradiography of Synthesis of (1→3)- β and (1→6)- β Linkages in a Wall Glucan during Hyphal Growth of *Schizophyllum commune*

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Labelling of the hyphal walls of basidiospore germlings of *Schizophyllum commune* with radioactive glucose resulted in immediate labelling of the glucans but labelling of chitin was delayed for about 20 min. Radioactive glucose could thus be used as a specific label for wall glucans in a 10 min labelling pulse. Special attention was given to the alkali-insoluble glucans containing (1→3)- β and (1→6)- β linkages and occurring in close association with chitin. Using [^3H]glucose and treating the labelled glucans with periodate, a distinction could be made between glucose residues incorporated in (1→3) and in (1→6) linkages and the spatial distribution of these synthetic activities could be followed by microautoradiography. The results indicated that in growing hyphae the alkali-insoluble glucan synthesized at the apex was very rich in (1→3) linkages and that synthesis of (1→6) linkages increased subapically from about 10% at the apex to about 60% just outside the extension zone. Inhibition of hyphal growth with cycloheximide or by low glucose concentrations inhibited glucan synthesis at the apex but not in subapical parts. At the same time a shift towards synthesis of (1→6) linkages was observed at the apex.

INTRODUCTION

Previous work on wall biogenesis in growing hyphae of *Schizophyllum commune* (Wessels *et al.*, 1983; Vermeulen & Wessels, 1984) has suggested that at the growing hyphal tip chitin and β -glucans are excreted as individual polymers but are then cross-linked *in situ* at a relatively slow rate. The arising chitin–glucan complex is thought to endow the wall with increasing rigidity, explaining the presumed gradient in rigidity required in the extension zone for the occurrence of hyphal morphogenesis (Green, 1974; Trinci & Saunders, 1977; Koch, 1982; Wessels, 1984).

The glucan chains in the alkali-insoluble chitin–glucan complex of mature walls contain both (1→3)- β and (1→6)- β linkages (Sietsma & Wessels, 1977, 1979). On the other hand, a hot-water-soluble glucan at the apex which becomes gradually insolubilized by linkage to chitin appeared to contain only (1→3)- β linkages (Wessels *et al.*, 1983).

We therefore examined the synthesis of the two types of linkages in the alkali-insoluble glucans along the hyphae by autoradiography. We made use of the fact, described in this paper, that radioactivity from added glucose rapidly appears in polymeric glucose but not in polymeric *N*-acetylglucosamine. Using a short labelling time (10 min) we could thus avoid labelling of chitin. For labelling we used glucose with ^3H attached to C-3. Autoradiography was then done before and after periodate oxidation, which removes the label at C-3 when the glucose is involved in (1→6) linkage. The results show that in growing hyphae the alkali-insoluble β -glucan synthesized at the tip is primarily (1→3)-linked but that (1→6) linkages appear subapically. This pattern is drastically changed when growth is inhibited by cycloheximide or low glucose in the medium.

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METHODS

Growth of hyphae. Basidiospores from a dikaryon of *Schizophyllum commune* 1-40 (CBS 344.81) $\times$ 1-50 (CBS 342.81) were grown on minimal medium in shaken cultures at 24 °C for 16 h (Wessels *et al.*, 1983).

Radioactive labelling and isolation of wall fraction. After 16 h of growth the hyphae were labelled by adding [^{14}C]glucose (150 mCi mmol $^{-1}$; 1 mCi = 37 MBq), *N*-acetyl[1- ^{14}C]glucosamine (50 mCi mmol $^{-1}$), [^3H]glucose (20 Ci mmol $^{-1}$), or [2- ^3H]glucose (20 Ci mmol $^{-1}$) (all obtained from New England Nuclear). Growth was stopped by adding 2 vols methanol, the precipitate was collected by centrifugation and washed with 66% ethanol after which cytoplasmic components were extracted with a 1 M-KOH/ethanol mixture (1:2, v/v) at 60 °C for 20 min and the residue was washed with 66% ethanol until neutral.

Labelling of glucose and glucosamine in the wall fraction. After addition of [^{14}C]glucose (7.5 $\mu\text{Ci ml}^{-1}$) or *N*-acetyl[1- ^{14}C]glucosamine (5.0 $\mu\text{Ci ml}^{-1}$) to basidiospore germlings, samples were taken at various times up to 60 min. The wall fraction was isolated, treated with 6 M-HCl at 120 °C for 30 min to effect dissolution and partial hydrolysis and the hydrolysate was taken to dryness over KOH and P_2O_5 . It was hydrolysed to completion with 0.25 M-HCl in the presence of 2 g per 5 ml of Dowex 50 W-X8 (200–400 mesh) in the H^+ form at 100 °C for 24 h. The Dowex was eluted with 2 M-HCl (2 vols) and the eluate was taken to dryness and dissolved in water. This procedure was effective in hydrolysing both the chitin and the glucan in the wall fraction with minimal destruction of the monomers. The hydrolysate was then applied to a cellulose thin-layer plate and chromatographed with ethyl acetate/pyridine/acetic acid/water (5:5:1:3, by vol.). Radioactive glucose and glucosamine were located with a scanner (Berthold) and the cellulose in the relevant areas was scraped from the plates and extracted with water. Radioactivity in the eluates was measured by liquid scintillation and the amount of glucose or glucosamine was determined by a glucose oxidase method (the GOD-PAP test; Boehringer) or the colorimetric method of Johnson (1971), respectively. The specific activities of glucose and glucosamine were then calculated.

Autoradiography of linkage synthesis along the hyphae. Basidiospore germlings were labelled for 10 min with [^3H]glucose or [2- ^3H]glucose (30 $\mu\text{Ci ml}^{-1}$). Where labelling at different glucose concentrations was compared, the glucose concentration at the time of labelling was first determined (GOD-PAP test; Boehringer) and radioactive glucose was added to obtain identical specific activities (3.5 Ci mol $^{-1}$). Incorporation was stopped by adding 2 vols methanol and the wall fractions were isolated as above. A part of these was immediately prepared for autoradiography. Another part was used to isolate the chitin-glucan fraction. This was done by extracting water-soluble material with water at 100 °C for 1 h and alkali-soluble material [mainly (1 $\rightarrow$ 3)- α -glucan] with 1 M-KOH at 60 °C for 20 min (Wessels *et al.*, 1983). To remove traces of contaminating glycogen-like material, the fractions were treated overnight with pancreatic α -amylase (EC 3.2.1.1.; Boehringer) (0.1 mg ml $^{-1}$) in 10 mM-phosphate buffer (pH 7.5), 3 mM-NaCl under toluene at 30 °C. After washings with water, half of the resulting chitin-glucan fraction was incubated with 50 mM-metaperiodate for 2 d at 4 °C in the dark, the other half with water under the same conditions. Samples were then taken and prepared for autoradiography as described by Wessels *et al.* (1983).

RESULTS

Delayed labelling of polymeric glucosamine by radioactive glucose

In previous experiments (Wessels *et al.*, 1983) it was demonstrated that autoradiograms of alkali-extracted walls show grains at the extreme tip of the hyphae after short (10 min) labelling with *N*-acetyl[^3H]glucosamine but not after labelling with [^3H]glucose when labelling was greater subapically. This suggests that during the short exposure to radioactive glucose the chitin in the wall was not labelled.

Since chitin is not oxidized by periodate, and we intended to use the removal of radioactivity by periodate as a measure for the percentage of (1 $\rightarrow$ 6) linkages in the glucan, it was essential to prove that chitin was not labelled by a short exposure to radioactive glucose. Basidiospore germlings were labelled for various times with either [^{14}C]glucose or *N*-acetyl[1- ^{14}C]glucosamine and glucose and glucosamine were recovered from the wall fraction as described in Methods. Fig. 1(a) shows that, after adding radioactive glucose to the medium, glucose monomers in the wall are labelled almost immediately and the specific radioactivity of the glucose rises linearly with time of labelling. However, labelling of the glucosamine in the wall has a lag of about 20 min. Such a lag is not observed when labelling is done with radioactive *N*-acetylglucosamine (Fig. 1b): the specific radioactivity in glucosamine rises linearly from the start of labelling while no radioactivity is observed in the glucose isolated from the walls. Apparently the radioactive glucose taken up is considerably diluted by pools of intermediates in the pathway leading from glucose to *N*-acetylglucosamine.

Fig. 1. Time course of incorporation of radioactive glucose and *N*-acetylglucosamine into glucose and glucosamine isolated from the hyphal wall by hydrolysis. Basidiospore germlings were labelled for various times with [U - ^{14}C]glucose (a) or *N*-acetyl[1 - ^{14}C]glucosamine (b). The wall fractions were isolated and from these the glucose and glucosamine monomers, as described in Methods. The specific radioactivity of glucosamine (●) and glucose (○) was then determined. Straight lines were calculated from regression analysis (correlation coefficient 0.98). The curved line was drawn by hand.

This experiment indicates that a 10 min pulse of radioactive glucose can be safely used to label the glucan component specifically. In addition to chitin (about 23%) the chitin–glucan complex also contains about 9.5% amino acids (Sietsma & Wessels, 1979). The time course of labelling of these amino acids by radioactive glucose is unknown but can be expected to be also delayed. If not, their small contribution to the labelling would not be significant.

A curious result from Fig. 1(a) is that, after the initial lag, the specific radioactivity in glucosamine rises faster than that in glucose. This could be due to a methodological artefact such as preferential hydrolysis of newly synthesized chitin (Vermeulen & Wessels, 1984). Alternatively it reflects a temporary intercalary synthesis of septa which are rich in chitin (van der Valk *et al.*, 1977). Autoradiograms of these young hyphae after labelling with *N*-acetyl[3H]glucosamine often show heavy incorporation of label at several septal sites simultaneously (results not shown).

Localization of the synthesis of (1→3) and (1→6) linkages in the chitin–glucan complex of growing hyphae

In glucan synthesized from [3 - 3H]glucose, periodate should remove the label as 3HCOOH if the glucose molecules are (1→6) linked but not if they are (1→3) linked. Basidiospore germlings (16 h) were labelled for 10 min with either [3 - 3H]glucose or [2 - 3H]glucose as a control. The chitin–glucan complex, retaining the shape of the hyphae, was isolated and treated with α -amylase to remove any contaminating glycogen. The radioactivity along the hyphae was then estimated by autoradiography before and after periodate oxidation. In the case of labelling with [3 - 3H]glucose the grains disappearing after periodate oxidation would represent glucose residues that are (1→6)-linked during the labelling period, the grains remaining being those involved in (1→3) linkage.

Periodate only removed label from the chitin–glucan complex when it was labelled with [3 - 3H]glucose and not when labelling was done with [2 - 3H]glucose. In Fig. 2(e,f) the label removed by periodate oxidation is given as a percentage of the label present before oxidation and thus Fig. 2(f) can be interpreted as expressing the percentage of (1→6) linkages synthesized in the chitin–glucan complex along the hyphae. Apparently the glucan which becomes associated with chitin in the apical region contains fewer (1→6) linkages than in the subapical region.

In the previous experiment the segments measured were 3 μm in length, larger than the apical extension zone (about 1.8 μm). Therefore the experiment was repeated measuring sections 0.6 μm in length. Fig. 3(b, c, j) suggests that the glucan in the chitin–glucan complex made near

Fig. 2. Autoradiography of the chitin–glucan complex after labelling of basidiospore germlings (16 h old) for 10 min with [2-³H]glucose (a, b, e) or [3-³H]glucose (c, d, f). Grains were counted in 3 μ m segments before (a, c) and after (b, d) periodate oxidation. The percentage of grains disappearing was then calculated (e, f). Grain counts represent the mean of counts on 120 hyphae.

the apex is mainly (1→3) linked but that the percentage of (1→6) linkages rapidly increases in the extension zone to become almost constant just outside the extension zone. Fig. 3(a, b, c) also shows that the amount of chitin-associated glucan synthesized increases just behind the extreme tip, to reach a maximum value in the extension zone and then drops again. This pattern of synthesis was observed earlier (Wessels *et al.*, 1983) and interpreted as the generation of the alkali-insoluble glucan from a water-soluble (1→3)- β -glucan precursor maximally synthesized at the extreme tip.

Localization of the synthesis of (1→3)- and (1→6)-linked glucan in the chitin–glucan complex of hyphae inhibited in growth

The growth of hyphae was inhibited by culturing in low-glucose medium or in high-glucose medium in the presence of cycloheximide. Basidiospore germlings (16 h old) were harvested by filtration and resuspended in minimal medium with either 2.5 mg glucose ml⁻¹ (high glucose) or no glucose (low glucose). After 2 h incubation the cultures were labelled for 10 min with [3-³H]glucose in such a way that the specific activity of glucose in both cultures was the same (3.5 μ Ci mol⁻¹) while the high-glucose and low-glucose cultures contained 2.3 and 0.23 mg glucose ml⁻¹, respectively. To part of the high-glucose culture 5 μ g cycloheximide ml⁻¹ was added before labelling.

Comparing the total amount of wall glucans synthesized (Fig. 3a, d, g) it is clear that under growth-inhibiting conditions apical synthesis is reduced but subapical synthesis may even be enhanced. Considering the grains caused by the chitin–glucan complex (Fig. 3b, e, h), the decrease in apical synthesis is again evident but there is still considerable subapical synthesis of the alkali-insoluble glucan in the growth-inhibited hyphae. However, compared to normal

Fig. 3. Autoradiography of total glucan and the chitin-glucan complex after labelling growing and growth-inhibited basidiospore germlings with $[3\text{-}^3\text{H}]\text{glucose}$ for 10 min. (a, b, c, j) Hyphae in $2.3\text{ mg glucose ml}^{-1}$; (d, e, f, k) hyphae in $0.23\text{ mg glucose ml}^{-1}$; (g, h, i, l) hyphae in $2.3\text{ mg glucose ml}^{-1}$ plus $5\text{ }\mu\text{g cycloheximide ml}^{-1}$. Grains were counted in $0.6\text{ }\mu\text{m}$ segments of hyphae immediately after alkali/ethanol extraction (total glucan) (a, d, g) and after extractions revealing the chitin-glucan complex before (b, e, h) and after (c, f, i) periodate oxidation. The percentage of grains disappearing by the periodate oxidation was calculated (j, k, l). Grain counts represent the mean of counts on 120 hyphae.

cultures, a larger part of the label in the alkali-insoluble glucan can be removed in these growth-inhibited cultures (Fig. 3c, f, i), indicating the synthesis of a preponderance of (1 $\rightarrow$ 6) linkages. Calculated over the $10\text{ }\mu\text{m}$ segment the periodate removed 32%, 70% and 53% of the label in hyphae of the high-glucose, low-glucose and cycloheximide-inhibited cultures, respectively. Moreover, in growth-inhibited hyphae the high percentage of synthesis of (1 $\rightarrow$ 6) linkages was particularly evident at the hyphal apex, contrary to the pattern observed in growing hyphae (Fig. 3j, k, l).

DISCUSSION

Fungal hyphae grow by apical extension and in microautoradiograms heavy labelling of the wall can be seen at the apex after administration of radioactive *N*-acetylglucosamine or glucose (Bartnicki-Garcia & Lippman, 1969; Gooday, 1971; Katz & Rosenberger, 1971; Wessels *et al.*, 1983). However, labelled wall polymers are not exclusively synthesized at the hyphal apex as has been tacitly assumed by various authors. For instance, Polacheck & Rosenberger (1975) have assumed that briefly labelled wall material, which they showed to be very susceptible to autolysins, must be equated with apical wall material. On the other hand, Girard & Fèvre (1984)

have suggested that apical and subapical parts of hyphae may differ in the kind of wall polymers that they synthesize.

As shown in the present and in previous autoradiographic studies with *S. commune* (Gooday, 1971; Wessels *et al.*, 1983), a considerable part of the wall glucans is actually synthesized subapically. This material may easily amount to more than 50% of the total, even in 50 μm long germings, because of the large area involved. It now appears that the alkali-insoluble β -glucan deposited at the apex in the extension zone is mainly (1 $\rightarrow$ 3)- β -linked and may be devoid of any (1 $\rightarrow$ 6)- β linkages at the extreme apex. The (1 $\rightarrow$ 6)- β -linked glucosyl residues occur in the extension zone but form the majority in the subapically synthesized glucan. These results do not permit firm conclusions concerning subapical synthesis of one or more glucans with variation in the (1 $\rightarrow$ 3)- β and (1 $\rightarrow$ 6)- β linkages that they contain. However, previous chemical studies on the accumulated alkali-insoluble β -glucan of *S. commune* (Sietsma & Wessels, 1977, 1979) suggest that the mature wall contains very little pure (1 $\rightarrow$ 3)- β -glucan chains. Thus it seems probable that any alkali-insoluble pure (1 $\rightarrow$ 3)- β -glucan chains deposited in the wall are subsequently modified by attachment of (1 $\rightarrow$ 6)- β -linked branches.

The spatial pattern of synthesis of the (1 $\rightarrow$ 3)- β and (1 $\rightarrow$ 6)- β linkages in the alkali-insoluble glucan may be relevant for the process of hyphal morphogenesis. Previously it was shown that the β -glucan at the very apex is in a water/alkali-soluble form and is gradually converted into an alkali-insoluble form, presumably by linkage to chitin (Wessels *et al.*, 1983), while at the same time chitin was shown to undergo a transition which decreased its susceptibility to chitinase and dilute acid (Vermeulen & Wessels, 1984). These changes, occurring in the extension zone, were interpreted as being due to linkages of the glucan chains to chitin chains concomitant with crystallization of chitin and glucan chains to produce a rigid chitin-glucan complex. The fact that the first glucan chains insolubilized at the apex are (1 $\rightarrow$ 3)- β -linked and contain few, if any (1 $\rightarrow$ 6)- β linkages is in line with the observation that the presumed water/alkali-soluble polymeric precursor for this glucan is also mainly (1 $\rightarrow$ 3)- β -linked (Wessels *et al.*, 1983). This structure of the alkali-insoluble glucan in the extension zone would also facilitate the formation of stable triple helices from the chains (Jelsma & Kreger, 1975; Marchessault *et al.*, 1977; Marchessault & Deslandes, 1980). This is required for cross-linking the chitin chains which may be involved in microfibril formation at the same time (Wessels *et al.*, 1983; Wessels, 1984). In the mature wall about half of the (1 $\rightarrow$ 3)- β -glucan chains attached to chitin contain single glucose branches that are (1 $\rightarrow$ 6)- β -linked to the main chain while the other half also contains longer branches of (1 $\rightarrow$ 6)- β -linked glucan chains (Sietsma & Wessels, 1979). At least the presence of single glucose branches does not eliminate the tendency of the (1 $\rightarrow$ 3)- β -glucans to form triple helices (Sato *et al.*, 1981). However, the role of the (1 $\rightarrow$ 6)- β linkages remains unknown.

At low glucose concentration or in the presence of cycloheximide, conditions that impair extension, the apical synthesis of the alkali-insoluble (1 $\rightarrow$ 3)- β -glucan ceases but subapical synthesis of alkali-insoluble (1 $\rightarrow$ 3)- β /(1 $\rightarrow$ 6)- β -glucan continues at a slightly lower rate. As shown in Fig. 3 the subapical synthesis of the alkali-soluble glucan [(1 $\rightarrow$ 3)- α -glucan] may even be enhanced. Apparently apical synthesis is much more sensitive to these conditions than deposition of glucans in subapical parts. Studying the incorporation of *N*-acetylglucosamine, Katz & Rosenberger (1971) also showed cessation of apical labelling of *Aspergillus nidulans* hyphae but increased labelling subapically after cycloheximide addition or osmotic shock. This change in the pattern of *N*-acetylglucosamine incorporation was interpreted as being due to displacement of chitin synthesis. We have observed a similar phenomenon in *S. commune* after addition of cycloheximide or puromycin (unpublished observation). In the case of incorporation of radioactive glucose into the alkali-insoluble glucan, it is not so much displacement of the synthetic machinery but a decrease in the synthesis of (1 $\rightarrow$ 3)- β linkages, particularly at the apex, which is noted after inhibition of growth. In the inhibited systems the synthesis of (1 $\rightarrow$ 6)- β linkages is hardly inhibited and even extends over the apices. In regenerating protoplasts of *S. commune* it has been shown that cycloheximide specifically inhibits the synthesis of the water-soluble (1 $\rightarrow$ 3)- β -glucan precursor, but not its subsequent linkage to chitin, whereas the synthesis of (1 $\rightarrow$ 3)- α -glucan and chitin were less affected (Sonnenberg *et al.*, 1982). Possibly this also applies to growing hyphae.

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