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Direct Photolabeling with [^{32}P]UDP-Glucose for Identification of a Subunit of Cotton Fiber Callose Synthase[1]

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ABSTRACT

We have identified a 52 kilodalton polypeptide as being a likely candidate for the catalytic subunit of the UDP-glucose: (1→3)-β-glucan (callose) synthase of developing fibers of *Gossypium hirsutum* (cotton). Such a polypeptide migrates coincident with callose synthase during glycerol gradient centrifugation in the presence of EDTA, and can be directly photolabeled with the radioactive substrate, α-[^{32}P]UDP-glucose. Interaction with the labeled probe requires Ca^{2+}, a specific activator of callose synthase which is known to lower the K_m of higher plant callose synthases for the substrate UDP-glucose. Using this probe and several other related ones, several other proteins which interact with UDP-glucose were also identified, but none satisfied all of the above criteria for being components of the callose synthase.

The glycosyltransferase UDP-glucose: (1→3)-β-glucan synthase (callose synthase) is an intriguing enzyme in higher plants for a variety of reasons. Located in the plasma membrane, it is normally latent and only becomes activated under conditions of stress such as mechanical damage or pathogen invasion; however, synthesis of callose can also occur without apparent stress, for example, at the cell plate and surrounding plasmodesmata, in pollen tubes, and in cotton fibers at one stage of development (for reviews, see refs. 3, 5, 14), and the question of whether this enzyme may share subunits with the elusive cellulose synthase of plants has been raised several times (3, 12). The callose synthase is clearly activated by micromolar levels of Ca^{2+} in combination with any of a variety of β-glucosides (2, 3, 5, 10, 14).

Callose synthase has proven difficult to purify by conventional techniques, and recently several different approaches have been taken toward identification of the polypeptides which comprise the enzyme. Read and Delmer (22) showed that the substrate analog UDP-pyridoxal, first used to identify the active site of glycogen synthase (25), is an effective inhibitor of callose synthase, apparently through formation of a Schiff's base with an active-site lysine; because inhibition was rendered irreversible after reduction of the Schiff's base with sodium borohydride, this analog offered the hope that the catalytic subunit might be identified by covalent coupling to the active site. There have been several reports of use of UDP-[^{3}H]pyridoxal as labeled probe; in the first (22), a polypeptide of 42 kD from mung bean membranes was labeled, but labeling did not meet all the criteria expected for the catalytic subunit of callose synthase; a second report (19) indicated that a number of membrane proteins from red beet could be labeled; when the specificity of labeling was improved by a substrate protection technique, these were reduced to labeling of polypeptides of 200, 76, 60, and 57 kD. Wasserman's group also demonstrated the feasibility of using 5-azido-[^{32}P]UDP-glucose as an affinity labeling probe (16) and, in subsequent work using this probe (9), concluded that the 57 kD polypeptide of red beet is the most likely candidate for catalytic subunit of the enzyme based upon its enrichment upon product entrapment (see 13), its pH optimum for labeling, and its effector requirements.

Several other techniques of labeling polypeptides may also provide information relevant to β-glucan synthesis in plants. Our group (23) reported that a 73 kD mung bean membrane polypeptide could bind with high affinity, but apparently not react with, UDP-[^{14}C]glucose, after renaturation of polypeptides from SDS gels; we also found a 44 kD polypeptide which was apparently self-glycosylated with one residue of glucose following incubation of mung bean membranes with Mg^{2+} and UDP-[^{14}C]glucose. Since the glucose moeity showed turnover in pulse-chase experiments, this polypeptide could be a candidate for a primer protein in glucan synthesis. This polypeptide may relate to a 40 kD self-glycosylating polypeptide found by Ingold and Seitz (11) in both soluble and membrane fractions of *Daucus carota* L, the soluble form of which has now been purified (21), but whose function remains unclear.

This paper reports our further, more detailed, characterization of cotton fiber membrane polypeptides which interact with UDP-glucose and/or UDP-[^{3}H]pyridoxal. UDP-[^{14}C]glucose was used to detect the glucosylation reaction, and α-[^{32}P]UDP-glucose was used in conjunction with UV irradiation for direct photolabeling of UDP-glucose-binding polypeptides. We have concentrated our efforts on identification of any polypeptides, the labeling of which meet certain specific criteria for being components of a callose synthase complex: (a) the polypeptide(s) should peak coincident with the profile of callose synthase activity during glycerol gradient centrifugation where callose synthase can be well-resolved from the bulk of solubilized membrane proteins; and (b) since Ca^{2+} is known to lower substantially the K_m for UDP-glucose (10),

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the interaction of a catalytic subunit with its substrate or substrate analog should be enhanced by this cation.

METHODS

Isolation, Separation, and Assay of Callose Synthase

Bolls were harvested 19 to 22 d postanthesis from *Gossypium hirsutum* Acala SJ-2 plants grown in the field in Israel in the summers of 1988 and 1989. Locules were quick-frozen in liquid nitrogen and stored at −80°C until used. Fibers were removed and ground to a powder in liquid nitrogen and then ground at 4°C with EB[3] containing 5 mM EDTA, 0.5 mM PMSF, and 0.05 mM leupeptin (2 mL per locule). After filtration through 3 layers of Miracloth, the filtrate was centrifuged at 100,000*g*, and the resulting membrane pellet was resuspended in one-tenth the original volume of EB containing 5 mM EDTA and 1% (w/v) digitonin (Serva), treated in a sonic bath at 4°C for 5 min, and centrifuged again for 1 h at 100,000*g*; this supernatant contained the digitonin-solubilized callose synthase (D) which was subsequently layered in 2 mL aliquots (1–2 mg protein/mL) onto 35 mL of 15 to 30% (w/v) glycerol gradients prepared in EB containing 1 mM EDTA and 1 mM DTT. The gradients were centrifuged at 100,000*g* for 20 to 22 h at 4°C in an SW-28 rotor, fractionated, and assayed for protein using the Bio-Rad Protein Assay Reagent. Callose synthase activity was detected by assaying 150 μL of each fraction in a final volume of 200 μL containing 5 mM $CaCl_2$, 20 mM cellobiose, and 0.2 mM UDP-[^{14}C]glucose (specific activity 5.0 cpm per pmol). [^{14}C]Glucan formed was quantified as described previously (10).

Direct Photolabeling With α-[^{32}P]UDP-Glucose

Synthesis of α-[^{32}P]UDP-glucose was carried out using 250 μCi of α-[^{32}P]UTP (Amersham; >400 Ci/mmol) which was evaporated to dryness and resuspended in a final volume of 250 μL containing 50 mM Hepes/KOH (pH 7.3), containing 8 mM $MgCl_2$, 0.8 mM EDTA, 13 mM glucose-1-P, 3.5 units of UDP-glucose pyrophosphorylase (Sigma), and 4 units of pyrophosphatase (Sigma). TLC and radioautography indicated that after 30 min at 30°C, >95% of the UTP was converted to UDP-glucose and which could be converted to UDP by reaction with callose synthase. The radioactive product was bound to washed activated charcoal (2 mg), the charcoal washed twice with water by centrifugation and the α-[^{32}P]UDP-glucose eluted by two extractions with 50% (v/v) ethanol containing 0.16 N NH_4OH. This extract was immediately evaporated to dryness under vacuum and resuspended in 50% (v/v) ethanol and stored at −20°C. When used for labeling, an aliquot was evaporated to dryness under vacuum and resuspended in 50 mM Hepes/KOH (pH 7.3).

Gradient fractions (190 μL containing 2–30 μg protein depending upon fraction labeled) were incubated at 4°C in a final volume of 250 μL containing 50 mM Hepes/KOH (pH 7.3), 0.5 μCi of α-[^{32}]UDP-glucose (approximate concentration 5 μM), and effectors as described in the text. Samples were irradiated for 20 min using a Mineralight short-wave UV lamp placed approximately 2 cm above the samples. The proteins were freed of the reaction mixture and concentrated as described by Wessel and Flugge (26). The resulting protein precipitates were resuspended in SDS sample buffer containing 2% (w/v) SDS, 5% (v/v) β-mercaptoethanol, and 0.5 mM leupeptin, heated at 100°C for 2 min and subjected to SDS-PAGE according to the procedure of Laemmli (15) using a 4.5 and 12.5% (w/v) acrylamide concentration in the stacking and separating gels, respectively. Electrophoresis was carried out using the Hoeffer Mighty Small gel apparatus and gels of 0.75 mm thickness. Gels were fixed 30 min in 50% (v/v) methanol/12.5% (v/v) glacial acetic acid, stained with Coomassie blue, destained, dried, and subjected to autoradiography for 2 to 7 d using an intensifying screen and Agfa-Gevaert Curix RP2 film.

Labeling with UDP-[^{3}H]Pyridoxal or UDP-[^{14}C]Glucose

UDP-[^{3}H]pyridoxal was synthesized as described (19, 22). Gradient fractions (190 μL) were incubated 10 min at 4°C and then 5 min at 25°C in a final volume of 250 μL containing 0.8 μCi of UDP-[^{3}H]pyridoxal (specific activity 3.2 Ci/mmol; final concentration 1.2 μM), 50 mM Hepes/KOH (pH 7.3), and effectors as described in the text. Any Schiff's bases formed were then reduced by addition of $NaBH_4$ to a final concentration of 10 mM and incubation for a further 5 min at 25°C. Incubation of gradient fractions with 0.1 μCi of UDP-[^{14}C]glucose (200 Ci/mol; final concentration 1.7 μM) with effectors as described was for 10 min at 25°C.

Following these labeling procedures all samples were cleaned, concentrated, and electrophoresed as described above. To prevent quenching, gels containing ^{3}H or ^{14}C were not stained, and were soaked in Amplify (Amersham) after fixation and prior to fluorography.

RESULTS

Glycerol Gradient Separation of Callose Synthase

Figure 1 shows the profile of total protein and callose synthase activity obtained after glycerol gradient centrifugation of digitonin-solubilized cotton fiber membrane proteins in the presence of 1 mM EDTA. Also shown are patterns of Coomassie blue staining of proteins after SDS-PAGE of each fraction, including a pellet fraction (P) from the gradient and a pattern for the total proteins prior to separation (D). Activity was determined under the two different conditions used in subsequent experiments for labeling: (a) with Ca^{2+} (as $CaCl_2$) as effector, or (b) with $MgCl_2$ and EGTA. The latter condition was considered to be analogous to a control, since many proteins might interact with nucleotides in the presence of Mg^{2+}, but the callose synthase is rather unique in requiring Ca^{2+}; in addition, we also considered that a catalytic subunit of cellulose synthase would probably require Mg^{2+} (as is known for the bacterial enzyme, see ref. 3), and it might be possible to label such a polypeptide even if glycosyltransferase activity is not detectable. A β-glucoside activator was not included in the assays, since the high concentration of glycerol present in the fractions is known to substitute for these effectors (10), and callose synthase activity profiles were not different when cellobiose or octylglucoside was included in

[3] Abbreviations: EB, extraction buffer containing 25 mM Hepes/KOH (pH 7.3); D, digitonin-solubilized membrane proteins.

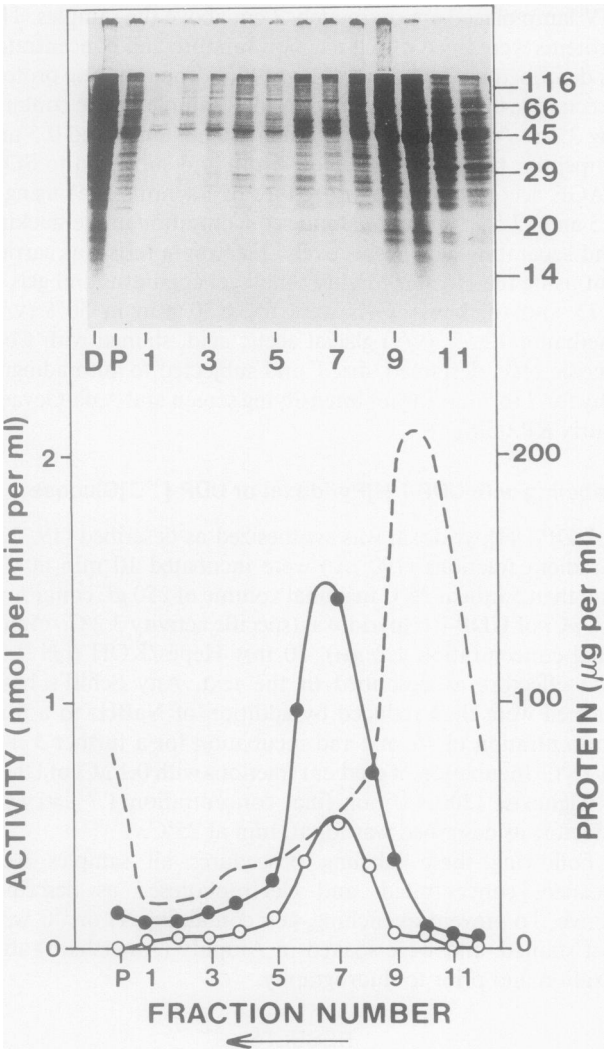

Figure 1. Glycerol gradient centrifugation of cotton fiber digitonin-solubilized proteins. The arrow shows the direction of sedimentation. The upper panel shows the Coomassie blue pattern of staining of polypeptides from equal volumes (190 μL, 2–30 μg protein depending upon the fraction) of the various gradient fractions after separation by SDS-PAGE. Numbers to the right of this panel indicate the position of molecular mass standards (in kD). (– – –), Total protein; (●), callose synthase activity assayed in the presence of 1.2 mM free $CaCl_2$; (○), callose synthase activity assayed in the presence of 5 mM each of $MgCl_2$ and EGTA. D, Total digitonin-solubilized proteins prior to centrifugation; P, pellet fraction obtained after gradient centrifugation.

the assay. As is consistent with previous kinetic studies with the enzyme (10), callose synthase activity is substantially higher with Ca^{2+} as effector, but methylation analyses indicate that the product of enzyme from the gradients is entirely (1→3)-β-glucan under either condition of assay (not shown). As reported previously (10), the callose synthase under these conditions behaves as a high molecular mass complex, migrating faster than the bulk of the soluble proteins. Note that two polypeptides, at about 52 and 46 kD, peak in concentration coincident with the peak of enzyme activity.

Labeling of Polypeptides with UDP-[^{14}C]Glucose

Figure 2 shows the results obtained when gradient fractions were incubated under the two different effector conditions with UDP-[^{14}C]glucose and subsequently separated by SDS-PAGE and subjected to fluorography. Most prominent was a polypeptide of 54 kD (a minor band just below this band is also faintly visible), which is labeled better in the presence of Mg^{2+} and EGTA than in the presence of Ca^{2+}; a different, less intense band of about 42 kD was observed in P under both conditions of labeling. Neither of these bands peaked coincident with callose synthase activity which peaks in fractions 6, 7, and 8 (see Fig. 1), nor were they labeled more effectively with Ca^{2+}.

Labeling of Polypeptides with UDP-[^{3}H]Pyridoxal

Figure 3 shows the profile obtained after fluorography when fractions were incubated with UDP-[^{3}H]pyridoxal followed by reduction with $NaBH_4$. A very prominent band at 34 kD (with several surrounding minor bands) was labeled, and labeling was observed only in the presence of Ca^{2+}. The interaction with Ca^{2+} is very specific; further experiments

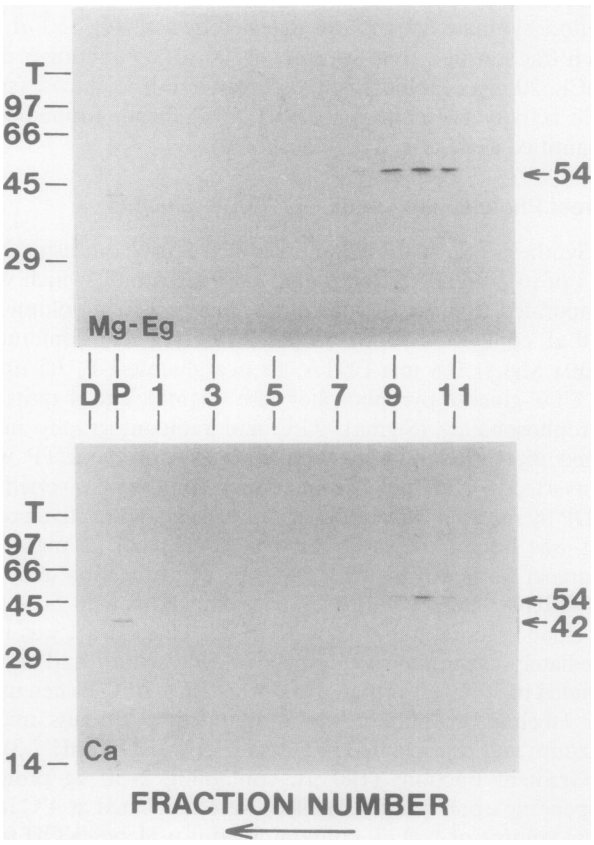

Figure 2. Fluorogram of polypeptides from gradient fractions (190 μL) following labeling with UDP-[^{14}C]glucose in the presence of 5 mM each of $MgCl_2$ and EGTA (Mg-Eg) or $CaCl_2$ (Ca) and separation by SDS-PAGE. Fraction numbers correspond to those of Figure 1. Numbers at left indicate position of molecular mass standards; T, top of the separating gel.

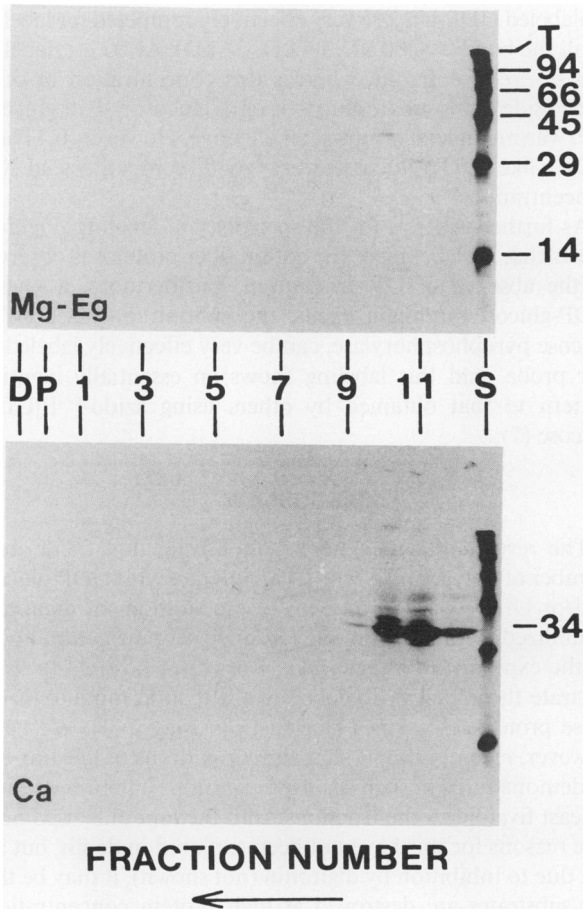

Figure 3. Fluorogram of polypeptides from gradient fractions following labeling with UDP-[^{3}H]pyridoxal. Lanes on far right of fluorogram contain radioactive molecular mass standards.

indicated that addition of Mg^{2+} with Ca^{2+} substantially inhibited labeling (not shown). Although labeling of this polypeptide met one important criterion (*i.e.* a requirement of Ca^{2+}) for callose synthase, the position of this polypeptide in the gradient did not coincide with callose synthase activity.

Direct Photolabeling of Polypeptides with α-[^{32}P]UDP-Glucose

Figure 4 shows that a more complex pattern of labeling of polypeptides is obtained with this probe. Under either condition of labeling, the most prominent band labeled was an 84 kD band which, although present in the peak fractions of callose synthase activity (fractions 6, 7, and 8), did not peak coincident with it. A similar pattern is also observed for a less-intensely labeled species which focuses at the top of the separating gel. A triplet of weakly labeled bands at 34, 39, and 42 kD are observed in the area where total protein peaks in the gradient; these bands are more heavily labeled in the presence of Ca^{2+} suggesting that at least the 34 kD band may be analogous to the one labeled by UDP-[^{3}H]pyridoxal (see Fig. 3).

Most interesting is a faint band at about 52 kD which peaks coincident with callose synthase activity and is only observed in the presence of Ca^{2+}. Thus, this band appears to meet the two criteria we suggested for the catalytic subunit of the callose synthase.

In order to study further the effector requirements for labeling of these bands, we pooled and concentrated by pressure dialysis the fractions of peak callose synthase activity (6–8); this preparation was then labeled under several different conditions as shown in Figure 5. As in the experiment with individual gradient fractions, the 84 kD band is again prominent, but labeling shows no strict dependence upon the presence of divalent cations. Upon concentration of the fractions for this experiment, the Ca^{2+}-dependent labeling of the 52 kD band is more clearly visible; as for the 34 kD band labeled with UDP-[^{3}H]pyridoxal, labeling of this 52 kD band is clearly inhibited by the presence of Mg^{2+}.

Also notable is the enhancement by Ca^{2+} of labeling of high mol wt species, some of which penetrates to the top of the separating gel and some of which does not even penetrate the stacking gel (stacking gel not shown here). These species are clearly >200 kD, since they run similarly even in 7.5 to 15% gradient gels (not shown).

The affinity for and specificity of interaction of these polypeptides with UDP-glucose was examined using unlabeled competitors in combination with the labeled probe (Fig. 6).

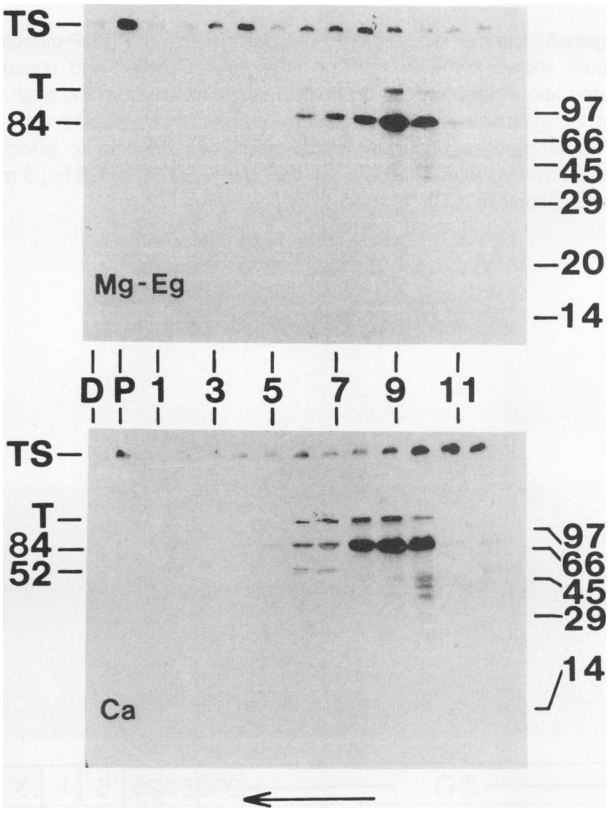

Figure 4. Autoradiogram of [^{32}P]polypeptides from gradient fractions following labeling with α-[^{32}P]UDP-glucose. Definitions and labeling conditions are as described in Figure 2. Numbers at right indicate position of mol wt standards; TS, Top of stacking gel; T, top of separating gel.

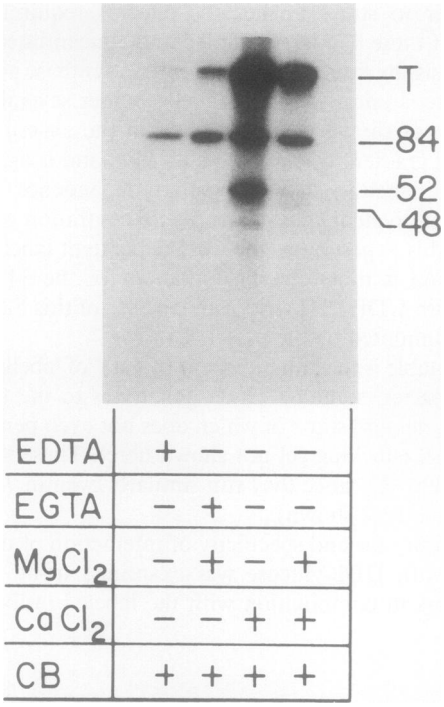

Figure 5. Effector requirements for labeling with α-[^{32}P]UDP-glucose. Figure shows autoradiogram of polypeptides pooled and concentrated about threefold (25 μg protein per reaction) from the peak of callose synthase activity on glycerol gradients and labeled with α-[^{32}P]UDP-glucose under a variety of effector conditions. Effector concentrations were: free Ca^{2+}, 1 mM; Mg^{2+}, EDTA, or EGTA, 5 mM each; cellobiose (CB), 10 mM.

Unlabeled UDP-glucose very effectively competed for labeling of all the bands (>200 kD, 84 kD, 52 kD). At 50 μM, labeling was severely repressed, whereas this concentration of other nucleoside diphosphate glucoses, or of glucose-1-P or glucose-6-P, was much less, or not at all effective. However, UTP and UDP, like UDP-glucose, were good competitors at this concentration.

As further controls for the specificity of labeling, Figure 7 shows that no labeling of the cotton fiber proteins is observed in the absence of UV irradiation. Furthermore, a known UDP-glucose-binding protein, the subunit of yeast UDP-glucose pyrophosphorylase, can be very effectively labeled by our probe, and this labeling shows an essentially identical pattern to that obtained by others using azido-[^{32}P]UDP-glucose (7).

DISCUSSION

The results presented here demonstrate that there are a number of polypeptides which can interact with UDP-glucose and/or UDP-pyridoxal present in preparations of digitonin-solubilized membrane proteins from developing cotton fibers. In the experiments shown here, it was not possible to demonstrate these by affinity labeling of the total mixture (D) of these proteins (see lanes labeled D in Figs. 1–4). We have, however, recently shown that similar patterns of labeling can be demonstrated in such crude preparations if they are diluted at least fivefold in the reactions from the amounts used here. The reasons for this have not been explored in depth, but are not due to inhibition by digitonin (not shown); it may be that the substrates are destroyed at high protein concentrations before they can be effectively covalently coupled to the polypeptides. However, after separation by glycerol gradient centrifugation, labeling patterns are most distinct, and a number of different polypeptides can be labeled by one or more of the techniques employed herein.

The polypeptides labeled by incubation of solubilized mem-

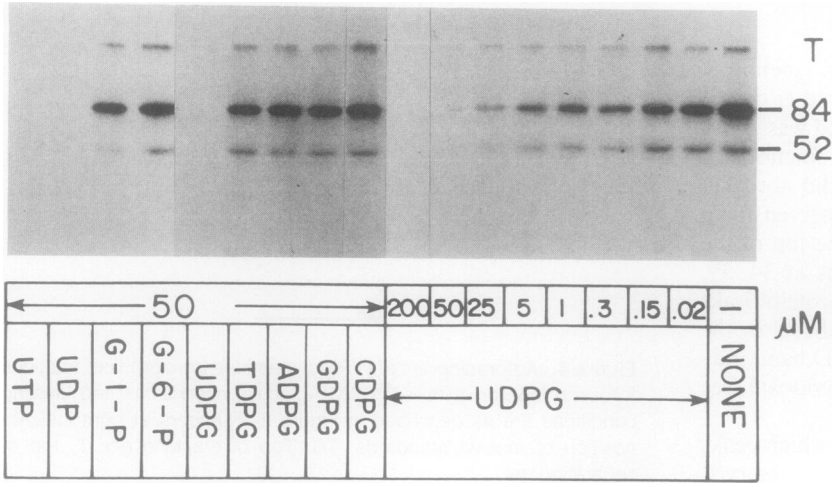

Figure 6. Specificity of labeling with α-[^{32}P]UDP-glucose. Preparations similar to those used in the experiment of Figure 5 were labeled with 5 μM α-[^{32}P]UDP-glucose, 1 mM free Ca^{2+}, and various concentrations of unlabeled competitors as indicated.

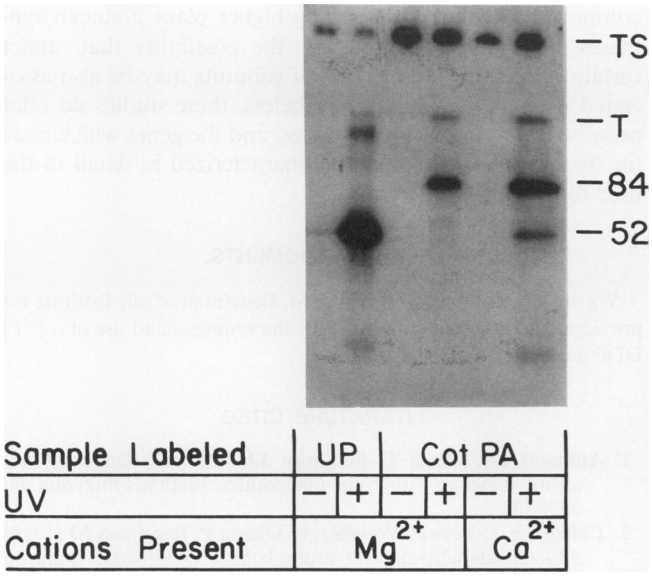

Figure 7. Further controls on specificity of labeling with α-[^{32}P]UDP-glucose. UP, Yeast UDP-glucose pyrophosphorylase (Sigma, 1.6 μg protein, 0.3–0.8 units per reaction); Cot PA, cotton fiber proteins concentrated 10-fold from peak of activity of callose synthase on glycerol gradients. $MgCl_2$ was present at 5 mM, $CaCl_2$ at 1 mM.

brane proteins with $MgCl_2$ and UDP-[^{14}C]glucose do not appear to be associated in any obvious way with callose synthase; they do not migrate coincident with the enzyme in gradients, nor do they show a preference for Ca^{2+} as effector. As suggested previously (23), these polypeptides may function in some other pathway of glucan synthesis, such as that for starch or xyloglucan. They also may be related to the self-glycosylating polypeptide studied by Ingold and Seitz (11) and Quentmeier *et al.* (21). If so, it is not clear why they do not effectively label also with α-[^{32}P]UDP-glucose (see Fig. 4), and raises the possibility that the polypeptides labeled with UDP-[^{14}C]-glucose do not interact directly with the substrate, but rather serve only as acceptors of the glucose moeity. Further work would be necessary to clarify this issue.

Direct photolabeling with α-[^{32}P]UDP-glucose revealed a 52 kD polypeptide which appears to have a direct relationship to the callose synthase. It meets the criteria we set for being a catalytic subunit of the enzyme: it labels only in the presence of Ca^{2+}, and, rather surprisingly, concomitant addition of Mg^{2+} inhibits labeling; it migrates coincident with the enzyme activity in glycerol gradients as demonstrated both by Coomassie blue staining (Fig. 1) and by labeling (Fig. 4).

Use of unlabeled competitors (Fig. 6) indicated that there is a high degree of specificity in the interaction of this and the other labeled species with UDP-glucose. The exact mechanism of direct photolabeling is not completely clear; upon irradiation, free radicals can be generated from UV-absorbing compounds which can lead to covalent coupling of that compound to its nearest-neighbor molecule (1, 18); thus, one can envisage a reaction of UDP-glucose with a UDP- or UDP-glucose-binding polypeptide as occurring either by a free radical generated in the uracil moeity or, less likely but also possible, within a tyr, phe, or trp moeity in the active site of the protein. It is clear from the results presented that coupling does occur after UV irradiation, but we cannot be certain of the mechanism. For example, we cannot totally exclude the possibility that a portion of the labeling could come from interaction with UDP which would be cleaved at the active site during catalysis. UDP is known to be a good competitive inhibitor of the enzyme (20) and would be expected also to label in a similar way to UDP-glucose. We have determined, however, that the majority of substrate does remain intact at the end of the labeling period at 4°C, and it would seem most probable that the predominant reactive species is UDP-glucose. As another indication of the validity of this method of labeling, we have also been able to photolabel a preparation of UDP-glucose pyrophosphorylase (Fig. 7) and show that its subunit is very effectively labeled.

A different species, the labeling of which is also markedly enhanced by Ca^{2+}, is a high molecular mass species of >200 kD. The nature of this high molecular mass species is not yet clear; in several experiments, we have observed that the ratio of labeling of this species relative to the 52 kD polypeptide is variable. It is possible that the high molecular mass species represents a form of the 52 kD subunit with associated glucan product. We also cannot exclude the possibility that this species is some aggregate of the labeled polypeptide(s) which is formed during concentration and preparation of the samples for SDS-PAGE, or which is formed by UV-mediated cross-linking during photolabeling. However, we have not observed dissociation of this species when samples were concentrated by other means or heated in SDS-PAGE sample buffer at lower temperatures. We have also studied the time-dependence of appearance of this species during labeling, thinking that if it is due to UV-mediated cross-linking, it may appear only at later times of irradiation. However, a time-course of labeling (not shown) indicated that the ratio of labeling of all bands remained approximately constant between 5 and 30 min of labeling; the intensity of labeling of all bands did increase up to about 20 min, and was not enhanced by further irradiation. We also tested if similar UV irradiation caused a shift in mobility of molecular mass standards, and it did not. However, if the holoenzyme is composed of many subunits, there may be enhanced potential for cross-linking in such a transmembrane complex where hydrophobic interactions involving aromatic amino acids may be important. Certainly, cross-linking during such photolabeling has been reported in literature (24). Resolution of the nature of the labeled high mol wt species must therefore await the availability of a specific antibody to the 52 kD species and/or other probes to test for relatedness between these species.

We have also seen a very variable labeling of a 46 to 48 kD species which is often, but not always stimulated in its interaction with UDP-glucose by inclusion of octylglucoside in the reactions. In Figure 5, labeling of this species can be seen, but is not as pronounced as in some other experiments which were not shown here. Due to the variable nature of labeling of this species, we are not able to conclude anything at this time about its role in glucan synthesis.

Lawson *et al.* (16) demonstrated photolabeling of a variety of polypeptides from membranes of carrot using azido-[^{32}P] UDP-glucose; the most prominent of these were at 150, 57, and 43 kD; in a subsequent study (9), they concluded that the

57 kD band was the most likely candidate for being the catalytic polypeptide of callose synthase. It is difficult to compare results, since they used a different plant species and different techniques of partial purification and labeling, and their SDS-PAGE separations were with gradient gels and a different set of mol wt standards; however, it seems reasonable to propose that the 57 kD band of carrot is indeed analogous to the 52 kD polypeptide of cotton fibers. The fact that labeling of the 57 kD polypeptide is also suppressed by Mg^{2+} (D Frost, personal communication) indicates another similarity between these polypeptides.

Additional results of ours concerning the selection of a monoclonal antibody which can immobilize detergent-solubilized callose synthase (4, 6, and DP Delmer, unpublished data) indicate that the structure of the digitonin-solubilized enzyme in the presence of Mg^{2+} may be more complex. It is clear that the enzyme is larger under these conditions (10), and we now know that the doublet of 60 to 62 kD recognized by this antibody associates with the enzyme in a Mg^{2+}-dependent manner (6, and DP Delmer, unpublished data). The studies presented here do not identify this doublet as being UDP-glucose-binding proteins, and we have had difficulty in proving whether the association of these polypeptides with the enzyme represents an event of physiological importance or is an artifact mediated by interaction of divalent cations with the detergent-lipid-protein micelles which comprise the enzyme.

The effects of Mg^{2+} on complex size may also be relevant to the results we obtained using the probe UDP-[^{3}H]pyridoxal (Fig. 3). Although this analog is a potent inhibitor of callose synthase (22), no condition was found wherein it labeled the 52 or >200 kD polypeptides. Instead, it labels, in a strictly Ca^{2+}-dependent manner, a 34 kD polypeptide which does not migrate coincident with callose synthase activity in gradients containing EDTA (Fig. 3). We have evidence now that such a 34 kD polypeptide is also associated in very high concentrations with the larger Mg^{2+}-induced complex (6). Fink *et al.* (8) have also found that a 32 kD polypeptide is heavily enriched in their most pure preparations of soybean callose synthase. Even so, this still does not explain why we find that the enzyme, taken from the peak of activity from EDTA gradients where the 34 kD polypeptide is virtually nondetectable, is still susceptible to inhibition by this analog.

The most predominant cotton fiber membrane polypeptide labeled by α-[^{32}P]UDP-glucose is that at 84 kD. Its relationship to the callose synthase is not obvious, although we do find it also sedimenting with callose synthase in the presence of Mg^{2+} and Ca^{2+} (6). Its size is essentially the same as that recently identified for the catalytic subunit of the bacterial cellulose synthase (17), and this prompted us recently to investigate whether it may also interact with the specific activator of this enzyme, c-di-GMP. Indeed, we have found that a polypeptide of identical mobility to the 84 kD polypeptide identified here can also be photolabeled with c-di-[^{32}P]GMP (Y Amor, R Mayer, M Benziman, DP Delmer, unpublished data), indicating this species may be involved in cellulose synthesis.

In sum, these results have identified a 52 kD polypeptide as a highly likely subunit of the cotton fiber callose synthase. However, our other results imply that there is considerable complexity involved in studying higher plant β-glucan synthases, and we cannot rule out the possibility that, under certain conditions, other types of subunits may be also associated with the enzyme. Nevertheless, these studies do offer promise that relevant polypeptides, and the genes which code for them, may be isolated and characterized in detail in the near future.

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