Heterogeneous Distribution of Glycosyltransferases in the Endoplasmic Reticulum of Castor Bean Endosperm

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ABSTRACT
Endoplasmic reticulum membranes stripped of attached ribosomes were isolated from homogenates of germinating castor bean (Ricinus communis L.) endosperm by sucrose density gradient centrifugation. The isolated endoplasmic reticulum fraction was further separated into two major membrane subfractions by centrifugation on a flotation gradient. Both subfractions appeared to be derived from the endoplasmic reticulum inasmuch as they share several enzymic markers including cholinephosphotransferase, NADH-cytochrome c reductase, and glycoprotein fucosyltransferase and phase separation of membrane polyptides using Triton X-114 revealed a striking similarity in both their hydrophilic and hydrophobic protein components. The endoplasmic reticulum membrane subfractions contain glycoproteins which were readily labeled by incubating intact endosperm tissue with radioactive sugars prior to fractionation.

Castor bean endosperm endoplasmic reticulum apparently exhibits a degree of enzymic heterogeneity, however, since the enzymes responsible for the synthesis of dolicholiproporphospho-N-acetylgalactosamine and dolicholphosphomonophosphate mannose together with their incorporation into the oligosaccharide-lipid precursor of protein N-glycosylation were largely recovered in a single endoplasmic reticulum subfraction.

The morphological heterogeneity of the ER in most eukaryotic cells is well known. Cellular fractionation studies, predominantly performed using animal tissues, have refined a variety of approaches for separating ER vesicles bearing attached ribosomes (rough microsomes) from those not associated with ribosomes (12). Analytical studies on these isolated membrane fractions have also revealed that the ER is biochemically heterogeneous in certain respects (1). The studies have largely focused on the distribution of ER marker enzymes among ER subfractions. In general, although it seems that qualitative differences, if present at all, are rare, quantitative differences in the relative distribution of certain enzymes may well occur (1). The possibility of enzymic heterogeneity within particular ER subfractions, membranes derived from either rough surfaced or smooth surfaced ER, has also been suggested (32).

In contrast to the situation with mammalian tissues, very few attempts have been made to isolate and characterize ER membranes from plant cells. ER membranes have been isolated from castor bean endosperm cells (20). Sucrose density gradient centrifugation of endosperm homogenates effectively separates the ER membranes from the other major organelle fractions. These membranes, stripped of attached ribosomes, form a discrete band on such gradients at a mean buoyant density of 1.12 g/ml (20).

In the present study, we have subjected ER membranes isolated from germinating castor bean endosperm as described above, to a second fractionation on a flotation gradient. In this way, two major membrane subfractions are obtained. Whereas it is clear that on the basis of several shared enzymic and structural features both membrane subfractions are derived from the ER, differences in their capacity to synthesize mono- and oligosaccharide lipid intermediates involved in protein glycosylation were observed.

MATERIALS AND METHODS

Materials. [35S]Metionine (750-1000 Ci/mmol), 14C-labeled amino acid mixture (>50 mCi/milliatom of carbon), CDP-[methyl-14C]choline (50 Ci/mol), N-acetyl-d-[1-14C]glucosamine (11 Ci/mmol), d-[1-14C]mannose (5 Ci/mmol), L-[1-14C]fucose (5.4 Ci/mmol), d-6[14C]glucose (12.8 Ci/mmol), UDP-N-acetyl-d-[U-14C]glucosamine (282 Ci/mol), GDP-[U-14C]mannose (191 Ci/mol), GDP-L-[1-14C]fucose (24.5 Ci/mol), and UDP-[U-14C]glucose (293 Ci/mol) were from Amershaam International Inc. (Amershaam, U. K.) and Triton X-114 was from Sigma (Poole, U. K.).

Plant Material. Dry castor bean (Ricinus communis L.) seeds were soaked overnight and germinated in moist vermiculite in darkness at 30°C. After 3 d, developing seedlings were selected for uniformity and the endosperm tissue was excised.

Organelle Isolation. Endosperm homogenates were prepared and fractionated as described previously (20). After centrifugation, gradients were either collected as 1-mL fractions using an ISCO model 185 gradient fractionator or the visible organelle bands were removed directly using a syringe.

Subfractionation of ER Vesicles. Three ml of an ER membrane fraction isolated as described above (in approximately 30% [w/w] sucrose) were mixed with 2.5 ml of 70% (w/w) sucrose. The mixture was placed in a 17.5-ml centrifuge tube and was successively overlaid with 3 ml of 40% (w/w) sucrose, 6.5 ml of 30% (w/w) sucrose, and 2 ml of 20% (w/w) sucrose. Flotation gradients were centrifuged at 24,000 rpm (75,000g) and 2°C for 17 h in an SW 27 rotor on a Beckman L5-40 centrifuge. After centrifugation, the gradients were collected as 1.0-mL fractions.

Labeling in Vivo of Intact Tissue. Endosperm halves were placed abaxial surface down on moistened filter paper in a Petri dish. Depending on the required labeling, each half was treated with (a) 25 μCi of [35S]methionine, (b) 2 μCi of 14C-labeled amino acids, (c) 10 μCi of 3H-labeled sugars, or (d) 25 nCi of CDP-[14C]choline which were added directly to the adaxial surface in volumes of 10 to 25 μL. For the subsequent determination of incorporated radioactivity present in isolated fractions, the labeled tissue was incubated at 30°C for 2 h, whereas for samples ultimately subjected to electrophoresis and fluorography the tissue was incubated at 20°C for 16 h.

Enzyme Assays. Cholinephosphotransferase, fumarase, isocitrate lyase, and NADH-Cyt c reductase were assayed as described previously (9, 20).

Glycoprotein Fucosyltransferase. The reaction mixture con-
tained 900 μl of collected gradient fraction and 100 mM Tris-HCl, pH 7.4, 10 mM MgCl₂, 2 mM β-mercaptoethanol, and 0.05% Triton X-100 in a final volume of 1.0 ml. After adding 0.05 μCi (2 μl) of GDP-[14C]glucose, the mixture was incubated at 30°C for 30 min. The reaction was stopped by adding 1 ml of methanol followed by cold TCA (10% [w/v] final concentration). Precipitated material was filtered on a Whatman GF/A disc, washed in 10% TCA, and counted after immersing the disc in 10 ml of scintillant (6). Controls established that radioactivity incorporated into the reaction product was not reduced by prior extraction with chloroform/methanol (2:1) or chloroform/methanol/water (10:10:3).

**Glycolipid Glycosyltransferases.** Two 400-μl aliquots were removed from each collected gradient fraction. These were added to a reaction mixture (final volume, 500 μl) containing either 100 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 2 mM β-mercaptoethanol, and 0.1% (v/v) Triton X-100, or these components supplemented with 15 μg dolichol monophosphate. Reactions were initiated by adding 0.05 μCi UDP-α-[14C]glucosamine, 0.05 μCi GDP-[14C]mannose, or 0.1 μCi UDP-[14C]glucose and were incubated at 30°C for 30 min. The reactions were stopped by adding 4 ml of chloroform/methanol (2:1). Incorporation of radioactive sugars into (a) product soluble in chloroform/methanol (2:1) (monosaccharide lipid) and (b) product soluble in chloroform/methanol/water (10:10:3) (oligosaccharide lipid) was determined as described previously (23).

**Phase Separation of Membrane Proteins.** Washed membranes were treated essentially as described by Bordier (5) in order to separate amphipathic integral membrane proteins from hydrophobic proteins. A solution of Triton X-114 is homogeneous at 0°C but separates into an aqueous phase and a detergent phase above 20°C; this is the basis of the method. Sucrose gradient fractions containing either glyoxysomes or ER vesicles were diluted with 50 mM Tricine, pH 7.5, and the membranes were recovered by centrifugation. The membrane pellets were washed by resuspension in 50 mM Tricine, pH 7.5. An aliquot of this suspension was transferred to a Microfuge tube and the membranes were collected by centrifugation (15 min in an Eppendorf model 5412 centrifuge). The pellet was resuspended in 200 μl of 10 mM Tris-HCl, pH 7.5, 0.5 mM KCl, and 14 μl of Triton X-114 was added. The Triton X-114 had been precondensed exactly as described (5) except that NaCl had been replaced by an equivalent concentration of KCl. The mixture was placed on ice for 5 min to form a single phase which was then sonicated and allowed to stand on ice for a further 1 h. Insoluble material was removed by 15-min centrifugation in a chilled Eppendorf model 5412 centrifuge. The clear supernatant represented the total proteins extracted from the membrane preparations.
Fig. 3. Distribution of glycolipid N-acetylglucosaminyltransferase between ER subfractions. Aliquots of collected flotation gradient fractions were incubated with UDP-[U-14C]mannose at 30°C in the presence (C) or absence (I) of exogenous dolicholmonophosphate. After 30 min, the incorporation of radioactivity into chloroform/methanol (2:1)-soluble monosaccharide lipid (a), or chloroform/methanol (2:1)-soluble monosaccharide lipid (b), or chloroform/methanol/water (10:10:3)-soluble oligosaccharide lipid (b) was determined.

To separate the proteins, the clear supernatant was layered over a 300-μl cushion of 6% (w/v) sucrose, 10 mM Tris-HCl, pH 7.4, 150 mM KCl, and 0.06% Triton X-114 contained in a 1.5-ml Microfuge tube. The tube was incubated at 30°C for 3 min when clumping of the upper phase occurred. The tube was centrifuged for 30 to 60 s at room temperature. After centrifugation, the detergent phase formed an oily droplet beneath the sucrose cushion. The upper aqueous phase was transferred to a clean Microfuge tube and was re-extracted by adding 8 μl of Triton X-114. The tube was cooled on ice to allow the formation of a single phase which was then reloaded onto the original sucrose cushion, incubated at 30°C for 3 min, and then centrifuged to combine the detergent phases. The aqueous phase was again removed and it was re-extracted in exactly the same manner after adding a further 30 μl of Triton X-114; the detergent phase from this extraction was discarded. The hydrophilic protein solution was mixed with an equivalent volume of cold 20% (w/w) TCA. After standing on ice for 30 min, the precipitated proteins were collected by centrifugation and retained for electrophoretic analysis.

After removing the sucrose cushion, the detergent phase was mixed with 300 μl of 100 mM Tris-HCl, pH 7.4, 0.5 mM KCl and allowed to stand on ice until a single phase had formed. After incubation at 30°C for 3 min, the detergent phase (which contained integral membrane proteins with an amphipathic nature) was recovered by centrifugation and the aqueous phase was discarded.

Prior to electrophoresis, the detergent was extracted with chloroform. The Triton X-114 extract (20-25 μl) was mixed with 80 μl of 50 mM Tris-HCl, pH 7.4, containing 40 μg/ml PMSF. This solution was placed on ice until a single phase had formed and then 1 ml of chloroform was added. After centrifugation, the upper aqueous phase plus interface material were removed and mixed with an equivalent volume of cold 20% (w/v) TCA. After standing on ice for 30 min, the precipitated proteins were recovered by centrifugation.

Dodecyl Sulfate/Polyacrylamide Gel Electrophoresis. Proteins were separated on a 10% acrylamide slab gel, fixed, and stained with Coomassie blue as described previously (29).

Other Methods. Protein was determined by the Lowry procedure (21) using BSA as the standard and sucrose concentration was determined refractometrically.

2 Abbreviation: PMSF, phenylmethylsulfonyl fluoride.
RESULTS AND DISCUSSION

Ribosome-denuded ER membranes were purified from castor bean endosperm homogenates by sucrose density gradient centrifugation (20). The distribution of organelle marker enzymes among fractions collected from such gradients suggested that the ER vesicles (located using the exclusive marker cholinephosphotransferase) were not contaminated by, and did not themselves contaminate, the mitochondria and glyoxysomes (located using fumarase and isocitrate lyase as respective markers, Fig. 1a).

Salt-washed membranes prepared from organelle fractions separated from [35S]methionine-labeled tissue were subjected to decyl sulfate-polyacrylamide gel electrophoresis. ER and mitoochondrial and glyoxysomal membranes contained characteristic 35S-labeled polypeptide profiles which also indicated that cross-contamination between these fractions was insignificant (data not shown).

The isolation gradients used here contain EDTA and the ER is isolated as smooth surfaced vesicles. These vesicles are thought to derive from rough surfaced ER since (a) electron micrographs of germinating castor bean endosperm cells show that the ER is predominantly rough surfaced (33), and (b) over 90% of the protein, phospholipid, and marker enzyme activity present in the smooth vesicle band (fractions 9 to 11, Fig. 1a) is recovered at higher sucrose densities under conditions which maintain ribosome-membrane attachment (20).

The ribosome-stripped ER membranes recovered from the initial fractionation gradient, suspended in approximately 30% (w/w) sucrose, were further subfractionated by flotation. The sucrose density of the collected membrane preparation was increased to approximately 50% (w/w) sucrose by the addition of 70% (w/w) sucrose solution. This suspension was overlaid by the successive addition of 40%, 30%, and 20% (w/w) sucrose layers. After centrifugation, two membrane bands were visible, at the 40 to 30% and the 30 to 20% sucrose interfaces (Fig. 2a). The uppermost band contained twice as much protein as the lower band. Proteins present in both membrane fractions were readily labeled by incubating intact tissue with radioactive amino acids or radioactive N-acetylglucosamine (Fig. 2b), mannose, glucose, or fucose (data not shown).

Both membrane subfractions contained the characteristic ER marker enzymes cholinephosphotransferase and NADH-Cyt c reductase (Fig. 2c) together with glycoprotein fucosyltransferase, which in the nondividing endosperm cell is confined to the ER rather than the Golgi apparatus (30) (data not shown). Both membrane subfractions accumulated [14C]phosphatidylcholine synthesized in vivo by incubating intact tissue with CDP-[methyl-14C]choline. The extent to which newly synthesized [14C]phosphatidylcholine accumulated in these membrane subfractions was directly proportional to their relative cholinephosphotransferase activity (data not shown).

Castor bean endosperm membrane glycoproteins contain N-glycosidically linked oligosaccharide chains and the incorporation of labeled glucosamine into TCA-insoluble membrane components is inhibited by prior treatment of the tissue with tunicamycin (Ref. 4 and M. J. Conder, unpublished data). The synthesis of dolichol-linked intermediates containing N-acetylglucosamine and mannos, their incorporation into a mannos-rich oligosaccharide lipid and the transfer of the oligosaccharide from its lipid carrier to asparagine residues in acceptor proteins are all catalyzed by enzymes located in the ER membrane (22, 24, 26). The ER subfractions separated in the present study were assayed for the presence of glycosyltransferases catalyzing the transfer of the sugar moiety from nucleotide diphosphate derivatives to the lipid acceptor and the assembly of the mannosaccharide lipids into an oligosaccharide lipid. The transferases catalyzing the synthesis of lipid

Table 1. Enzyme Distribution between ER Subfractions

The distribution of enzymic activity between the lower gradient ER band and the upper gradient ER band is compared. With the exception of NADH-Cyt c reductase, for which activity is expressed as μmol/min, all other enzyme activities are given as pmol/h.

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Lower ER Band</th>
<th>Upper ER Band</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Activity</td>
<td>Activity/mg protein</td>
</tr>
<tr>
<td>Protein</td>
<td>380 μg</td>
<td>730 μg</td>
</tr>
<tr>
<td>Cholinephosphotransferase</td>
<td>78.9</td>
<td>70</td>
</tr>
<tr>
<td>NADH-Cyt c reductase</td>
<td>11.3</td>
<td>14.6</td>
</tr>
<tr>
<td>Fucosyltransferase</td>
<td>24.7</td>
<td>23.8</td>
</tr>
<tr>
<td>Glucosyltransferase</td>
<td>21.7</td>
<td>25.2</td>
</tr>
<tr>
<td>Mannosyltransferase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oligosaccharide</td>
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<td>600</td>
</tr>
<tr>
<td>Monosaccharide lipids</td>
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<td>54.8</td>
</tr>
<tr>
<td>Oligosaccharide</td>
<td>16.8</td>
<td>61.3</td>
</tr>
<tr>
<td>N-Acetylglucosaminyltransferase</td>
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intermediates containing either N-acetylglucosamine (Fig. 3) or mannose (Fig. 4) were predominantly located in the upper ER subtraction. The monosaccharide lipid intermediates have previously been identified as dolicholpyrophosphate N-acetylglucosamine and the dolicholmonophosphate mannose in castor bean endosperm (25) as they have been in other plant tissues (2, 13, 14). Both sugars are probably incorporated into the same dolichol-linked oligosaccharide (14, 15). The involvement of dolichol as the lipid carrier was confirmed by its stimulatory effect when added exogenously to the assay systems; the synthesis of monosaccharide lipids in particular was increased 20- to 30-fold by adding dolicholmonophosphate to a final concentration of 30 \( \mu \)g/ml (Figs. 3a and 4a). In contrast, the enzymic activity catalyzing glucose transfer from UDP-glucose to chloroform/methanol-soluble glucolipid was present in both ER subfractions (Fig. 5). At present we have not characterized the glucolipid(s) formed in this reaction. Recent experimental findings have shown that the core oligosaccharide involved in the initial N-glycosylation reaction of plants, like its counterpart in animal cells (8, 27), contains glucose in a characteristic (glucose)(mannose)(N-acetylglucosamine)\(_2\) structure (17, 31). The synthesis of dolicholmonophosphate glucose in plant tissues has been described and it apparently functions as an intermediate in both protein glycosylation (28) and polysaccharide synthesis (7). Glucolipid synthesis observed here was not stimulated by exogenous dolicholmonophosphate (Fig. 5) and the glucolipid itself is stable during acid hydrolysis, suggesting that it is not dolicholmonophosphate glucose (M. J. Conder, unpublished data). It is possible that the glucolipid synthesized by the castor bean enzyme is a sterlyglucoside. In any event, glucose is clearly not incorporated into the oligosaccharide lipid (Fig. 5).

The heterogeneity in the distribution of N-acetylglucosaminyl- and mannosyltransferases between the two ER subfractions is emphasized by the data in Table I. The upper fraction contained approximately twice as much protein as the lower fraction. Like-
wise, it contained 65 to 70% of the total cholinephosphotransferase, NADH-Cyt c reductase, and fucosyl- and glucosyltransferases which therefore have similar activities in each subfraction. In contrast, over 90% of the total N-acetylglucosaminyl- and mannosyltransferases were present in the uppermost subfraction at specific activities some 5-fold higher than those of the lower subfraction.

The quantitative differences in the distribution of the glucosyltransferases between the subfractions (Table I) probably represents a corresponding difference in the distribution of active enzymes. The total activity measured was not significantly reduced when aliquots of the two subfractions were mixed prior to assay. This suggests that the reduced activity present in the lower ER subfraction cannot be attributed to destruction of nucleotide sugars by microsomal pyrophosphatase or glycosidase (3).

The contention that the two microsomal fractions obtained in the present study represent genuine ER subfractions was strengthened by characterizing and comparing their constituent polypeptides. Prior to dodecyl sulfate-polyacrylamide gel electrophoresis, membrane-associated polypeptides were separated into hydrophilic and hydrophobic classes by phase separation in Triton X-114 according to the procedure of Bordier (5). The ER subfractions were strikingly similar in both their water-soluble and Triton X-114-soluble polypeptide profiles (Fig. 6). Whereas some similarity in the hydrophilic polypeptides associated with each subfraction could conceivably be due to nonspecific adsorption of the same contaminating soluble proteins, the identical profiles exhibited by the subfractions (Fig. 6, lanes 2 and 3) strongly suggest that they also share identical peripherally bound or relatively hydrophilic membrane proteins. The subfractions also shared virtually identical Triton X-114-soluble hydrophobic integral membrane protein polypeptide profiles (Fig. 6, lanes 6 and 7) although some possibly significant differences were noted (polypeptides predominantly confined to a particular subfraction are denoted by the arrows in Fig. 6, lanes 6 and 7). Both the water-soluble and Triton-soluble polypeptides present in the ER subfractions were clearly different from those present in corresponding fractions prepared from glyoxysomes (Fig. 6).

Demonstrations of biochemical heterogeneity in microsomal and submicrosomal vesicles have previously been restricted largely to results obtained using rat liver. Numerous quantitative differences in enzyme distribution between rough and smooth ER and in subfractions derived therefrom have been reported (1, 11, 32). This heterogeneity suggests that there may be distinct regions of the ER which are specialized for the performance of a given function. In castor bean endosperm ER, one specialized region may be enriched in dolichol-linked glycosyltransferases involved in assembling the core oligosaccharide of N-glycosylated proteins. We may further speculate that this region may be derived from rough ER since (a) it is thought that the core sugars are added to nascent proteins before polypeptide chain termination (18, 19) and (b) rough ER has been identified as the intracellular location of dolichol utilizing glycosyltransferases (10, 16, 26).

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