Fucosyl transferase activity and fucose incorporation in vivo as markers for subfractionating cucumber microsomes

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Golgi apparatus only rarely seen in electromicroscopic examinations of post-germinative storage tissues such as cucumber cotyledons could be detected by biochemical means. Enzyme activity transferring the L-fucosyl moiety from GDP fucose to membrane-glycoproteins was almost exclusively confined to golgi vesicles. This was demonstrated by comparing highly purified preparations of the ER, golgi and other organelles. The findings in vitro were supplemented by feeding experiments in vivo, establishing vesicles of golgi and plasma membrane as the by far dominating intracellular sites characterizable by fucose-containing glycoproteins. Detailed analysis including the single peptides within the fractions revealed that the ER can be easily distinguished from golgi apparatus on the basis of glycoproteins.

1. INTRODUCTION

Cucumber cotyledons, a gluconeogenic tissue [1,2] with storage organelles and compartments actively degrading proteins and lipids, are known to contain glycoproteins. A few glycoproteins have been shown to be constituents of the membranes of protein bodies in cucumber cotyledons [3,4] and in those of glyoxysomes of *Ricinus* endosperm [5,6]. Glycosyl transferases have been localized in the endoplasmic reticulum (ER) of pea cotyledons [7] and in castor bean endosperm [8], a tissue biochemically comparable to cucumber cotyledons.

In unravelling the biosynthetic pathway responsible for synthesis, ripening and vectorial transport of glycoproteins into various intracellular membranes, membranes of the ER have been investigated as a probable site of glycoprotein biosynthesis. In addition, irrespective of glycoprotein biosynthesis, there is continuous interest in subfractionating and characterizing the hitherto hardly studied microsomal fraction from plant cells. By confining fucosyl transferase activity to the golgi fraction and demonstrating fucose incorporation into the golgi fraction by short pulses in vivo, we have provided further evidence for the function of golgi apparatus as the primary site of fucose transfer and incorporation into the glycomoieties of glycoproteins.

2. EXPERIMENTAL

Cucumber seeds (*Cucumis sativus* L.) were germinated in the dark on vermiculite at 27°C. Cotyledons from 3-day old seedlings were homogenized as in [6,9]. The homogenate was filtered through miracloth and centrifuged at 5000 × *g* for 15 min in the SW-28 rotor of the Beckman ultracentrifuge. As in [10], 22 ml supernatant containing the microsomal membranes was layered on a gradient consisting of 3.5 ml 52% sucrose (w/w), 3.5 ml 46% sucrose and 6 ml 20% sucrose. Following centrifugation for 2 h at 27000 × *g* in the SW-28 rotor, microsomal membranes were contained in the 20%/46% sucrose interface. For purification of the membranes, viz. by density gradient flotation, the upper 28 ml of the former gradient were aspirated. The remaining portion (8 ml) of this gradient was used as part of a new gradient constructed by layering 10 ml 34.5% sucrose, 10 ml 29% sucrose, 4 ml 25% sucrose and 4 ml

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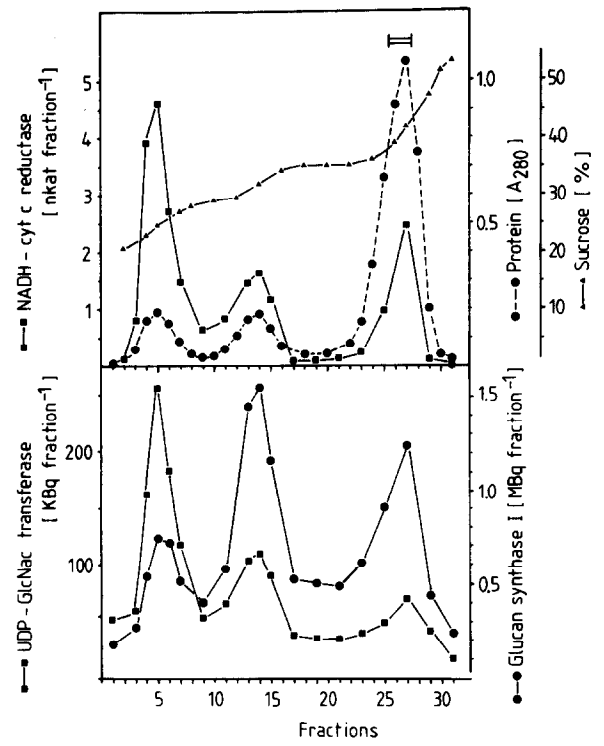

Fig.1. Separation of the ER membranes and golgi vesicles by flotation in a sucrose gradient (detailed in section 2). Starting from 10 g cotyledons, a crude membrane fraction was collected at a 20/46% sucrose interphase (primary treatment, not shown). This fraction was then placed within the gradient at the range indicated by the open bar (upper right side). Membranes reached their equilibrium density during flotation for 18 h. The peak in fraction 5 indicates the range of the ER, the zone in the middle of the gradient (fractions 13,14) contains golgi vesicles. Some enzyme activities remained at the position where the crude microsomal preparation was applied within the gradient before flotation (fractions 26,27). The profile of inosine diphosphatase activity (not shown) was essentially the same as drawn here for glucan synthase I.

20% sucrose above the already existing denser zone. With this gradient, flotation of the membranes was then performed in the SW-28 rotor by centrifugation at 28000 rev./min for 18 h. After centrifugation, 1.2 ml fractions were collected (fig.1) using an ISCO model-640 density gradient fractionator. Glycoproteins in intact cotyledons were labelled by incubating 10 g of excised cucumber cotyledons with 29.6 GBq of L-[6-^{3}H]fucose (740 GBq/mmol) for 20 h at 20°C.

Glycosyl transferase activities were determined as in [7]; the samples were incubated with the labelled substrate at 38°C for 1 h. Fractions from the gradient (0.3 ml) were incubated with 0.1 ml of the reaction mixture. The final concentrations were as follows: 50 mM Tris–HCl (pH 7.0), 10 mM KCl, 10 mM $MgCl_2$ and 2.5 mM mercaptoethanol; 37 kBq of UDP-*N*-acetyl-D-[U-^{14}C]glucosamine (10.8 GBq/mmol) or of GDP-L-[U-^{14}C]fucose (10.0 GBq/mmol). Reactions were terminated by adding 1 ml of a solution of bovine serum albumin (fraction V; 2 mg/ml) and subsequent precipitation with trichloroacetic acid. The precipitated material was washed 3-times with 5% trichloroacetic acid. Radiochemicals were from Amersham International (Bucks).

Glucan synthase and NADH:cytochrome *c* reductase were assayed as in [11,12]. Membrane fractions were analyzed by gel electrophoresis and fluorography [13,14] after separating, in each gradient fraction, the matrix proteins from the membrane constituents. This was achieved by exposing the organelle fraction to osmotic shock in diluted buffer, by treatment of the sedimented membranes with 100 mM sodium carbonate [15] and centrifugation at 100000 × *g* for 1 h. Matrix proteins were recovered from the supernatant while the pellet contained the membrane proteins.

3. RESULTS AND DISCUSSION

For subfractionation of microsomal membranes from cucumber cotyledons we developed a procedure which takes into consideration the particular intracellular structures in this tissue. The procedure allows to detect golgi vesicles besides the ER and to separate them from the membranes of oleosomes, plastids, microbodies, mitochondria and protein bodies.

For the optimal separation of golgi vesicles, we found it advantageous not to pellet the crude microsomes but rather to collect the membranes at an interface of a stepwise sucrose density gradient (not shown) and to subject them to isopycnic gradient flotation (fig.1).

Fig.1 shows the separation of markers of the ER membrane (NADH–cytochrome *c* reductase; fraction 5; banding at 25% sucrose) and the golgi vesicles (glucan synthase I; fraction 14; banding at

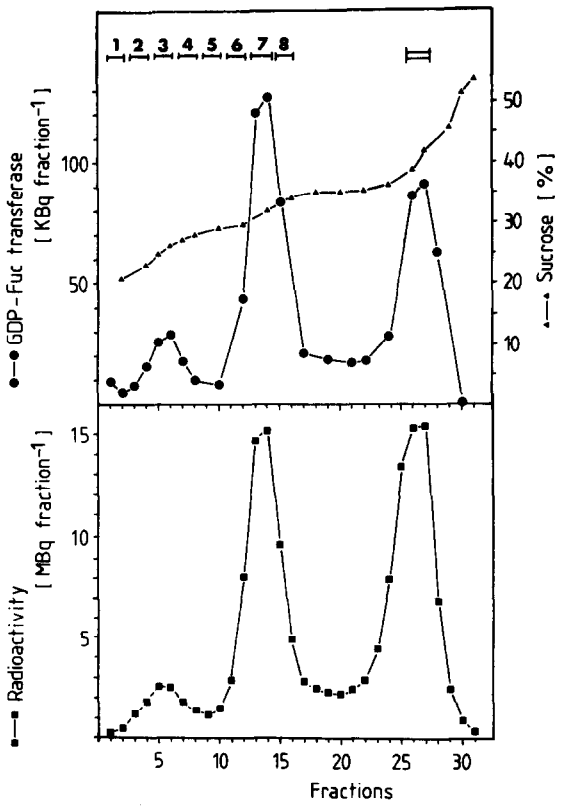

Fig.2. Separation of the ER membranes and golgi vesicles. Enrichment of microsomes and separation by flotation was as in fig.1. The open bar indicates the position within the gradient where the crude membranes were located before centrifugation. The upper part shows the profile of the fucosyl transferase activity (GDP-Fuc transferase), tested with GDP-L-[U-^{14}C]fucose as substrate and unlabelled gradient fraction as enzyme source. The lower part outlines the results of an in vivo experiment. Prior to cell fractionation, cotyledons were labelled in vivo with L-[6-^{3}H]fucose. The numbers at the upper left side indicate the parts of the gradient that were subsequently analyzed by electrophoresis (see fig.3).

32% sucrose). On the basis of these markers, the profiles of the glycosyl transferase activities suggest that GlcNAc transferase (UDP-2-actoamido-2-deoxy-D-glucose:lipopolysaccharide-2-acetamido-2-deoxy-D-glucosyltransferase; EC 2.4.1.56) is located primarily in the ER fraction. In contrast, fucosyl transferase (GDP fucose:glycoprotein L-fucosyltransferase, EC 2.4.1.68) was found to be located in the fractions containing golgi vesicles (fig.2). A comparison of specific enzyme activities supports the idea of fucose-transfer being a marker of the golgi apparatus.

In vivo isotope feeding experiments were carried out to corroborate that the golgi vesicles are the prime site of fucosylation. L-[^{3}H]Fucose was administered to 3-day old cotyledons of cucumber seedlings and the organelles were separated as described before. It became evident that L-fucose was preferentially incorporated into the membranes of golgi vesicles while the ER membrane seemed to lack fucosylated glycoproteins (fig.2).

In order to establish the appearance of

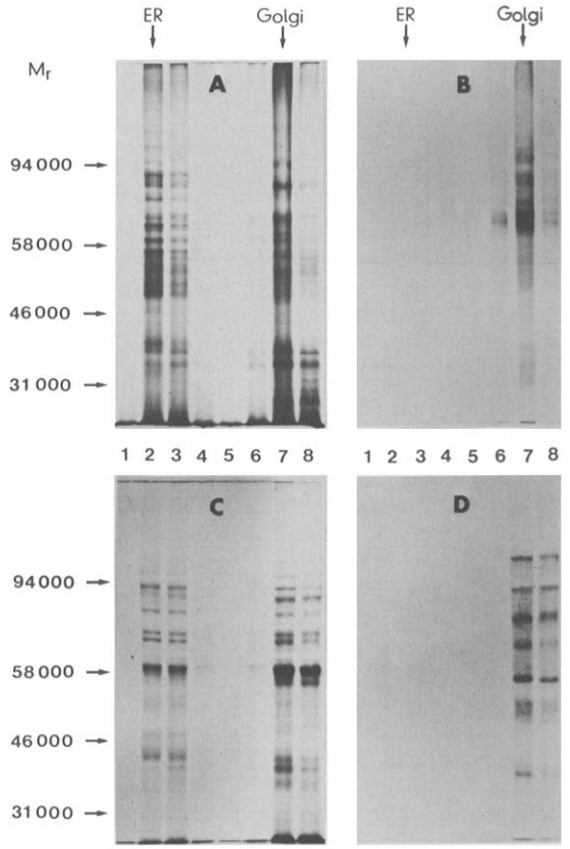

Fig.3. Electrophoretic analysis of microsomal fractions obtained by flotation. Combined fractions taken from the gradient (fig.2) were separated into membrane (A,B) and matrix (C,D) by osmotic shock. The figure presents protein-staining (A,C) as well as fluorography (B,D) of 8% polyacrylamide gels after electrophoreses in the presence of sodium dodecyl sulfate, 6 M urea and 15% glycerol.

Table 1

Quantitative comparison of radioactivity in cell-fractions after administration of L-[^{3}H]fucose to etiolated cotyledons of 3-day old cucumber seedlings

Fraction	Radio-activity (%)
Membranes of oleosomes	3
Golgi vesicles	25
Plasma membrane-enriched fraction	60
Glyoxysomes + protein bodies	10

Radioactivity in the subfractions was surveyed after separating a homogenate on an isopycnic density gradient ranging from 10–60% sucrose. The sedimentable radioactivity in the gradient supernatant was as high as the sum of organelle-bound radioactivities listed

fucosylated glycoproteins in the golgi vesicles, we analyzed, by electrophoresis, the fractions obtained from the flotation gradient (fig.3). It could thus be demonstrated, also for individual glycopeptides, that the golgi fraction contained the glycoproteins with the highest fucose label. Comparison (table 1) of these glycoproteins in the golgi fraction with the labelled glycoproteins in the cytosol, plasma membrane fraction and protein body membrane fraction, respectively, indicated that the dominating glycoproteins of the golgi fraction differ in molecular mass from the preferentially labelled glycoproteins of the other fractions (fluorograms not shown).

These studies establish that the golgi apparatus, although not well developed and only very rarely seen in electron microscopic examinations [1,16] of post-germinative storage tissues such as cucumber cotyledons or castor bean endosperm, can be detected by biochemical means. Unlike secretory cells, cotyledons heterotrophically growing on their own storage compounds were thought not to depend on golgi activity. In cucumber cotyledons, cells do not divide during growth in the dark but expand and are assumed to build a lytic compartment; both processes may require cooperation with the golgi membranes. In addition, golgi activity seems to be required for the biosynthesis of complex glycoproteins, which contain, among other sugars, L-fucose and D-galactose.

It is, in fact, possible to subfractionate microsomal membranes and then to attain an unequivocal assignment of marker activities and marker constituents; e.g., fucose-containing glycoproteins. These data differ from the findings in [8] in which all fucosyl transferase activity of castor bean endosperm cells was attributed to the endoplasmic reticulum and no significant role was assigned to the golgi apparatus in glycoprotein formation.

Our findings provide data that allow us to utilize fucosyl transferase activity, as well as fucosylated glycoproteins, as markers of the golgi membranes.

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REFERENCES

[1] Trelease, R.N., Becker, W.M., Gruber, P.J. and Newcomb, E.H. (1971) Plant Physiol. 48, 461–475.
[2] Kindl, H. (1983) in: Comprehensive Biochemistry, vol.36 new ser., Elsevier, Amsterdam, New York, in press.
[3] Kara, U.A.K. and Kindl, H. (1982) Eur. J. Biochem. 121, 533–538.
[4] Kara, U.A.K. and Kindl, H. (1982) Eur. J. Biochem. 121, 539–544.
[5] Bergner, U. and Tanner, W. (1981) FEBS Lett. 131, 68–72.
[6] Kindl, H. and Kruse, C. (1983) Methods Enzymol. 96, 700–715.
[7] Nagahashi, J. and Beevers, L. (1978) Plant Physiol. 61, 451–459.
[8] Roberts, L.M., Mellor, R.B. and Lord, J.M. (1980) FEBS Lett. 113, 90–94.
[9] Kruse, C., Frevert, J. and Kindl, H. (1981) FEBS Lett. 129, 36–38.
[10] Morré, D.J., Lembi, C.A. and Van der Woude, W.J. (1974) in: Praktikum der Zytology (Jacobi, G. ed) pp.146–171, Thieme, Stuttgart.
[11] Ray, P.M. (1977) Plant Physiol. 59, 594–599.
[12] Lord, J.M., Kagawa, T., Moore, T.S. and Beevers, H. (1973) J. Cell Biol. 57, 659–667.
[13] Laemmli, U.K. (1970) Nature 227, 680–684.
[14] Bonner, W.M. and Laskey, R.A. (1974) Eur. J. Biochem. 46, 83–88.
[15] Fujiki, Y., Hubbard, L., Fowler, S. and Lazarow, P.B. (1982) J. Cell Biol. 93, 97–102.
[16] Vigil, E.L. (1970) J. Cell Biol. 46, 435–454.