SPATIAL ORIENTATION OF GLYCOPEPTIDES IN MEMBRANES OF RAT LIVER ROUGH MICROSOMES

I. Localization of Lectin-Binding Sites in Microsomal Membranes

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ABSTRACT

Carbohydrate-containing structures in rat liver rough microsomes (RM) were localized and characterized using iodinated lectins of defined specificity. Binding of $[^{125}\text{I}]$Con A increased six- to sevenfold in the presence of low DOC (0.04-0.05%) which opens the vesicles and allows the penetration of the lectins. On the other hand, binding of $[^{125}\text{I}]$WGA and $[^{125}\text{I}]$RCA increased only slightly when the microsomal vesicles were opened by DOC. Sites available in the intact microsomal fraction had an affinity for $[^{125}\text{I}]$Con A 14 times higher than sites for lectin binding which were exposed by the detergent treatment. Lectin-binding sites in RM were also localized electron microscopically with lectins covalently bound to biotin, which, in turn, were visualized after their reaction with ferritin-avidin (F-Av) markers. Using this method, it was demonstrated that in untreated RM samples, binding sites for lectins are not present on the cytoplasmic face of the microsomal vesicles, even after removal of ribosomes by treatment with high salt buffer and puromycin, but are located on smooth membranes which contaminate the rough microsomal fraction. Combining this technique with procedures which render the interior of the microsomal vesicles accessible to lectins and remove luminal proteins, it was found that RM membranes contain binding sites for Con A and for Lens culinaris agglutinin (LCA) located exclusively on the cisternal face of the membrane. No sites for WGA, RCA, soybean (SBA) and Lotus tetragonobulus (LTA) agglutinins were detected on either the cytoplasmic or the luminal faces of the rough microsomes. These observations demonstrate that: (a) sugar moieties of microsomal glycoproteins are exposed only on the luminal surface of the membranes and (b) microsomal membrane glycoproteins have incomplete carbohydrate chains without the characteristic terminal trisaccharides N-acetylgalactosamine $\rightarrow$ galactose $\rightarrow$ sialic acid or fucose present in most glycoproteins secreted by the liver. The orientation and composition of the carbohydrate chains in microsomal glycoproteins indicate that the passage of these glycoproteins through the Golgi apparatus, followed by their return to the endoplasmic reticulum, is not required for their biogenesis and insertion into the endoplasmic reticulum (ER) membrane.
Secretory proteins are synthesized in membrane-bound polysomes and are transferred through the lumen of the endoplasmic reticulum (ER) and the Golgi apparatus to secretory vesicles for storage or direct discharge to the exterior by exocytosis (c.f. reference 58). Membrane proteins, and probably proteins that are retained within organelles such as lysosomes and peroxisomes, are thought to follow a subcellular pathway which parallels that of secretory proteins (cf. reference 65). Several ER membrane proteins, such as cytochrome \( b_5 \) (57), NADPH cytochrome \( c \) reductase (54), and cytochrome P-450 (53), have been shown to be synthesized in bound polysomes and to be directly inserted into rough ER membranes. Recent studies with viral envelope glycoproteins also indicate that proteins that are synthesized in membrane-bound ribosomes and are first incorporated into ER membranes (30-32) may be ultimately transferred to the plasma membrane.

During their passage through the various subcellular compartments, secretory and membrane proteins may undergo glycosidation and other post-translational modifications, which are carried out by membrane-associated enzymatic systems specific for the different segments of the secretory pathway (4, 63, 66, 59, 56). Most glycosylated proteins produced by the hepatocyte are rich in mannose units which are linked, via \( N \)-acetylglucosamine, to asparagine residues in the polypeptide chain (cf. reference 67). Completed secretory polypeptides contain, in addition, attached to the mannose core, two or three of the terminal trisaccharides (\( N \)-acetylglucosamine \( \rightarrow \) galactose \( \rightarrow \) sialic acid or fucose), at different degrees of completion (70, 34, 5; see reference 71 for review). These terminal sugars are also present in carbohydrate chains of plasma membrane glycoproteins exposed on the cell surface (35, 72).

The subcellular distribution of the glycosylating enzymes poses interesting questions concerning the biosynthesis and distribution of ER membrane glycoproteins. Whereas enzymes participating in the synthesis of the carbohydrate core in glycoproteins (proximal glycosylation) are found in microsomal fractions (8, 41), those involved in the addition of the terminal trisaccharides (terminal glycosylation) appear to be localized in the Golgi apparatus (66) and in the plasma membrane (64).

The presence of terminal trisaccharides in ER membrane glycoproteins would therefore imply that they are transferred to the Golgi apparatus before reaching their final location in the ER membrane. On the other hand, if proteins inserted into ER membranes are retained in the ER or are transferred unidirectionally to other subcellular compartments, it should be expected that they lack these terminal trisaccharides. It has been recently reported, on the basis of experiments in which the time-course of incorporation of labeled glucosamine into sialic acid was measured in several cell fractions (6, 7, 16), that microsomal membrane glycoproteins contain sugars of the terminal trisaccharides. It was also proposed that these are added after the glycoproteins are transferred to the Golgi apparatus, from where they must return to the ER via a cytoplasmic intermediate stage.

A major difficulty in the interpretation of experiments designed to determine the distribution of carbohydrates in intracellular membranes arises from the frequent cross-contamination of subcellular fractions and from their possible contamination with glycosylated extracellular components. Carbohydrates represent a relatively small fraction of the microsomal membrane mass; therefore, a slight contamination with fragments of other membranes rich in glycosidic residues, such as the plasma membrane or lysosomal membranes, or with highly glycosylated extracellular or secretory proteins, may seriously affect the validity of the results of chemical analysis.

The use of lectins to identify the presence of specific sugars in membranes provides a useful complement to the results of chemical carbohydrate analysis. Because of their relatively narrow specificity and their high affinity for specific carbohydrate configurations (cf. reference 69), lectins can be used as probes to assess the availability and exposure of specific sugars in complex cell fractions. Moreover, methods are available to localize membrane-bound lectins electron microscopically. It is therefore possible to correlate the presence of lectin-binding sites in membranous structures with the morphological appearance which reveals the subcellular origin of these structures.

In this study, we have used biochemical and cytochemical techniques to characterize and localize lectin-binding structures in rough microsomal membranes. Our observations with iodinated lectins and with lectins coupled covalently to biotin,
which were visualized electron microscopically using ferritin-avidin (F-Av) (23), indicate that sugar residues of ER membrane components are exposed on the cisternal side of the membranes, in agreement with reports by Hirano et al. (25) and Virtanen and Wartiowaara (74). The lectin-binding characteristics of the carbohydrate chains in rough ER membranes indicate that, although the glycoproteins in these membranes contain mannose-rich cores, they lack the terminal trisaccharides present in completed secretory proteins and plasma glycoproteins.

MATERIALS AND METHODS

Materials

Enzyme grade sucrose was obtained from Schwartz/Mann Div., Becton, Dickinson & Co. (Orangeburg, N. Y.); Tris base (Trienza), biotin, avidin, and N-acetylgalactosamine from Sigma Chemical Co. (St. Louis, Mo.); horse spleen ferritin (2 x crystallized) obtained from Miles Laboratories Inc. (Eikhart, Ind.), was purified further according to Morimoto et al. (51); NCS and [14C]biotin were purchased from Amersham Corp. (Arlington Heights, Ill.); [3H]choline, [14C]leucine, [131I], and Liquifluor from New England Nuclear (Boston, Mass.); D-galactose and α-methyl-D-mannoside from Calbiochem (La Jolla, Calif.); DOC (sodium form) from Mann Labs (Rutherford, N. J.); concanavalin A from Pharmacia Inc., (Piscataway, N. J.); Ricinus communis agglutinin (RCA) was a generous gift from Dr. Joel Oppenheim (Department of Microbiology, N. Y. U. Medical Center), and was also purchased from Miles Laboratories Inc. (Elkhart, Ind.). The levels of iodination obtained, after removing free iodine by chroma-

**Solutions**

Low salt buffer (LSB) is 50 mM KCl, 50 mM Tris HCl (pH 7.5), and 5 mM MgCl₂; high salt buffer (HSB) is 500 mM KCl, 50 mM Tris HCl (pH 7.5), and 10 mM MgCl₂; TKM is 52 mM KCl, 50 mM Tris HCl (pH 7.5), and 5 mM MgCl₂, 0.25 M SLSB or 0.25 M SHSB are LSB or HSB containing 0.25 M sucrose. When necessary, Tris was replaced by triethanolamine in LSB or HSB. This is indicated as LSB (TEA) or HSB (TEA). PBS is 0.15 M NaCl in 0.01 M phosphate buffer at pH 7.4. DOC solutions were prepared from sodium deoxycholate or from the free acid DOC and adjusted to pH 7.5 with NaOH. Modifications are indicated in the text.

**Fractionation of Rough Microsomes**

Rat liver rough microsomes (RM) were prepared by a modification of the procedure of Adelman et al., (1), from Sprague-Dawley male albino rats (120-150 g) fasted for 20 h before sacrifice. A postmitochondrial supernate was adjusted to 1.35 M sucrose and layered over a step gradient consisting of 5 ml of 1.55 M sucrose and 5 ml of 2 M STKM. After centrifugation (5 h at 27,000 rpm in SW27 rotor) the smooth microsomes (SM) floated on top of the 1.35 M sucrose. Two layers of RM were obtained: one between the 1.35- and 1.55-M sucrose layers (RM₁), and another, with a higher ribosome content, between the 1.55- and 2.0-M sucrose layers (RM₂). The latter fraction is of higher purity (63) and was used for the experiments reported in this and the following paper. RM (10 mg of protein in 5 ml) were stored in 1:2 LSB:glycerol at −70°C for up to 2 mo. Before use, microsomes were diluted five times with HSB and recovered by centrifugation for 20 min at 30,000 rpm in a Ti60 rotor. (This and other centrifugation conditions are abbreviated; as an example: 20 min, 30 K, Ti60.) Removal of ribosomes from RM by treatment with puromycin in high salt medium was carried out as described by Adelman et al., (2).

**Sucrose Gradient Centrifugation**

Linear sucrose density gradients (12 ml) were prepared in nitrocellulose tubes (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). Centrifugation was done at 4°C and the centrifuge was stopped without braking. Gradients were withdrawn from the bottom with an auto densiflow probe (Buchler Instruments Div., Searle Diagnostics Inc., Fort Lee, N. J.), and absorbancy at 254 nm was monitored with an LKB Uvicord II (LKB Produkter, Bromma, Sweden). When necessary, fractions of about 0.5 ml were collected from the pump effluent for determination of the radioactivity distribution throughout the gradient.

**Chemical Analysis and Determinations of Radioactivity**

Protein was determined according to Lowry (43) using BSA as a standard (19). [131I] radioactivity was measured at 70% efficiency in a Nuclear Chicago model 1185 gamma counter (Searle Radiographics, Inc., Des Plaines, Ill.). Samples containing 3H and 14C were dissolved with NCS reagent (Amersham Corp.), and the radioactivity measured with Toluene-Liquifluor in a Beckman LS250 Scintillation counter (Beckman Instruments, Inc., Schles Park, Ill.).

**Lectin Labeling**

Lectins were labeled with [125I] by the iodine monochloride method of McFarlane (46). The levels of iodination obtained, after removing free iodine by chroma-
tography in a Dowex AG1X column (Bio-Rad Laboratories, Richmond, Calif.), were between 0.5 and 1.5 x 10^7 cpn/mg protein. The following percentages of each lectin radioactivity were precipitable by TCA: [%^{125}I]Con A = 93%, [%^{125}I]WGA = 86.5%, and [%^{125}I]RCA = 88.6%. These determinations were made on Whatman GF/C glass fiber filters processed as described for RNA precipitation (44). The capacity of Con A to bind to dextran was unimpaired by iodination, since 96% of [%^{125}I]Con A was retained by a Sephadex G-100 column, and could be eluted quantitatively with 0.1 M glucose containing 1 M NaCl. Hemagglutination filters of labeled Con A, WGA, and RCA were similar to those of unlabeled lectins.

**Binding of Iodinated Lectins to RM**

For the binding assay, RM were incubated in LSB with iodinated lectins (1 mg/ml) for 1 h at room temperature. Aliquots (50-100 µl) were pipetted onto 0.2-µm Cellulose filters (Millipore Corp., Bedford, Mass.), which retained >95% of the microsomal vesicles. The free [%^{125}I]lectin was removed by passing 20 ml of LSB through the filter. Background retention of [%^{125}I]Con A was lowered considerably by preincubating the filters with unlabeled Con A, followed by a wash with LSB to remove unbound Con A. A similar procedure has been used by Wallach and Schmidt-Ullrich (75). Specific binding of Con A, WGA, or RCA to RM refers to the difference between binding in the absence and binding in the presence of the specific inhibitors (α-methyl-D-mannoside, N-acetyl-D-glucosamine, and D-galactose, respectively). Further experimental conditions are described in the figure legends and in Results.

**Electron Microscope Localization of Lectin-Binding Sites in RM**

For these experiments, the method of Heintzmann and Richards (23) was adapted. Binding sites bearing lectins to which avidin had been covalently bound were visualized in the electron microscope by coupling to F-Av complexes, taking advantage of the high affinity of avidin for biotin.

Ferritin, crystallized 10× according to Morimoto et al. (51), was coupled to avidin with toluene 2,4-diisocyanate (68) using iodinated avidin to follow the coupling. F-Av complexes were separated from free avidin, ferritin, and ferritin aggregates by gel filtration on a Biogel A 1.5 (Bio-Rad Laboratories) column (100 x 2 cm). In different preparations, the amount of avidin bound to ferritin was estimated using [%^{125}I]avidin or, alternatively, measuring the amount of [%^{14}C]biotin bound to the F-Av complexes, which were separated from free biotin by sucrose density gradient centrifugation (Fig. 1). Assuming three binding sites for biotin per molecule of avidin (47), results by both methods were in close agreement, indicating that the ratio of avidin to ferritin varied between 1:1 and 3:1. These measurements also showed that avidin within the F-Av complexes retained its full capacity for biotin binding (Fig. 1 b). The binding of [%^{14}C]biotin to F-Av complexes was specific since it was suppressed by an excess of unlabeled biotin (Fig. 1c). Sucrose density gradient analysis (Fig. 1) showed that the sedimentation rate of F-Av complexes was similar to that of free ferritin (compare position of peak in Fig. 1a with peak in Fig. 1b).

Double diffusion tests in 1% agar containing 0.3 M K phosphate, pH 7.5 (Figs. 2 and 3) demonstrated that avidin was able to form complexes and co-precipitate with the biotinized lectins after incubation for 20 h (Fig. 2a). Precipitation between Con A-biotin and F-Av came evident after longer incubation periods (60 h), possibly due to the slower diffusion of F-Av complexes in the agar (Fig. 2b). The reaction between the biotinized lectins and avidin was inhibited by the presence of biotin in the medium (Figs. 2c and 3, bottom) except for WGA-biotin and Con A-biotin which still reacted with avidin under these conditions. This should be expected since avidin is a glycoprotein (47) with a mannos-rich core and terminal N-acetylgalactosamine (14).

Two different procedures were used to make the interior of the RM vesicles accessible to the lectins and the F-Av complexes:

(a) Low detergent treatment: RM (3 mg protein/ml in LSB) were treated with 0.05% DOC to remove luminal proteins (36), and recovered by centrifugation. The washed RM were mildly fixed in suspension with 0.01% glutaraldehyde in LSB (TEA) for 15 min at 4°C. After sedimentation and washing in LSB, to destroy free aldehyde groups, the resuspended RM were incubated...
Double diffusion test showing the avidin-binding capacity of Con A coupled to biotin. Wells (3 mm) in the Ouchterlony plates (1% agar containing 0.3 M K phosphate, pH 7.5) (a, b, and c) received Con A-biotin (nos. 1 and 5), ovalbumin (no. 2), ovalbumin-biotin (no. 3), Con A (no. 4), F-AV (no. 6), and avidin (no. 7). After 20 h of incubation at room temperature (Fig. 2a), precipitation of avidin with ovalbumin-biotin and Con A-biotin was apparent. At 60 h (Fig. 2b), new precipitation lines between Con A-biotin and F-AV appeared. The time lag observed for the precipitation of F-AV may be related to its slow diffusion in the agar. Addition of biotin to the agar medium used to prepare the Ouchterlony plates inhibited the precipitation reaction (Fig. 2c).

Double diffusion test demonstrating the avidin-binding activity of several lectins covalently linked to biotin. Samples 1-7 were loaded into wells (2 mm) of Ouchterlony plates made of 1% agar containing 0.3 M K phosphate, pH 7.5. After 20 h of incubation, precipitation lines were apparent (top) between the wells containing avidin (no. 2) and wells containing SBA-biotin (no. 1), LCA-biotin (no. 3), RCA-biotin (no. 4), WGA-biotin (no. 5), LTA-biotin (no. 6), and Con A-biotin (no. 7). The addition of biotin to the medium (bottom) inhibited the reaction of avidin with all biotinized lectins except for Con A and WGA which have affinity for avidin, a glycoprotein.

for 1 h at room temperature with the appropriate lectin-biotin in LSB containing 0.05% DOC. The RM were then recovered and incubated with F-AV in HSB containing 0.05% DOC; this concentration of DOC was higher than the minimum required at this high ionic strength to release the microsomal content (36). This level of detergent, however, was necessary to produce larger openings which facilitate the penetration of F-Av complexes. The prefixation in 0.01% glutaraldehyde was introduced to prevent release of ribosomes which, to some extent, takes place when 0.05% DOC is added in HSB (36). It will be shown that the mild glutaraldehyde prefixation did not prevent the effect of DOC in allowing passage of macromolecules into the vesicles.

(a) Disruption in a high pressure cell: RM prefixed in 2% glutaraldehyde for 15 min at 0°C were submitted to a compression-decompression cycle at 1,500 lb/ in² in a Yeda press (Rehovot, Israel). Electron microscope examination showed that this treatment resulted in a large proportion of permanently open RM vesicles (experiments with Dr. G. Ojakian), especially when very large microsomes, such as those present in the rapidly sedimenting ER fraction (RSER) described by Lewis and Tata (42), were used. Prefixation prevented reclosing and extensive vesiculation of the microsomes, which otherwise is caused by the compression-decompression cycle in the Yeda press.

For electron microscopy, all microsomal samples were fixed for 30 min at 4°C in LSB (TEA) containing 2% glutaraldehyde and sedimented. The pellets obtained were washed overnight in 0.1 M cacodylate buffer, pH 7.0, postfixed for 1 h with 1% OsO4 in 0.1 M cacodylate and stained with 1% uranyl acetate before dehydration and embedding in Epon 812. The sections were examined in a Philips 301 electron microscope at 80 kV.

RESULTS

Effect of DOC on Con A Binding to RM

It has been shown that incubation of rough microsomes with low detergent concentrations

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leads to the selective release of a subset of microsomal proteins, which represents the luminal content of the vesicles (36, 37). Since some of the released proteins have molecular weights higher than that of Con A (108,000 at pH 7.4; reference 3), it was reasonable to expect that the same treatment would render the microsomal vesicles permeable to this lectin. Lectin-binding sites on the luminal face of microsomal vesicles, if existent, could then be detected. Fig. 4 shows the effect of a low DOC concentration on the capacity of RM to bind [\[^{125}\text{I}\]Con A. While only small amounts of radioactive Con A were bound to control RM (Fig. 4a), suggesting that few, if any, binding sites for Con A are present on the outer face of the microsomal vesicles, treatment with 0.05% DOC caused a dramatic increase (greater than six times) in the amount of Con A bound to the microsomes (Fig. 4c).

To determine whether the increase in Con A binding caused by the low DOC concentration paralleled the change in permeability brought about by the detergent, the extent of [\[^{125}\text{I}\]Con A bound at different detergent concentrations was compared with the release of phospholipids under the same conditions. As is shown in Fig. 5, the curve describing specific Con A binding (i.e. the binding susceptible to \(\alpha\)-methylmannoside [\(\alpha\)-MM]) as a function of DOC concentration followed closely the curve of release of \([^{14}\text{C}]\)leucine-labeled content proteins. Moreover, binding of Con A to RM reached a maximum at DOC concentrations which did not cause a significant solubilization of the \[^{3}\text{H}\]choline-labeled membrane phospholipids. These observations suggest that Con A-binding sites are located either in membrane proteins which are exposed on the luminal side of the membranes, or in proteins of the vesicular lumen which are not released from the microsomes in the presence of Con A.

Since Con A is tetravalent at the pH used in these experiments (7.5) (3), it might cause cross-linking of content glycoproteins to membrane glycoproteins. Complexes of this type, or the presence of aggregates of luminal proteins caused by Con A, could lead to an overestimate of the amount of Con A bound directly to membrane proteins. Therefore, an attempt to measure the contribution of content proteins to the binding of Con A to RM in the presence of DOC was made. Content proteins were first released by treatment with 0.05% DOC, and the lectin-binding capacity of the content-free RM was then measured in the presence of DOC. It was found (Table 1, column E) that 10-20% of the Con A-binding capacity of RM was due to content proteins, since binding was reduced in this proportion by pretreatment with 0.05% DOC. It should also be noted that RM previously freed of content showed higher basal levels of binding (in the absence of DOC).
DOC (~7.5°C) Binding of [125I]Con A to RM occurs in parallel with the release of content and is completed before solubilization of membrane phospholipids begins. RM were prepared from rats which received [3H]choline (4 h) and [14C]leucine (30 min) to label microsomal phospholipid and content proteins, respectively (see Materials and Methods). Microsomes suspended in LSB (1 mg protein/ml) received DOC to final concentrations varying from 0 to 0.1%. Aliquots were incubated with [125I]Con A (1 mg/ml) or [125I]Con A in the presence of α-MM (0.1 M) for 45 min at 25°C. Radioactivity in membrane phospholipids ([3H]choline) or microsomal content ([14C]leucine), which remained associated with the sedimentable microsomes, was determined in fractions recovered after centrifugation through a 1-M SLSB cushion (20 min, 50K, SW50.1). Nitrocellulose tubes were frozen at -70°C, bottoms were cut with a razor blade, and pellets were dissolved in NCS to determine the radioactivity. For each DOC concentration, the nonsedimentable radioactivity was expressed as percent of radioactivity in the control sample (no DOC). A filtration assay was used (see Materials and Methods) to separate the free [125I]Con A and to determine binding of [125I]Con A to RM at different DOC concentrations. Specific binding of [125I]Con A was computed from the difference between binding without and with α-MM.

Figure 5 Binding of [125I]Con A to RM was measured as a function of the time of incubation in the presence of a low DOC concentration. RM were preincubated for 1 h at room temperature with [125I]Con A to saturate all binding sites which might be accessible on the outer surface of the vesicles or in contaminating membranes. It was found (Fig. 6) that Con A binding increased rapidly after addition of 0.05% DOC, approaching a plateau after ~40 min at room temperature.

Specific binding of [125I]Con A was also measured in the presence and in the absence of DOC (0.04%), as a function of Con A concentration, to determine the affinity of the microsomal sites for Con A and the number of binding sites. It was found (Fig. 7a) that the binding of [125I]Con A to intact RM reached saturation at relatively low lectin concentrations (2 μM), in the presence of 0.04% DOC the specific binding of [125I]Con A to RM increased throughout the whole range of Con A concentrations up to 10 μM. Scatchard plots showed that binding to intact RM can be represented by a single straight line (Fig. 7b), as would be expected from an homogeneous population of sites (n = 0.35 × 10⁻⁹ mol/mg RM protein) with a high affinity for the lectin (K = 13.8 × 10⁶ M⁻¹). In the presence of 0.04% DOC, however, the binding may be interpreted as a composite function with two linear components. One of these corresponds to a small number of sites with a high affinity, similar to that of the sites available in intact RM, the number of these sites in the presence of 0.04% DOC was only slightly higher (~30%) than in intact RM, indicating that most of these sites are normally exposed and are unaffected by the DOC treatment. The other component in the Scatchard plots corresponds to sites with lower affinity (K = 0.97 × 10⁶ M⁻¹), which are present in larger number (n = 1.65 × 10⁻⁹ mol/mg RM protein). These lower affinity sites are exposed by the treatment with 0.04% DOC. Similar biphasic curves were obtained when RM, which had been previously treated with a low DOC concentration to remove the content proteins, were used.

It should be noted that in Fig. 7, measurements of Con A binding as a function of Con A concentration are expressed as amount of Con A bound per milligram of microsomal protein. On this basis Con A binding in the presence of 0.04% DOC.

Characterization of the Con A-Binding Sites

than control RM (compare columns C and A in Table 1). This is likely to reflect the incomplete resealing of some microsomal vesicles after the first DOC treatment; the proportion of unsealed vesicles could then be estimated to range between 11 and 37% (Table 1, column F). Contamination of resealed vesicles with content glycoproteins or with membrane glycoproteins from partially dissolved vesicles may also contribute to the higher basal levels of [125I]Con A binding.
| Exp no | Content-free RM | Control RM | RM Content | Unsealed content free RM |
|-------|----------------|------------|------------|--------------------------|
|       | A | B  | C   | D  | E | F |
| 1     | 83.8 | 496.8 | 123.2 | 356.8 | 28.2 | 11.0 |
| 2     | 89.7 | 515.7 | 232.2 | 386.4 | 25.1 | 36.8 |
| 3     | 101.1 | 439.5 | 187.8 | 394.0 | 10.1 | 20.4 |
| 4     | 125.4 | 666.9 | 275.9 | 484.4 | 27.4 | 31.1 |

* Results are expressed in μg [125I]Con A bound/mg microsomal phospholipid (columns A, B, C, and D).

† Content-free RM were prepared by treatment of RM (3 mg protein/ml) with 0.05% DOC in LSB (exp 1, 2, and 3) or with 0.025% DOC in HSB (exp 4). Treated RM were separated from content proteins by centrifugation through 1 M SLSB followed by two washes in LSB to remove the sucrose. Binding of [125I]Con A to control RM and content-free RM, with or without 0.04% DOC, was measured by the filtration assay described in Materials and Methods. For control RM, a phospholipid/protein ratio of 0.37 was used (10). For content-free RM, a phospholipid/protein ratio of 0.37/(1-0.15) = 0.435 was used since content proteins represent 15% of total RM protein (38).

§ Values in column E represent percent of microsomal Con A-binding sites provided by content proteins.

∥ Values in column F correspond to the fraction of content-free RM vesicles which remained unsealed after the first low DOC treatment.

**TABLE 1**

*Specific Binding of [125I]Con A to the Membranes and Content of Rough Microsomal Fractions*

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**FIGURE 6** Kinetics of [125I]Con A binding to RM in presence of 0.05% DOC. RM (1 mg protein/ml) were preincubated in LSB with [125I]Con A (1 mg/ml). One aliquot had also received 0.3 M α-MM (○). After 1 h at room temperature (arrow at 0 time), the incubation mixture was divided into two samples which received 0.05% DOC with (x) or without (○) 0.03 M α-MM. At different times, Con A binding was determined by filtration in duplicate 50-μl aliquots.

**FIGURE 7** Binding of [125I]Con A to RM as a function of the Con A concentration. RM (1 mg/ml) were incubated in LSB containing different Con A concentrations (0.1-10 × 10^{-6} M) with (○) and without (△) 0.04% DOC. Binding of [125I]Con A in the presence of 0.04% DOC to RM from which content proteins had been previously removed with low DOC is represented by x (Fig. 7a and b). The binding of [125I]Con A at different lectin concentrations is represented directly (Fig. 7a) and as a Scatchard plot (Fig. 7b).
appears to be unaffected by previous removal of content (compare x and ○ in Fig. 7). On the other hand, if binding is expressed as amount of Con A bound per milligram of phospholipid, a denominator which is almost unchanged by the treatment with a low DOC concentration (see description of Table III), it becomes apparent that RM freed of content have a slightly lower binding capacity than control RM. This observation suggests that the density of Con A-binding sites in proteins of the content fraction released by DOC is similar to the density of sites in membrane proteins.

As will be shown below, sites exposed by low DOC treatment are located on the inner (luminal) aspect of the microsomal membranes. Current information on the structure of Con A receptors suggests that the relatively low affinity for Con A of these sites reflects the presence of incomplete carbohydrate chains in microsomal glycoproteins (see Discussion).

**Binding of [125I]WGA and [125I]RCA to RM**

To assess the degree of completion of carbohydrate chains in microsomal glycoproteins, we used iodinated lectins specific for sugar moieties distal to the mannose core, which is responsible for Con A binding. Table II allows a comparison to be made of the effect of 0.04% DOC on the binding of [125I]WGA and [125I]RCA to RM and its effect on the binding of [125I]Con A. Intact microsomal fractions showed basal levels of binding for WGA and RCA comparable to those of Con A. On the other hand, the DOC-promoted increase in WGA- and RCA-specific binding was much smaller than for Con A and amounted to only a fraction of the original binding in control RM. These results indicate that most sites for WGA and RCA present in the RM fraction are normally available for binding. Therefore, sites for WGA and RCA behave like the small number of high affinity sites for Con A, which did not increase substantially after treatment with DOC (Fig. 7).

RCA-binding sites in the carbohydrate chains of glycoproteins are thought to contain α-galactose as a terminal sugar. The fact that in some membranes WGA, which has affinity for N-acetylgalactosamine (52), partially competes with RCA for binding (55) suggests that WGA has a preference for N-acetylgalactosamine residues which are near the terminal position. Since galactose and distal N-acetylgalactosamine residues are thought to be added to glycoproteins by enzymes present in membranes of the Golgi apparatus or plasma membrane (66, 64), the observations in this section raise the possibility that binding sites for WGA and RCA present in RM fractions are located in membranes derived from organelles other than the ER.

**Electron Microscope Localization of Lectin-Binding Sites**

These experiments were carried out in two steps (see Materials and Methods). In the first step, microsomes were incubated with lectins (Con A, WGA, RCA, SBA, etc.) covalently linked to biotin, under conditions similar to those employed in the radioactive lectin-binding assays. After the

| Table II
| Binding of [125I]Ricin and [125I]WGA to RM* |

| No DOC | 0.04% DOC |
|--------|-----------|
| WGA    |           |
| (1)    | 54.6      |
|        | 66.4      |
| (2)    | 33.2      |
|        | 46.2      |
| RCA    |           |
| (1)    | 31.2      |
|        | 41.4      |
| (2)    | 29.3      |
|        | 47.5      |
| Con A  |           |
| (1)    | 56.8      |
|        | 306.0     |

* Binding assays were carried out by the filter assay described in Materials and Methods.

† The inhibitors used were N-acetylgalactosamine, α-galactose, and α-MM (0.2 M each) for WGA, RCA, and Con A, respectively. Results are expressed in μg [125I]lectin bound/mg of RM protein.

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TABLE III

Binding of $^{[125]}$Con A and $^{[125]}$Con A-Biotin to RM

| DOC Type | $^{[125]}$Con A | $^{[125]}$Con A-Biotin |
|----------|----------------|-----------------------|
| No DOC   | 78.3           | 71.2                  |
| $-\alpha$-MM | 11.2           | 14.1                  |
| Specific | 67.1           | 57.1                  |
| 0.05% DOC| 224.2          | 214.5                 |
| $-\alpha$-MM | 13.6           | 15.4                  |
| Specific | 210.1          | 199.1                 |

* Binding was measured by the filter assay described in Materials and Methods. Results are expressed in µg Con A (Con A-biotin)/mg RM protein.

RM were washed by centrifugation, the incubation with F-Av complexes needed to visualize the bound lectins was performed in a medium of high salt concentration (HSB, 30–60 min, at room temperature) to prevent unspecific binding of F-Av to the membranes. A comparison between values of $^{[125]}$Con A and $^{[125]}$Con A-biotin bound to RM (Table III), indicated that the binding capacity of the lectin was not impaired by the attachment of biotin.

In intact unfixed microsomal fractions (Fig. 8), only membranes without attached ribosomes, which were present as vesicles or planar sheets contaminating the RM preparation, showed significant binding of Con A-biotin, WGA-biotin and RCA-biotin. For none of the lectins used did the cytoplasmic face of the RM, i.e., the face which bears the ribosomes, show any binding. This indicates that the high affinity sites for $^{[125]}$Con A as well as the sites for the $^{[125]}$RCA and $^{[125]}$WGA detected in untreated RM fractions are not present in membranes derived from the rough ER.

No binding sites for Con A were detected on the cytoplasmic face of the RM vesicles after removal of the ribosomes by treatment with HSB and puromycin (Fig. 9). In preparations of RM stripped of ribosomes, as in the original RM fraction, only smooth membrane vesicles with a well-defined unit membrane appearance showed binding of Con A on the outer surface (Fig. 9, arrow).

The two procedures described in Materials and Methods were used to demonstrate that Con A-binding sites are located on the luminal surface of microsomal membranes:

(a) RM from which luminal proteins had been previously removed with low DOC (Fig. 10) were incubated by the two-step procedure, with Con A-biotin and F-Av, in the presence of DOC. This method allowed the demonstration of significant binding of F-Av molecules to the luminal face of the microsomal vesicles, i.e., the face of RM opposite to that which has attached ribosomes (Fig. 10B). To facilitate penetration of F-Av complexes into the vesicles, the second incubation in these experiments was carried out in the presence of 0.05% DOC and in a medium of high salt concentration (HSB). It has been shown that under these conditions a partial solubilization of phospholipids occurs and large openings are produced in the vesicles (36).

(b) In the alternative procedure, RM were disrupted by sudden decompression in a high pressure cell to allow better exposure of the inner face of RM to F-Av complexes. These experiments were carried out with large fragments of rough ER membranes present in the RSER described by Lewis and Tata (42). No significant binding of F-Av was detected when untreated microsomal preparations were incubated in the presence of F-Av (Fig. 11A). However, RM that had received Con A-biotin in the first step bound large numbers of F-Av complexes during the second step of this process. The F-Av complexes were always found in the luminal face of the RM, while the cytoplasmic faces were completely free of ferritin (Fig. 11B). No WGA- or RCA-binding sites were detected by these procedures in either the outer or the inner aspects of RM vesicles (Fig. 11C and D).

Inner or outer mitochondrial membranes, which were identifiable as contaminants of the RM preparation and appeared accessible to the lectins, showed no binding sites for any of the lectins used (62).

LCA, LTA, and SBA coupled covalently to biotin were also used in electron microscopy experiments, to broaden the characterization of lectin-binding sites present in RM. When RSER fractions, submitted to a compression-decompression cycle, were incubated with biotinized LCA, a lectin with carbohydrate affinities comparable to those of Con A (73), and subsequently treated with F-Av, the presence of binding to sites on the luminal face of the RM membrane was apparent (Fig. 12). Lens culinaris sites were detected as aggregates containing three to five ferritin molecules, which were not uniformly distributed in...
FIGURE 8 Binding of RCA, WGA, and Con A to RM. RM were incubated with the biotinylated lectins for 1 h at room temperature, recovered, and washed with LSB by centrifugation in an Eppendorf 3200 microfuge (Brinkmann Instruments, Westbury, N.Y.) and incubated with the F-Av complexes in HSB. (A) Con A, (B) RCA, and (C) WGA. Only membrane vesicles without ribosomes, with a well-defined unit membrane appearance which is not characteristic of the ER (A and B) or large membrane sheets of plasma membrane origin (C) have occupied lectin-binding sites which bind F-Av complexes (arrows). × 112,000.
FIGURE 9 Binding of Con A to RM stripped of ribosomes. RM stripped of ribosomes (RMstr) (1 mg RM protein/ml) by treatment with puromycin in a high salt medium were incubated with Con A-biotin (1 mg/ml) in LSB. The RMstr were washed by centrifugation (2×) and reincubated in HSB containing ferritin coupled to avidin. The luminal surface of a microsome which contains residual ribosomes attached to its outer face (arrowheads) shows ferritin binding. A vesicle which contaminates the fraction and can be recognized by its well-defined unit membrane appearance (arrow) shows ferritin on its outer surface. × 62,000.

different RM vesicles. In some vesicles numerous aggregates were found, while others contained few ferritin molecules. Therefore, although the location of Lens culinaris binding sites with respect to the plane of the membranes is similar to that of Con A sites, both lectins appear to bind to different receptors. It is possible that although Con A and LCA have similar primary specificities, their affinity varies with the length of the carbohydrate chains exposed on the luminal face of the membranes. No binding of LTA (specific for L-fucose) or SBA (specific for N-acetylgalactosamine-like residues) (69) to either face of RM membranes was observed (data not shown).

DISCUSSION

The results in this report demonstrate the existence, in rough microsomal fractions, of two classes of binding sites to which Con A binds specifically: (a) high affinity sites (20–25% of the total)—which are normally accessible to the lectin in untreated microsomal fractions, and (b) low affinity sites (75–80% of the total)—which become available to Con A only when microsomes are treated with low concentrations of DOC, allowing the penetration of the lectin into the vesicles without dissolving the membranes (36). Visualization of the bound lectins in the electron microscope by means of the F-Av labeling technique (23) showed that the high affinity sites are present in membranes of smooth vesicles and in large plasma membrane fragments which contaminate the RM. On the other hand, the set of low affinity sites exposed by DOC was found to be truly microsomal. This includes sites located in the content proteins (15–25%) which can be removed by a previous treatment of RM with detergent, and sites which are part of the microsomal membranes and are exposed only on the luminal surface of the vesicles, where the bound lectins could be demonstrated with F-Av markers. In the following paper (63), it will be shown that microsomal glycoproteins purified by affinity chromatography on Con A Sepharose columns from content and membrane subfractions are two distinct molecular subsets. The possibility that Con A-binding sites exist on the cytoplasmic face of RM but are not available to the lectin-F-Av system because of steric hindrance by the ribosomes was excluded by the results of experiments...
Figure 10 Con A-binding sites on the luminal face of RM vesicles opened with a low DOC concentration. RM content proteins were removed by the low DOC treatment. RM vesicles were mildly fixed with 0.01% glutaraldehyde and subsequently incubated (1 mg RM protein/ml) in the presence of 0.04% DOC with 1 mg/ml Con A-biotin. After this first step, the vesicles were recovered, washed by centrifugation, and reincubated with 0.05% DOC in HSB containing the F-Av complexes. (A) Sample in which specific binding was suppressed by addition of 0.2 M α-MM during the incubation with Con A-biotin and with the F-Av complexes. (B) Specific sites for Con A-binding are present only on the luminal face of the RM membranes. × 112,000.
in which RM stripped of ribosomes with puromycin in a high salt medium did not show lectin binding on their outer surface.

The orientation with respect to the plane of the membrane of the Con A-binding sites found in rat liver microsomes is in agreement with that for Con A sites in microsomes of myeloma tumor cells, established by Hirano et al. (25), using ferritin coupled directly to Con A, and with recent reports by Keenan et al. (28) and Virtanen and Wartiovaara (74) on Con A-binding sites in liver cell fractions. Since the lumen of ER cisternae can be regarded as topologically equivalent to the extracellular space, glycoproteins capable of Con A binding in ER membranes have the same orientation of their carbohydrates with respect to the plane of the membrane as glycoproteins bearing Con A receptors in plasma membranes (61).

Our results allow an estimate to be made of the density of Con A-binding sites present in the cisternal face of ER membranes. The amount of Con A bound to the inner surface may be obtained from Table I by subtracting the average of values in column A (A = 100.0 µg Con A/mg RM phospholipid) from the average of values in column D of the same table (D = 405 µg Con A/mg RM phospholipid). The results (305.5 µg Con A bound/mg RM phospholipid) is in good agreement with that given by the number of binding sites determined from Scatchard plots (Fig. 7). If the thickness of the RM membrane is taken as 60 Å, its phospholipid/protein ratio as 0.435 (10) and the isopinic density as 1.18 g/cm³ (13), the density of binding sites obtained is 0.38 × 10¹² sites/cm², for a mol wt of 108,000 for Con A at pH 7.4 (3). Edelman and Millette (15), using a mol wt of 27,000 for Con A, have calculated that in liver plasma membranes there are 3 × 10⁹ Con A sites/cell. Taking the surface of liver cells as 1,680 µm² (76) and the mol wt of Con A as 108,000, the density of sites in liver plasma membranes can be computed as 0.45 × 10¹² sites/cm². This value is remarkably close to that for the density of sites in the luminal surface of ER membranes.

In spite of the similar orientation and density of distribution of binding sites in ER and plasma membranes, carbohydrate side chains in microsomal membranes have a much lower affinity for Con A than sites found in the liver plasma membrane (K = 6.3 × 10⁶ M⁻¹, our own unpublished results) and in plasma membranes from other cells (21, 75). This, of course, must reflect specific compositional differences, at least some of which may be related to the stage of growth of the carbohydrate chain (33). A common feature of many glycoproteins secreted by the liver, which appears to be shared by membrane glycoproteins, is the presence of a core composed of several mannose and N-acetylglucosamine residues linked to the peptide backbone via an N-acetylglucosamine-asparagine linkage (cf. reference 71). The mannose core provides binding sites for Con A, and LCA, the affinity of which depends on the sugar residues distal to the core (73, 33). Usually, two or three trisaccharides (N-acetylglucosamine ~ galactose ~ sialic acid or fucose) emerge like antennae from the mannose core, constituting "bi- or triantennary" glycopeptides (50). It appears that the "ideal" Con A-binding site is a biantennary asparagine-linked glycopeptide (39), from which terminal sialic acid or fucose and galactose residues have been removed (33). The high affinity for Con A of sites in membranes which are not derived from the rough ER and are present as contaminants in the RM fraction suggests that the structure of these carbohydrates approaches that of ideal Con A receptors. On the other hand, sites in the luminal face of the RM have lower affinity for Con A, which is expected for carbohydrate chains in glycoproteins which contain mannose cores but lack N-acetylglucosamine in the distal positions (33).

Treatment of RM with low DOC concentrations led only to a slight increase in the binding of WGA and RCA. In contrast with the fivefold increase in the number of low affinity Con A sites which was caused by the detergent treatment, the increase in WGA and RCA binding did not represent more than a fraction of the binding observed in absence of detergent. In this respect, the behavior of WGA- and RCA-binding sites was similar to that of the high affinity Con A-binding sites. Electron microscope observations with the F-Av-biotin method showed that, as is the case with the high affinity sites for Con A, WGA- and RCA-binding sites are not present in either the outer or inner face of microsomal vesicles, but are located exclusively in contaminating smooth vesicles and in plasma membrane fragments. Hirano et al. (25) have reported that binding sites for RCA-ferritin are not present in rough microsomal fragments from myeloma cells. Recently, the presence of RCA-binding sites has been demonstrated in liver plasma membranes (62, 12) and in the outer face of lysosomal membranes (17).
FIGURE 11 Binding of RCA, WGA, and Con A to large fragments of the rough endoplasmic reticulum. A fraction containing rapidly sedimenting ER fragments (RSER) was lightly fixed with glutaraldehyde and disrupted by compression-decompression as described in Materials and Methods, to render the vesicles permanently open. The opened RSER vesicles were incubated with lectin-biotin, washed by centrifugation, and reincubated with F-Av. (A) No lectin-biotin was present in the first incubation. (B) Con A-biotin, (C) WGA-biotin, and (D) RCA-biotin in the first incubation. × 112,000.
together, these observations suggest that the slight increase in the number of RCA, WGA, and high affinity Con A-binding sites present in rat liver microsomal fractions caused by treatment with low DOC concentrations only reflects the opening of contaminating membrane vesicles, some of which contain the appropriate sugar residues on their inner face.

Our observations concerning the distribution of lectin-binding sites in ER membranes, which reflect the presence of specific residues in the carbohydrate chains of glycoproteins, must be considered in the light of current knowledge on the mechanism of biosynthesis of carbohydrate chains in glycoproteins and the subcellular distribution of the glycosyl transferases which participate in this process. Current evidence suggests that, during the synthesis of the glycoprotein, the carbohydrate core is transferred to the polypeptide in block from a lipid intermediate found in the microsomal membranes, on which the proximal sugars have been previously added (cf. reference 41). On the other hand, the distal sugar residues are thought to be added to the core sequentially by glycosyl transferases located at the level of the Golgi apparatus (66). The absence of ricin-binding sites in RM membranes correlates very well with the fact that the activity of galactosyl transferase is confined to membranes which do not bear ribosomes (18, 66) and probably do not represent remains of the ER cisternae. In most animal glycoproteins (cf. reference 71), the first sugar of the carbohydrate chain is N-acetylglucosamine, a good inhibitor for WGA binding. It might therefore be regarded as a puzzling observation that binding sites for this lectin were not detectable in the microsomal content or in the limiting membranes. It is likely, however, that this only reflects the fact that potential sites for WGA in RM, which may be provided by the proximal N-acetylglucosamine residues, are always covered by the other proximal sugar residues. N-acetylglucosamine appears to be linked to the peptide backbone during growth of the nascent polypeptide chain (48, 24, 40, 49, 22, 60). Current evidence indicates that this sugar is transferred in block as part of a mannose-rich core from a lipid intermediate (29, see reference 41 for review). Therefore,
proximal N-acetylgalactosamine residues, the only ones present in RM, are likely to be always masked by mannose residues, which have no affinity for WGA. These considerations should explain why only N-acetylgalactosamine residues located in a position distal to the mannose core, which are added by N-acetylgalactosaminyl transferases present in the Golgi apparatus, may participate in WGA binding. Indeed, we have shown that avidin, which has a carbohydrate configuration containing a mannose core and terminal N-acetylgalactosamine residues, reacts strongly with WGA.

The absence of WGA-binding sites in RM also suggests that sialic acid, which is always a terminal sugar in glycoproteins, is not present in ER membranes. Sialic acid is thought to be involved in the binding of WGA to membranes (11, 9). WGA binds sialic acid (20), and treatment of transformed cells with neuraminidase has been reported to abolish their ability to be agglutinated by WGA (11), although this effect may depend on the specificity of the neuraminidase (9). Sialic acid involvement in WGA binding, however, does not seem to be a direct one, as part of the site, since it has not been found in direct linkage to N-acetylgalactosamine (a good hapten sugar for WGA). Furthermore, when added alone, N-acetyl-neuraminic acid (NANA) does not inhibit WGA-mediated agglutination of cells (11).

In glycoproteins, sialic acid is usually linked to galactose or N-acetylgalactosamine residues (71). Our observations indicate that these two sugars are also absent from RM glycoproteins. The absence of galactose from RM is inferred from the absence of RCA-binding sites and the absence of N-acetylgalactosamine from the lack of binding sites for SBA. Biochemical analysis has also failed to show significant amounts of galactosamine in RM fractions (26, 27). Since these two sugars are the only ones participating in O-glycosidic linkages in glycoproteins (71), it might be inferred that glycoproteins with this type of linkage are not components of the rough ER.

The findings reported in this paper are compatible with the notion that membrane proteins once inserted into rough ER membranes are distributed subcellularly by mechanisms similar to those used for secretory proteins. The observation that carbohydrates typically found in the terminal trisaccharide portion of glycoprotein are not present in rough ER membranes or in content glycoproteins does not support the proposal (6, 7, 16) that a retrograde migration of glycoproteins from the Golgi apparatus to the rough ER plays a major role in the biogenesis of ER membranes. Indeed, the observations in this report suggest that glycoproteins move unidirectionally from the rough ER to the Golgi apparatus, as secretory proteins do (15).

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