

INSULIN BINDING TO RAT LIVER GOLGI FRACTIONS

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INTRODUCTION

Although Golgi elements were first obtained from rat liver homogenates as a single fraction (1, 2) a new procedure was recently developed (3) which yielded three fractions which accounted for all of the Golgi elements present in the microsomal fraction, as indicated by recovery of UDP-galactosyl transferase¹ (4). Morphological analysis showed that the Golgi "light" and "intermediate" subfractions contained a large number of large and small vesicles filled with very low density lipoprotein (VLDL) particles, whereas in the Golgi "heavy" subfraction, flat cisternal elements derived from the cis face of the Golgi apparatus predominated (3, 5). Low activities of enzymes (e.g., glucose-6-phosphatase) associated with the microsomal fraction were detected, but all fractions possessed 5'-nucleotidase activity (4). Cytochemical tests indicated that the presence of 5'-nucleotidase (normally considered as a plasma-

lemma marker) in the Golgi subfractions was not due to contamination by plasmalemma membranes, but that 5'-nucleotidase was an indigenous component of the Golgi (5). We now report the detection of insulin-binding activity in the Golgi subfractions.

METHODS

Preparation of Subcellular Fractions

Rat liver plasmalemma fractions were isolated by the rate-zonal centrifugation method of Evans (6). Final purification was achieved by centrifugation in a discontinuous sucrose gradient (7). Golgi subfractions and a residual microsomal fraction were isolated as described previously (3, 4) but a Beckman SW27 rotor (95,000 g_{av} for 3 h) (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) replaced the SW25.2 rotor previously used (3, 4) in the final centrifugation step.

The *smooth* microsomal fraction free of Golgi elements was obtained by subjecting the microsomal preparation freed of Golgi to the Rothschild procedure (8). This consisted of obtaining a microsomal

¹ *Abbreviations used in this paper:* SDS, sodium dodecyl sulfate; UDP-galactosyl transferase, UDP galactose; *N*-acetyl glucosamine galactosyl transferase; VLDL, very low density lipoprotein.

preparation freed of Golgi (3, 4), and resuspending to a concentration of 1.32 M sucrose. This suspension was layered beneath 0.25 M sucrose and centrifuged for 12 h in the Beckman SW27 rotor. Smooth microsomes freed of Golgi elements were collected at the 0.25/1.32 M sucrose interface.

Iodination of Insulin and Binding Assay

Crystalline porcine insulin (Novo Industries, Copenhagen; batch 66/1, sp act 22.6 U/mg) was iodinated with [¹²⁵I]Na (carrier free, The Radiochemical Centre, Amersham, England) to a specific activity of 1.25 Ci/μmol (9). Hormone binding was determined by incubating membrane fractions at 25°C for 40 min in the following mixture (9) of: 10% bovine serum albumin in 4 times concentrated Krebs-Ringer bicarbonate (40 μl); [¹²⁵I]insulin (final concentration, 1.8×10^{-9} M = 300,000 cpm); fractions (10–500 μg of protein); and distilled water to a final volume of 150 μl. Portions (50 μl) were removed from each tube and filtered through prewashed Whatman GF/A filters. The filters were rapidly washed with 10 ml ice-cold 0.25% bovine serum albumin in Krebs-Ringer bicarbonate. Filters were air dried, then removed, and ¹²⁵I measured in a Nuclear Enterprises γ-spectrometer (efficiency, 40%) (Nuclear Enterprises, Inc., San Carlos, Calif.).

To determine specificity of insulin binding, control tubes differing from the above only by the presence of native insulin (9.2×10^{-6} M) were incubated. The amount of [¹²⁵I]insulin binding in the presence

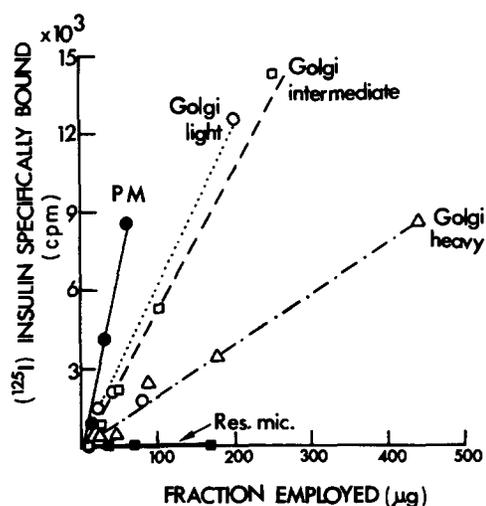


FIGURE 1 Binding of insulin to rat liver subcellular fractions. Increasing amounts of each fraction (microgram protein) were added to the assay mixture as described in Methods. Specific binding was determined as described in Methods.

of native insulin is designated “nonspecific.” “Specific binding” is the difference between total binding and nonspecific binding. Approximately 0.7% of the total radioactivity was attached to the filters in the absence of membraneous fractions.

Protein content of membranes was determined by the method of Lowry et al. (10).

Electron Microscopy

Frozen aliquots of the Golgi fractions were thawed and mixed with an equal volume of 2% OsO₄ in water and placed in the bottom of a Spinco 40 centrifuge tube which was filled with water. After centrifuging at 105,000 *g*_{av} for 30 min, the supernate was removed and the pellet block stained in uranyl acetate (11), then dehydrated, and embedded into Epon, oriented as described previously (3). Thin sections were cut on an LKB III microtome with a glass knife and mounted on naked grids. The sections were stained for 1 min in ethanolic uranyl acetate (12) and then in lead citrate for 5–10 min (13). They were examined in a Philips electron microscope operated at 80 kV.

Sodium Dodecyl Sulfate (SDS)

Gel Electrophoresis

SDS slab electrophoresis was carried out by using an apparatus modelled on the commercially available E-C apparatus (E-C Apparatus Corp., St. Petersburg, Fla.). SDS polyacrylamide gels were made essentially as described by Maizel (14). Gels contained 0.1% SDS and were discontinuous with a 3.6% acrylamide spacer (pH 6.7) and a 8.5% acrylamide resolving gel (pH 8.3). The electrode buffer consisted of Tris HCl (0.6%), glycine (2.88%), SDS (0.1%), pH 8.3. Samples were prepared for electro-

TABLE I
Binding of [¹²⁵I]Insulin to Rat Liver
Subcellular Fractions

Subcellular fraction	Moles bound/mg protein
Plasmalemma	1.3×10^{-13}
Golgi light	6.4×10^{-14}
Golgi intermediate	4.6×10^{-14}
Golgi heavy	1.7×10^{-14}
Microsomes residual	Not detectable
Plasmalemma	1.1×10^{-13} *
Cell membrane	1×10^{-13} †

Saturation binding calculated from data of:

* Freychet et al. (19),

† Cuatrecasas et al. (21).

phoresis as follows: to 30 μ l of each required sub-cellular fraction was added 10 μ l of 0.5 M Tris HCl (pH 6.7); 10 μ l of β -mercaptoethanol; 10 μ l of bromophenol blue; 10 μ l of SDS; 10 μ l of glycerol. Each sample was boiled for 1 min and the entire aliquot applied to the gel. The gels were stained for protein with Coomassie brilliant blue as described by Maizel (14). Reovirus, type 3, was used to calibrate the gel. The virus contains the following well-characterized (15) polypeptides λ_2 (mol wt 140,000), μm_1 (mol wt 80,000), μm_2 (mol wt 72,000), σ_2 (mol wt 38,000), σ_3 (mol wt 34,000).

RESULTS AND DISCUSSION

The specific binding of [125 I]insulin to the three Golgi subfractions and the plasmalemma fraction

is shown in Fig. 1. A summary of the binding is given in Table I. Of the Golgi fractions the secretory vacuole fraction (Golgi light) showed highest insulin binding followed by the Golgi intermediate fraction. The fraction containing flattened cisternal elements (Golgi heavy) was the least active of the Golgi subfractions. A "residual" microsomal fraction (i.e., the microsomal fraction remaining after the Golgi was removed by flotation [3, 4]) failed to bind insulin specifically and plasmalemma membranes showed binding activity which was higher than Golgi light. The relative binding capacities of the fractions (Table I) were obtained from the linear portions of Fig. 1.

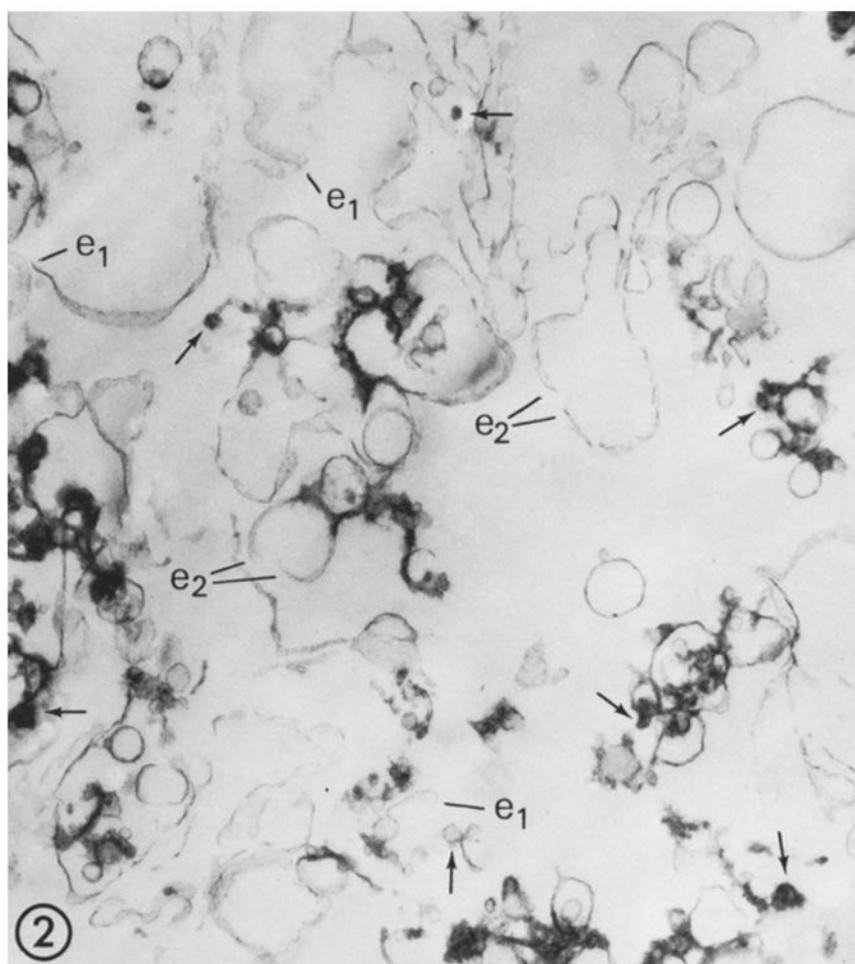


FIGURE 2 Intermediate Golgi fraction after freezing and thawing. Field in the middle of the pellet showing that the fraction consists of membrane profiles with VLDL particles (arrows) in close apposition. Most of the membrane profiles have free ends (e_1) and show a number of "breaks" (e_2). The Golgi light fraction is identical. $\times 36,000$.

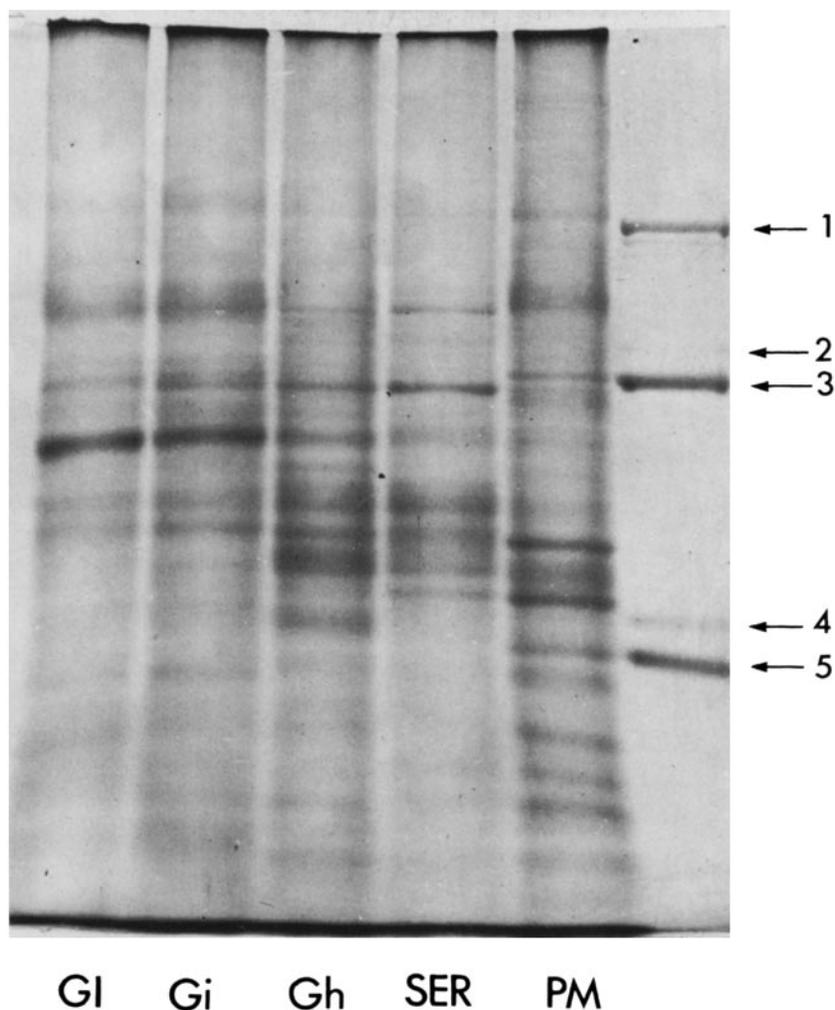


FIGURE 3 Slab gel electrophoretic patterns of subcellular fractions. 125 μ g of Golgi light (*Gl*), Golgi intermediate (*Gi*), Golgi heavy (*Gh*), Golgi-free smooth microsomes (*SER*), and plasmalemma (*PM*) were applied to the corresponding slots and electrophoresed. The final slot contains reovirus viral polypeptides as molecular weight markers. 1, 2, 3, 4, and 5, represent the λ_2 , μm_1 , μm_2 , σ_2 , and σ_3 polypeptides of reovirus, respectively. The major band common to the Golgi light and intermediate fractions corresponds to the molecular weight of rat serum albumin (64,000). Parallel gels with purified rat serum albumin have confirmed the identification.

All fractions were stored frozen and then thawed before use in the insulin-binding assay (see Methods). Fig. 2 shows that this action disrupts the integrity of the Golgi fractions.² The fractions now

² The separation of Golgi membrane from content has been accomplished by the use of a hypotonic treatment followed by the French press and centrifugation (3). We have tested the ability of these membranes to bind insulin but have found only a 10–20%

consist mainly of open membrane elements, many with free ends. This is in contrast to the closed vesicles filled with 30–80 nm VLDL particles (3) comprising the undisrupted Golgi light and inter-

enrichment in binding activity. We do not know if this is due to a removal or inactivation of receptors by the French press or some other reason. Freezing and thawing with subsequent centrifugation will not separate membrane from content (3).

mediate fractions. The possibility of permeability barriers accounting for the lower degree of binding in the thawed fractions (Fig. 2) is therefore unlikely, since insulin receptors, whether they be

present on either side of the membrane, are available for binding with the $[^{125}\text{I}]$ insulin. Furthermore, as the binding occurs with (a) low concentrations of insulin, (b) takes place in the presence of a large excess of albumin, and (c) can be removed by native insulin, the present results suggest that an insulin receptor is present within the Golgi fractions.

It has already been demonstrated by both morphological (3) and biochemical (4) criteria, that the Golgi fractions are free of significant contamination by other subcellular organelles including the plasmalemma. In addition we now show a comparison by SDS polyacrylamide gel (Fig. 3) electrophoresis. The protein pattern of the three Golgi fractions used in the binding studies shows that no major bands are common between the Golgi fractions and the plasmalemma fraction. Therefore we may conclude that the insulin binding demonstrated with the Golgi fractions cannot be explained by a contamination of these fractions by plasmalemma-derived elements.

The primary biological action of insulin is exerted at the cell surface (16) and a number of studies have localized the insulin receptor on the surface membrane of cells (17-22). The present evidence shows that insulin-binding activity is also present within Golgi elements derived from the hepatocyte. As insulin covalently coupled to Sepharose beads was shown to mimic the biologic response to the hormone (16) then intracellular binding to the Golgi cannot be metabolically meaningful. We therefore interpret the results as indicative of the biosynthetic route of the insulin receptor.

Cytochemical and biochemical work (4, 5) has clearly demonstrated the presence of 5'-nucleotidase within the Golgi fractions, and that glycosylating enzymes (in particular UDP-galactosyl transferase) are primarily located in the Golgi (4, 23-25). It is noteworthy that the insulin receptor and 5'-nucleotidase are both glycoproteins (21, 26, 27) and the scheme we envisage (Fig. 4) may be applicable to the insertion and assembly of all plasmalemma membrane glycoproteins.

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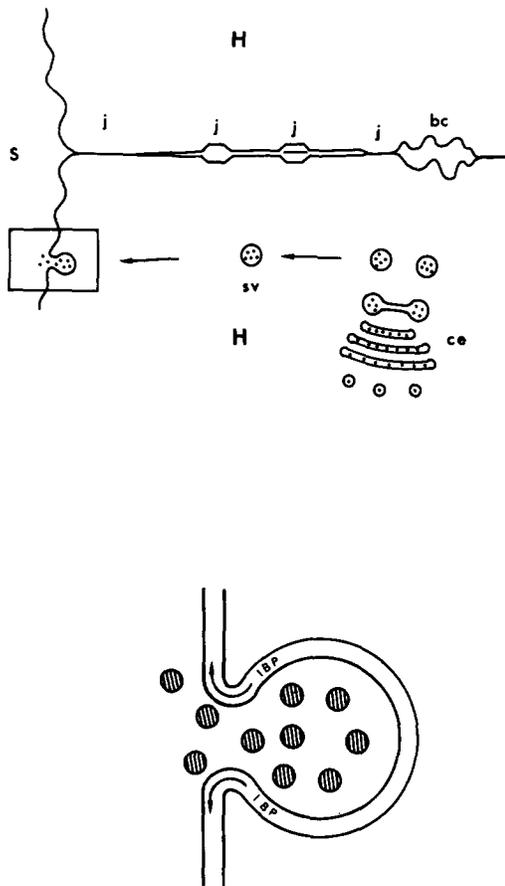


FIGURE 4 A diagrammatic representation of secretion in the hepatocyte (*H*). Secretory material (black dots) is processed in the flattened cisternal elements (*ce*) on the cis side of the Golgi. The secretory material passes into the Golgi secretory vacuoles (*sv*) on the trans side of the Golgi. The secretory vacuoles (*sv*) fuse with the plasmalemma discharging secretory content. The sinusoidal face of the plasmalemma membrane is represented (*s*) as are the various intercellular junctions (*j*) and the bile canaliculus (*bc*). The higher magnification view of fusion between the Golgi secretory vacuoles and the plasmalemma represent the *selective* diffusion of the insulin receptor (*IBP*) along the plane of the fluid (28, 29) fused membranes onto its position in the plasmalemma. To maintain specificity of the Golgi membranes and the plasmalemma (4, 23, 30, 31) the secretory vacuole membrane must be internalized within the cell (32, 33, 34).

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