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Intracellular hormone receptors: Evidence for insulin and lactogen receptors in a unique vesicle sedimenting in lysosome fractions of rat liver

(Golgi/tritosomes/metrizamide/Percoll/galactosyltransferase)

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ABSTRACT **Previous studies have established the presence of polypeptide hormone receptors in Golgi fractions from rodent liver. In this study we attempted to identify peptide hormone receptors in other intracellular elements, particularly lysosomes. Tritosomes were prepared by a standard procedure, and highly purified secondary lysosomes were prepared by fractionating the L fraction of rat liver in a discontinuous metrizamide gradient into subfractions L1 to L4. Binding of ^{125}I-labeled insulin and ^{125}I-labeled somatotropin was studied with membranes prepared from osmotically shocked fractions. The L2 and L3 fractions, virtually devoid of galactosyltransferase (UDP galactose:2-acetamido-2-deoxy-D-glucosylglycopeptide galactosyltransferase, EC 2.4.1.38) but highly enriched in acid phosphatase [orthophosphoric-monoester phosphohydrolase (acid optimum), EC 3.1.3.2], appeared as classical secondary lysosomes by electron microscopy. When compared with Golgi fractions, the level of specific binding per 50 μg of protein of ^{125}I-labeled somatotropin in L2 and L3 was 1/3, whereas that of ^{125}I-labeled insulin was comparable. L1, which was reduced in acid phosphatase and increased in galactosyltransferase activities, showed higher hormone binding than did L2 and L3. This was not attributable to Golgi fraction contamination, as evident by specific binding/galactosyltransferase ratios. Binding to tritosome membranes could be largely accounted for by variable contamination with Golgi fractions as judged by specific binding/galactosyltransferase ratios. To clarify the distribution of receptor sites in lysosomal preparations, we fractionated the entire L fraction on a continuous Percoll gradient. Acid phosphatase and galactosyltransferase activities were segregated to the high and low density ranges of the gradient, respectively; however, the fractions enriched in hormone binding were of intermediate density, distinct from Golgi and lysosomal biochemical markers. We conclude that intracellular receptors are found not only in galactosyltransferase-containing very low density lipoprotein-marked Golgi vesicles but also in a unique vesicle of intermediate density between classical Golgi and lysosomal structures.**

We have identified and characterized polypeptide hormone receptors in Golgi elements from rodent liver (1–7). We also have shown that after the binding of hormone to the plasmalemma of the rat hepatocyte, it is internalized through a receptor-mediated process and concentrated in Golgi vesicles (8–10). Other investigators, using electron-microscope radioautography to study the internalization of polypeptide hormones in cultured cells, have claimed that the hormone and probably the receptor as well are internalized directly and exclusively into lysosomes (11, 12). To resolve this controversy, we have attempted to identify receptors for insulin and lactogen in lysosome fractions from rat liver. Our results indicate that a structure intermediate in density between Golgi vesicles and lysosomes is especially enriched in receptors.

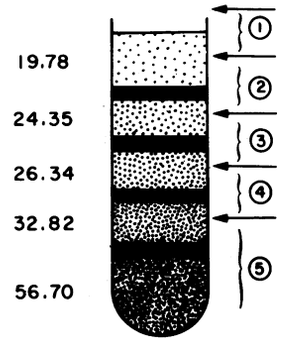

FIG. 1. Diagrammatic representation of the discontinuous metrizamide gradient used to purify secondary lysosomes. Percentage of metrizamide is shown on left. The volume of metrizamide solution in each successive zone from the top was 9, 6, 6, 6, and 9 ml, respectively. Arrows indicate the regions of the lysosomal subfractions.

MATERIALS AND METHODS

Female Sprague–Dawley rats (age 10 wk with body weight approximately 200 g each) were purchased from Canadian Breeding Laboratories (St. Constant, Quebec). Porcine insulin (24.5 units/mg) was a gift from the Connaught Laboratories, Willowdale, Ontario. Human somatotropin (2.0 units/mg) was kindly provided by the Pituitary Hormone Distribution Program of the National Institute of Arthritis, Metabolism and Digestive Diseases. Most reagents were purchased from Sigma. ^{14}C-Labeled UDP galactose was obtained from New England Nuclear. Percoll was supplied by Pharmacia Fine Chemicals and metrizamide was from Aldrich.

Subcellular Fractionations. Tritosomes (13) were isolated from the livers of rats injected with Triton WR 1339 (85 mg/100 g of body weight) 4 days prior to isolation using discontinuous sucrose density gradient centrifugation (14). A tritosome membrane preparation was recovered by centrifugation after osmotic shock and was resuspended in 25 mM Tris·HCl/10 mM $MgCl_2$, pH 7.4. Such preparations were assayed immediately for enzymes and protein or were frozen at −20°C prior to assaying for hormone binding. Golgi fractions (light, intermediate, and heavy) were prepared from rats 90 min after alcohol loading and assayed for enzymes, protein, and hormone binding as described (1, 2). Lysosomes from normal rats were isolated and subfractionated on discontinuous metrizamide gradients by the method of Wattiaux *et al.* (15) with minor modifications. L fraction was mixed 1:2 (vol/vol) with 85.6% (wt/vol) metrizamide solution, and 9 ml of this mixture was placed by needle and syringe beneath a discontinuous metrizamide gradient (Fig. 1). Subsequent centrifugation (82,000 × g_{av} for 2.5 hr) at 4°C yielded protein bands as noted in Fig. 1. The top four fractions were individually removed, diluted with 0.25 M sucrose, and

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Abbreviation: VLDL, very low density lipoprotein.

Table 1. Enzymatic characterization of rat liver subcellular fractions

Cell fraction	Protein, mg/g of liver	Acid phosphatase		Galactosyltransferase		5'-Nucleotidase	
		Yield	RSA	Yield	RSA	Yield	RSA
Golgi							
Light	0.18 ± 0.02	0.31 ± 0.03	2.9 ± 0.3	3.93 ± 0.10	37.3 ± 1.8	0.11	0.7
Intermediate	0.33 ± 0.02	0.76 ± 0.02	4.0 ± 0.1	6.30 ± 0.39	33.4 ± 1.5	1.68	5.9
Heavy	1.18 ± 0.06	1.41 ± 0.04	2.1 ± 0.1	12.11 ± 0.74	17.7 ± 1.4	4.27	3.0
Lysosomes							
Total L fraction	7.03 ± 0.84	24.21 ± 2.69	5.7 ± 1.2	1.52 ± 0.70	0.4 ± 0.2	10.59 ± 5.72	2.7 ± 1.7
Subfractions							
L1	0.13 ± 0.03	0.99 ± 0.27	12.2 ± 1.2	0.15 ± 0.03	2.0 ± 0.2	0.52 ± 0.02	5.8 ± 0.8
L2	0.22 ± 0.03	3.30 ± 0.70	24.1 ± 2.8	0.10 ± 0.04	0.7 ± 0.2	0.90 ± 0.20	6.1 ± 1.0
L3	0.24 ± 0.05	2.77 ± 0.82	18.3 ± 2.0	0.10 ± 0.03	0.7 ± 0.3	0.79 ± 0.28	4.6 ± 0.9
L4	2.53 ± 1.13	5.07 ± 1.44	3.8 ± 1.0	0.25 ± 0.05	0.3 ± 0.1	1.21 ± 0.32	1.0 ± 0.4
Tritosomes	0.33 ± 0.01	8.71 ± 0.44	38.1 ± 2.7	1.65 ± 0.33	7.5 ± 1.8	1.80 ± 0.63	8.9 ± 3.2

Golgi, lysosome, and tritosome fractions were isolated as described. The activity of 5'-nucleotidase was determined by the method of Heppel and Hilmoe (19) and acid phosphatase was assayed as described by Gianetto and de Duve (20) with minor modifications. Inorganic phosphate released in these assays was measured by the method of Chen *et al.* (21). Galactosyltransferase activity was determined with ovomucoid as acceptor, as described (22). Yields were calculated from specific activities and protein recoveries with the results expressed as a percent of total homogenate activity. Relative specific activity (RSA) is the ratio of fraction to homogenate specific activity. Average homogenate values for protein content, and the specific activities of acid phosphatase, galactosyltransferase, and 5'-nucleotidase were, respectively, 158.7 mg of protein per g wet weight of liver, 3.42 ± 0.22 μmol of P_i per hr per mg, 1.79 ± 0.1 nmol of galactose transferred per hr per mg of protein, and 2.81 ± 0.31 μmol of P_i per hr per mg of protein. These values represent the mean ± SEM of three (L and its subfractions) or six (Golgi elements and tritosomes) separate fractionations except for 5'-nucleotidase (one experiment for Golgi fractions and two for tritosome and lysosome fractions, where values represent the mean ± 1/2 range).

centrifuged at 170,000 × g_{av} for 35 min. The pellets were suspended in Tris/$MgCl_2$ buffer and used for enzyme assays immediately or were frozen at −20°C before assaying for hormone binding.

Further fractionation of the L fraction was also accomplished by Percoll density gradient centrifugation. The fraction (≈10–15 mg of protein) was diluted with Percoll in 0.25 M sucrose to a final 24-ml homogeneous suspension [density 1.07 g/ml; final Percoll concentration, 32.2% (vol/vol)]. A continuous gradient was generated *in situ* by centrifugation at 40,000 × g_{av} for 60 min at 4°C in a fixed angle rotor. Ten fractions (2–4 ml) were sequentially collected from the top by pumping a 62% (wt/vol) sucrose solution into the bottom of the centrifuge tube. Percoll was removed by diluting fractions by a factor of three with Tris/$MgCl_2$ buffer followed by centrifugation at 4°C (82,000 × g_{av} for 120 min). Particulate material above the Percoll pellet was removed, suspended in 1–3 ml of Tris/$MgCl_2$ buffer, and assayed for protein and enzymes immediately. A portion was frozen at −20°C for hormone binding assays.

Protein Determinations. Protein concentration was determined by the procedure of Lowry *et al.* (16) with bovine serum albumin as standard. Metrizamide interference was minimized by trichloroacetic acid precipitation of protein, followed by resolubilization in 0.5 M NaOH. The interference from remaining Percoll was accounted for by running controls at appropriate Percoll concentrations.

Electron Microscopy. Tritosome fractions were fixed in glutaraldehyde [2.5% (wt /vol) in 0.05 M cacodylate buffer, pH 7.4] followed by reduced OsO_4 (17) and stained *en bloc* with uranyl acetate (18). Lysosome subfractions from metrizamide gradients were fixed in unbuffered OsO_4. Pelleted fractions were block-stained in uranyl acetate and embedded in Epon 812 after dehydration in ascending alcohols and propylene oxide. Through-the-pellet thin sections were cut on an LKB-Huxley Ultramicrotome and viewed in a Philips 400 electron microscope after grid-staining in uranyl acetate and lead citrate.

RESULTS

Table 1 summarizes the results of experiments on enzymatic characteristics of Golgi, lysosome, and tritosome preparations. The marker enzymes for Golgi elements [galactosyltransferase (UDP galactose:2-acetamido-2-deoxy-D-glucosylglycopeptide galactosyltransferase, EC 2.4.1.38.)], lysosomes [acid phosphatase (orthophosphoric-monoester phosphohydrolase, acid optimum, EC 3.1.3.2.)], and plasma membrane [5'-nucleotidase (5'-ribonucleotide phosphohydrolase, EC 3.1.3.5.)] were measured. Only 0.3–1.4% of homogenate acid phosphatase activity was recovered in Golgi fractions, whereas recovery was much higher in the L fraction and tritosome preparations. Maximum enrichment for acid phosphatase in Golgi fractions (intermediate) was 4-fold more than homogenate activity. This was much lower than that for tritosomes and purified lysosomal subfractions L1 to L3. In contrast, the recovery of galactosyltransferase in Golgi fractions was high, totaling about 22% of homogenate activity, whereas in both tritosome and L fractions the yield was less than 2% of homogenate activity. In L subfractions, only L1 showed enrichment of galactosyltransferase activity (specific activity of 2.0 relative to homogenate activity). Characteristically, galactosyltransferase activity was concentrated (relative specific activity of 17.7–37.3) in the Golgi subfractions (23). A substantial enrichment (7.5-fold) of this enzyme was observed in tritosomes. The yield of 5'-nucleotidase activity was highest in the L fraction (10.6%) and the heavy Golgi fraction (4.3%). Relative enrichment of 5'-nucleotidase was comparable in Golgi (except for the light fraction) and those L fractions (L1 and L3) enriched in acid phosphatase (relative specific activity of 3.0–6.1).

The tritosomes and L subfractions (L1 to L4) were examined by electron microscopy. The center of pellet micrographs are shown in Fig. 2 *A–F*. There were very low density lipoprotein (VLDL)-containing vesicles seen in all fractions, but they constituted a minor component. These elements were more abundant in tritosomes and L1 than in L2 and L3. L2 and L3 were composed overwhelmingly of characteristic secondary lysosomes. L4 and L5 (not shown) were also examined and found to consist largely of mitochondria and smooth membranous elements.

The binding of ^{125}I-labeled somatotropin and ^{125}I-labeled insulin was studied in each of the subcellular fractions (Fig. 3). As previously observed, the binding of ^{125}I-labeled somatotro-

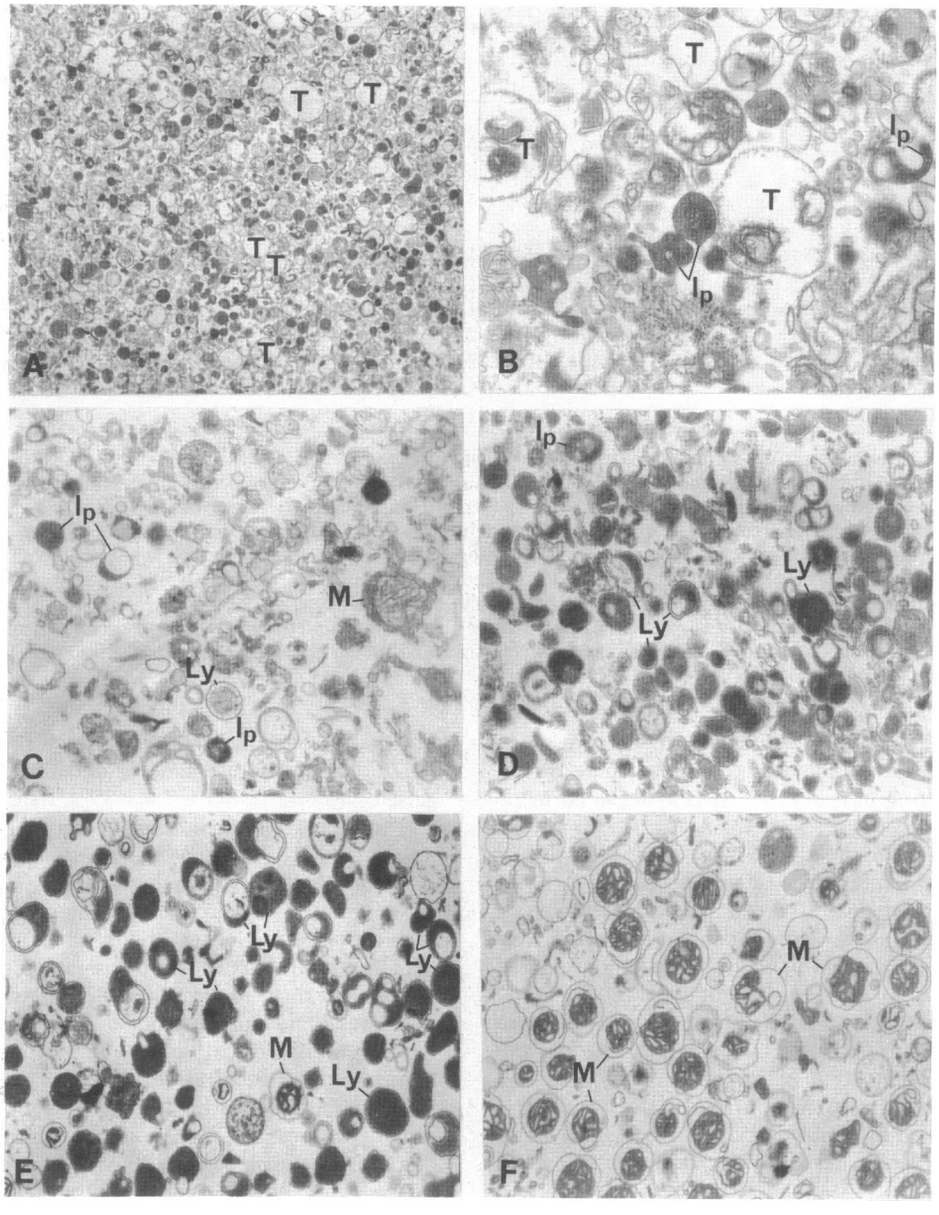

FIG. 2. Electron micrographs. (*A*) Tritosome fraction. (×3900.) (*B*) Tritosome fraction. (×12,500.) (*C–F*) Lysosome fractions L1, L2, L3, L4, respectively. (×10,500.) Low power views reveal circular profiles of tritosomes interspersed with smaller vesicular structures containing a denser intraluminal content (*A*). At higher power characteristic, large tritosomes with fine punctate material lining the intraluminal membrane and vesicles and attached tubules with a lipoprotein-like content are found (*B*). The lysosome fraction L1 (*C*) is heterogenous with vesicular and signet-ring structures containing lipoprotein-like content; disrupted mitochondria and lysosomes with a thick membrane and halo demarcating the intraluminal content are seen. The L2 fraction (*D*) is less heterogenous and consists mainly of apparent secondary lysosomes with a dense content, halo, and thick membrane; lipoprotein-containing vesicles are also observed. The L3 fraction (*E*) consists mainly of lysosomal structures, although rare mitochondria are also observed. The L4 fraction (*F*) is mainly mitochondrial. T, tritosomes; lp, lipoprotein-like structure; ly, lysosomes; M, mitochondria.

pin was maximum in Golgi light and intermediate fractions and was much greater than insulin binding to these fractions (2, 3, 5). Substantial binding of both ligands was seen in the lysosomal subfractions, with the greatest binding in L1, intermediate levels in L2 and L3, and low levels in L4. [Binding in L1 was greater than in L2 ($P < 0.02$, t test for paired data).] Tritosome membranes also showed high levels of ^{125}I-labeled somatotropin binding and easily measurable, though lower, levels of ^{125}I-labeled insulin binding. By using enzyme activity data from Table 1, ratios of specific hormone binding to relative specific activity of galactosyltransferase were computed (Table 2). The ratios for Golgi fractions were 0.6–1.3 for somatotropin binding and 0.2–0.3 for insulin binding. In contrast, the ratios for the L subfractions were much higher: 17–27.2 for somatotropin binding and 11.2–29.7 for insulin binding. It is of interest that the ratios for tritosomes were much closer to those in Golgi elements.

The L fraction also was resolved into 10 subfractions by Percoll density gradient centrifugation (24). Protein distributed in two main peaks with the greater being in fraction 10 ($\rho = 1.126$) and the lesser in fraction 2 ($\rho = 1.038$) (Fig. 4). Galactosyltransferase activity was found largely at low density (fractions 1–3). There was no enrichment of this enzyme over the homogenate, as maximum relative specific activity in fraction 2 was only 1.0. In contrast, acid phosphatase activity was found largely in heavier density fractions 6–10, with peak relative specific activity of about 12 in fractions 7 and 8. Binding sites for somatotropin were distributed over a broad density range, with the bulk ac-

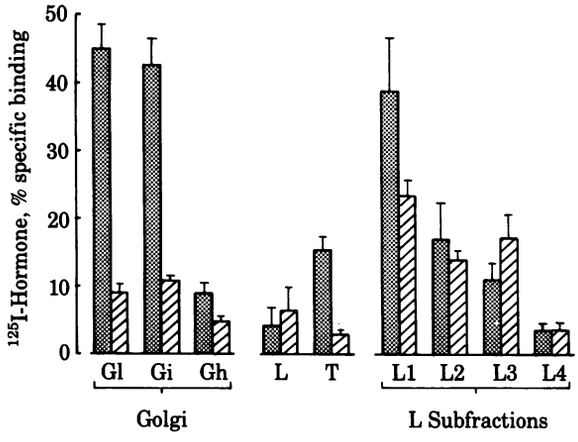

FIG. 3. Binding of ^{125}I-labeled insulin (▨) and ^{125}I-labeled somatotropin (▩) to different subcellular fractions from female rat liver. For binding studies ^{125}I-labeled insulin and ^{125}I-labeled somatotropin were prepared, and incubations with membrane preparations were performed as described (3, 5). Binding is expressed per 50 μg of cell fraction protein. Each bar with its vertical line represents the mean ± SEM of three (L and L subfractions) or six (Golgi elements and tritosomes) separate subcellular fraction preparations. ^{125}I-Hormone, ^{125}I-labeled hormone; T, tritosomes; Gl, light Golgi fraction; Gi, intermediate Golgi fraction; Gh, heavy Golgi fraction.

counted for by fractions 2–6. The maximum specific binding was seen in fractions 5 and 6 and did not conform to the peak of either acid phosphatase or galactosyltransferase activity. Most of the insulin binding sites were recovered at low density (fractions 1–3); and maximum specific binding was seen in fraction 1. It is of interest that fraction 1 showed notably high binding of ^{125}I-labeled somatotropin as well, though not quite as marked as that seen in fractions 5 and 6.

DISCUSSION

We have identified peptide hormone binding sites in Golgi elements from rat liver (1–7). In this study we examined both insulin and somatotropin binding in morphologically and enzymatically characterized tritosomes and lysosomes and compared

Table 2. Ratio of binding of ^{125}I-labeled somatotropin and ^{125}I-labeled insulin to the relative specific activities of galactosyltransferase and acid phosphatase in Golgi, lysosome, and tritosome membrane fractions isolated from female rat liver

	Galactosyltransferase		Acid phosphatase	
Cell fraction	Somatotropin	Insulin	Somatotropin	Insulin
Golgi				
Light	1.3 ± 0.1	0.2 ± 0.0	16.6 ± 2.0	3.5 ± 0.9
Intermediate	1.3 ± 0.1	0.3 ± 0.0	10.7 ± 1.2	2.7 ± 0.1
Heavy	0.6 ± 0.1	0.3 ± 0.0	4.4 ± 0.7	2.3 ± 0.1
Lysosomes				
Subfractions				
L1	20.3 ± 6.0	11.2 ± 0.0	3.2 ± 0.6	2.0 ± 0.2
L2	27.2 ± 9.6	22.8 ± 5.0	0.7 ± 0.3	0.6 ± 0.0
L3	17.0 ± 4.8	29.7 ± 12.5	0.6 ± 0.2	1.0 ± 0.3
L4	18.0 ± 4.6	16.7 ± 4.4	1.0 ± 0.3	0.9 ± 0.3
Tritosomes	2.6 ± 0.5	0.5 ± 0.2	0.4 ± 0.0	0.1 ± 0.0

Ratios were formed from enzymatic data summarized in Table 1 and binding data shown in Fig. 3. Each value is the mean ± SEM of three (L and its subfractions) or six (Golgi elements and tritosomes) separate fractionations.

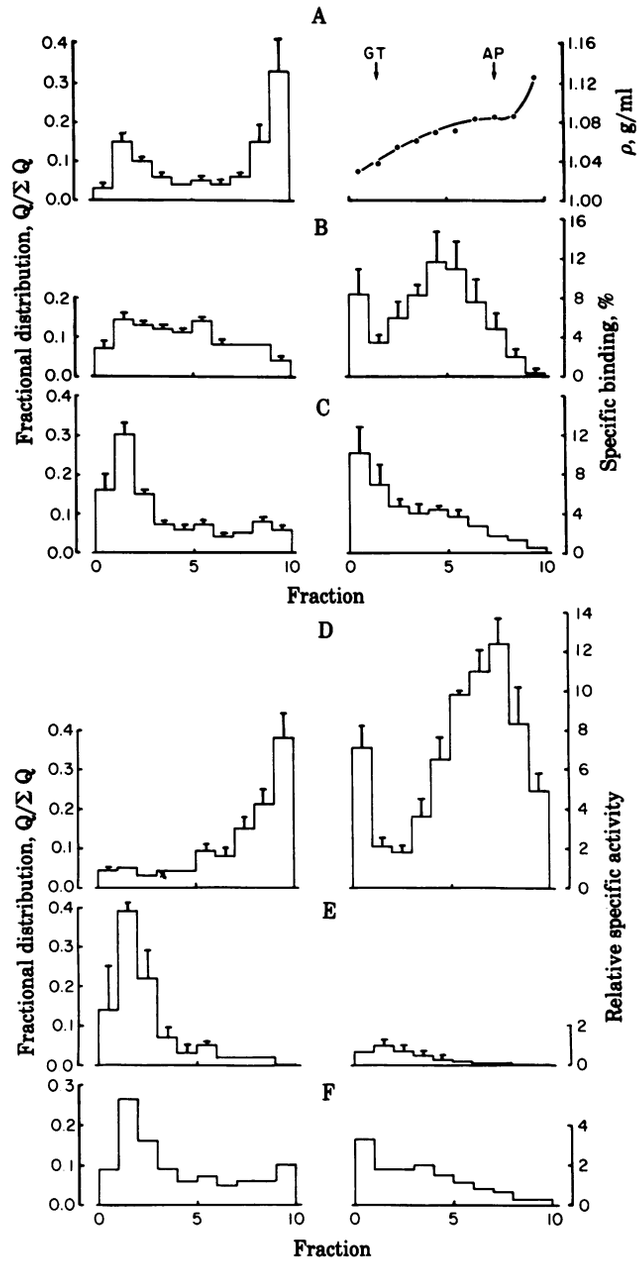

FIG. 4. Distribution of protein (*A*), hormone binding sites (*B* and *C*), and marker enzymes: acid phosphatase (AP) (*D*) and galactosyltransferase (GT) (*E*) and 5′-nucleotidase (*F*) after subfractionation of rat liver L fraction on Percoll gradient. The L fraction was isolated and subfractionated on iso-osmotic, continuous Percoll gradient as described. Density of fractions was measured by weighing 100-μl samples in capped Microfuge tubes and expressed as g/ml. All other assays were done on membrane preparations after removal of Percoll. (*Left*) Ordinates of panels are the fractional distribution (Q/ΣQ) of protein content (mg per fraction), insulin (*B*) binding sites (fmol of insulin bound),§ somatotropin (*C*) binding sites (fmol of somatotropin bound), and marker enzyme activities recovered in the fractions; Q represents the amount found in the fraction and ΣQ the total recovered amount. (*Right*) Ordinates of panels (from the top) are density of fractions (g/ml), percentage specific binding of ^{125}I-labeled somatotropin per 50 μg of protein, percentage specific binding of ^{125}I-labeled insulin per 50 μg of protein, and relative specific activities of acid phosphatase, galactosyltransferase, and 5′-nucleotidase. Protein content and enzyme activities were measured in fresh fractions. Hormone binding was determined in fractions frozen and thawed four times. Vertical lines, SEM of three to six fractionations.

§ (mg of protein per fraction) × (fmol of hormone bound per mg of protein) = fmol of hormone bound per fraction.

these new observations with those in Golgi fractions. The Golgi fractions differed from lysosomes in being enriched in galactosyltransferase and low in acid phosphatase (23). Lysosomal fractions were enriched in acid phosphatase and correspondingly contained low levels of galactosyltransferase. Tritosomes were relatively more enriched in the latter enzyme. Electron microscopy of the lysosomal subfractions (L2 and L3) revealed vesicles of varying size with a thick membrane, clear halo, and amorphous matrix. These had the appearance of typical secondary lysosomes as originally shown by Wattiaux *et al.* (15). The difference in appearance between these structures and the VLDL-filled Golgi vesicles is readily appreciated. Small quantities of VLDL-containing vesicles were seen in all of the lysosomal subfractions but most notably in L1, which correspondingly showed a higher relative specific activity for galactosyltransferase. We presume that the contamination of tritosomes by VLDL-containing vesicles (Fig. 2) contributed to the considerable galactosyltransferase content here as well (Table 1).

This study has demonstrated both ^{125}I-labeled insulin and ^{125}I-labeled somatotropin specific binding to both lysosomal subfraction and tritosome membrane preparations. On the basis of previous observations, we infer that ^{125}I-labeled somatotropin binding reflects binding to the lactogen receptor of female rat liver (5). The binding to the acid phosphatase-enriched fraction cannot be explained by galactosyltransferase contamination (Golgi element) because the ratios of specific binding to relative specific activity of galactosyltransferase far exceeded that of Golgi fractions (Table 2). However, specific binding to tritosome membrane preparations can largely, if not completely, be explained by Golgi element contamination. Closer examination of the pattern of binding in the lysosomal subfractions reveals that maximum binding was in L1, whereas higher acid phosphatase activity was in L2 and L3 (Table 1). This disparity is especially marked when binding to acid phosphatase ratios are examined. L1 showed significantly higher ratios than did L2 and L3 (Table 2). These observations indicate that binding sites in the lysosomes are, at least in part, located in components distinct from Golgi elements or lysosomes. If tritosome membranes accurately reflect lysosomal membranes, it can be inferred that there is little binding to *bona fide* lysosomes as such.

To test this hypothesis further, the L fraction prepared from rat liver was fractionated on a continuous iso-osmotic Percoll gradient (Fig. 4). Neither insulin nor somatotropin binding corresponded to the peak of acid phosphatase activity. The bulk of somatotropin binding did not correspond to the peak activity of any of the enzymes studied. The peak of insulin binding sedimented at a lower density than that of somatotropin and was maximal in fractions containing the bulk of galactosyltransferase and 5′-nucleotidase (Fig. 4). The ratio of either insulin or somatotropin binding to the relative specific activity of 5′-nucleotidase was much higher in the unique vesicle fraction‡ than in plasma membrane or smooth microsomes (2), indicating that the observed binding was not likely to be explained by plasma membrane or endoplasmic reticulum contamination. Thus, hormone receptors appear to be found in a new subpopulation of vesicles that do not correspond to either lysosomes, Golgi, or other defined subcellular elements. These structures are not especially enriched in the Golgi apparatus marker enzyme galactosyltransferase. The difference in the distribution of binding sites for insulin and somatotropin suggests that there is a substantial heterogeneity in hormone receptor content within these vesicles. Recent studies with both cell fractionation and morphological approaches to examine ^{125}I-labeled hormone uptake suggest that these vesicles of intermediate density between typical Golgi and lysosomal elements are VLDL-containing and are involved in receptor-mediated hormone internalization (unpublished data). The relevance of this apparently new class of vesicular structures to hormone action remains to be elucidated, but these vesicles may play a role in hormone receptor turnover or processing of internalized hormone, or both.

‡ In fractions 5 and 6 of Fig. 4, the ratios of hormone binding (fmol of hormone bound per mg of protein) to the relative specific activity of 5′-nucleotidase were: insulin, 53.0:1; somatotropin, 42.5:1. For plasma membrane, the ratios for insulin and somatotropin were 19.6:1 and 2.6:1, respectively (2). For smooth microsomes the ratios for insulin and somatotropin were shown to be 12.3:1 and 2.4:1, respectively (2). In addition, the binding to smooth microsomes of insulin was greater than that of somatotropin (insulin/somatotropin binding ratio, >5:1), whereas the converse was seen in fractions 5 and 6 (Fig. 4, insulin/somatotropin binding ratio, <0.5).

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