Coated Vesicles Participate in the Receptor-mediated Endocytosis of Insulin

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ABSTRACT We have purified coated vesicles from rat liver by differential ultracentrifugation. Electron micrographs of these preparations reveal only the polyhedral structures typical of coated vesicles. SDS PAGE of the coated vesicle preparation followed by Coomassie Blue staining of proteins reveals a protein composition also typical of coated vesicles. We determined that these rat liver coated vesicles possess a latent insulin binding capability. That is, little if any specific binding of ^{125}I-insulin to coated vesicles is observed in the absence of detergent. However, coated vesicles treated with the detergent octyl glucoside exhibit a substantial specific ^{125}I-insulin binding capacity. We visualized the insulin binding structure of coated vesicles by cross-linking ^{125}I-insulin to detergent-solubilized coated vesicles using the bifunctional reagent disuccinimidyl suberate followed by electrophoresis and autoradiography. The receptor structure thus identified is identical to that of the high-affinity insulin receptor present in a variety of tissues. We isolated liver coated vesicles from rats which had received injections of ^{125}I-insulin in the hepatic portal vein. We found that insulin administered in this fashion was rapidly and specifically taken up by liver coated vesicles. Taken together, these data are compatible with a functional role for coated vesicles in the receptor-mediated endocytosis of insulin.

Insulin is rapidly taken up by rat liver in a receptor-mediated process (1, 2). The physiological significance of this uptake has not been completely established, but a major role of hormone internalization is likely to be the clearance of plasma insulin (1, 3). In addition, internalization of the hormone-receptor complex may play a part in the regulation of cell surface receptor number (4) or may occur as a result of a constant process involving receptor cycling from plasma membranes to and from the membranes of internal organelles (5, 6). Insulin internalized by rat liver or isolated rat hepatocytes can subsequently be found in the Golgi apparatus (2), in lysosomes (7), or in lysosome-like vesicles of undetermined origin (8, 9). It is very likely that receptor-bound insulin follows a pathway from the plasma membrane to these intracellular organelles involving coated pits and coated vesicles (reviewed in references 10 and 11). Such a pathway involves initial clustering of insulin-receptor complexes in clathrin-coated domains of the plasma membrane, and this clustering has been demonstrated (12, 13). The coated pits then invaginate to form coated vesicles which are proposed to migrate within the cell and mediate the transport of membrane proteins, including ligand-receptor complexes, to and from their target membranes (10, 11, 14).

To establish the role of coated vesicles in the internalization of insulin, we purified these structures from rat liver. We determined that rat liver coated vesicles possess characteristics consistent with their having a role in the intracellular transport of insulin and insulin receptors. These characteristics are: (*a*) the ability to specifically and latently bind insulin to a receptor structure identical to that established for the insulin receptor in a variety of other tissues, and (*b*) coated vesicles isolated at various times after hepatic portal vein injection of ^{125}I-insulin contain the labeled hormone. The kinetics of hepatic insulin uptake and the specificity of this process are consistent with a role for coated vesicles in the receptor-mediated endocytosis of the hormone-receptor complex by rat liver.

MATERIALS AND METHODS

Coated Vesicle Isolation: Coated vesicles were purified from rat livers obtained from 150–300 g male Sprague-Dawley rats by the procedure of Pearse (15) essentially as modified by Blitz et al. (16). Rat livers (6–10) were excised and immediately cooled in an ice-cold buffer consisting of 10 mM HEPES, pH 7.4 with 0.25 M sucrose and 0.5 mM benzamidine. The livers were blotted, minced, and homogenized in 1–2 vol of the above buffer using 4–6 strokes of a motor-driven Potter-Elvehjem tissue grinder. 1 mM phenylmethylsulfonyl fluoride (PMSF) was added to some preparations at this point but no

THE JOURNAL OF CELL BIOLOGY · VOLUME 93 January 1983 133-138

difference in coated vesicle yield or integrity was observed due to the PMSF. Additional buffer was added to the vesicle preparation to achieve a 4 to 1 ratio of buffer volume to tissue weight. The homogenate was filtered through cheesecloth and spun for 20 min at 12,000 *g*. The resultant supernatant was saved and adjusted to 0.1 M 2-(*N*-morpholino-) ethane sulfonic acid (MES) sodium salt, pH 6.5, 1 mM EGTA, 1.5 mM magnesium chloride, and 0.02% sodium azide (MES buffer). This supernatant was then centrifuged for 70 min at 125,000 *g* to yield a microsomal fraction. The microsomal fraction was resuspended in a minimum of MES buffer and subjected to two or three successive separations by discontinuous sucrose density gradient centrifugation. The first gradient consisted of 5 ml each of 10, 20, 30, 40, and 50% (wt/vol) sucrose in MES buffer in a 36-ml ultracentrifuge tube. The microsomes were carefully layered onto the top of the gradient and spun for 45 min at 21,000 rpm in either a Beckman SW 27 rotor (Beckman Instruments, Inc., Fullerton, CA) or a Sorvall AH 627 rotor (Sorvall-DuPont, Newtown, CT). The portion of the gradient from just above the 30–40% sucrose interface up to and including the 10% sucrose layer was collected, diluted two- to threefold with MES buffer, and pelleted for 70 min at 125,000 *g*. This pellet was resuspended in a minimum of MES buffer and layered onto a discontinuous gradient consisting of 5.5 or 6.0 ml each of 40, 45, 50, 55, and 60% sucrose (wt/vol) in MES buffer. The coated-vesicle preparation was centrifuged overnight (16–18 h) at 21,000 rpm in the rotors indicated above. Coated vesicles were recovered from the 45–50% and 50–55% sucrose interfaces by dilution with MES buffer and centrifugation for 70 min at 128,000 *g*. Agarose gel electrophoresis and/or negative stain electron microscopy (see below) indicated ≥90% purity of the coated vesicles at this point. For the experiment depicted in Fig. 4, the coated vesicles were used at this point without further purification. For the experiments of Table I and Figs. 1, 2, and 3, an additional gradient was performed. This gradient consisted of 4 ml each of 5, 10, 20, and 30% sucrose (wt/vol) in MES buffer and was spun for 45 min at 21,000 *g* in 16-ml buckets for either an SW 27 or an AH 627 rotor. The coated vesicles were collected from above the 20–30% interface, diluted, and concentrated by centrifugation as in previous steps. Electron micrographs of thin-sectioned material (Fig. 1 *a*) indicated this vesicle preparation to be essentially homogeneous. Typically, 100 g of rat liver yielded 2–3 mg of coated-vesicle protein. Animals subjected to hepatic portal vein injection were anesthetized with pentobarbital before injection of ^{125}I-insulin (2×10^7 cpm/animal), and coated vesicles were prepared as described above.

Liver Plasma Membrane Isolation: Rat livers were excised and homogenized as described above for coated vesicle preparation, and plasma membranes were prepared essentially as described by Carey and Hirschberg (17). The homogenate was filtered through cheesecloth and spun at 2,000 *g* for 10 min. The 2,000 *g* pellet was dispersed in a minimum of 10 mM HEPES, 0.25 M sucrose, pH 7.4 and adjusted to 47% (wt/wt) sucrose by the addition of 66% (wt/wt) sucrose as monitored by refractive index. The homogenate was then overlayed with 41% (wt/wt) sucrose in 3½ × 1-in. centrifuge tubes. These tubes were spun at 25,000 rpm for 90 min in a Sorvall AH 627 rotor (Sorvall-DuPont). The plasma membranes were collected from the top of the 41% sucrose layer and were washed once in 10 mM HEPES, 0.25 M sucrose, pH 7.4. The membranes were dispersed in this same buffer and stored at −20°C before use.

TABLE I

Insulin Binding to Rat Liver Coated Vesicles and Plasma Membranes

	fmoles Insulin bound/milligram protein			
Preparation	Coated vesicles*		Latency	Plasma membranes
	−	+	%	
1	2	61	97	—
2	21	202	89	92
3	24	239	89	95

Rat liver coated vesicles and plasma membranes were isolated as described in Materials and Methods. The vesicles, suspended in MES buffer at 0.5–2.0 mg/ml, were diluted in PBS and then spun for 1 h at 100,000 *g*. The coated vesicles were resuspended in PBS at 1–2 mg/ml and treated or not with 1% octyl glucoside for 20 min at 23°C. Portions (20–40 μg) were then removed for insulin binding exactly as described (19). Binding of insulin to plasma membranes was performed in the absence of octyl glucoside by the same precipitation assay (19) used for coated vesicles as described in detail in Materials and Methods. Tracer insulin concentrations were 3×10^{-10} M (*Preparation 1*) or 1.2×10^{-9} M (*Preparations 2* and *3*). Nonspecific binding (always less than 20% of the total binding) was determined in the presence of 7×10^{-7} M unlabeled insulin and was subtracted from total binding to obtain specific binding. Percentage latency is defined as specific binding in the presence of detergent (total binding) minus specific binding in the absence of detergent divided by total specific binding.

* Not treated (−), or treated (+) with 1% octyl glucoside.

Agarose Gel Electrophoresis of Coated Vesicles: Coated vesicles obtained after two successive sucrose gradients (see above) were further purified essentially as described by Rubenstein et al. (18). Samples consisting of 50–100 μg of coated vesicle proteins were applied to a 0.15% agarose gel and electrophoresed for 24 h at 25 Volts in 50 mM MES, pH 6.5. The coated vesicles could be directly visualized by their turbidity and were dried for autoradiography and stained with Coomassie Blue exactly as previously described (18).

Insulin Binding to Coated Vesicles: Monocomponent porcine insulin was obtained from Eli Lilly Corporation as a gift from Dr. Ronald Chance. ^{125}I-insulin was prepared from insulin and Na^{125}I (New England Nuclear, Boston, MA) to a specific activity of 60–100 μCi/μg using Enzymobeads (Bio-Rad Laboratories, Richmond, CA) according to the protocol supplied with the Enzymobeads. Coated vesicles purified through three successive sucrose density gradients (see above) were suspended at 1:2 mg/ml protein in a buffer consisting of 10 mM phosphate, 142 mM NaCl, and 7 mM NaCl, pH 7.4 (PBS). Octyl glucoside (10% wt/vol in PBS) was then added to a final concentration of 1%. The vesicles were incubated with the detergent for 20 min at 23°C. The vesicles (20–40 μg protein) were then incubated with ^{125}I-insulin (3×10^{-10} M or 1.2×10^{-9} M, see Table I) in the presence or absence of 7×10^{-7} M unlabeled insulin in a total volume of 200 μl of PBS, pH 7.4, containing 1 mg/ml albumin. Liver plasma membranes were assayed for insulin binding by adding 20 μl of membrane (80–160 μg protein) in 10 mM HEPES, 0.25 M sucrose, pH 7.4 to 180 μl of PBS adjusted to attain the same final insulin and albumin concentrations indicated above. Binding was terminated after 30 min by the addition of 0.5 ml of an ice-cold solution of 0.1% gamma globulin in 50 mM sodium phosphate pH 7.4, followed immediately by 0.5 ml of ice-cold 25% polyethyleneglycol (6,000–8,000 mol wt) in 10 mM sodium phosphate, pH 7.4. After 15 min, the precipitated ^{125}I-insulin-receptor complex was collected and separated from free hormone by filtration exactly as previously described (19).

Electron Microscopy: A sample of rat-liver coated vesicles purified by three sucrose gradients and containing ~150 μg of proteins was centrifuged in a Beckman Airfuge (Beckman Instruments) at 23 psi for 15 min. The resulting pellet was fixed for 1 h in a fixative containing 4% paraformaldehyde and 1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. After fixation, the pellets were rinsed in three 15-min changes of 0.1 M phosphate buffer and postfixed for 1 h in 1% OsO_4, 1.5% potassium ferricyanide. The postfixed pellet was rinsed three times (15 min each) in phosphate buffer and removed from the airfuge tube to a glass test tube. The pellet was then dehydrated in graded ethanols to propylene oxide and placed in a 1:1 propylene oxide:Araldite mixture in a vacuum dessicator overnight. The next day the pellet was embedded in Araldite 502 and allowed to cure for 48 h at 60°C. Silver sections were taken on a Porter:Blum MT:2B ultramicrotome and stained with alcoholic uranyl acetate and lead citrate. Sections were viewed on an AEI-6B electron microscope.

Other Methods: Affinity labeling of the insulin receptor was performed exactly as previously described using disuccinimidyl suberate (Pierce Chemical) (20, 21). Protein was determined by the method of Lowry et al. (22). SDS PAGE was performed according to Laemmli (23).

RESULTS

Purity of Coated Vesicles

An electron micrograph of coated vesicles obtained from rat liver (see Materials and Methods) is shown in Fig. 1 *a*. There are no detectable structures in Fig. 1 *a* other than the cagelike coated vesicle particles, indicating a highly purified preparation. Others have reported the same degree of purification (>95%) using essentially the same purification protocol as applied to rabbit brain (16) and bovine adrenal cortex (24). Fig. 1 *b* is a Coomassie Blue-stained polyacrylamide gel of the coated vesicle preparation. The predominant band of M_r 180,000 is clathrin (15), and other coated vesicle associated bands are seen at M_r 100,000 and M_r 50,000. This electrophoretic pattern is similar to that of coated vesicles obtained from other tissues although the number and exact molecular weight of the nonclathrin bands differ somewhat (15, 16, 24). Thus the coated vesicle preparation we obtain from rat liver is typical of coated vesicles in general and is highly purified.

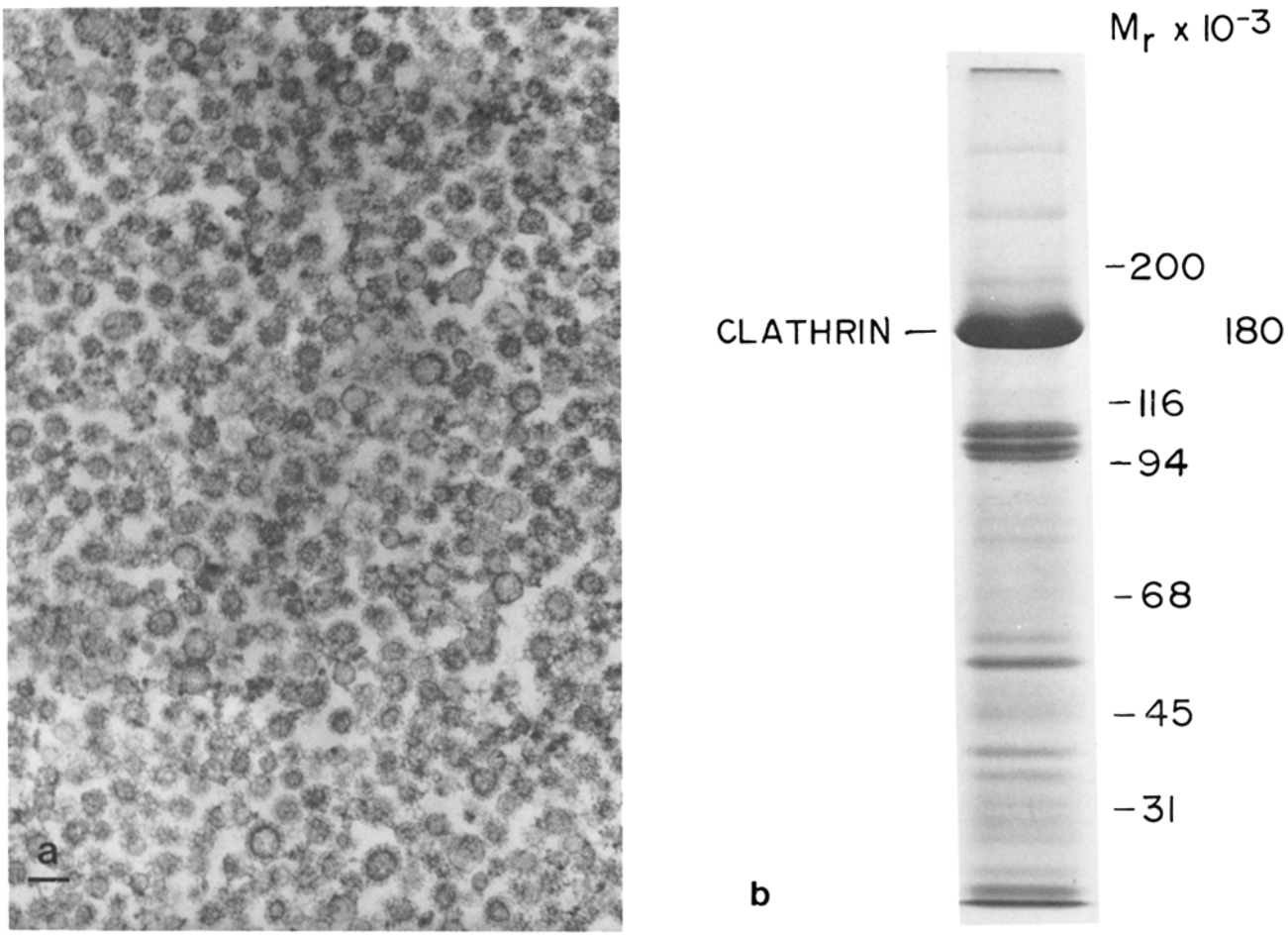

FIGURE 1 (a) Electron micrograph of rat-liver coated vesicles. Rat liver coated vesicles were purified and prepared for microscopy as described in Materials and Methods. Bar, 0.1 μm. (b) SDS PAGE of rat-liver coated vesicles. Purified coated vesicles (25 μg) were solubilized in SDS and run on a 6–12% polyacrylamide gradient gel according the Laemmli procedure (23). Depicted are the Coomassie Blue-stained gel and the migration positions of various molecular weight standards.

Insulin Binding to Coated Vesicles

Insulin binding protocols were performed in the presence and absence of the detergent octyl glucoside to determine whether purified coated vesicles contain specific insulin binding capacity, which would imply the presence of insulin receptors. As shown in Table I, significant insulin binding to coated vesicles is seen only in the presence of detergent. These data are from three separate coated-vesicle preparations, each showing latent binding. This binding latency is exactly what we expected since the insulin binding domain should be facing the lumen of the vesicle and would not, therefore, be insulin-accessible in the absence of detergent. In the first preparation, 3×10^{-10} M ^{125}I-insulin was used to assess insulin binding. In the second and third preparations, 1.25×10^{-9} M ^{125}I-insulin was used to obtain a higher level of specific binding. When the data from the first preparation are normalized to the same degree of receptor occupancy as in the other preparations by assuming a K_d of 3×10^{-9} M (20), essentially the same degree of specific binding is calculated for preparation one (Table I) as is observed for preparations two and three. In these latter preparations, insulin binding to liver plasma membranes was assessed in parallel. Interestingly, specific insulin binding to coated vesicles exceeded binding to plasma membranes (see Discussion). Latent insulin binding to plasma membranes was very low (10–20%) in comparison to coated vesicle latency and presumably reflects microsomal contamination of plasma membranes (data not shown).

Coated-Vesicle Insulin Receptor Subunit Structure

Affinity cross-linking of ^{125}I-insulin to coated-vesicle insulin receptors was performed (20, 21) to compare receptor labeling patterns in coated vesicles to plasma membrane receptors. Both in the presence and in the absence of reductant, the coated-vesicle insulin receptor exhibits a labeling pattern identical to that of the liver plasma membrane insulin receptor (Fig. 2). In the absence of reductant, three bands of M_r 350,000, 320,000, and 290,000 are visible from coated vesicles, and these collapse to an M_r 125,000 band upon dithiothreitol treatment. This labeling pattern is the typical pattern of insulin receptors from a number of rat tissues (25).

Internalization of Insulin in Coated Vesicles

The data shown in Fig. 2 and in Table I demonstrate that rat liver coated vesicles contain insulin receptors. To determine

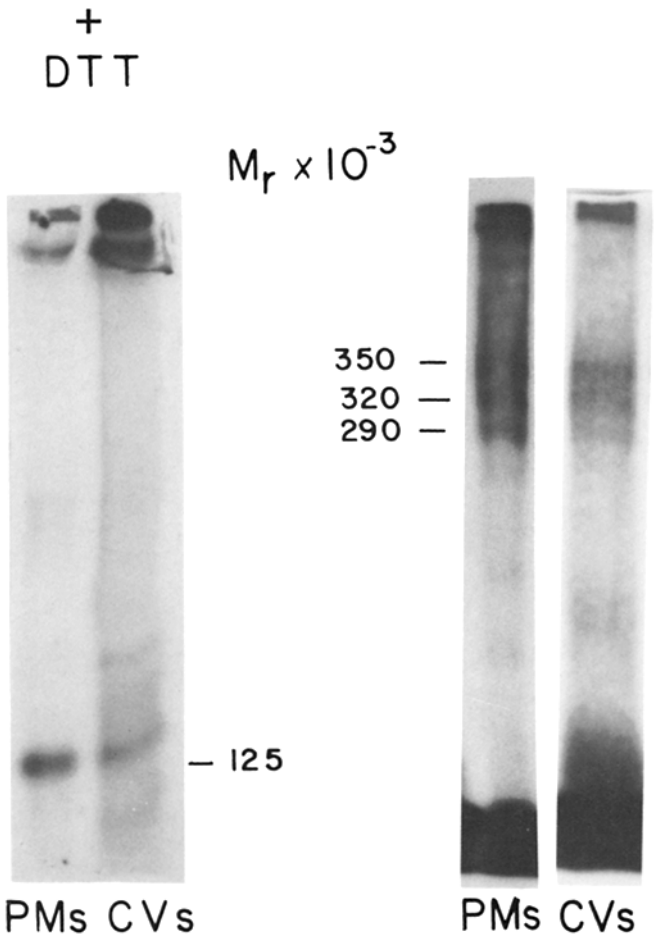

FIGURE 2 Affinity cross-linking of insulin receptors in coated vesicles and plasma membranes. Coated vesicles (*CVs*) (300 μg) suspended in 300 μl of PBS were incubated with 1% octyl glucoside for 20 min at 23°C and then further incubated for 30 min at 23°C with 2×10^{-8} M ^{125}I-insulin in the presence of 0.1% albumin in a total volume of 0.5 ml. The mixture was cooled to 0°C, and 0.25 mM disuccinimidyl suberate was added at a 1:40 dilution in dimethyl sulfoxide. Cross-linking was terminated after 15 min by the addition of 0.05 ml of 0.5 M Tris, pH 6.8. To remove most of the unreacted insulin, the affinity labeled coated vesicle preparation was then centrifuged through a 1-ml tuberculin syringe containing Sephadex G-50 equilibrated with PBS and 1% octyl glucoside. A portion of the material eluting from the syringe was electrophoresed on a 5% acrylamide gel (23). Depicted is the autoradiograph of that gel run with a liver plasma-membrane preparation (*PMs*) affinity labeled as previously described (20, 21).

whether these coated vesicle receptors might function in the internalization of hormone, ^{125}I-insulin was injected into the hepatic portal vein of rats and then the livers were excised and homogenized at various times after injection. Coated vesicles were purified from these preparations, and the time course of insulin uptake into coated vesicles and microsomes is shown in Fig. 3 together with the total radioactivity in the homogenate. Significant ^{125}I-insulin incorporation into coated vesicles is observed after 30 s of exposure to hormone and there is a rapid peaking of label incorporation at 3 min. Importantly, 10 min after hormone administration, the label associated with coated vesicles has dropped to a background level. In contrast, the total radioactivity in the homogenate has dropped only from ~45% of the total radioactivity administered (0.5–5 min) to ~25% of the total (10 min). Microsomal ^{125}I-insulin has also fallen off with time much less rapidly than coated vesicle insulin. The time course of radioactivity associated with the homogenate and the microsomes shown in Fig. 3 is essentially identical to that described by others (2, 3). In addition, Posner et al. (2) reported a 60% decrease in plasma membrane-associated ^{125}I-insulin in the postinjection time period of 30 s to 5 min (2). Thus, the peak of ^{125}I-insulin incorporation into coated vesicles at 3 min shown in Fig. 3 is consistent with the rapid internalization of hormone during the period when plasma membrane receptors show maximal or near maximal occupancy. The kinetics of insulin uptake into rat liver coated vesicles are similar to the kinetics of epidermal growth factor uptake into 3T3-cell coated vesicles (26).

The uptake of insulin into coated vesicles as shown in Fig. 3 could be due to nonspecific fluid-phase endocytosis of hormone as the coated pit budded off into a coated vesicle rather than to receptor-mediated uptake. Accordingly, rats were given a hepatic portal vein injection of ^{125}I-insulin in the presence and absence of excess unlabeled insulin to determine the specificity of uptake. Coated vesicles were then purified through two sucrose gradients (see Materials and Methods) from livers (two each condition) excised after 3 min of hormone exposure, the peak of label incorporation (Fig. 3). A portion of the coated vesicles was then subjected to agarose gel electrophoresis as depicted in Fig. 4. The dried gel was sliced into 0.5-cm fractions and the radioactivity was counted (lower panel). After determination of counts per fraction, the gel slices were boiled in SDS PAGE sample buffer (23) and subjected to electrophoresis on a 7.5% polyacrylamide gel as depicted in the upper panel of Fig. 4. In coated vesicles obtained from rats injected with ^{125}I-insulin only, a peak of radioactivity is seen in fractions 8, 9, and 10 (closed circles). This corresponds exactly to the peak of clathrin shown in the upper panel of Fig. 4. When excess unlabeled insulin is coinjected with ^{125}I-insulin,

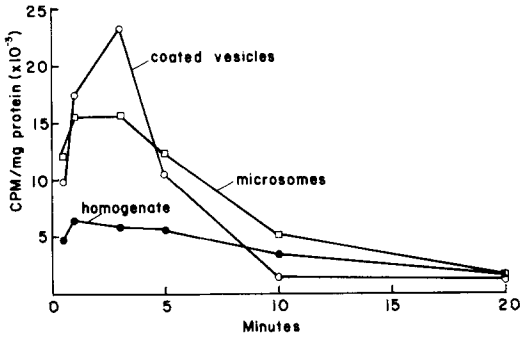

FIGURE 3 Time course of insulin uptake into liver microsomes and coated vesicles. Rats were anesthetized with pentobarbital, and 2×10^7 cpm ^{125}I-insulin was injected into the hepatic portal vein. At the times indicated, the livers were rapidly excised and immediately homogenized in ice-cold buffer (see Materials and Methods). The homogenate was sampled for protein and radioactivity and was then centrifuged for 20 min at 12,000 *g*. The pellet was discarded and the supernatant was centrifuged for 10 min at 125,000 *g* to yield a microsomal fraction. A portion of the microsomes was removed for determination of radioactivity and protein content after resuspension in MES buffer. Coated vesicles were then prepared from the microsomes as described in Materials and Methods and previously (6). Radioactivity was determined by gamma counting and normalized to protein yield (22) for both coated vesicles (open circles), microsomes (open squares) and homogenate (closed circles).

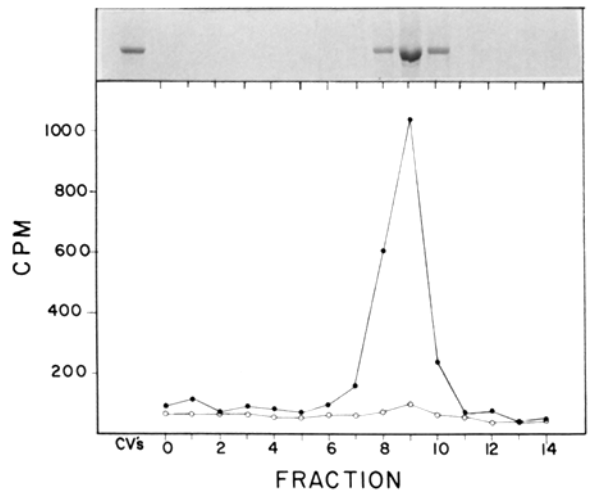

FIGURE 4 Agarose gel electrophoresis of rat-liver coated vesicles. Two rats each were given hepatic portal vein injections of 0.4 μg of ^{125}I-insulin (closed circles) or 0.4 μg of ^{125}I-insulin plus 150 μg of unlabeled insulin (open circles). The livers were excised at 3 min postinjection and immediately homogenized in ice-cold buffer. Coated vesicles were prepared from the homogenate as described in Materials and Methods and elsewhere (16). The coated vesicles obtained from the rats injected with ^{125}I-insulin alone (open circles) yielded 35,000 cpm ^{125}I-insulin whereas those obtained from rats injected with both labeled and unlabeled insulin yielded 1,800 cpm. A portion of each of the above coated-vesicle preparations was subjected to agarose gel electrophoresis as previously described (18). The agarose gel was then dried by blotting into Whatman 3 M paper and sliced into 0.5-cm pieces. The pieces were counted by gamma counting and the results are depicted above. After counting, the fractions were boiled in 100 μl of Laemmli sample buffer (23) and electrophoresed on a 7.5% acrylamide gel (top panel). The 180-kilodalton region containing the clathrin band is shown and the left-most lane designated *CV's* (coated vesicles) indicates a sample of the vesicle preparation before agarose gel electrophoresis.

virtually no label is incorporated into coated vesicles (open circles). Thus we conclude that the uptake of insulin into coated vesicles is a specific, receptor-mediated process.

DISCUSSION

The receptor-mediated internalization of insulin by a variety of tissues is a well-described biological phenomenon. These tissues include whole rat liver (1–3, 8, 9), isolated hepatocytes (7), adipocytes (27), and human lymphocytes (7, 13). The recent studies of human lymphocytes have suggested that insulin receptors can cluster in coated areas of the plasma membrane before internalization (13). This clustering in coated pits is typical of the process of receptor-mediated endocytosis involving a multitude of ligands including low-density lipoprotein, epidermal growth factor, 2-macroglobulin, asialoglycoproteins, and viruses (see references 10 and 11 for reviews). After clustering in coated pits, the ligand-receptor complexes bud from the plasma membrane to form coated vesicles which may deliver the ligand-receptor complex to an intracellular organelle. The present studies provide biochemical evidence for the participation of coated vesicles in the pathway of receptor-mediated endocytosis of insulin. Rat-liver coated vesicles are shown to specifically bind insulin (Table I) to a receptor structure typical of the insulin receptor (Fig. 2). Most importantly, the kinetics of insulin incorporation into coated vesicles (Fig. 3) and the specificity of uptake into these structures (Fig. 4) suggest that coated vesicles are intermediates in the pathway of hormone internalization into various organelles (2, 7–9). These data provide a convincing biochemical demonstration of a role for coated vesicles in receptor-mediated endocytosis where all the following criteria are met: (*a*) Demonstration of specific hormone binding in coated vesicles (Table I) (*b*) Demonstration of a receptor structure in coated vesicles (Fig. 2) (*c*) Demonstration that coated vesicles function in hormone internalization (Figs. 3 and 4).

In the experiment shown in Fig. 4, 3.3×10^4 cpm of ^{125}I-insulin were specifically taken up into coated vesicles, and this is 0.09% of the injected insulin. The rapid uptake of insulin into coated vesicles and the fact that all of coated vesicle-associated radioactivity is TCA-precipitable (data not shown) suggest that these coated vesicles derive from the plasma membrane and are transporting insulin into intracellular organelles (2, 7–9). The microsomes contain considerably more radioactivity than the coated vesicles (7.4×10^6 cpm or 19.3% of the total injected). Liver microsomes prepared as described in Materials and Methods are known to contain considerable plasma membrane (28). In addition, our yield of coated vesicles is likely to be low. Others have estimated a yield from 3% (29) to 10% (24, 29) in protocols for isolating coated vesicles that are essentially identical to those used by us. If the half-life of a coated vesicle is less than a minute (11), three or more internalization cycles may have occurred during the 3-min course of the experiment described in Fig. 4. Thus, whereas coated-vesicle insulin content is approximately 200-fold lower than microsomal content, it is still likely that these vesicles represent the principal vehicle for receptor-mediated endocytosis. We have probably underestimated coated-vesicle insulin content by 20-fold because of our low yield. In addition, we have overestimated microsomal content by twofold and have time for three or more internalization cycles during the protocol. These considerations can roughly account for the observed differences in radioactivity between microsomes and coated vesicles. Until such time as technology permits a more accurate determination of coated vesicle life-time and a more quantitative preparative technique for the structures, we can only approximate the quantitative role of coated vesicles in receptor-mediated endocytosis. Nevertheless, a large body of biochemical and morphological evidence (reviewed in references 10 and 11) supports the notion that the coated pit-coated vesicle pathway is primarily responsible for internalization of hormones and other ligands rather than fluid phase endocytosis (30).

It has been proposed that coated pits act as selective filters for certain membrane proteins such as receptors (31). This selection would allow the specific endocytosis of receptors while excluding other proteins and implies a potential concentration of receptors in coated pits, and in coated vesicles as they bud from coated pits. Insulin receptors may therefore be concentrated in coated pits and coated vesicles as has previously been suggested (12, 13). When we compare insulin binding to coated vesicles *vs.* insulin binding to plasma membranes prepared as described by Carey and Hirschberg (17), a two- to threefold higher specific binding is observed for the coated vesicles when normalized for equal protein (Table I). Most of the coated vesicle protein is clathrin (15), suggesting an even higher ratio of coated vesicle insulin binding capacity to plasma membrane binding capacity. These results are therefore consistent with the proposed concentration of receptors in coated

pits and coated vesicles (12, 13, 31) but must be interpreted with caution. Our plasma membrane preparation (17) is not derived exclusively from the blood sinusoidal surface of the liver where insulin receptors should be predominantly, if not exclusively, located. There exists some controversy as to what, if any, liver plasma membrane preparation constitutes an enrichment of the blood sinusoidal surface with enhanced specific binding of insulin (reviewed by Evans 32). In addition, we do not know the origin of our coated vesicles. They may derive in substantial proportion from the Golgi complex (14) though some of them must derive from plasma membrane (Figs. 3 and 4). The low yield of the coated-vesicle preparations also makes unequivocal assignment of their origin difficult.

The fate of insulin receptor after internalization is not known with certainty, but some proportion of receptors are likely to recycle back to the plasma membrane (33) as do the receptors for low density lipoproteins (5) and asialoglycoproteins (6). The exact pathway of the receptor within the cell is likely to be complex in view of the diverse organelles reported to contain either receptors and/or internalized insulin (2, 7–9, 34, 35). One direct way of following the fate of the receptor as it is cycled through the cell is to follow the fate of a covalent hormone-receptor complex after generation of this complex by photoaffinity labeling at the cell surface. Several studies of this nature have recently been performed which directly demonstrate that insulin receptor is internalized along with the ligand (36–38) and that a large portion of these complexes eventually return to the cell surface (36). It may be feasible to use this methodology to determine whether coated vesicles are involved in the various stages of membrane-to-membrane transfer of this important receptor.

We wisk to thank Joseph Squicciarini and Katherine Porter-Jordan for assistance with the agarose gel electrophoresis and Michaelle Montgomery for excellent secretarial assistance.

This research was supported by grants from The American Diabetes Association and The Juvenile Diabetes Foundation (P. F. Pilch) and by grants NSCD-10582 (R. E. Fine) and AM-30425 (P. F. Pilch) from the National Institutes of Health.

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Received for publication 30 August 1982, and in revised form 27 September 1982.

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