Biochemical and Functional Characterization of Three Types of Coated Vesicles in Bovine Adrenocortical Cells: Implication in the Intracellular Traffic†

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ABSTRACT: Three populations of pure coated vesicles from adrenocortical cells, differing in their density, i.e., 1.125–1.155, 1.155–1.175, and 1.175–1.210 g/cm^3, are obtained after separation on two successive sucrose-^2H_2O gradients. They are involved in LDL internalization and in the receptor cycle as confirmed by the presence, in each population, of the LDL receptor. Electron micrographs confirm the existence of three homogeneous populations exhibiting the typical polygonal structure of the clathrin coat. They differ in their size distribution (small, ≃70-nm diameter; medium, ≃90-nm diameter; large, ≃110-nm diameter) and in the organization of clathrin and of the coat proteins as evidenced on electrophoreses carried out under nondenaturing and denaturing conditions. Activity measurements of marker enzymes, phosphodiesterase and galactosyltransferase, suggest that medium coated vesicles might originate from plasma membranes and small ones from the Golgi complex. Large coated vesicles exhibit phosphokinase enzyme and substrate polypeptides different from those of the two other populations, tubulins being the preferred kinase substrates for the small and medium coated vesicles. These kinases are autophosphorylating enzymes and are revealed, by nondenaturing electrophoreses, as different high molecular mass complexes in the three populations. Clathrin and coat proteins are not part of these complexes.

In adrenocortical cells where corticosteroid biosynthesis is the main function, the transport process achieved by the CV[1] follows different intracellular routes. The LDL carry the cholesterol and enter the cell by receptor-mediated endocytosis. The LDL receptor is delivered into endosomes where it dissociates. The receptor is cycled back to the plasma membrane, whereas LDL are degraded in lysosomes. De novo synthesized LDL receptor is transported from the endoplasmic reticulum to the Golgi complex and then to the plasma membrane through CV (Goldstein et al., 1985; Bomsel et al., 1986). In this study we report the purification in adrenocortical cells of three morphologically distinct populations of CV and address the questions of their respective origin and of the biochemically different characteristics which might participate to their targeting signal. The complex protein composition of the clathrin coat in CV prepared from mammalian brain has been evidenced recently by Keen (1987). The so-called 100-kDa polypeptides, involved in the binding of the coat to the lipidic vesicle, contain at least three different components (Robinson, 1987). The clathrin molecule by itself seems to be variable in its light chains (Bar-Zvi, 1987; Kirchhausen et al., 1987; Kohtz et al., 1987; Mooibroek et al., 1987). These chains are related to the protein family of apparent mass of 36 kDa which binds Ca^{2+} and interacts with the cytoskeleton (Burgoyne, 1987; Cooper et al., 1987; Glenney et al., 1987). The LDL receptor interacts with some parts of the clathrin coat through its cytoplasmic domain (Davis et al., 1986, 1987), essential for its internalization. It was therefore important to analyze in the different types of CV isolated from the adrenocortical cells the chemical characteristics of the coat as well as those of the lipidic vesicle.

The present work is a quantitative and qualitative description of proteins from large (LCV), medium (MCV), and small (SCV) coated vesicles. The marker enzyme of plasma membranes, alkaline phosphodiesterase, and that of the Golgi complex, galactosyltransferase, have been measured. Protein kinase activity which has been described in brain CV (Pauloin et al., 1982, 1984; Bar-Zvi & Branton, 1986) has also been measured in the three types of adrenal vesicles.

It appears from all these studies that each type of CV exhibits structural and functional characteristics and could originate from different membranes to mediate specific transport and targeting in the intracellular traffic.

Materials and Methods

Chemicals. Chemicals were of reagent grade and obtained from Sigma Chemical Co, St. Louis, MO, Riedel, Hannover, West Germany, or Bio-Rad, Richmond, CA. [γ-^{32}P]ATP was from NEN Dupont, France.

Biological Material from Bovine Adrenocortical Cells. (A) Plasma and Golgi Membrane Preparations. The plasma membrane fraction was obtained as previously described (Bomsel et al., 1986). The Golgi membrane fraction was obtained according to Dunphy and Rothman (1983).

(B) Coated Vesicle Preparations. Coated vesicles were isolated according to a previously described method (Weintraub et al., 1985; Bomsel et al., 1986). Briefly, about 20 adrenal glands (80–100 g wet wt of cortex tissue) were homogenized by the addition of 300 mL of buffer A [100 mM MES (pH 6.5), 1 mM EGTA, 0.5 mM $MgCl_2$, 200 mM NaCl, and 3 mM NaN_3]. The suspension was centrifuged at 15000g for 45 min. The pellet was discarded and the supernatant centrifuged at 105000g for 1 h. The supernatant was discarded and the pellet homogenized in 300 mL of buffer A with a Dounce. The homogenate was centrifuged at 10000g

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[1] Abbreviations: LDL, low-density lipoprotein(s); CV, coated vesicle(s); LCV, large coated vesicle(s); MCV, medium coated vesicle(s); SCV, small coated vesicle(s); SDS, sodium dodecyl sulfate; SDS–PAGE, SDS–polyacrylamide gel electrophoresis; ND-PAGE, nondenaturing PAGE.

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for 10 min. The pellet was discarded. The 105000*g* and 10000*g* steps were repeated twice. Aliquots of 2.5 mL of the last 10000*g* supernatant were layered on the top of a 31-mL discontinuous gradient. The first layer was made only with buffer A in H_2O/buffer A in ^2H_2O (1:1 v/v), 5 mL. The following layers were made with buffer A in ^2H_2O and sucrose (w/w): 5%, 6 mL; 12%, 7 mL; 16%, 7 mL; 24%, 6 mL. Gradients were centrifuged at 90000*g* for 1.5 h. Three fractions were collected at the 5%–12% interface (1.125–1.155 density range), 12%–16% interface (1.155–1.175 density range), and 16%–24% interface (1.175–1.210 density range). Each fraction was separately layered on a second identical gradient leading to three well-separated fractions corresponding to the density ranges 1.125–1.155, 1.155–1.175, and 1.175–1.210. Each of these fractions was extensively dialyzed against buffer A.

(*C*) *Clathrin and Uncoated Vesicle Preparations.* Uncoating was performed in two steps. In the first step CV were dialyzed overnight against 500 mM Tris-HCl at pH 8 and centrifuged at 105000*g* for 1 h. The supernatant was termed clathrin 1 (cla 1). The corresponding pellet was resuspended in 10 mM Tris-HCl at pH 8, dialyzed overnight against the same buffer supplemented with 2 M urea, and centrifuged as above. The second supernatant was termed clathrin 2 (cla 2). The corresponding pellet was resuspended in 10 mM Tris-HCl at pH 8 (UV 2) and dialyzed overnight against the same buffer.

Electron microscopy was as previously reported (Bomsel et al., 1986).

Protein content was determined according to Peterson (1971).

SDS–PAGE. SDS–PAGE was carried out on either 6 or 8% acrylamide gels according to Laemmli (1970), stained with Coomassie brilliant blue R250 (0.2%) and destained with a methanol–acetic acid solution (10:10 v/v).

Scans were carried out on gels on which the same amount of protein [as measured according to Peterson (1971)] from different preparations was layered in each well for each measurement. Measurements have been repeated on four different preparations. Averaged intensities of the main peaks were taken as a measure of the component amount instead of areas under the peak, since large bands represent more than one component.

Immunoblotting. Following SDS–PAGE, proteins were electrotransferred to nitrocellulose according to Burnette (1981). For detection of the LDL receptor, ligand blotting was performed according to the procedure previously described (Weintraub et al., 1985). For the identification of tubulin, the immunoblotting technique was performed with monoclonal β-tubulin antibodies either from a commercial source (Sanbio) or kindly given by Dr. A. Fellous (Bicêtre, France). Purified tubulin from rat brain was also a gift from Dr. A. Fellous. Tubulin was detected with horseradish peroxidase conjugated IgG, and 3,3′-diaminobenzidine tetrachloride was used as substrate.

ND-PAGE. ND-PAGE was performed according to Kuonen et al. (1986) on acrylamide gradient gels (2% or 5%–15%). Samples were solubilized by the addition of 0.5 volume of 20% (w/v) Triton X-100 solution. After incubation for 20 min on ice, insoluble material was removed by centrifugation at 100000*g* for 45 min. Electrophoreses were run in 100 mM Tris–glycine buffer, pH 9.0, which contained 0.1% (w/v) Triton X-100, at 4 °C. Proteins were stained and destained as for SDS–PAGE. To obtain an estimation of molecular mass for the main protein components evidenced by ND-PAGE, the bands of each one of the lanes corresponding to plasma and Golgi membranes, LCV, MCV, and SCV were excised, solubilized in the Laemmli sample buffer, reloaded, and analyzed by SDS–PAGE. For all the gels given in the figures for comparison of the component migration rate, all the polypeptides shown were run on a single slab gel.

Enzyme Assays. All the enzyme assays were performed with the post nuclear supernatant from the initial homogenate, the CV before the two successive gradients, and the LCV, MCV, and SCV.

(*A*) *Galactosyltransferase.* Activity measurements were made according to Stojanovic et al. (1984). Calculations were made according to Marsh et al. (1987) by using the total protein content of each sample analyzed. The post nuclear supernatant was regarded as 100% of enzymatic activity. The values on Figure 7 are given in arbitrary units (AU): 1 AU representing 1000 cpm.

(*B*) *Alkaline Phosphodiesterase.* Activity measurements were made according to Razzell (1963); 100% enzymatic activity was taken as described above; 1 AU represents 1000 A_{490nm}.

(*C*) *Phosphokinase.* Activity measurements were made according to Pauloin et al. (1984). The reaction was initiated by the addition of 0.15 nmol of [γ-^{32}P]ATP with a specific activity of 30–40 Ci/mmol. SDS or ND-PAGE was carried out, and the gels were stained, destained, and dried. They were exposed at −70 °C to Kodak Royal X-Omat films in cassettes with intensifying screens for 1 h.

Renaturation and in Situ Phosphorylation after PAGE. Membranous preparations (100 μg of membranous protein/lane) fractionated by SDS or ND-PAGE were processed as described by Bost and Blalock (1986), to allow renaturation of the proteins prior to the addition of [γ-^{32}P]ATP. Briefly, the gels were washed 12 h in 700 mL of 10% ethanol and then rinsed for 15 min in PBS–0.1% NaN_3–0.1% gelatin. Radioactive phosphorylation was performed on the gels by incubation with 0.3 nmol of [γ-^{32}P]ATP (sp act. 30–40 Ci/mmol) per lane of PAGE.

To control if the kinase was an autophosphorylating enzyme and still active after separation of the CV components on the gels, in denaturing or nondenaturing conditions, a CV preparation was overlaid on the gels (250 μL/lane of PAGE). Before overlay, this preparation was saturated with 4 nmol of unlabeled ATP and the phosphorylation reaction performed at 37 °C during 10 min. To eliminate the free ATP, the suspension was filtered through a Sephadex G-25 column. The gels incubated with the CV preparation and [γ-^{32}P]ATP were then washed extensively (5 × 2 h), stained, destained, dried, and exposed at −70 °C to Kodak Royal X-Omat films in cassettes with intensifying screens for 8 days.

RESULTS

Separation of Three Populations of Pure Coated Vesicles. The isolation procedure of CV from adrenocortical cells, reported in previous works (Weintraub et al., 1985; Bomsel et al., 1986), produced a pure preparation of CV without contamination by plasma or microsomal membranes, as shown by electron microscopy examination. The biochemical characteristics of these CV exhibited small differences with those of the brain CV, namely, in their lower content of clathrin relative to other coat proteins. After two identicl density gradients, three populations of CV distinct by density criteria were obtained from adrenocortical cells. The contamination of each type of CV by the two others was $\leq$3%, as shown on the size distribution pattern (Figure 1b). The third coated vesicle population was collected at the 1.155–1.125 g/cm^3

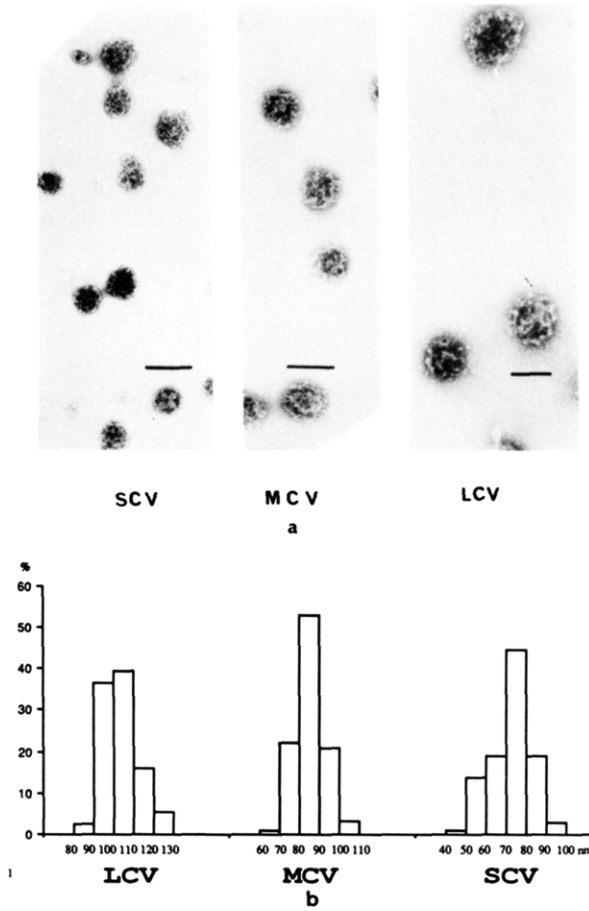

FIGURE 1: (a) Electron micrographs of LCV, MCV, and SCV negatively stained with 2% uranyl acetate. A drop of the sample was placed on a 200-mesh Formvar carbon-coated grid and allowed to air-dry. Micrographs were taken on a Jeol 100 CX electron microscope. The three classes of CV were obtained after two successive ^2H_2O–sucrose gradients: LCV were collected at the 1.210–1.175 g/cm^3 density interface, SCV at the 1.175–1.155 g/cm^3 density interface, and MCV at the 1.155–1.125 g/cm^3 density interface. Bars represent 100 nm. (b) Size distribution pattern of the three populations of CV: LCV, MSV, and SCV. More than 900 diameter measurements were made on each class.

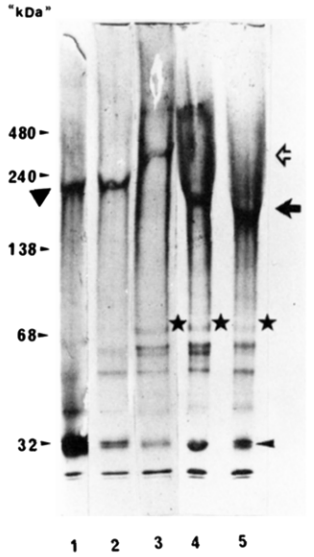

FIGURE 2: The 2–15% acrylamide gradient Coomassie-stained ND-PAGE of plasma- (lane 1) and Golgi-enriched membrane fractions (lane 2), LCV (lane 3), MCV (lane 4), and SCV (lane 5). Molecular mass indications shown are not comparable with molecular mass determinations in SDS–PAGE. The values were obtained from a set of soluble protein markers (apoferritin, urease, bovine serum albumin, and pepsin) and are given to assess the reproducibility of this system between different samples. Symbols: (▼) the major proteins of plasma- (lane 1) and Golgi-enriched membrane fractions (lane 2) of "220" kDa, (open arrowhead) the protein of LCV (lane 3) of "300" kDa, and (solid arrowhead) the protein of MCV (lane 4) and SCV (lane 5) of "200" kDa. (★) The proteins present only in the CV.

density interface. Figure 1a shows an electron micrograph of each class of CV termed according to their diameter: the large, LCV, with diameters ranging from 120 to 90 nm and a density between 1.210 and 1.175 g/cm^3, the medium, MCV, with diameters ranging from 100 to 75 nm and a density between 1.155 and 1.125 g/cm^3, and the small, SCV, with diameters ranging from 85 to 60 nm and a density between 1.175 and 1.155 g/cm^3. The characteristic polygonal structure of the clathrin surrounding lipid vesicles is observed for the three populations of CV. The size distribution pattern of each class of CV is shown in Figure 1b, and more than 900 diameter measurements were made for each CV population.

Protein Organization of the Three Populations of Coated Vesicles. Analyses were made by gel electrophoresis under nondenaturing conditions to examine the proteins in the coat and in the lipid membrane of the vesicles. Figure 2 shows a ND-PAGE of plasma (lane 1) and Golgi membranes (lane 2), LCV (lane 3), MCV (lane 4), and SCV (lane 5). Under nondenaturing conditions, the resolution of the different proteins is not as well defined as that under the conditions of SDS–PAGE. Their migration is a function not only of their molecular mass but also of their net charge (Yarden & Schlessinger, 1987). The molecular masses are given only as indications referring to the migration of protein markers. All types of membranes exhibit a high molecular mass complex between "300" and "200" kDa. This complex migrates with a similar rate for the plasma [(▼) lane 1] and the Golgi membrane [(▼) lane 2]. For LCV, MCV, and SCV, an increasing migration rate is evidenced [(solid arrowhead, open arrowhead) lanes 3–5]. A group of proteins around "70" kDa (★) appears to be characteristic of all CV (lanes 3, 4, and 5). A "32"-kDa protein appears more strongly stained in plasma membrane and MCV as compared with other components of the same lane. Clathrin does not penetrate the gel in the absence of SDS due to its high molecular mass (600 kDa) and low net charge at pH 9. Indeed, the bands excised at the top of the gel lanes and reloaded on SDS–PAGE generate all the polypeptides of the clathrin coat (clathrin heavy and light chains and the 100-kDa polypeptide family), which do not dissociate under conditions of the ND-PAGE (data not shown).

For an estimation of the molecular mass of the proteins corresponding to high molecular mass components evidenced by ND-PAGE, these bands were excised, reloaded, and analyzed by SDS–PAGE. The results shown on Figure 3 indicate that the "300"-kDa protein of LCV is actually formed of six polypeptides of molecular mass between 100 and 45 kDa (lane 1). Unequivocally, LCV differ from SCV and MCV, which are similar. Their common "200"-kDa protein is formed of one polypeptide of 65 kDa and two polypeptides of 54–56 kDa (lanes 2 and 3).

The polypeptide patterns characteristic of each type of CV were also analyzed directly by SDS–PAGE (Figure 4b). All the polypeptide components already described for the clathrin coat were also present on each of the CV lanes, namely, 180, 110, 33, and 36 kDa. The clathrin heavy chain (H) is present at a lower amount in adrenocortical CV (≃30%) than in brain CV [between 42% (Woodward & Roth, 1978) and 70% (Pearse, 1976)]. The identity of the clathrin heavy chain (180 kDa) and of the 33- and 36-kDa light chains has been con-

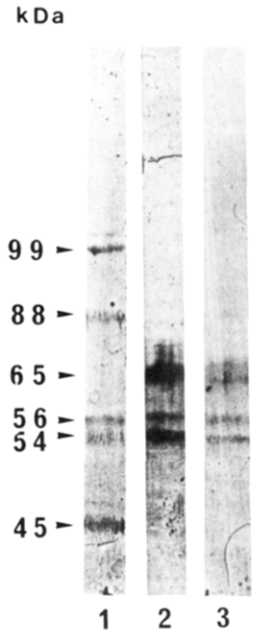

FIGURE 3: The 8% acrylamide Coomassie-stained SDS–PAGE. The major protein bands are excised from the ND-PAGE and electrophoresed on SDS–PAGE. (Lane 1) The "300" kDa of LCV; (lane 2) the "200" kDa of MCV; (lane 3) the "200" kDa of SCV.

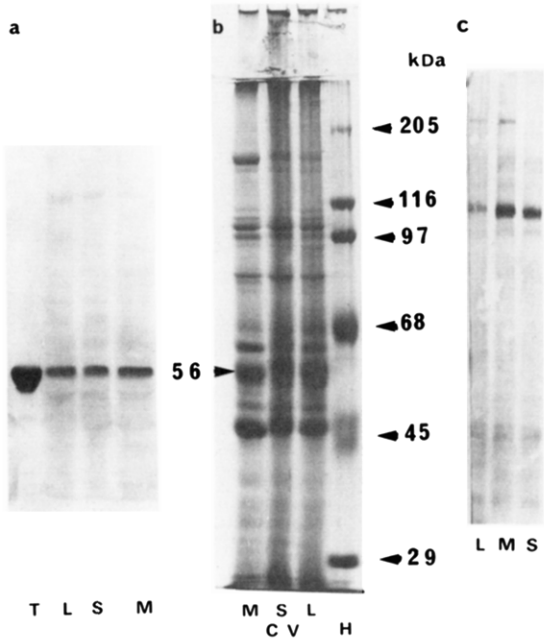

FIGURE 4: The 8% acrylamide Coomassie-stained SDS–PAGE and immunoblot. (Panel a) Identification of tubulin with anti-β-tubulin monoclonal antibodies: (lane T) tubulin; (lane L) LCV; (lane M) MCV; (lane S) SCV. (Panel b) Polypeptides electrophoresed: (lane L) LCV; (lane M) MCV; (lane S) SCV; (lane H) high molecular weight markers. (Panel c) Identification of LDL receptor with anti-LDL polyclonal antibodies: (lane L) LCV; (lane M) MCV; (lane S) SCV.

firmed by immunoblot carried out with polyclonal antibodies raised to bovine brain clathrin in goat and to bovine adrenocortical clathrin in rabbit, as previously published (Huang et al., 1987). To emphasize the differences, not detectable by visual inspection of the gels, between each class of CV, the SDS–PAGE were scanned (Figure 6a), and the intensity of each peak was measured in absorbance units (Table I). LCV contain a low amount of clathrin heavy chain (H, 180 kDa; $I = 2$) as compared with SCV ($I = 4$) and MCV ($I = 4$). For the clathrin light chains, a 33-kDa component has a higher

Table I: Intensity Values in Absorbance Units of the Main Peaks Obtained on SDS–PAGE Scans

	LCV	SCV	MCV
H, 180 kDa	2	4	4
Lcb, 33 kDa		2	2
Lca, 36 kDa		1.3	1.3
B/C, 101/99 kDa	0.5	1.4	1.4
54 + 56 kDa	7	15	10

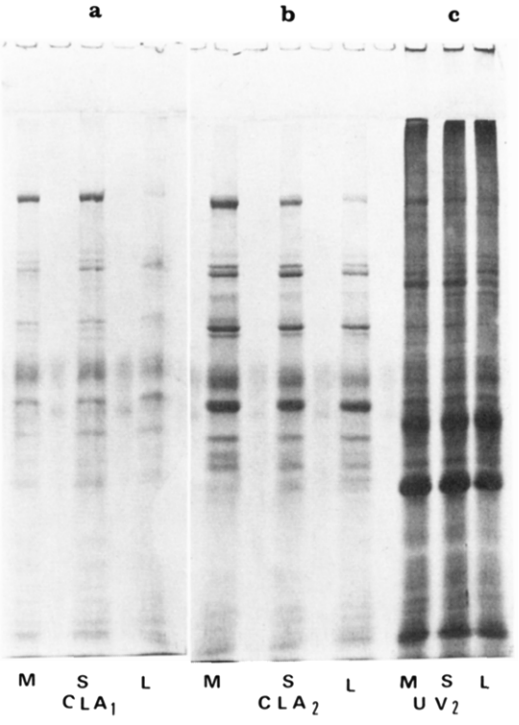

FIGURE 5: The 8% acrylamide Coomassie-stained SDS–PAGE. (Panel a) Cla_1: (lane M) MCV; (lane S) SCV; (lane L) LCV. (Panel b) Cla_2. (Panel c) UV_2.

intensity in MCV and SCV (LCb, $I = 2$) than the 36-kDa component (LCa, $I = 1.3$). A 100-kDa family of polypeptides is present in the three populations of CV as 107- (A), 101- (B), and 99- (C) kDa components, but their respective amount is different for each population. The intensity ratio of the 101/99 components is 0.5 for LCV and 1.4 for the two other populations. The amount of the polypeptides of 54 and 56 kDa is higher in SCV (sum of $I = 15$) than in the MCV (sum of $I = 10$) and in LCV (sum of $I = 7$). LCV exhibit a 45- (p) kDa component, distinct on the scans from the 46-kDa component present in the three CV populations.

The presence of the LDL receptor which is not detectable by Coomassie blue staining of SDS–PAGE was visualized on SCV, LCV, and MCV by immunoblotting with LDL and anti-LDL antibodies, as previously published (Weintraub et al., 1985; Bomsel et al., 1986). The amount of LDL receptor becomes progressively higher in LCV, SCV, and MCV (Figure 4c).

Clathrin Coat. In adrenal cortex, the coat of the vesicles is composed, as in any other tissue studied, of the clathrin heavy and light chains and of a family of 100-kDa proteins positioned between clathrin and the vesicle membrane (Vigers et al., 1986). To analyze all these coat proteins, SDS–PAGE were performed on the supernatant of each uncoating step leading to Cla_1 and Cla_2 and on the pellet of the second step leading to UV_2 as shown in Figure 5 (cf. Materials and Methods). The scanning of these gels (Figure 6b) evidences that the clathrin heavy chain (H) of LCV is poorly extracted in the first step (row LCV, column Cla_1; $I = 2$) and almost

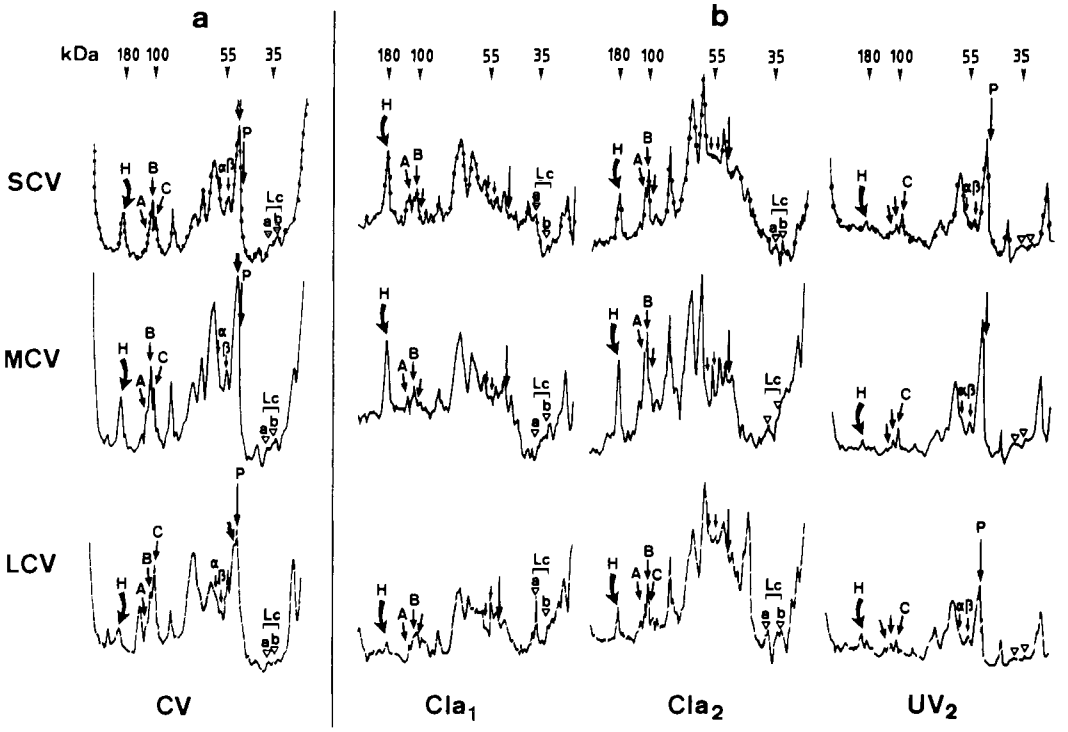

FIGURE 6: Scanning of the Coomassie-stained SDS–PAGE. (Panel a) Scans of the polypeptides obtained from the three classes of CV: LCV; MCV; SCV. (Panel b) Scans of the polypeptides obtained after uncoating each class of CV in a two-step procedure. First, CV are dialyzed overnight against 500 mM Tris-HCl, at pH 8, and centrifuged for 1 h at 105000*g*. The supernatant is termed clathrin 1 (Cla_1). The pellet is then resuspended in 10 mM Tris-HCl at pH 8, dialyzed overnight against the same buffer supplemented with 2 M urea, and centrifuged as above. The second supernatant is termed clathrin 2 (Cla_2). The remaining pellets are rehomogenized with 10 mM Tris, pH 8, giving uncoated vesicles (UV_2). Molecular mass standards are indicated in kilodaltons. The different arrows indicate the location on the scans of the main polypeptides analyzed in the present study. (H) Clathrin heavy chain at 180 kDa; (A–C) polypeptides at 107, 101, and 99 kDa, respectively; (α and β) α- and β-tubulins at 56 and 54 kDa, respectively; (p) polypeptide specific of LCV at 45 kDa; [Lc(a,b)] clathrin light chains a and b at 36 and 33 kDa, respectively.

completely recovered by the second extraction (row LCV, column Cla_2; $I = 3.7$). For SCV and MCV both extractions yield large amounts of clathrin heavy chains [H; row SCV, column Cla_1 ($I = 5.8$), column Cla_2 ($I = 4$)]; however, they yield less from SCV than from MCV [H; row MCV, column Cla_1 ($I = 6.3$), column Cla_2 ($I = 5.7$)].

For the clathrin light chains, the 36-kDa peptide (Lca) is largely extracted under mild conditions from LCV (row LCV, column Cla_1, $I = 4.5$) and practically not removed from MCV (row MCV, column Cla_1). This component is released from MCV after urea extraction only (row MCV, column Cla_2, $I = 1.5$). As expected, the 100-kDa proteins are better extracted from CV under drastic conditions except for the 107-kDa component in LCV (rows LCV, MCV, and SCV, columns Cla_1 and Cla_2). The 101- and 99-kDa components are present in all three populations of CV but with a higher intensity in MCV ($I_{101} = 7.2$ and $I_{99} = 4.1$, as compared to $I_{101} = 5.4$ and $I_{99} = 2.5$ for SCV and $I_{101} = 3$ and $I_{99} = 2.4$ for LCV). The 54–56-kDa polypeptides which are present in the three populations of CV are not released into the CV clathrin, Cla_1, or Cla_2. By immunoblotting with monoclonal anti-β-tubulin antibodies, the 56-kDa polypeptide was identified as β-tubulin, (Figure 4a).

Marker Enzymes. Marker enzymes, phosphodiesterase and galactosyltransferase, have been measured to determine the possible origin of each population of CV.

(A) Phosphodiesterase Activity as Plasma Membrane Marker. The phosphodiesterase activity, when assayed with the three populations of CV, showed a specificity for the 5′-(*p*-nitrophenyl) substrate but did not recognize the 3′ isomer as a substrate. The MCV exhibit a much higher activity than SCV and LCV (Figure 7).

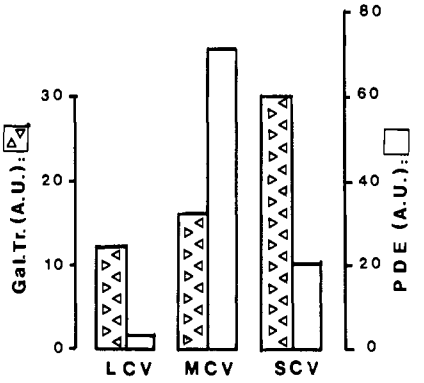

FIGURE 7: Marker enzyme activities measured in LCV, MCV, and SCV. Galactosyltransferase (Gal-Tr) is measured as a Golgi membrane marker and 5′-phosphodiesterase (PDE) as a plasma membrane marker. The values are expressed in arbitrary units (AU), 1 AU representing 1000 cpm for Gal-Tr and 1000 A_{490nm} for PDE.

Table II: Marker Enzyme Activities Expressed as a Percent of the Post Nuclear Supernatant (Relative to the Total Protein Content)

	Gal-Tr[a] (%)	PDE[b] (%)	total protein (mg)
post nuclear supernatant	100	100	10×10^3
CV[c]	1.5	30	152
LCV	0.014	0.18	4.8
MCV	0.015	1.7	17
SCV	0.035	0.27	9

[a] Galactosyltransferase. [b] Phosphodiesterase. [c] The CV mixture obtained before the two successive sucrose-^2H_2O gradients.

(B) Galactosyltransferase Activity as Golgi Membrane Marker. From Table II it can be seen that the total activity measured in the post nuclear supernatant is recovered with

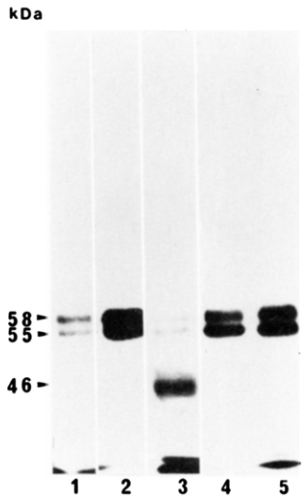

FIGURE 8: Phosphorylation of polypeptides from plasma- and Golgi-enriched membrane fractions (lanes 1 and 2), LCV (lane 3), MCV (lane 4), and SCV (lane 5). The samples are incubated for 10 min at 37 °C with [γ-^{32}P]ATP and loaded on an 8% acrylamide SDS–PAGE. The phosphorylated compounds are revealed by autoradiography.

a very low yield in the whole CV, due to the multiple steps of the purification procedure. This is in agreement with the data obtained by Marsh et al. (1987) on purified endosomes. However, the galactosyltransferase activity becomes progressively higher in LCV, MCV and SCV.

Phosphokinase Activities in the Three Populations of CV. (*A*) *Substrate Detection.* The endogenous substrates of phosphokinases were first characterized. The CV as well as the plasma and Golgi membrane fractions were incubated with [γ-^{32}P]ATP at 37 °C for 10 min, a time short enough to avoid nonspecific phosphorylation (Cooper & Hunter, 1983). The phosphorylated substrates were then detected by PAGE followed by autoradiography. After SDS–PAGE, two strongly phosphorylated polypeptides appeared at 55 and 58 kDa in MCV (Figure 8, lane 4) and SCV (lane 5) as well as in the Golgi membrane fraction (Figure 8, lane 2). These two polypeptides were also substrates of the kinase(s) from LCV (lane 3) and plasma membranes (lane 1) but to a lower extent. This doublet of 55–58 kDa corresponds to the α- and β-tubulins as identified by immunoblotting (Figure 4a). Furthermore, a third polypeptide of 46 kDa is strongly phosphorylated in LCV (Figure 8, lane 3). The phosphorylated protein pattern analyzed after ND-PAGE (Figure 9) indicates on one side a similarity between Golgi (lane 2) and plasma membrane fractions (lane 1) which both exhibit a phosphorylated "220"-kDa component (▼). In MCV (lane 4) and SCV (lane 5), proteins of ≃"200" kDa (solid arrowhead) and "210" kDa are strongly labeled. On the other side, LCV (lane 3) exhibits a ≃"300"-kDa protein (open arrowhead) strongly labeled, while the "200"-kDa protein is only faintly labeled. Furthermore, a protein of ≃"900" kDa (open arrowhead) nondetectable by Coomassie blue staining is phosphorylated only in LCV.

(*B*) *Autophosphorylation.* PAGE were performed on each fraction, and after protein fixation and renaturation, [γ-^{32}P]ATP was overlaid on the gel, allowing autophosphorylation if any to proceed.

When [γ-^{32}P]ATP only was overlaid after ND-PAGE and renaturation of the proteins, the phosphorylated pattern for all the fractions except the LCV was the same as the one observed when the phosphorylation reaction was carried out before ND-PAGE (Figure 9). This demonstrate that the phosphorylated component is comprised by the enzyme and

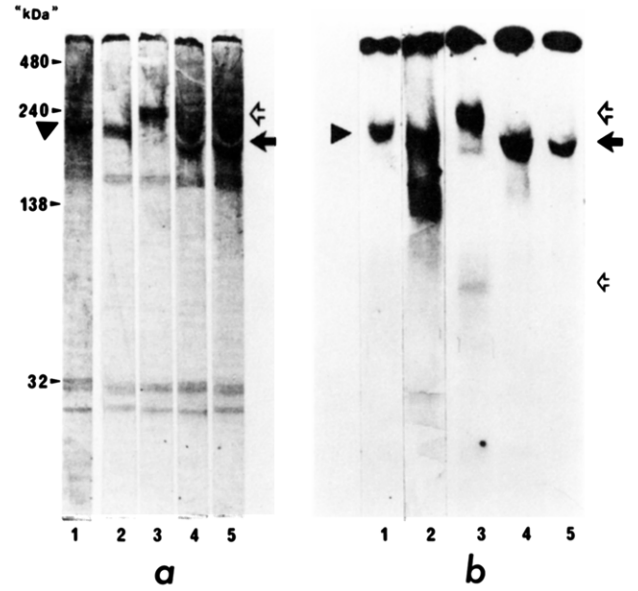

FIGURE 9: Phosphorylation of polypeptides from plasma- (lane 1) and Golgi-enriched membrane fractions (lane 2), LCV (lane 3), MCV (lane 4), and SCV (lane 5). The samples are incubated for 10 min at 37 °C with [γ-^{32}P]ATP and loaded on a 5–15% acrylamide ND-PAGE. (a) Coomassie blue stained gel. (b) Autoradiography. Molecular masses are given in kilodaltons. (▼) The major proteins of plasma- (lane 1) and Golgi-enriched membrane fractions (lane 2) at "220" kDa; (open arrowhead) the protein of LCV at "300" kDa (lane 3); (solid arrowhead) the major protein of MCV (lane 4) and SCV (lane 5) at "200" kDa.

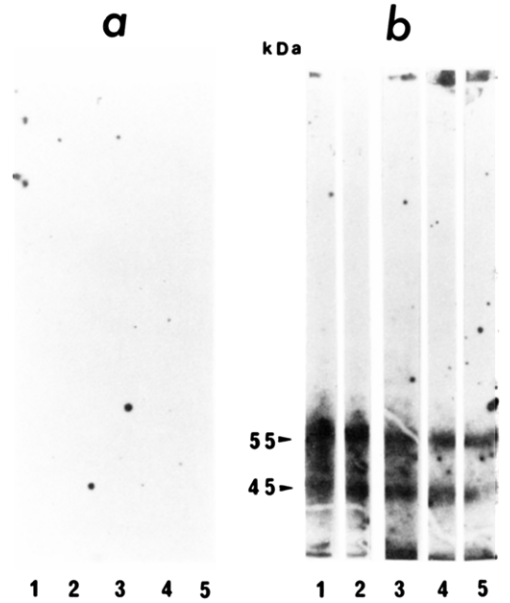

FIGURE 10: Autoradiography of an 8% acrylamide SDS–PAGE of plasma- (lane 1) and Golgi-enriched membrane fractions (lane 2), LCV (lane 3), MCV (lane 4), and SCV (lane 5). After electrophoresis the gel is treated for polypeptide renaturation. (a) The gel is incubated with [γ-^{32}P]ATP to detect autophosphorylation. (b) The gel is overlaid with a mixture of [γ-^{32}P]ATP and an exogenous CV kinase preparation saturated with unlabeled ATP. The numbers to the left indicate SDS molecular mass calibration markers.

its substrate and also that the enzyme is still active under ND-PAGE conditions. For LCV the phosphorylated substrate detected at ≃"90" kDa was not autophosphorylated.

After SDS–PAGE and overlay with [γ-^{32}P]ATP only, no phosphorylated polypeptides could be detected in any fraction (Figure 10a), indicating that the kinase enzymes are polypeptides distinct from their substrates and separated under these conditions on the gels. When a CV preparation which

contains both the kinase and its substrates in the overlay medium was added, these internal substrates have to be saturated with unlabeled ATP. In these conditions and after addition of [γ-^{32}P]ATP to start the phosphorylation reaction, phosphorylated polypeptides were observed on SDS gels. They were the same as those observed when the phosphorylation reaction was carried out before SDS–PAGE (Figure 10b).

In conclusion, MCV and SCV contain a protein kinase of "200" kDa with autophosphorylating activity on nondenaturing gels. The substrates α- and β-tubulins are two polypeptides distinct from the enzyme polypeptide itself. LCV contain a protein kinase of $\simeq$"300" kDa with autophosphorylating activity. The three substrates, i.e., the two tubulins and the 45-kDa polypeptide, are also peptides distinct from the enzyme. The enzyme polypeptides might be of 65 kDa in MCV and SCV and of 88 kDa for LCV, as deduced from analyses of the high molecular mass components observed on SDS–PAGE (Figure 3).

Discussion

Coated vesicles are the only intracellular carrier organelles which can be purified in their native state (Pearse, 1987). The separation of pure CV from adrenocortical cells into three different populations has been obtained, on the basis of their different densities. If different subpopulations of CV have been already separated in bovine brain (Pfeffer & Kelly, 1985) and in rat hepatocytes (Baenzinger & Fiete, 1986; Helmy et al., 1986; Kedersha et al., 1986), the method used in the present work allowed CV to be obtained still biologically active. The presence of the LDL receptor in the three populations establishes their involvement in the intracellular LDL trafficking and receptor recycling.

The marker enzyme of Golgi membrane, galactosyltransferase, is mainly found in SCV. These data support the hypothesis previously suggested and based on lipid analysis (de Paillerets et al., 1987) that SCV might originate from Golgi membranes. The phosphodiesterase activity, marker of the plasma membrane, mainly found in MCV, suggests that MCV might originate from this membrane. No other difference between MCV and LCV permit further suggestion.

It is however worth questioning the actual meaning of marker enzymes for organelles like CV, involved in the transport and recycling of molecules from one cell membrane to another. During such a cycle the CV, after uncoating, fuse with other endosomes and some with lysosomes. They also bud off from Golgi complex (Goldstein et al., 1985). If recent data (de Paillerets et al., 1987) evidence a selection and a segregation of lipids and proteins during the formation of coated pits and CV, the presence of a small but not negligible activity of each of the so-called marker enzymes in all the CV types demonstrates that such a selection is not absolute at least for the enzymes studied. Micrograph controls and the high reproducibility of quantitative data as analysis of fatty acids in each preparation of CV preclude the possibility of contamination by either other membranes or the two other types of CV.

The simultaneous and successive examinations of the samples by ND-PAGE and SDS–PAGE evidence differences in the actual structure of the clathrin and coat proteins among the three types of isolated CV, as well as differences in the kinase enzyme and substrates. From SDS–PAGE analysis, it appears that the clathrin heavy chain (180 kDa) is present in a much lower amount in LCV than in SCV or MCV. For the coat proteins the most significant difference is in the ratio of 101/99-kDa components which is lower in LCV than in the two others. Moreover, a 45-kDa component appears characteristic of LCV. These differences between LCV and the other types of vesicles are further emphasized by the examination of the high molecular mass proteins separated on ND-PAGE. Indeed, a LCV protein of apparent molecular mass of "300" appears after SDS–PAGE analysis to be formed of six peptides: two of 100 kDa, one of 88 kDa, the tubulins of 54 and 56 kDa, and one of 45 kDa. The MCV and SCV high molecular mass proteins are comprised by only three peptides of 65 and 54–56 kDa. An additional result obtained from the ND-PAGE is that all the clathrin and coat proteins migrate as a unique complex of very high molecular mass which is only dissociated by SDS. Therefore, all the peptides forming the "300"- or "220"-kDa components are distinct from the coat proteins. Uncoating experiments show that the 54–56-kDa components are not coat but rather are integral membrane proteins from CV. These results suggest that there is a clear difference between LCV and the SCV and MCV.

The kinase activity of each population of CV confirms this difference. The same high molecular mass complexes, revealed by Coomassie blue staining and able to undergo autophosphorylation, are resolved into two substrates of 55 and 58 kDa, not able to undergo autophosphorylation, for SCV and MCV on SDS–PAGE. This doublet was shown to be integral membrane protein and identified as tubulin by immunoblotting in agreement with the data of Pfeffer et al. (1983), Wiedenmann and Mimms (1983), and Pfeffer and Kelly (1986) on bovine brain coated vesicles. Campbell et al. (1984) reported different results on rat liver coated vesicles suggesting that coated vesicle protein kinase of molecular mass of 53 and 51 kDa catalyzes autophosphorylation. These polypeptides are coat and not membrane integral proteins and cannot in the present state of investigation be identified as tubulin in contrast with the data obtained in adrenal cortex. Three substrates are resolved on SDS–PAGE for LCV: 55, 58, and 46 kDa. This last component can be identified with the 45-kDa component found in LCV only by scanning of the gels (Figure 6). All these substrates are able to be phosphorylated by overlay of the gel with a CV preparation. This set of data further support the data and hypotheses of Manfredi and Bazari (1987), about coat assembly polypeptides from calf brain coated vesicles. Moreover, in LCV a 88-kDa component is present on the SDS gel while in MCV and SCV a 65-kDa component is observed. None of these components are phosphorylated in the gel overlay, suggesting that they are the enzyme part of the complex.

In conclusion, the present data lead us to suggest the following:

(1) The organization of the clathrin coat already demonstrated as complex and composed of numerous polypeptides (Robinson, 1987) appears different for each type of CV. These polypeptides might have a role in the direction and distribution of the different populations of CV, in addition to the role of specific lipids previously suggested (de Paillerets et al., 1987).

(2) Protein phosphorylation through CV kinase could be the source of the potential energy for the motion of the vesicles (Pfeffer et al., 1983). The phosphorylated substrates, tubulins and the other LCV kinase substrate, might be the signal for the interaction with a part of the cytoskeleton, targeting the vesicles toward the right cell organelle related to its function.

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Registry No. Phosphokinase, 9031-44-1.

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