# Ligand-blotting visualization of the LDL receptor in plasma membranes and in two classes of coated vesicles from adrenocortical cells

Hadassa Weintraub, Morgane Bomsel, Christine de Paillerets, Isabelle Néant, Maryse Ayrault-Jarrier* and Annette Alfsen

*E.R. 64 CNRS – 45, rue des Saints-Pères, 75270 Paris Cédex 6,* and **U.A. 524, CNRS, C.H.U. Saint-Antoine, 27, rue de Chaligny, 75571 Paris Cédex 12, France*

Received 4 March 1985

Two populations of coated vesicles, different in size, have been isolated from the bovine adrenal cortex. The enrichment of the LDL receptor from the plasma membrane to the large coated vesicles and then to the small ones was evidenced by ligand-blotting ELISA assays. The LDL receptor has been characterized as a 130-kDa proteic component which retains the binding specificity and structural features in plasma membranes as well as in the two classes of coated vesicles.

*Adrenal cortex* *Receptor-mediated endocytosis* *LDL receptor* *Coated vesicle* *Ligand blotting*

## 1. INTRODUCTION

In adrenocortical cells, biosynthesis of corticosteroid hormones requires unesterified cholesterol provided by LDL, the major cholesterol-carrying lipoprotein in human plasma, internalized via receptor-mediated endocytosis (RME) [1]. Internalization is initiated by interaction with the specific high-activity LDL membrane receptor clustered in coated pits (CP), which on their cytoplasmic side are characterized by a lattice of clathrin [2]. Coated vesicles involved in the internalization and recycling process are morphologically different [3,4]. According to Anderson et al. [5], the large coated vesicles (LCV) which derive from pinching off of CP participate in the endocytic transport of the LDL-receptor complex to lysosomes. There, unesterified cholesterol is liberated, and further, converted to corticosteroid hormones in other organelles. Small coated vesicles (SCV) are involved in the receptor recycling. In this cyclic process, the LDL receptor participates in the regulation of the cholesterol level in adrenocortical cells [6]. Therefore, characterization of the LDL receptor at different steps of this cyclic process appears important in the understanding of the regulation mechanism.

Identification of the LDL receptor as a glycoprotein of 160 kDa under reducing conditions and 130 kDa under mild non-reducing conditions has been made with the purified receptor or with plasma membrane preparations [7]. In these membranes the LDL receptor is topologically heterogeneous, i.e., mainly concentrated in CP which represent only 2% of the plasma membrane surface [1,2] and randomly redistributed by the recycling process.

Here, for the first time, two populations of coated vesicles, LCV and SCV, have been isolated, uniform in composition and morphologically highly homogeneous. Ligand-blotting ELISA assays have allowed demonstration of not only the

*Abbreviations:* LDL, low density lipoprotein; ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin; NC, nitrocellulose paper; Mes, 2-(*N*-morpholino)ethanesulfonic acid; SDS-PAGE, SDS-polyacrylamide gel electrophoresis

*Published by Elsevier Science Publishers B.V. (Biomedical Division)*
00145793/85/$3.30 

presence of the LDL receptor in LCV and SCV but also an LDL receptor enrichment in these coated vesicles as compared to its concentration in partially purified plasma membrane extracts.

The binding specificity and the apparent $M_r$ of the LDL receptor observed with the 3 fractions studied are in agreement with previously reported results achieved under similar experimental conditions.

## 2. MATERIALS AND METHODS

### 2.1. *Chemicals*

Suramin (germanin) was the Bayer 205 product. Triton X-100, amido black 10B, and BSA (fraction V) were Sigma products. SDS was a BDH product. All other chemicals were analytical grade Merck products. LDL and rabbit LDL antibodies were kindly provided by M. Ayrault-Jarrier. Goat anti-rabbit IgG horseradish peroxidase conjugate was obtained from Pasteur Production. 3,3′-Diaminobenzidine tetrahydrochloride was from Interchim. NC (0.45 $\mu$m pore size) was from Biorad.

### 2.2. *Adrenal membrane preparation*

The extraction step giving the crude membrane fractions was carried out as described by Schlegel and Schwyzer [8]. This fraction was layered on top of a sucrose density discontinuous gradient (15, 19, 22.5, 26, 35, 45, 50%; w/w) and centrifuged at 90000 × $g$ for 1.5 h. The membranes were collected at the 22.5–26% interface and extensively dialysed against 1 mM $NaHCO_3$, pH 7.4.

### 2.3. *Coated vesicles*

These were purified according to Nandi et al. [9] with a modification in the gradient step. The crude vesicles were layered on top of a $D_2O$ sucrose


Fig.1. Electron micrographs of LCV and SCV. Separation was obtained by a discontinuous $D_2O$ sucrose gradient. Bars: 100 nm.

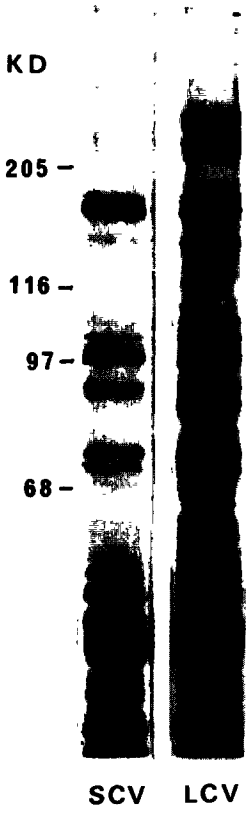


Fig.2. SDS-PAGE electrophoresis of LCV and SCV. Coomassie blue staining.

discontinuous gradient (0, 8, 12, 16, 24%) and centrifuged at 90000 × *g* for 1.5 h in a swinging rotor. SCV were collected at the 12–16% interface and LCV at the 16–24% interface. Fractions were dialyzed against Mes buffer, pH 6.5.

2.4. *Proteins*

The protein content of samples was determined by the method of Petterson [10].

2.5. *Electron microscopy*

A drop of material was placed on a 200 mesh Formwar carbon-coated grid and allowed to air-dry. Samples were negatively stained with 2% uranyl acetate. Micrographs were taken on a Jeol 100 CX electron microscope.

2.6. *SDS-PAGE*

0.1% SDS-PAGE was performed in 7.5% polyacrylamide slab gels according to Laemmli [11] with or without a reducing agent (β-mercaptoethanol). A high-$M_r$ kit (Sigma) was used for $M_r$ calibration.

2.7. *Immunoblotting*

Proteins were transferred from SDS-PAGE to NC paper according to Towbin et al. [12]. The NC paper was incubated with the ligand. A second incubation after washing was performed with rabbit LDL antibodies. The bound antibodies were detected by ELISA using peroxidase-labeled goat anti-rabbit IgG.

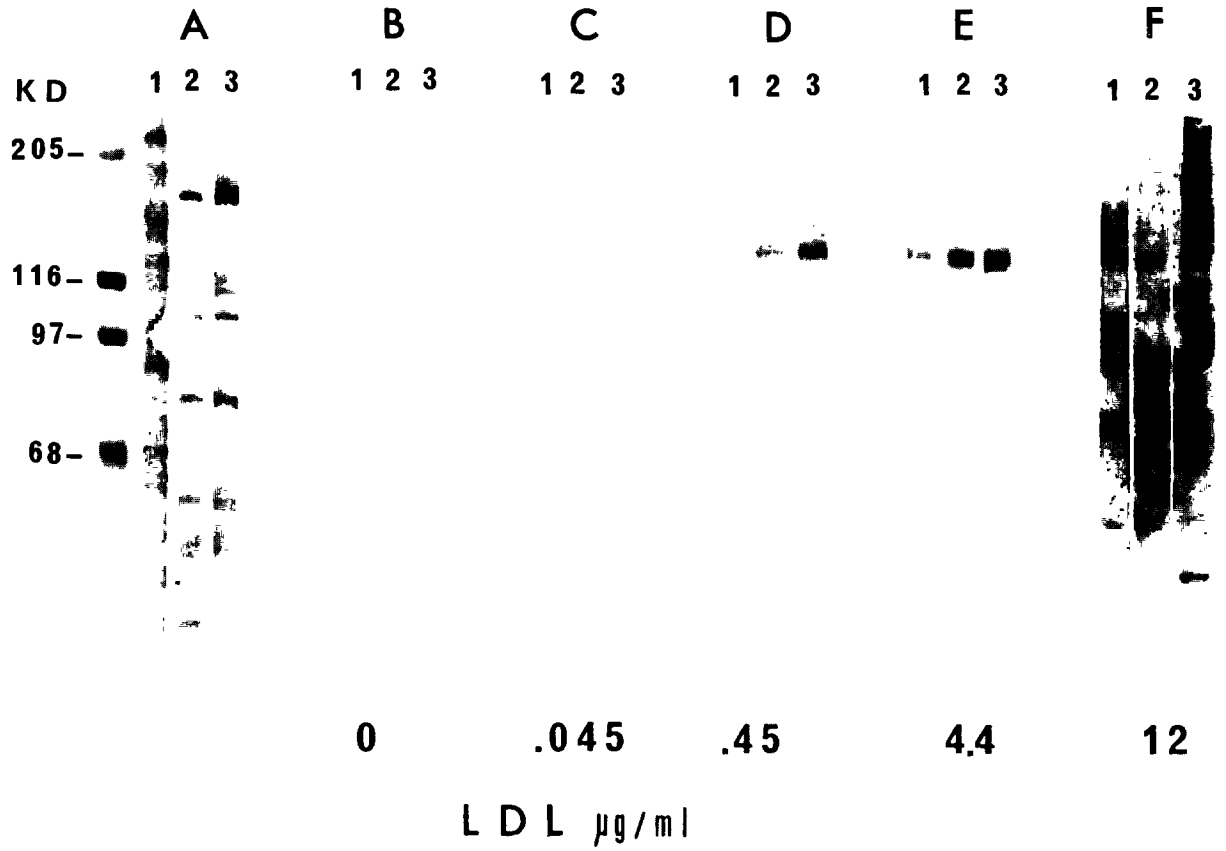


Fig.3. Quantitative estimation by ligand-blotting ELISA of the LDL receptor binding in plasma membranes, SCV and LCV. Each fraction was subjected to 7.5% SDS-PAGE and transferred to NC paper. (1) Plasma membranes (140 μg protein), (2) LCV (70 μg protein), (3) SCV (70 μg protein). Lane A (1–3) was stained with amido black 10B. Lanes B–F were incubated with human LDL at the concentrations given below each lane, followed by ELISA detection.

## 3. RESULTS AND DISCUSSION

Two populations of coated vesicles have been isolated from bovine adrenal cortex using the modified $D_2O$ sucrose discontinuous gradient described above. As shown by the electron micrographs (fig.1), these two populations have different sizes corresponding to differences in density as revealed by the gradient: LCV have a density range of 1.18–1.21, with diameters from 90 to 120 nm; SCV, a density range of 1.16–1.17 and diameters from 50 to 80 nm.

The major protein components analyzed by Coomassie blue stained SDS-PAGE (fig.2) are the same in LCV and SCV and as those previously described in the mixture of coated vesicles from adrenal cortex and other tissues [13]. However, a difference in the staining intensity appears between the respective components of SCV and LCV. Moreover, two high molecular mass species, 210 and 230 kDa, seem to be characteristic of LCV. These components could belong to the lipoprotein peptides as suggested by a comparison with SDS-PAGE of LDL under denaturing conditions [14]. Such a hypothesis would be consistent with the endocytic function recognized in situ for the LCV [3] and is furthermore supported by the absence of these components in the SCV (fig.2). A relatively high proportion of the isolated SCV (80%) as compared with the low one of LCV (20%) suggests a plasma membrane origin for LCV which are known to have a transient lifetime and to disappear in a time range of a minute [2] by losing their clathrin coat.

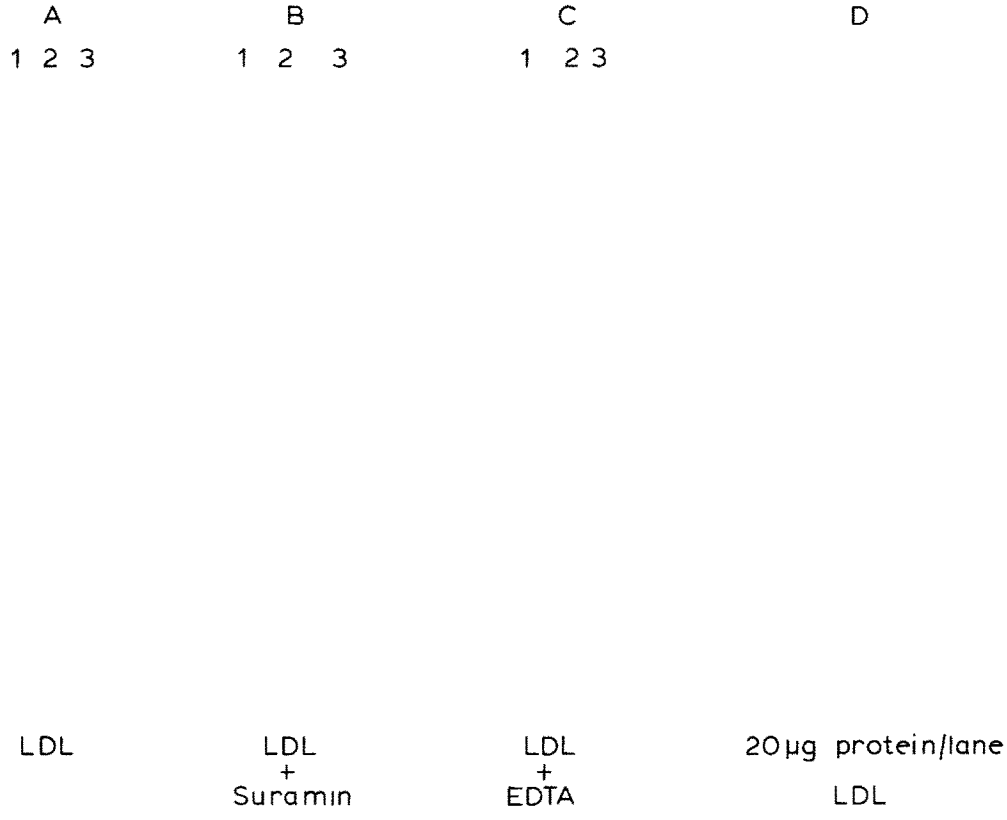


Fig.4. Visualization of LDL receptor in plasma membrane, LCV and SCV by ligand-blotting ELISA. Each fraction was subjected to 7.5% SDS-PAGE and transferred to NC. (1) Plasma membranes (140 $\mu$g protein), (2) LCV (70 $\mu$g protein), (3) SCV (70 $\mu$g protein). The NC strips were incubated with, respectively: lane A (1–3), 4.5 $\mu$g protein/ml of human LDL; lane B (1–3), 4.5 $\mu$g protein/ml of human LDL + 7.5 mM Suramin; lane C (1–3), 4.5 $\mu$g protein/ml of human LDL + 10 mM EDTA; lane D, only 20 $\mu$g protein/lane, same conditions as A. Binding of LDL was detected by ELISA.

The presence of the LDL receptor and its activity have been determined qualitatively and estimated quantitatively in the plasma membrane and in the LCV and SCV. The ligand-blotting ELISA method has been used for the study of plasma membranes, LCV and SCV. The limit of detection was 20 $\mu$g total protein, in coated vesicles layered on SDS-PAGE, an amount insufficient to be revealed by Coomassie blue staining. Thus we have been able to characterize a component which binds LDL in a specific way and is present in all 3 preparations. This receptor with an apparent molecular mass (in the absence of reducing agent) of ~130 kDa binds LDL in increasing amounts (fig.3) between 0.045 and 4.4 $\mu$g/ml protein and reaches saturation at higher concentrations as seen by visual inspection. Inhibition of LDL binding by Suramin or EDTA was observed (fig.4) indicating the specificity of the LDL receptor for its ligand.

These results fit in with the data obtained with the affinity-purified LDL receptor from adrenal cortex membranes [15–17] and with the LDL receptor in crude plasma extracts visualized by immuno or ligand blotting [18,19]. This report demonstrates for the first time the presence of the LDL receptor in coated vesicles by the ligand-blotting technique.

It is worth noting that isolation of LCV and SCV has allowed the demonstration of a higher amount of LDL receptor in SCV than in LCV (figs 3–5) and a higher amount in both types of coated vesicles as compared with the plasma membranes. The binding specificity and structural characteristics (130 kDa) of the LDL receptor, preserved in all fractions studied (plasma membranes, LCV, SCV) under the experimental conditions of SDS-PAGE and ligand blotting, indicate that the receptor remains unchanged in these different cell organelles. The origin of the SCV remains to be determined before assigning a specific biological role to this class in the LDL receptor cycle.

## REFERENCES

[1] Brown, M.S. and Goldstein, J.L. (1979) Proc. Natl. Acad. Sci. USA 76, 3330–3337.
[2] Bretscher, M.S. (1982) Science 224, 681–686.
[3] Friend, D.S. and Farquar, M.G. (1967) J. Cell Biol. 35, 357–376.
[4] Steven, A.C., Hainfeld, F.J. and Wall, J.S. (1983) J. Cell Biol. 97, 1714–1723.
[5] Anderson, R.G.W., Brown, M.S. and Goldstein, J.L. (1981) J. Cell Biol. 88, 441–452.
[6] Tolleshaug, H., Hobgood, K.K., Brown, M.S. and Goldstein, J.L. (1983) Cell 32, 941–951.
[7] Daniel, T.O., Schneider, W.J., Goldstein, J.L. and Brown, M.S. (1983) J. Biol. Chem. 258, 4606–4611.
[8] Schlegel, W. and Schwyzer, R. (1977) Eur. J. Biochem. 72, 415–424.
[9] Nandi, P.K., Irace, G., Van Jaarsveld, P.P., Lippoldt, R.E. and Edelhoch, H. (1982) Proc. Natl. Acad. Sci. USA 79, 5881–5885.
[10] Petterson, G.L. (1977) Anal. Biochem. 23, 346–357.
[11] Laemmli, U.K. (1970) Nature 227, 680–685.
[12] Towbin, H., Staehlin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[13] Woods, J.W., Woodward, M.P. and Roth, T.F. (1978) J. Cell Sci. 30, 87–97.
[14] Salmon, S., Theron, L., Beucler, I., Ayrault-Jarrier, M. and Polonovski, J. (1984) Biochimie 66, 159–170.
[15] Mello, R.J., Brown, M.S., Goldstein, J.L. and Anderson, G.W. (1980) Cell 70, 829–837.
[16] Schneider, W.J., Goldstein, J.L. and Brown, M.S. (1980) J. Biol. Chem. 255, 11442–11447.
[17] Schneider, W.J., Beisiegel, U., Goldstein, J.L. and Brown, M.S. (1982) J. Biol. Chem. 257, 2664–2673.
[18] Beisiegel, U., Schneider, W.J., Brown, M.S. and Goldstein, J.L. (1982) J. Biol. Chem. 257, 13150–13156.
[19] Dresel, H., Otto, I., Weighel, H., Schettler, G. and Via, D.P. (1984) Biochim. Biophys. Acta 795, 452–457.