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The LDL receptor and LRP are receptors for βVLDL on pigeon monocyte-derived macrophages

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Abstract Receptors for the lipoprotein, beta very low density lipoprotein (βVLDL), have been identified through the binding of βVLDL-gold conjugates on two ligand-induced regions of pigeon monocyte-derived macrophages. These regions were microvilli/retraction fibers and membrane ruffles. The present study investigated the location and identity of βVLDL receptors using an antiserum directed against the epidermal growth factor (EGF) precursor region of the human low density lipoprotein (LDL) receptor. The anti-receptor serum recognized two membrane proteins from pigeon monocyte-derived macrophages, a 116 kDa (LDL receptor) protein and a 600 kDa (low density lipoprotein receptor-related protein; LRP) protein. Ligand blot analysis demonstrated that pigeon βVLDL bound to both the LDL receptor and LRP. Immuno-gold electron microscopy using the anti-receptor serum resulted in immunoglobulin localization on the same two ligand-induced regions, microvilli/retraction fibers and membrane ruffles, to which the ligand had bound. Furthermore, simultaneous immunogold localization of the lipoprotein receptor antigens and βVLDL-gold (ligand) binding substantiated co-localization of the receptor antigens and βVLDL on the ligand-induced regions. Cross-competition studies with the anti-receptor serum and βVLDL-gold conjugates documented that increasing concentration of the anti-receptor serum resulted in 70% inhibition of βVLDL-gold conjugate binding. These data suggest that pigeon monocyte-derived macrophages utilize both the LDL receptor and LRP as receptors for pigeon βVLDL.

Key words Beta very low density lipoprotein receptors · Receptor-mediated endocytosis · Lipoprotein

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Introduction

Monocyte-derived macrophage foam cells, a hallmark of atherosclerosis, can be formed in vitro by cell exposure to either beta-migrating very low density lipoprotein (βVLDL), a naturally occurring lipoprotein, or experimentally modified lipoproteins such as acetylated LDL [7, 16, 17]. Pigeon blood monocytes have been cultured in vitro as a model for monocyte-derived foam cells. Like human macrophages, the pigeon cells can be stimulated by endocytosis of either βVLDL or acetylated-LDL (AcLDL) to accumulate cholesterol esters resulting in a foam cell-like appearance [16, 17].

Lipoproteins are internalized by specific receptors, several of which have been characterized. The LDL receptor, in addition to binding LDL, also binds aggregated LDL, particulate LDL and βVLDL [15]. Recently an LDL receptor-related protein (LRP) has been identified. Lipoprotein receptor-related protein is the $\alpha_2$macroglobulin receptor, although it was first identified through homology with the EGF precursor region to the LDL receptor [18]. *In vitro* LRP binds multiple apo E-enriched lipoproteins, and recent *in vivo* evidence suggests that LRP also serves as the chylomicron receptor [19]. Macrophages also possess scavenger receptors [3, 28] that bind modified forms of LDL such as AcLDL [12], malondialdehyde-modified LDL [13] and oxidized LDL [7].

Previously, two kinetically defined βVLDL binding sites have been identified on pigeon monocyte-derived macrophages [16] and peritoneal macrophages [2].One of these sites, the "pigeon βVLDL receptor", is believed to be a form of the native LDL receptor [2]. This "pigeon βVLDL receptor", like the mammalian macrophage βVLDL receptor, shares many characteristics with the LDL receptor. It binds βVLDL and LDL with high affinity, requires calcium, and is destroyed by the proteolytic enzyme, pronase. This βVLDL/LDL receptor relationship on pigeon monocyte-derived macrophages is evidenced by 40% competition between LDL and βVLDL for the receptor [16]. The second lipoprotein receptor on pigeon macrophages is a low affinity binding site, which

is relatively nonspecific, recognizes multiple lipoproteins and is insensitive to pronase treatment [2]. This report, utilizing an anti-serum directed against the EGF sequence of the LDL receptor, studied possible receptors involved in pigeon βVLDL binding to pigeon monocyte-derived macrophages.

Materials and methods

Random bred White Carneau (RBWC) pigeons were maintained by the Department of Comparative Medicine with appropriate veterinary care. Monocyte isolation and lipoprotein isolation procedures were approved by the Animal Care and Use Committee of Bowman Gray School of Medicine, which are in compliance with the Public Health Service's Policy on Humane Care and Use of Laboratory Animals.

Monocytes from RBWC pigeons were isolated from anticoagulated blood by differential centrifugation over isolymph as previously reported [20]. The monocytes were plated in 35 mm dishes or in dishes containing formvar-coated gold grids mounted on glass coverslips [20]. The monocytes were cultured in Eagle's Minimum Essential Medium (MEM), (Hazelton, Denver, Pa.) containing 10% (V/V) heat-inactivated chicken serum (Hazelton, Denver, Pa.). The cultures were maintained at 37° C in a 5% carbon dioxide humidified incubator for 7 to 10 days. These monocyte-derived macrophages will be referred to as macrophages throughout the rest of the manuscript.

Low density lipoprotein (LDL), high density lipoprotein (HDL) and βVLDL were obtained from hypercholesterolaemic RBWC pigeons by plasma fractionation using differential centrifugation [1]. Briefly, βVLDL was isolated from plasma by ultracentrifugation at d<1.006 g/ml and H-LDL at d>1.016 g/ml. The lipoprotein stocks were spot checked for the presence of endotoxin (Endotect, Swartz/Mann Biotech, Cleveland, Ohio) and were consistently negative in the range of 0.06 to 0.10 ng/ml endotoxin. Beta very low density lipoprotein was conjugated to 40 nm colloidal gold or 35 nm gold colloids which had been prepared from sodium citrate and gold chloride [17]. Our laboratory previously verified that βVLDL-gold conjugates have binding characteristics identical to native lipoproteins by effectively competing a double-labelled ^{125}I-gold conjugated βVLDL with unlabelled βVLDL [16, 17]. Two sources of human $\alpha_2$macroglobulin were used in these studies; (Sigma, St. Louis, Mo.) and native and active $\alpha_2$macroglobulin (a generous gift from Dr. Steven Feldman, Bowman Gray School of Medicine) prepared as described in Feldman and Pizzo [11].

For immunogold localization macrophages were washed two times with Gey's balanced salt solution (GBSS; Gibco, Grand Island, N.Y.) incubated with primary antibodies for 7 min at room temperature at a 1 to 500 dilution. The anti-receptor serum was a gift from Dr. L. Rudel, Bowman Gray School of Medicine (see Table 1 for the peptide sequence used to make this antiserum). Macrophages were then washed three times with GBSS. The secondary antibody treatment was a mixture of two Amersham auroprobes (Amersham, Buckinghamshire, England) diluted 1:4 for each antibody with GBSS. Since the primary antibodies were raised in rabbits, the second stage antibodies included both specific goat anti-rabbit (GAR), and nonspecific goat anti-mouse (GAM) secondary antibodies. The gold colloidal sizes were principally 15 nm for GAR and 5 nm for GAM; however in select experiments, the gold colloid sizes were reversed to controls for steric effects.

Table 1 Peptide sequence of the LDL receptor used to make the anti-receptor serum.

[sequence 573 ASN-ARG-LYS-THR-ILE-GLU-ASP-GLU-LYS-ARG-LEU-ALA-(TYR)]

Macrophages after immuno(gold)-localization were fixed with 2.5% glutaraldehyde (TAAB) and processed for whole mount microscopy [20]. Following primary fixation, the cells were post-fixed with 1% osmium tetroxide in 0.1 M phosphate buffer (pH 7.3) for 5 min and dehydrated stepwise in ethanol. The dehydrated cells were then dried from carbon dioxide by the critical point method, coated with carbon and observed directly at 300 keV in the Phillips CM-30 IVEM [20]. Samples to be sectioned for thin section TEM were infiltrated and embedded in Spurr Resin (Polysciences, Warrington Pa.) according to the manufacturer's specifications. Thin sections (0.1 μm) were visualized at 80 keV in the Phillips EM-400.

Competition Studies are described in some detail. Cells were incubated with 25 μg/ml of βVLDL-40 nm gold conjugate in buffer, or buffer containing various concentrations of anti-receptor serum (1:500, 1:50 and 1:10 dilutions). A 40 fold excess of unconjugated βVLDL was incubated with βVLDL-40 nm gold conjugate as a positive control for competition with βVLDL-gold conjugates. After incubating the macrophages for 8 min at room temperature with one of the above treatments, the cells were washed then incubated for 7 min at room temperature with second stage antibodies as described above for immunocytochemistry. Ultimately, the cells were fixed and processed for standard thin section TEM. In order to ensure a non-biased selection of cells, a predetermined, non-random pattern was used in which the first 20 consecutive cells were analysed. Partial cell profiles were excluded.

For receptor localization cells were incubated with 1:50 dilution of anti-receptor serum in either buffer or buffer containing 1 mg/ml pigeon LDL, βVLDL, HDL, or human $\alpha_2$macroglobulin (Sigma). After incubating macrophages for 8 min at room temperature, the cells were washed and incubated for 7 min at room temperature with the second stage antibodies. The cells were fixed and processed for standard thin section TEM.

Membranes were isolated from 7 day macrophages cultured from 10–20 birds. Cells were washed twice with PBS, scrapped, and collected by centrifugation. The pellet of cells was resuspended in sucrose (11.2%) in CAT buffer (CAT buffer: 0.5 mM calcium chloride, 10 mM TRIS pH 7.4, containing the protease inhibitors, 1 mM leupeptin, 1 mM aprotinin, and 0.2 mM phenyl-sulphonyl-fluoride). Macrophages were disrupted by sonication (Ultrasonic devices, 3x10 seconds) on ice prior to loading on a 15 and 40% step gradient in CAT buffer. The samples were ultracentrifuged for 30 min at 100,000×g in a SW 55 rotor. The opaque membrane band at the 15 and 40% sucrose interface was collected, diluted with CAT buffer to 10% sucrose and pelleted by ultracentrifugation for 60 min at 160,000×g in a 50 Ti rotor. The membrane pellet was resuspended in CAT buffer and stored at –70° C.

For *SDS-PAGE*, proteins were solubilized in 2% SDS and were electrophoresed on 4–15% or 4–20% gradient acrylamide gels according to the method of Laemmlie [24] using the Biorad minigel system (Biorad, Richmond, Calif.). Prestained molecular weight standards (Sigma, St. Louis, Mo.) were used. Additionally, either pigeon LDL or biotinylated LDL was used as a high molecular weight standard (pigeon apo-B, MW 513,000 kDa [4]). Biotinylated protein molecular weight markers (Vector Laboratories, Burlingame, Calif.) were run on gels that were later transblotted for ligand blotting. Gels were stained with Coomassie brilliant blue or transblotted to either polyvinylidene difluoride (Immobilon-P, PVDF, 0.45 μm; Millipore Corp., Bedford, Mass.) or nitrocellulose as described below.

Gels were transblotted to either PVDF (immuno- and ligand blotting) or nitrocellulose (calcium blotting) using a Biorad transblot apparatus in buffer containing 25 mM TRIS and 192 mM glycine, pH 8.3. The efficiency of transfer was assessed by visualization of the prestained standards on PVDF paper and staining the gels with coomassie blue after transfer.

For immunoblotting, the paper was placed in blocking buffer (10 mM TRIS, pH 7.4, 0.15 M sodium chloride, 3% casein) either overnight at 4° C or 1 h at room temperature, and incubated for 2 h at room temperature with the anti-receptor serum at a 1:500 dilution. After washing twice with TRIS buffered saline (10 mM TRIS,

0.15 M sodium chloride, pH 7.4), the paper was incubated for 2 h at room temperature with 1:1000 dilution of the secondary antibody, horseradish peroxidase (HRP)-linked goat anti-rabbit IgG.

Biotin was conjugated to lipoproteins via sialic acid residues on the apoproteins as described by Kowal et al. [21]. Briefly, lipoproteins (2 mg/ml) were oxidized by sodium metaperiodate (final concentration equaled 4 mM) for 30 min at 4° C in total darkness. The reaction was quenched by the addition of Na_2SO_3 to a final concentration of 8 mM. Two milligrams of biotin hydrazide was added and the combination incubated for 30 min at room temperature. This reaction was quenched with the addition of 0.1 ml of 1 M sodium glycine (pH 8.5) for 1 h at 4° C. The lipoproteins were then dialysed overnight against 20 mM TRIS-HCl, 150 mM sodium chloride and0.25 mM EDTA, pH 7.4.

Ligand blotting was done essentially as described by Wade et al. [30]. The PVDF transblotted paper was placed in blocking buffer (Buffer A: 10 mM TRIS, pH 8.0, 0.15 M sodium chloride and 50 mg/ml bovine serum albumin [Sigma, St. Louis, Mo.]) for 30 min at 37° C. The PVDF paper was incubated with biotinylated βVLDL (20 μg protein/ml) at room temperature for 30 min on a rocking platform. Subsequently, the paper was washed twice with buffer A and incubated with avidin-linked HRP (Sigma, St. Louis, Mo.) or streptavidin-biotin-peroxidase complex (Amersham, Arlington Heights, Ill.)

Dot blot analysis was done according to the method of Hawkes [4]. Twenty micrograms of each of the following lipoproteins; pigeon βVLDL, LDL, and HDL and rabbit βVLDL (gift from Dr. Dawn Schwenke, Bowman Gray School of Medicine) were boiled in SDS electrophoretic buffer and applied to nitrocellulose paper using the Minifold dot blot apparatus (Schleicher and Schuell, Keene, N.H.). After incubation for 1 h at room temperature in blocking buffer, the paper was incubated for 1 h at room temperature with a 1:500 dilution of the polyclonal rabbit anti-bovine milk lipoprotein lipase (7551, a generous gift from Dr. Ira Goldberg, Columbia University). After washing, the paper was incubated with 1:1000 dilution of the secondary antibody horse peroxidase labelled anti-rabbit IgG (Sigma, St. Louis, Mo.). The paper was developed as described below.

Immuno-, ligand and dot blots were developed in TRIS-buffered saline containing 3.4 μM 4-chloro-1-naphthol (Sigma, St. Louis, Mo.) and 0.2% hydrogen peroxide until blue bands appeared. The development was stopped by rinsing in distilled water.

Calcium blotting was done as described by Herz et al. [18]. The nitrocellulose transblot of pigeon macrophage membranes was rinsed with a buffer comprised of 50 mM sodium chloride, 5 mM magnesium chloride, and 15 mM TRIS-HCl, pH 7.0, then incubated in the same buffer with ^{45}Ca (1 mCi/l) (Amersham, Arlington, Heights, Ill.) for 10 min at room temperature. The transblot was rinsed with 25% ethanol and air dried. Radiographic film was exposed to the blot for 14 days at –70° C with an image intensifier screen and developed in an automated developer.

Results

Characterization of pigeon macrophage membrane βVLDL receptors

Previously, two kinetic binding sites for βVLDL have been identified for pigeon monocyte-derived macrophages [16] and pigeon peritoneal macrophages [2]. One of the receptors, implicated to be involved with lipoprotein binding in both types of pigeon macrophages, was the LDL receptor. An initial study was done to determine if proteins antigenically similar to the mammalian LDL receptor are on pigeon macrophages. Pigeon macrophage membranes were isolated and used as the source of receptor proteins. The isolated macrophage membranes

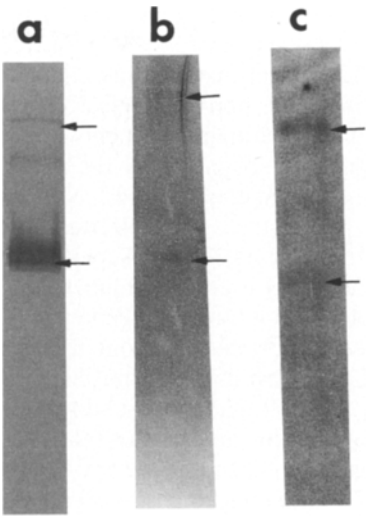

Fig. 1 Immuno-, ligand, and calcium blots of pigeon macrophage membrane proteins. **a** Pigeon macrophage membranes were electrophoresed on a 4–20% polyacrylamide gradient gel and transblotted to PVDF. The blot was incubated with the rabbit anti-receptor serum (1:500 dilution) prior to washing and incubation with an HRP-conjugated anti-rabbit antibody. Three proteins are recognized by the anti-receptor serum, a large molecular weight protein at approximately 600 kDa and a smaller protein of 116 kDa (*arrows*). A third band was detected which is similar to the dimer of LDL receptor reported by Esser et al. [10]. **b** A transblotted PVDF paper of pigeon macrophage membranes that had been incubated with biotinylated βVLDL (20 μg/ml). After βVLDL binding and washing, the blot was incubated with avidin-HRP and developed as described in materials and methods. Paralleling the immunoblot in panel **a**, two major bands a 600 kDa and 116 kDa (*arrows*) bound βVLDL. **c** Pigeon macrophage membranes were electrophoresed as above, but then were transblotted to nitrocellulose. A ^{45}Ca blot was done on pigeon macrophage membranes as described in the materials and methods. Both a 600 kDa and 116 kDa protein bound calcium (*arrows*). A third band similar to the LDL dimer was detected

were SDS-electrophoresed, blotted to PVDF paper and then probed with an anti-serum against the LDL receptor. The anti-serum was made against a polypeptide from the internal hydrophobic EGF region of the human LDL receptor (Table 1). This polypeptide was also homologous to sequences of the EGF precursor region of LRP. Two major proteins were recognized by the serum. These were a 116 kDa MW protein and 600 kDa MW protein (Fig. 1a). The 116 kDa pigeon protein corresponded to the size reported for the mammalian LDL receptor. The higher molecular weight protein, approximately 600 kDa, had a size which corresponded to molecular weights reported for LRP. Presumably the serum recognized LRP because the homologous EGF precursor region has been conserved in pigeon LRP. A third protein was sometimes recognized by the anti-receptor serum. This protein migrated in the range reported for dimers of the LDL receptor (see Fig. 1a) [10].

These studies indicated that peptide(s) antigenically similar to the mammalian LDL receptor were present on pigeon macrophage membranes. Although peptide(s), antigenically similar to the EGF precursor region of the LDL

receptor were present on pigeon macrophages, it was possible that the "pigeon macrophage LDL receptor" would not bind βVLDL, and that the binding of βVLDL to pigeon cells involved non-antigenically related proteins. Therefore, ligand blotting experiments were done to determine if the protein recognized by the anti-receptor serum was able to bind pigeon βVLDL. Non-reduced pigeon macrophage membranes were electrophoresed on SDS-PAGE then transblotted to PVDF paper. Pigeon βVLDL was biotinylated and used in conjunction with the transblotted receptors. Biotinylated pigeon βVLDL (20 μg/ml) was incubated for 30 min at room temperature prior to washing and detection using avidin-linked HRP. As illustrated in Fig. 1b, βVLDL was bound to both the 116 kDa LDL receptor protein and to the 600 kDa LRP protein which had been recognized by the anti-receptor serum.

Both the mammalian LDL receptor and LRP bind calcium [18]. Therefore, the two pigeon macrophage βVLDL receptors were examined to determine if they had similar calcium binding characteristics. Pigeon macrophage membranes were electrophoresed as above, then they were transferred to nitrocellulose filters and were incubated with ^{45}Ca. The resulting autoradiograph showed that both the 116 kDa protein (LDL receptor) and the high molecular weight-600 kDa protein (LRP) bound calcium (Fig. 1c). The protein which migrated as an LDL receptor dimer also bound calcium. When taken together these studies documented that the two antigenically related proteins bound both calcium and βVLDL.

Immuno(gold)-localization of the LDL receptor and LRP

Ultrastructural studies to determine the location of binding sites for pigeon βVLDL previously determined that the binding was associated with microvilli and membrane ruffles on pigeon macrophages [20]. In the present study, immuno(gold)-localization was done using the anti-receptor serum to determine if the receptor antigens were localized on the same ligand-induced binding regions. Whole mount electron microscopy was chosen because the technology provided the ability to identify microvilli and membrane ruffles while simultaneously localizing the receptor antigens. Macrophages were grown on formvar coated gold grids. After 7 days in culture the primary antibody, rabbit anti-receptor serum, was added to the living macrophages at either room temperature or at 4° C for 7–10 min. After primary antibody incubation, the cells were washed and incubated with a gold conjugated second stage antibody mixture for 7–10 min, then the cells were prepared for whole mount EM as described in the Materials and Methods. The anti-receptor serum was detected through the secondary antibody-gold conjugate. Consistent with the ligand binding studies, the receptor antigens were localized primarily to microvilli and membrane ruffles (Fig. 2). Throughout the experiments, non-specific labelling was monitored through the use of a non-related, gold labelled, second stage antibody. This control antibody and was rarely observed. An additional control, using non-immune rabbit serum as the primary antibody, also resulted in very little immunogold labelling (Fig. 2).

Assuming that the ligand and antiserum were recognizing the same protein, it seemed conceivable that the antigen and ligand could be co-localized to the same ligand binding regions. Following the exact experimental design as the receptor immunolocalization experiments, gold-conjugated βVLDL (20 μg/ml) was added to the cells in the presence of a limited quantity of the anti-receptor serum. The cells subsequently were washed, and

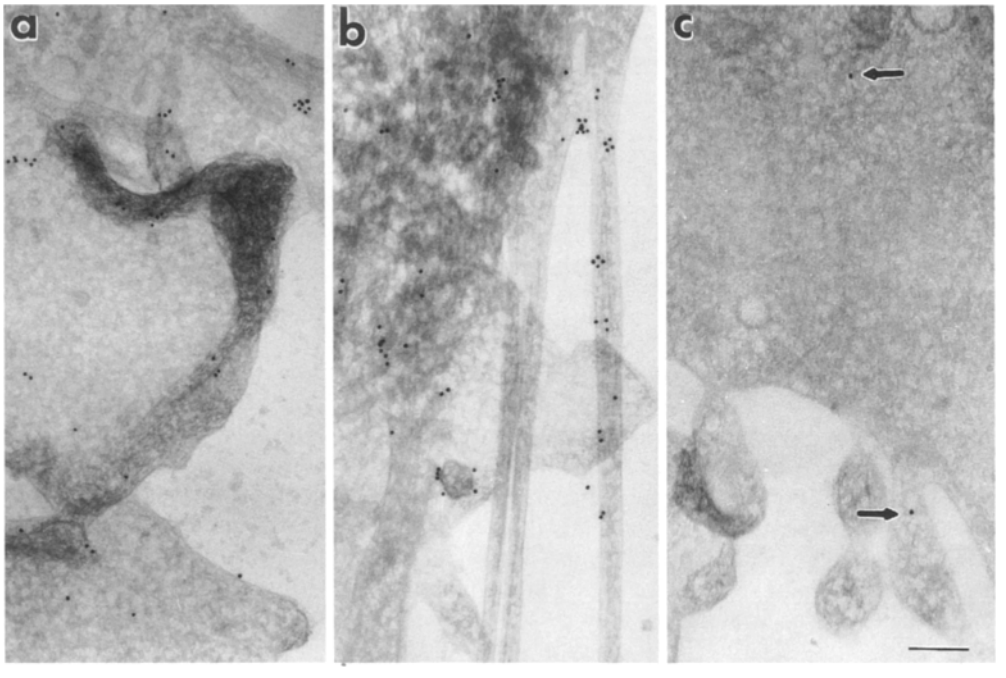

Fig. 2 Whole mount IVEM immuno(gold) localization of LDL receptor antigens on pigeon macrophages. **a** and **b** Macrophages were incubated with the primary anti-receptor serum, washed, then incubated with a second stage antibody mixture containing GAR-15 nm gold conjugate (to recognize the anti-receptor antibody) and GAM-5nm gold conjugate (which served as an internal control for non-specific binding). Receptor antigens were present on membrane ruffles (**a**) and microvilli (**b**). Little non-specific binding was detected. **c** Using a rabbit non-immune serum as the primary antibody resulted in minimal binding. (Bars=0.2 μm)

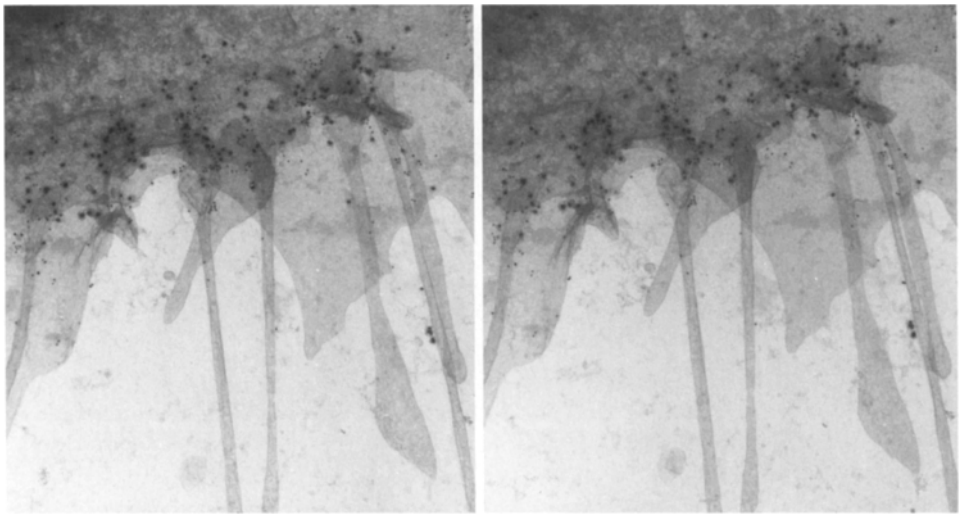

Fig. 3 Stereo pair electron micrographs illustrating co-localization of the receptor antigens and βVLDL ligand on whole mount pigeon macrophages. The primary anti-receptor serum and βVLDL conjugated to 25 nm colloidal gold were incubated for 7 min at 25° C prior to washing and incubating with the second stage antibody. The second stage antibody was a mixture of GAR-15nm gold conjugate (to recognize the anti-receptor antibody) and GAM-5 nm gold conjugate (which served as an internal control for non-specific binding). The cells were then washed, fixed and processed for whole mount IVEM. Both the lipoprotein receptors (15 nm gold) and βVLDL (25 nm gold) co-localized at the base of microvilli. Note the presence of pits lined with both the receptor and ligand at the base of the microvillus region. (Bar=0.2 μm)

the secondary antibody mixture was added to detect the anti-receptor antibodies.When the anti-receptor serum and the ligand were simultaneously used on pigeon macrophages, the ligand and receptor were co-localized on both microvilli and membrane ruffles (Fig. 3). Plasma membrane invaginations containing numerous immunogold and ligand gold labels were seen at the base of microvilli. Although the receptor antigens and gold-conjugated βVLDL co-localized to the same general microvilli and membrane ruffle regions, one to one localization did not occur. The co-localization of βVLDL-gold and the receptor antigen were also examined by conventional thin section TEM. Co-localization of the ligand and receptor antigen was observed on microvilli and membranes ruffles as well as in various compartments of endocytosis including coated pits, coated vesicles, uncoated vesicles and endosomes (see Fig. 4).

Competition studies

Competition studies were done to test the hypothesis that the anti-receptor serum recognized the active receptor for βVLDL on pigeon macrophages. The hypothesis was that if either the "LDL receptor" or "LRP" were βVLDL receptors, the anti-serum should block βVLDL binding to the macrophages. Therefore, the contribution of these potential receptors to βVLDL binding was studied by treating the cells with βVLDL-gold in the presence of increasing concentrations of the anti-receptor serum. In matched experiments, colloidal gold conjugated βVLDL was co-incubated with either a control buffer, or buffer containing increasing concentrations of the rabbit anti-receptor serum (1:500, 1:50 and 1:10 dilutions). Following this treatment, the cells were washed then incubated with second stage immunogold which was a mixture which contained GAR-15nm gold conjugate to recognize the anti-receptor serum and GAM-5nm gold conjugate which served as an internal control for non-specific binding. The cells then were embedded, and thin section TEM was done. A representative experiment is shown in Fig. 4. When βVLDL-gold conjugates were incubated with buffer which did not contain the primary antibody, the greatest binding of βVLDL was observed. There was little or no immunogold background binding as indicated by the paucity of both the specific secondary antisera (15 nm gold particles) and the non-specific control antisera (5 nm gold particles) (Fig. 4A). When anti-receptor serum was included in the primary incubation with gold conjugated βVLDL, the βVLDL binding decreased (35 nm gold) and the specific secondary antisera labelling increased (15 nm gold) (Fig. 4B, C). At the greatest concentration of anti-receptor serum (1:10 dilution) little βVLDL binding was observed, but there was heavy labelling of the anti-receptor antigens especially in the microvilli and membrane ruffle regions (Fig. 4D).

These general competition observations were quantitated by counting twenty cell profiles from three separate experiments for the presence of βVLDL (35–40 nm gold particles), the receptor antigens (15 nm gold particles) and non-specific immunogold (5 nm gold particles). The quantitation of ligand and antigen labelling is shown for a single experiment in Fig. 5A. The greatest βVLDL-gold conjugate labelling occurred when only the lipoprotein and buffer compromised the primary incubation. Although anti-receptor serum was not included in the βVLDL-gold buffer primary incubation, a limited amount of background labelling with the specific secondary antibody was noted (2 gold particles per cell pro-

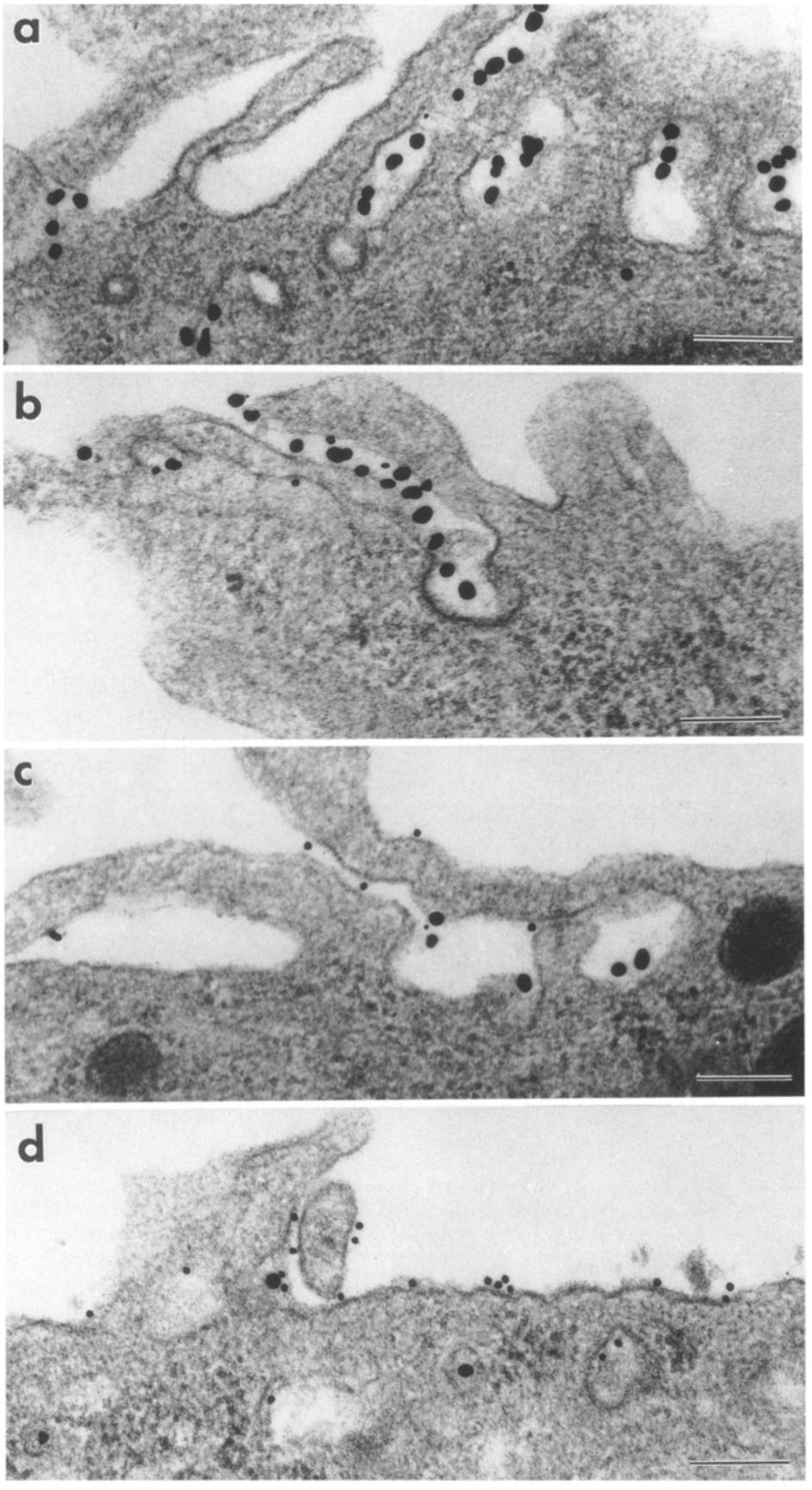

Fig. 4 Thin section TEM demonstrating competition of βVLDL-gold by the antiserum against the LDL receptor/LRP. The primary anti-receptor serum and βVLDL conjugated to 40 nm colloidal gold were applied for 7 min at 25° C, prior to washing and incubating the cells with the second stage antibody. The second stage antibody was a mixture of GAR-15 nm gold conjugate (to recognize the anti-receptor antibody) and GAM-5 nm gold conjugate (which served as an internal control for non-specific binding). The cells were then washed, fixed and processed for TEM. As shown in representative matched micrographs, increasing concentrations of the antiserum resulted in decreased binding of βVLDL-gold (large 40 nm colloidal gold) and an increase in labelling of the LDL receptor (smaller 15 nm gold colloidal gold). Few or no 5 nm gold particles were present in any experiment. **a** βVLDL-gold with buffer, **b** βVLDL-gold 1:500 dilution of the anti-receptor serum, **c** βVLDL-gold with a 1:50 dilution of the anti-receptor serum, and **d** βVLDL-gold with a 1:10 dilution of the anti-receptor serum

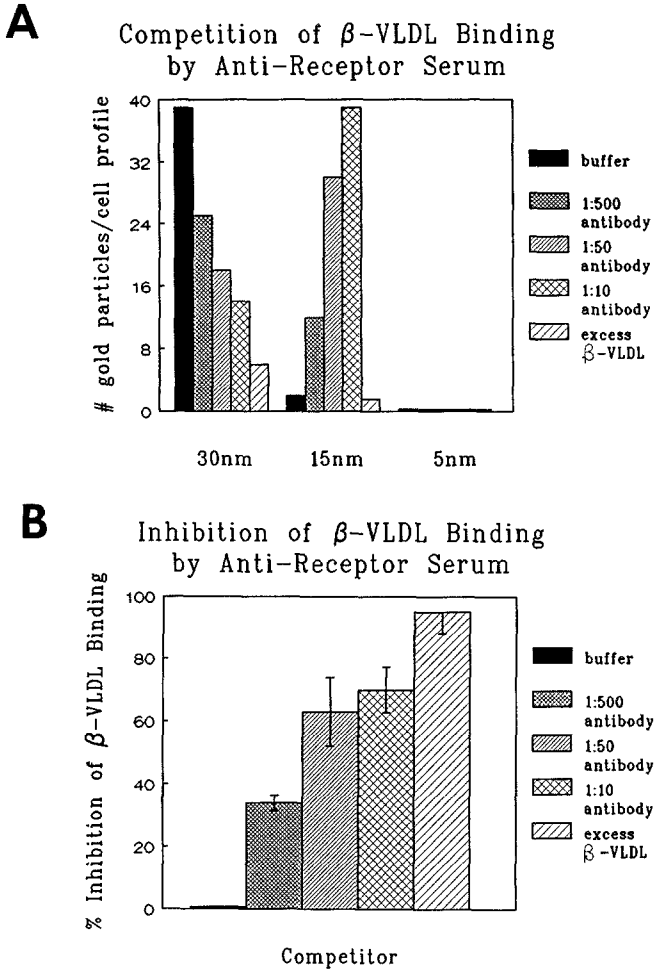

Fig. 5 Competition of βVLDL binding by the anti-receptor. **A** Competition for βVLDL-gold conjugate binding was as described in Fig. 4. Twenty cell profiles were quantitated for the presence of βVLDL (30 nm gold particles), the lipoprotein receptors (15 nm gold particles) and non-specific immuno(gold) binding (5 nm gold particles). A representative experiment is shown. βVLDL binding to the cells was competitively decreased with both increasing concentrations of the antibody against the lipoprotein receptors and with a 40 fold excess of unlabelled βVLDL. Conversely, increasing concentrations of the anti-receptor serum resulted in increased labelling of the LDL receptor. However, the non-specific immunolabelling remained constant in all conditions (0–2 gold per cell profile). **B** Three experiments were averaged for their inhibition of βVLDL-gold conjugates by various concentrations of the antiserum against the anti-receptor serum. Increasing concentrations of antiserum resulted in increasing inhibition of βVLDL-gold conjugates

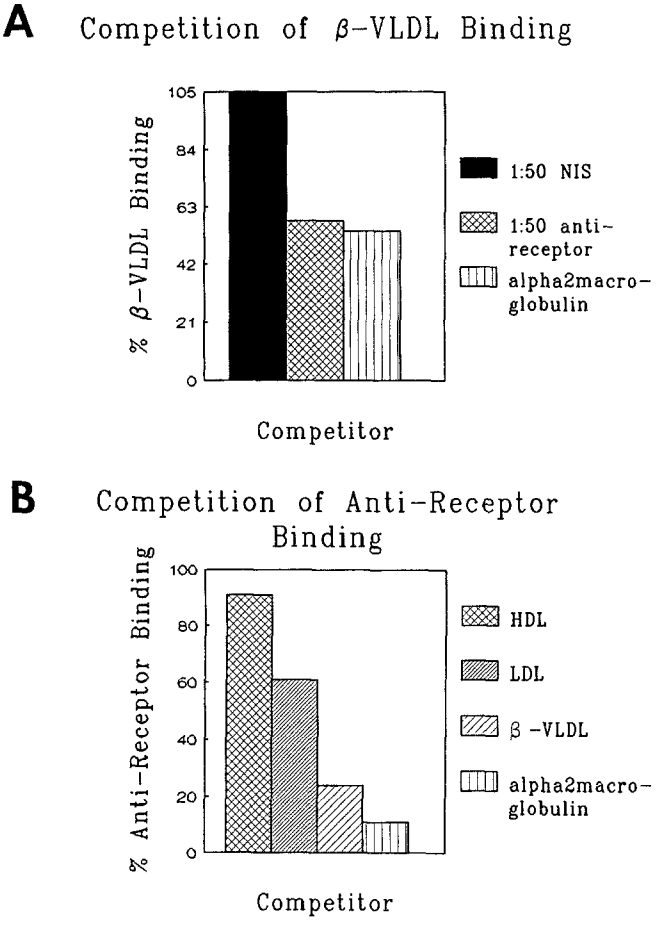

Fig. 6 *Competition studies* **A** 25 μg/ml of βVLDL conjugated to 40 nm gold colloids was incubated with either buffer (not shown), anti-receptor (1:50 dilution), an equal protein concentration of rabbit anti-NIS (1:50 dilution) or 0.2 mg/ml $\alpha_2$macroglobulin. Following this treatment, the cells were washed and then incubated with a second stage antibody which would identify the anti-receptor immunoglobulin (Materials and Methods). The cells were fixed and processed for standard thin sections TEM and quantitated as described in Fig. 5. The NIS did not inhibit βVLDL-gold binding while both the anti-receptor serum (anti-receptor) and $\alpha_2$macroglobulin inhibited βVLDL-gold binding. The results are the mean of two experiments. **B** The primary antibody, anti-receptor (1:50 dilution) was incubated with either buffer, 1 mg/ml of H-HDL, βVLDL, H-LDL, or 0.2 mg/ml of $\alpha_2$macroglobulin. Following this treatment, the cells were washed and then incubated with a second stage antibody which would identify the anti-receptor immunoglobulin and quantitated (see Fig. 5). A single representative experiment is shown. HDL did not inhibit anti-receptor binding while LDL, βVLDL and $\alpha_2$macroglobulin did compete for the receptor antiserum binding

file). The non-specific labelling (GAR-5nm) averaged less than 1 gold particle per cell profile. This was less than 2% of the positive βVLDL control and remained constant for all conditions. When the anti-receptor serum was added to the primary incubation, the number of βVLDL-gold conjugates per cell profile was reduced, while the specific secondary antibody labelling increased. As the concentrations of the anti-receptor serum were increased, the βVLDL labelling proportionally was reduced and the receptor labelling proportionally increased. Excess unlabelled βVLDL was added to the primary incubation to serve as a positive control. When a forty-fold excess of βVLDL was added, the βVLDL-gold conjugate labelling was inhibited by 85%. The anti-receptor label also returned to background levels.

The experiments had different absolute labelling of the receptor antigens and ligand, therefore the three competition experiments were analysed by averaging the percentage inhibition of βVLDL binding for the various conditions. Although the labelling efficiency differed among the three experiments, comparible trends were found. The βVLDL-buffer incubation was set as 100%

binding or 0% inhibition (Fig. 5b). Each increase in concentration of the antisera resulted in an proportional increase in inhibition of βVLDL-gold conjugate binding (Fig. 5). The greatest inhibition of βVLDL binding (70%) was achieved at the anti-receptor serum dilution of 1:10. A 95% inhibition of βVLDL was obtained with a forty-fold excess of unlabelled βVLDL.

It was possible that the anti-receptor serum was inhibiting βVLDL binding by non-specific steric blocking, rather than by typing up receptor specific epitopes. Therefore, a control experiment was done to determine if non-specific immunoglobulins would block βVLDL-gold conjugate binding. Experiments were similar to the above competition studies. Conjugates of βVLDL-gold were co-incubated with either a control buffer, the anti-receptor serum (1:50 dilution) or a matched protein concentration of the non-immune rabbit serum (NIS-1:50 dilution). When compared to control βVLDL binding, the NIS did not inhibit βVLDL-gold conjugate binding (Fig. 6),while the matched protein concentration of the anti-receptor serum inhibited βVLDL-cold conjugate binding by 50%.

The anti-receptor serum recognized two antigens, the LDL receptor and LRP. Binding of the anti-receptor serum to either one of these antigens could have contributed to the inhibition of βVLDL binding. Previous studies have determined that the principal ligand, LDL, inhibits the binding of βVLDL to pigeon macrophages by 40% [16, 17]. We confirmed this observation by competing βVLDL-gold conjugate binding with excess unlabelled LDL (data not shown). These results resubstantiated that the LDL receptor was a receptor for βVLDL on pigeon macrophages. In order to determine the potential contribution of LRP to βVLDL binding, competition experiments pitting βVLDL-gold conjugates against an LRP ligand were done. LRP has previously been shown to not only bind lipoproteins, but is the receptor for $\alpha_2$macroglobulin. Therefore βVLDL-gold conjugate binding was competed using an excess of $\alpha_2$macroglobulin. When $\alpha_2$macroglobulin (0.2 mg/ml) was added during the primary incubation with βVLDL, βVLDL-gold conjugate binding was inhibited by 50% (Fig. 6A). These data suggested that some βVLDL binding to pigeon macrophages is due to the LRP receptor.

Although LDL and $\alpha_2$macroglobulin both compete for βVLDL binding, and the anti-receptor serum, which recognizes both of the receptors for these ligands, inhibits βVLDL binding, it was still possible that the competition with the anti-receptor serum might have been due to only one of these receptors. Therefore, the specificity of the anti-receptor serum was further characterized by competition studies with several pigeon lipoproteins (Fig. 6B). In these studies, the primary incubation included the anti-receptor serum (1:50 dilution) with buffer or buffers containing various pigeon lipoproteins or $\alpha_2$macroglobulin. This was followed by incubation with secondary antibodies to detect the anti-receptor serum. Pigeon LDL was used to determine if the anti-receptor serum bound to the LDL receptor. The LDL (1 mg/ml) competed for anti-receptor serum labelling by 40%, suggesting that the anti-serum recognized the LDL receptor (Fig. 6B). The converse was also true. When gold-conjugated LDL binding was competed by increasing concentrations of the anti-receptor serum, the gold-conjugated LDL binding was likewise inhibited (not shown). Alpha$_2$macroglobulin (1 mg/ml), which binds to LRP, blocked labelling of the anti-receptor serum by 90%. This suggested that anti-receptor serum also blocked the binding of βVLDL to LRP as well as the LDL receptor. Pigeon HDL which does not bind to the LDL receptor or LRP was used as a control lipoprotein. Co-incubation with HDL (1 mg/ml) inhibited the anti-receptor serum labelling by less than 10%. In a matched experiment, βVLDL (1 mg/ml) also competed for the anti-receptor binding by 80%.

In contrast to mammalian βVLDL, pigeon βVLDL does not contain proteins with molecular weights similar to apo E; however, the lipoprotein does contain apo B and apo A1 [4]. Using a series of antibodies against apo E in the present study we were also unable to detect proteins antigenically similar to apo E in the pigeon βVLDL particle (data not shown). The presence of apo B presumably explains the ability of pigeon βVLDL to bind to the LDL receptor. However, the absence of apo E-like protein raises question regarding the mechanism for the binding of the βVLDL particle to LRP. Conceivably, another protein, lipoprotein lipase could play a role since it has been shown to enhance the binding of mammalian lipoproteins to mammalian LRP [6, 8]. Therefore, we tested pigeon βVLDL for the presence of lipoprotein lipase. Equal concentrations of pigeon βVLDL, LDL, HDL and rabbit βVLDL were dot blotted to nitrocellulose paper and probed with antibodies against bovine milk lipoprotein lipase. Lipoprotein lipase was present in pigeon βVLDL, LDL and rabbit βVLDL, but not in pigeon HDL. Rabbit βVLDL had the greatest reaction for lipoprotein lipase. However, lipoprotein lipase was consistently present on several isolates of pigeon βVLDL and LDL.

Discussion

Beta-VLDL and LDL receptor antigens co-localize to microvilli and membrane ruffles which are induced on monocyte-derived macrophages by the ligand (βVLDL). The anti-serum made against the EGF portion of the LDL receptor recognized these peptides isolated from pigeon monocyte-derived macrophage membranes. Included were the LDL receptor, LRP, and what appeared to be a dimer of the LDL receptor [10]. The pigeon LDL receptor, its dimer and LRP also were identified by their ability to bind calcium. Competition with a forty-fold excess native βVLDL during the incubation of biotinylated pigeon βVLDL inhibited recognition of these two proteins on ligand blots. The binding of biotinylated βVLDL to the 116 kDa and 600 kDa proteins was also inhibited when a 1:50 dilution of the anti-receptor serum was added during the ligand binding (not shown). These

results suggest that the 116 kDa and 600 kDa protein, respectively are the LDL receptor and LRP.

Pigeon βVLDL binds to both the pigeon LDL receptor and LRP. The mammalian LDL receptor has binding sites for two apoproteins, apo B and apo E and, consequently, two lipoproteins, LDL and βVLDL [10, 27]. In contrast to mammalian βVLDL, pigeon βVLDL contains no apo E; however, it does contain apo B and apo A1 [4]. Although apo A1 may potentially contribute to the binding of βVLDL to receptors, the inability of pigeon HDL to compete with the binding of either βVLDL [16] or the anti-receptor serum to pigeon macrophages makes this possibility unlikely. This suggests by default that the apo B component of pigeon βVLDL is responsible for mediating the binding of the βVLDL particle to the pigeon LDL receptor [2]. The ability of pigeon βVLDL to bind LRP was surprising due to the absence of apo E in pigeon lipoproteins. Based upon two lines of evidence apo E has been reported to be involved in lipoprotein binding to mammalian LRP. These two lines are; through ligand blotting of apo E enriched VLDL and chemical cross-linking of apo E-phospholipid liposomes to LRP (respectively, [5, 22]. Lund et al. [26], however, indicated through the use of ligand blotting involving apo E depleted rabbit βVLDL and LRP, that apo E may not be required. Therefore, it is possible that βVLDL binding to LRP is mediated through apo B.

Another candidate protein to facilitate binding of pigeon βVLDL or LRP is lipoprotein lipase. More than a decade ago, lipoprotein lipase was shown to increase the uptake of VLDL particles by macrophages. This enhancement was independent of lipolytic activity [25]. Recently, lipoprotein lipase has been shown to enhance binding of normal human triglyceride-rich lipoprotein particles to fibroblasts lacking the LDL receptor [8]. This binding was blocked by competitors for LRP, $\alpha_2$macroglobulin and the 39-kDa receptor-associated protein. Furthermore, lipoprotein lipase promoted binding of lipoproteins to LRP in an in vitro assay [8]. Beisiegel et al. [6] demonstrated that lipoprotein lipase enhanced binding of chylomicrons and βVLDL to LRP, while Willnow et al. [32] showed that LPL-enhanced βVLDL binding to FH fibroblasts (lacking the LDL receptor) could be blocked by a recombinant 39 kDa receptor associated protein as well as an antibody against LRP. These studies again suggest that lipoprotein lipase plays a role in the binding of lipoproteins to LRP. Therefore, it is feasible that pigeon βVLDL binding to pigeon LRP could be via lipoprotein lipase.

The anti-receptor serum used in the present study not only recognized both the LDL receptor and LRP, but it also inhibited binding of the ligand, βVLDL. This antiserum however, recognized the EGF precursor region (motif B) rather than the ligand "binding" region. The ligand "binding" domain of the LDL receptor is in the class A motif, which is the region with homology to complement proteins [10, 27]. Previous studies, however, have shown that the EGF region has affinity for apo B (LDL). Substitutions or deletions of the EGF portion of the mammalian LDL receptor resulted in a loss of recognition for LDL (apo B) but retained recognition for βVLDL (apo B/apoE) on the cell surface [10]. An LDL receptor mutant which lacked the EGF precursor region lost the ability to bind LDL on the cell surface, but the denatured protein bound LDL in a ligand blotting experiment [9]. Davis et al. [9] suggested that the EGF portion, may not actually bind LDL, but is necessary for facilitating the apo B (LDL) interaction with the ligand-binding region of the LDL receptor. The present study suggests that the EGF portion of the receptor is necessary for pigeon βVLDL (apo B) binding. However, this study could not separate whether EGF precursor region was a ligand binding domain or facilitated the apo B interaction with the ligand binding region.

Although we did not separate contributions of the LDL receptor from LRP, both appear to be involved in pigeon βVLDL binding. Recently, LRP has also been reported to be the $\alpha_2$macroglobulin receptor [23, 29]. This result is very interesting in light of the morphometric data of βVLDL binding and receptor binding. Previously, the $\alpha_2$macroglobulin receptors were shown to localize in coated pits at the base of microvilli [31]. This pattern of localization for $\alpha_2$macroglobulin is similar to the distribution we have found for both the ligand βVLDL and its receptor antigens on pigeon macrophages. Our results indicate that $\alpha_2$macroglobulin can inhibit gold conjugated βVLDL. These results suggest that βVLDL utilizes both LRP and the LDL receptor on pigeon macrophages.

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