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Very low density lipoprotein receptor from mammary gland and mammary epithelial cell lines binds and mediates endocytosis of M_r 40,000 receptor associated protein

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Abstract We here report that the M_r 40,000 receptor associated protein (RAP), previously found to bind to α_2-macroglobulin receptor/low density lipoprotein receptor related protein (α_2MR/LRP) and glycoprotein 330 (gp330), binds to an M_r 105,000 membrane protein from bovine mammary gland, human mamma tumors and mammary epithelial cell lines. We have purified this protein from bovine and human sources. N-terminal amino acid sequencing and immunoblotting analyses showed that the protein was identical or closely related to very low density lipoprotein receptor (VLDL-R). Experiments with the human mamma carcinoma cell line MCF-7 showed that this receptor was able to mediate an efficient endocytosis of RAP. These novel findings strongly suggest that RAP functions as a modulator of ligand binding to VLDL-R, similarly to α_2MR/LRP and gp330.

Key words: Endocytosis; Cell membrane; Ligand blotting; MCF-7 cell

1. Introduction

The low density lipoprotein receptor (LDL-R) family of cell membrane receptors currently includes LDL-R itself, α_2-macroglobulin receptor/low density lipoprotein receptor related protein (α_2MR/LRP), glycoprotein 330 (gp330), and very low density lipoprotein receptor (VLDL-R). They are endocytosis receptors and participate in clearance of lipoproteins, proteinase/inhibitor complexes and a variety of other compounds from the extracellular space and cell surfaces. LDL-R and VLDL-R have M_rs between 100,000 and 200,000, while α_2MR/LRP and gp330 have M_rs around 600,000. All have a small, C-terminal cytoplasmic domain, a single trans-membrane domain, and a large, ligand-binding extracellular part. Alpha$_2$MR/LRP differs from the others by being split into two non-covalently linked polypeptide chains, encoded by a single mRNA: an M_r 515,000 α-chain, containing most of the extracellular portion and being responsible for ligand binding, and an M_r 85,000 β-chain, spanning a small part of the extracellular domain, the trans-membrane domain and the cytoplasmic portion (for reviews see [1,2]).

During purification of α_2MR/LRP, a copurifying M_r 40,000 receptor associated protein (RAP) was discovered [3,4]. A cDNA representing human RAP was cloned by Strickland et al. [5]. RAP is now known to bind with high affinity to α_2MR/LRP and gp330. Two or more RAP molecules are bound per receptor molecule (see [2]). A low affinity binding to LDL-R has been reported [6]. RAP inhibits the binding of all other currently known ligands to α_2MR/LRP and gp330, but its physiological function remains to be established (see [2]).

In the present communication, we describe that RAP binds to VLDL-R from mammary tissue and cell lines, and that VLDL-R is able to mediate endocytosis of RAP in a cell line derived from mammary epithelium.

2. Materials and methods

2.1. Proteins, antibodies and various materials

Recombinant human RAP was prepared as described earlier [7]. Human α_2-macroglobulin-methyl amine (α_2M*) [8] was a gift from Dr. L. Sottrup-Jensen, Department of Molecular Biology, University of Aarhus, Denmark. Alpha$_2$MR/LRP [7] was a gift from Drs. A. Nykjær and J. Gliemann, Department of Medical Biochemistry, University of Aarhus, Denmark.

Labelling of proteins with ^{125}I was done as described [7,9]. The specific activities were around 2.5×10^6 Ci/mol.

A rabbit polyclonal antibody, raised against a synthetic peptide ASVGHTYPAISVVSTDDDLA, which represents the C-terminus of rabbit and human VLDL-R [10,11], was a gift from Drs. M.Z. Kounnas and D.K. Strickland, American Red Cross, Washington, USA.

Human tumor specimens were obtained from Dr. L. Christensen, Rigshospitalet, Copenhagen, Denmark.

ProBlott membranes and reagents used for protein sequencing were purchased from Applied Biosystems (Foster City, CA, USA). The ECL immunoblotting detection kit was from Amersham, Little Chalfont, UK. All other chemicals and materials were those previously described [7,9,12–14], or of the best grade commercially available.

2.2. Preparation of cell membranes

Large scale-preparation of cell membranes from bovine mammary gland was performed with tissue obtained from newly slaughtered cows at the local slaughterhouse. All procedures were performed at 0–4°C. Approximately 0.5 kg of tissue was rinsed with phosphate-buffered saline (PBS; 10 mM NaH_2PO_4, 0.15 M NaCl), finely minced and then homogenized by the use of an Ultra-Turrax (25,000 rpm, 3 min) in a

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Abbreviations: α_2M*, α_2-macroglobulin-methyl amine; α_2MR/LRP, α_2-macroglobulin receptor/low density lipoprotein receptor-related protein; CHAPS, 3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propanesulfonate; gp330, glycoprotein 330; LDL-R, low density lipoprotein receptor; RAP, M_r 40,000 receptor associated protein; PBS, phosphate-buffered saline; PMSF, phenyl-methyl-sulfonyl flouride; PVDF, polyvinylidine diflouride; SDS-PAGE, sodium dodecyl sulphate polyacryl amide gel electrophoresis; TCA, trichloroacetic acid; VLDL-R, very low density lipoprotein receptor.

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buffer of 10 mM HEPES, pH 7.4, 250 mM sucrose, 5 mM EDTA and 0.5 mM phenyl-methyl-sulfonyl flouride (PMSF), using 2 ml buffer per 1 g wet weight of tissue. Cell debris and nuclei were sedimented by centrifugation at 2,300 × *g* for 5 min. Membrane fragments were precipitated by centrifugation at 48,000 × *g* for 40 min. The pellet was rehomogenized in 200 ml of a buffer of 10 mM NaH_2PO_4, pH 7.8, 140 mM NaCl, 0.6 mM $CaCl_2$ and 0.5 mM PMSF, and centrifuged as above. The pellet represented the membrane preparation.

Small-scale preparation of cell membranes from cultured cells and samples of human tissues were performed similarly, but using 10 ml buffer per 1 g wet weight of cells and tissues during homogenization.

2.3. Purification of RAP-binding proteins from cell membranes

Cell membranes were solubilized by homogenization in 1–2 l of the following buffer: 20 mM HEPES, 2.5 mM NaH_2PO_4, pH 7.4, 124 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$ and 0.5 mM PMSF, with 0.6% 3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propanesulfonate (CHAPS). The solution was centrifuged at 10,000 × *g* and passed through a 1.2 μm filter to remove insoluble material.

Cell membrane extract was applied to a 4 ml column of Sepharose 4B coupled with approximately 10 mg RAP. The column was equilibrated in the buffer also used for solubilizing the membranes. The column was washed with 0.1 M Tris, pH 7.8, 0.14 M NaCl, 2 mM $CaCl_2$, 0.6% CHAPS. Bound protein was eluted with 0.1 M CH_3COOH, pH 4.0, 0.5 M NaCl, 10 mM EDTA and 0.6% CHAPS. The eluate was neutralized with 0.1 volumes of 1.0 M Tris, pH 9.0. Approximately 0.5 mg protein was obtained from 0.5 kg of bovine mammary gland by 8 consecutive runs of the membrane extract over the column.

2.4. Ligand blotting and immunoblotting analyses

Proteins were resolved by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE; see below) and transferred electrophoretically to polyvinylidene diflouride (PVDF) filters, which were then incubated with approximately 30 pM of ^{125}I-labeled RAP. After washing, bound ligands were visualized by autoradiography [14].

The same filters were used for immunoblotting analysis, following a standard procedure with a primary polyclonal rabbit antibody, a secondary peroxidase-conjugated antibody and visualization of bound antibody with the ECL immunoblotting detection kit.

2.5. Cell culture, whole cell ligand binding, and degradation experiments

The following cell lines were cultured as described previously [14,15]: the human breast carcinoma cell line MCF-7 (ATCC HTB 22); the human breast carcinoma cell line T47D (ATCC HTB 133); the bovine mamma epithelial cell line BMGE (a gift from Dr. W.W. Franke, German Cancer Research Center, Heidelberg, Germany [16]); COS-1 african green monkey kidney cells (ATCC CRL 1651).

For whole cell binding assays of RAP, cell monolayers were incubated with 5 pM [^{125}I]RAP with or without 100 nM non-radioactive RAP at 4°C for 16 h, by which time the cell associated radioactivity was determined [14].

Cellular endocytosis of RAP was estimated from the degradation by the cells of radiolabelled RAP into trichloroacetic acid (TCA)-soluble material. Briefly, serum-free cultures of confluent cells were incubated with around 5 pM [^{125}I]RAP for 16 h at 4°C, in order to allow the ligand to bind to the cells. The cells were then transferred to 37°C, and at various time points thereafter, intact and degraded ligand in the media were determined as the TCA-precipitable and -soluble radioactivity, respectively [14].

Trypsin treatment of MCF-7 cells was performed by exposing confluent cultures to 200 μg/ml trypsin in PBS at 37°C for 20 min. The trypsin was then inactivated by soy bean trypsin inhibitor (500 μg/ml). Control cultures were incubated in parallel in the same buffer without trypsin. Both sets of cultures were harvested by scraping and centrifugation.

2.6. Various procedures

SDS-PAGE and silver staining were performed by standard methods in 4–16% gradient gels. The following M_r markers were used: myosin (M_r 200,000); *E. coli* β-galactosidase (M_r 116,000); phosphorylase b (M_r 97,400); bovine serum albumin (M_r 66,200); ovalbumin (M_r 43,000). For N-terminal amino acid sequencing, proteins were resolved by SDS-PAGE, electroblotted onto a ProBlott filter, and sequenced directly [17]. Automated Edman degradation was carried out with an ABI 477A/120A protein sequencer (Applied Biosystems), using standard programs.

3. Results

3.1. RAP-binding proteins in cell membranes from mammary gland and mammary epithelial cell lines

Fig. 1 shows ligand blotting analysis of RAP binding to membrane proteins from bovine mammary gland, human primary breast tumors and lymph node metastases, and human and bovine mammary epithelial cell lines. The breast tumor samples shown in Fig. 1 were representative of a total of 7 primary tumors, 2 lymph node metastases and 5 benign tumors analyzed.

Most samples showed a RAP binding activity caused by the α_2MR/LRP α-chain, as judged from comigration with the α-chain of purified α_2MR/LRP. In addition, a previously uncharacterized RAP binding activity migrating with an apparent M_r of 105,000 was present in all samples in varying amounts. The binding of ^{125}I-RAP to this protein was, similarly to the binding to α_2MR/LRP α-chain, Ca^{2+} dependent and abolished by 100 nM non-radioactive ligand (data not shown).

Minor bands between the two major ones may represent degradation products of α_2MR/LRP α-chain; the weak RAP binding activity of LDL-R, migrating at M_r 130,000 [18]; or other, unknown RAP-binding proteins. gp330, migrating slower than α_2MR/LRP α-chain [19], was not observed.

3.2. Purification and characterization of RAP-binding proteins from bovine mammary gland and MCF-7 cells

The RAP-binding proteins from bovine mammary gland and from MCF-7 cells were purified by single step affinity chromatography with RAP immobilized on Sepharose. Analysing the purified fractions by RAP ligand blotting analysis, a band pattern similar to that in the cell membrane preparations was found (Fig. 2A).

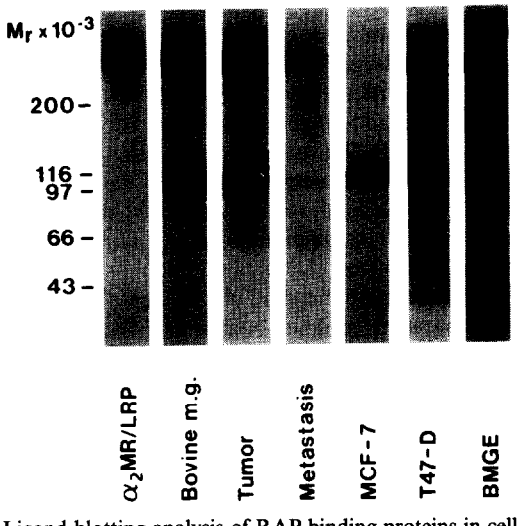

Fig. 1. Ligand blotting analysis of RAP binding proteins in cell membranes from mammary glands and mammary epithelial cell lines. Fifty ng α_2MR/LRP and cell membrane samples corresponding to 5 μg protein from the indicated sources were resolved by SDS-PAGE in 4–16% gradient gels and assayed for [^{125}I]RAP binding by ligand blotting analysis. M_r markers are indicated on the left. m.g. = mammary gland.

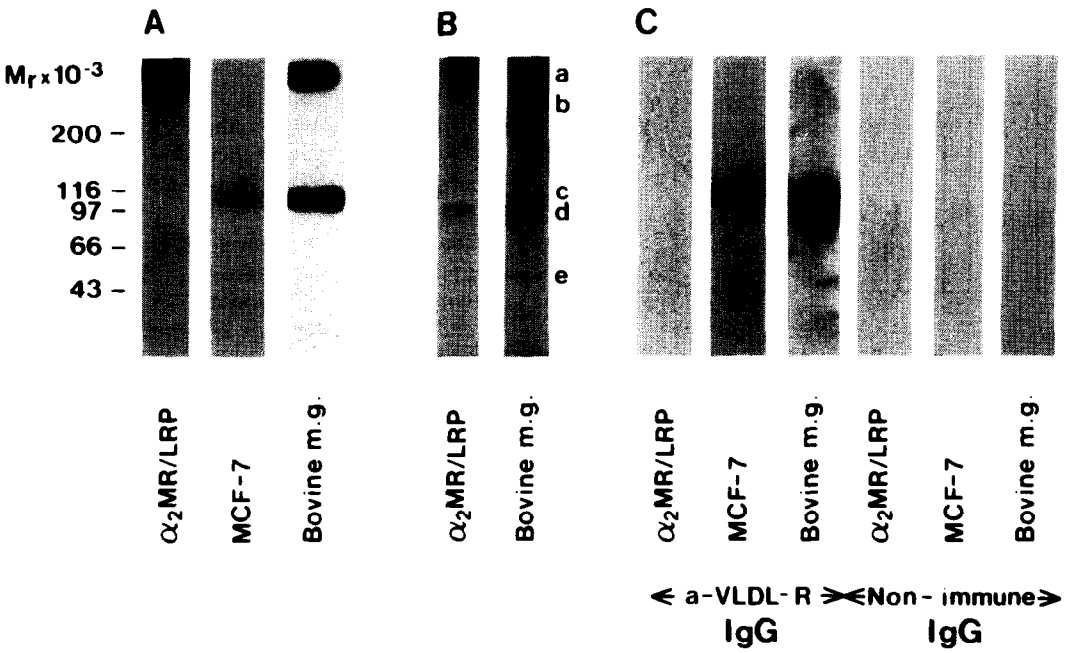

Fig. 2. Purification and characterization of RAP-binding proteins from bovine mammary gland and MCF-7 cells. Purified α_2MR/LRP, purified RAP binding proteins from MCF-7 cells and from bovine mammary gland were subjected to SDS-PAGE in 4–16% gradient gels. The lanes contain 50 ng α_2MR/LRP, 65 ng RAP-binding protein from bovine mammary gland or 8 ng RAP-binding protein from MCF-7 cells. (A) Shows ligand blotting analysis with ^{125}I-RAP, (B) silver staining and (C) immunoblotting analysis with rabbit polyclonal anti-VLDL-R IgG or non-immune rabbit IgG. The lettering of the bands on the silver stained gels of purified RAP-binding proteins from bovine mammary gland (a–e) refer to discussion in the text. M_r markers are indicated on the left. m.g. = mammary gland.

Due to the ready access to large quantities of tissue, the preparation of RAP binding proteins from bovine mammary gland was used for protein sequencing. SDS-PAGE and silver staining of the preparation showed 5 polypeptides (Fig. 2B). Bands a and d comigrated with the α- and the β-chain of purified α_2MR/LRP, respectively. The identity of band d was confirmed by N-terminal amino acid sequencing, the N-terminal 9 amino acids showing a 100% identity to the β-chain of human α_2MR/LRP (data not shown). Band e migrates close to recombinant human RAP (not shown), and this band probably represents copurifying RAP. Band b (M_r 400,000) was not identified.

The sequence of the N-terminal 14 amino acids of band *c*, comigrating with the M_r 105,000 RAP binding activity, is shown in Table 1. No PTH derivatives were detected in cycle 9. Of the remaining 13 amino acids, 10 and 9 were identical to the amino acids in the corresponding positions in human and rabbit VLDL-R, respectively [10,11]. Bovine VLDL-R has not yet been cloned. All differences between the bovine M_r 105,000 RAP binding protein and human and rabbit VLDL-R could be accounted for by single nucleotide changes. The fact that no PTH derivative was detected in cycle 9 was in agreement with expectances if the amino acid in that position is a Cys involved in a disulfide bridge, as in human and rabbit VLDL-R. The purified protein shows a much lower identity to bovine, human, rabbit, and hamster LDL-R. The sequence was not compatible

Table 1
N-terminal amino acid sequences of bovine M_r 105,000 RAP binding protein and VLDL-R and LDL-R from various species

Protein	Cycle													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Bovine M_r 105,000 RAP binding protein	T	G	A	G	R	K	A	K	X*	E	A	N	Q	F
Human VLDL-R**	T***	G	T	G	R	K	A	K	C	E	P	S	Q	F
Rabbit VLDL-R	T	A	T	G	R	K	T	K	C	E	A	S	Q	F
Bovine LDL-R				A	V	E	D	N	C	G	R	N	E	F
Human LDL-R	A	G	T	A	V	G	D	R	C	E	R	N	E	F
Rabbit LDL-R	A	G	A	A	A	G	D	K	C	G	R	N	E	F
Hamster LDL-R	A	G	A	A	A	E	D	T	C	D	R	N	E	F

*No PTH derivative was found in step 9; see the text for a further discussion.
**The sequences of the VLDL-Rs and LDL-Rs were obtained from the following sources: human VLDL-R cDNA [11]; rabbit VLDL-R cDNA [10]; bovine LDL-R protein [26]; human LDL-R cDNA [27]; rabbit LDL-R cDNA [28]; hamster LDL-R cDNA [29].
***The underlined amino acids in the VLDL-Rs and LDL-Rs are those that are identical to the amino acids in the corresponding positions in bovine M_r 105,000 RAP binding protein.

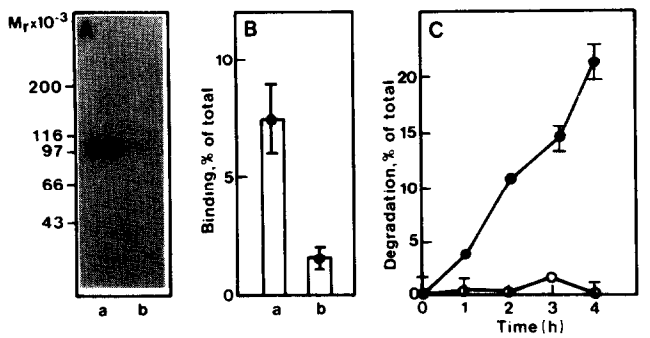

Fig. 3. Binding and degradation of RAP in MCF-7 cells. (A) Effect of trypsin treatment on the content of VLDL-R in MCF-7 cell membranes. Cell membrane samples corresponding to 5 μg of total protein from control MCF-7 cells (a) or trypsin-treated MCF-7 cells (b) were resolved by SDS-PAGE in 4–16% gradient gels and assayed for RAP binding by ligand blotting analysis. M_r markers are indicated on the left. (B) Whole cell binding of ^{125}I-RAP to MCF-7 cells. 5 pM ^{125}I-RAP was incubated with MCF-7 cell monolayers without (a) or with (b) 100 nM non-radioactive RAP for 16 h at 4°C. The cell bound fraction (mean ± S.D. of triple determinations) are indicated. (C) Assay of degradation of RAP and α_2M* by MCF-7 cells. Serum-free cultures of confluent MCF-7 cells were incubated for 16 h at 4°C with 5 pM ^{125}I-RAP (●) or 5 pM ^{125}I-α_2M* (○). After transfer to 37°C, the percentages of degraded ligand in the media was determined at the indicated time points. Means and standard deviations of triple determinations are shown. The experiment shown is a typical one out of a total of 6. The amounts degraded in the presence of 100 nM non-radioactive RAP has been subtracted from all values.

with the protein being a degradation product of α_2MR/LRP or gp330. No sequences besides those of the M_r 105,000 RAP binding protein and α_2MR/LRP β-chain was detectable in the M_r 100,000 region of the gel.

Immunoblotting analysis with a polyclonal rabbit peptide antibody against the C-terminus of human and rabbit VLDL-R revealed specific immunoreactivity comigrating with the M_r 105,000 RAP binding activity in the preparations from bovine mammary gland and MCF-7 cells (Fig. 2C).

We conclude that the purified M_r 105,000 RAP binding protein from bovine mammary gland and MCF-7 cells is identical or closely related to VLDL-R.

3.3. Binding and endocytosis of RAP by MCF-7 cells

In ligand blotting analysis (see Figs. 1 and 2), all detectable RAP binding activity in MCF-7 cells comigrated with the VLDL-R immunoreactivity, even with prolonged exposures of autoradiographic films. We estimate from these data that VLDL-R constitutes more than 95% of the RAP binding activity of MCF-7 cells, and the data are in agreement with MCF-7 cells being devoid of α_2MR/LRP, gp330 and putative RAP binding activity of LDL-R. α_2MR/LRP was also undetectable in MCF-7 cells by immunoblotting analysis with monoclonal antibodies against both the α-chain as well as the β-chain (data not shown). Trypsin treatment of MCF-7 cells led to digestion of VLDL-R, as revealed by RAP ligand blotting analysis (Fig. 3A). This shows that this receptor is exposed at the cell surface. Whole cell binding assays revealed that the cells were able to bind RAP at 4°C (Fig. 3B). In contrast, there was no measurable binding (< 3% of the RAP binding) of α_2M rendered α_2MR/LRP-binding by methyl amine treatment (α_2M*) [20,21]. Parallel control experiments were performed with the same preparations of radiolabelled RAP and α_2M* and COS-1 cells, expressing abundant α_2MR/LRP and devoid of other RAP binding proteins [12]. The binding of α_2M* to COS-1 cells amounted to 55% of the RAP binding (data not shown). These results confirm the absence of α_2MR/LRP in MCF-7 cells. These cells therefore seemed well suited for investigating whether VLDL-R was able to mediate endocytosis of RAP.

The ability of the cells to endocytose RAP was estimated from the ensuing conversion of [^{125}I]RAP to TCA-soluble radioactivity during incubation at 37°C. MCF-7 cells were found to endocytose RAP quite efficiently, around 20% of the total amount added being degraded in 4 h. There was no measurable endocytosis (< 1%) of α_2M* (Fig. 3C), in agreement with the binding results. In control experiments with COS-1 cells, the α_2M* degradation was found to be 75% of that of RAP. We conclude that VLDL-R is able to mediate endocytosis of RAP.

4. Discussion

In the present communication, we describe an M_r 105,000 RAP binding protein from mammary gland and mammary epithelial cell lines, which is identical or closely related to VLDL-R, as judged by its N-terminal amino acid sequence and its reactivity with a peptide antibody against the C-terminus of VLDL-R. The observed M_r agrees with that expected from the cDNAs for rabbit and human VLDL-R [10,11].

VLDL-R was the fourth mammalian member of the LDL-R family to be identified [10,11], after LDL-R itself, α_2MR/LRP and gp330 (see [2]). The extracellular ligand-binding part of the prototype of the family, LDL-R, contains, mentioned from the N-terminus, a cluster of 7 complement type repeats, an EGF precursor homology domain, and a domain with O-linked sugars. VLDL-R has a similar structure, the overall amino acid identity between the two receptors being around 40%. However, VLDL-R has an additional complement type repeat [10,11]. gp330 has only been partially sequenced. The ligand binding α_2MR/LRP α-chain has several clusters of complement type repeats and EGF precursor domains (see [2]). A large cluster of 8 complement type repeats near the N-terminus of a2MR/LRP has been shown to be indispensable for binding of RAP [22,23]. Although LDL-R, with 7 complement-type repeats, has been reported to possess a weak RAP-binding activity [6], the presence of a cluster of 8 repeats like in VLDL-R, α_2MR/LRP and possibly gp330, may be a crucial feature in conferring high RAP binding affinity.

In the cytoplasmic portion, LDL-R and VLDL-R have one, and α_2MR/LRP and gp330 two copies of the NPXY sequence needed for rapid endocytosis via clathrin-coated pits [2], in agreement with the functions of these receptors in endocytosis. One function of VLDL-R is believed to be endocytosis of apolipoprotein E-containing lipoprotein particles [10,11], but the ligand binding specificity of VLDL-R has only been incompletely elucidated. The observation that VLDL-R resembles α_2MR/LRP and gp330 with respect to RAP binding suggest that the three receptors may share other ligands too.

The physiological function of RAP is unknown. Sequence analysis of RAP protein and cDNA shows that it has a signal peptide that is cleaved post-translationally [5]. RAP has been demonstrated in endoplasmic reticulum, but is not found in blood plasma or extracellularly in RAP synthesizing cell lines

(see [2]). This is in agreement with the fact that RAP has a C-terminal endoplasmic reticulum retrieval signal (see [24]). As judged from pulse-chase studies, RAP interacts with α_2MR/LRP and gp330 before they reach the cell surface [5,25]. It therefore appears that its function in relation to these receptors may be to act as a chaperone or an intracellular modulator of ligand/receptor interaction. It does not appear to be a normal extracellular ligand for the receptors. Whatever the function may be, the present findings suggest that it involves interactions with VLDL-R as well as with α_2MR/LRP and gp330.

The present description of VLDL-R in mammary epithelial cell lines does not necessarily imply its presence in breast epithelium in vivo. Immunohistochemical studies will be able to reveal the relative cellular localization of VLDL-R and α_2MR/LRP in normal and malignant mammary tissue. In general, VLDL-R may function as an endocytosis receptor for a variety of ligands in cell types different from those expressing α_2MR/LRP and gp330.

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Note added in proof

After submission of this manuscript, another paper was published describing similar findings (Battey et al. (1994) The 39-kDa receptor-associated protein regulates ligand binding by the very low density lipoprotein receptor, J. Biol. Chem. 269, 23268–23273).