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Functional domains of the very low density lipoprotein receptor: molecular analysis of ligand binding and acid-dependent ligand dissociation mechanisms

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SUMMARY

The very low density lipoprotein (VLDL) receptor is closely related in structure to the low density lipoprotein receptor. The ectodomain of these endocytic receptors is composed of modules which include clusters of cysteine-rich class A repeats, epidermal growth factor (EGF)-like repeats, tyrosine-tryptophan-threonine-aspartic acid (YWTD) repeats and an O-linked sugar domain. To identify important functional regions within the ectodomain of the VLDL receptor, we produced a mutant receptor in which the EGF, YWTD and O-linked sugar domains were deleted. Cells transfected with the mutant receptor were able to bind and internalize ^{125}I-labeled receptor associated protein (RAP). In contrast to the wild-type receptor, however, RAP did not dissociate from the mutant receptor and consequently was not degraded. Immunofluoresence data indicated that once bound to the mutant receptor, fluorescent-labeled RAP co-localized with markers of the endosomal pathway, whereas, in cells expressing the wild-type receptor, RAP fluorescence co-localized with lysosomal markers. Thus this deleted region is responsible for ligand uncoupling within the endosomes. To identify regions responsible for ligand recognition, soluble receptor fragments containing the eight cysteine-rich class A repeats were produced. ^{125}I-RAP and ^{125}I-labeled urokinase-type plasminogen activator:plasminogen activator inhibitor type I (uPA:PAI-1) complexes bound to the soluble fragment with $K_{D,app}$ values of 0.3 and 14 nM, respectively. Deletion analysis demonstrate that high affinity RAP binding requires the first four cysteine-rich class A repeats (L1-4) in the VLDL receptor while the second repeat (L2) appears responsible for binding uPA:PAI-1 complexes. Together, these results confirm that ligand uncoupling occurs via an allosteric-type mechanism in which pH induced changes in the EGF and/or YWTD repeats alter the ligand binding properties at the amino-terminal portion of the molecule.

Key words: VLDL receptor, Urokinase, RAP, Endocytosis, Immunofluorescence

INTRODUCTION

The low density lipoprotein (LDL) receptor family consists of structurally related endocytic receptors that include the LDL receptor, apoE receptor 2, LDL receptor-related protein (LRP also know as LRP-1), megalin (also known as gp330 or LRP-2) and the very low density lipoprotein (VLDL) receptor. Certain members of this receptor family appear to be highly conserved, and *Caenorhabditis elegans* forms of megalin (Yochem and Greenwald, 1993), LRP (Ainscough et al., 1998) and the VLDL receptor (Ainscough et al., 1998) have been described.

The VLDL receptor was identified by homology cloning (Takahashi et al., 1992) and is most abundant in skeletal muscle, heart, adipose tissue, and brain (Gafvels et al., 1993; Sakai et al., 1994; Webb et al., 1992). Subsequent studies confirmed that this receptor is expressed in the endothelium (Wyne et al., 1996; Multhaupt et al., 1996) and in macrophages present in human atherosclerotic lesions (Multhaupt et al., 1996; Argraves et al., 1997). Initially, the VLDL receptor was thought to function in the delivery of triglyceride-rich lipoproteins to peripheral tissues (Takahashi et al., 1992; Sakai et al., 1994). However, studies in homozygous knockout mice that lack the VLDL receptor gene revealed that the animals had normal levels of plasma triglyceride and cholesterol indicating that in mice the VLDL receptor is likely not a major receptor

involved in lipoprotein catabolism (Frykman et al., 1995). The mice were, however, somewhat smaller than their wild-type littermates. The chicken homologue of the human VLDL receptor, termed LR8 has been shown to play a critical role in mediating the transport of triglycerides into growing oocytes since mutant hens defective in this receptor are characterized by hereditary hyperlipidemia and are unable to lay eggs (Bujo et al., 1994).

All members of the LDL receptor family contain five distinct domains: clusters of cysteine-rich class A repeats, epidermal growth factor (EGF)-like repeats, six modules of approximately 50 amino acid residues containing a YWTD motif that are proposed to form a β-propeller domain (Springer, 1998), a transmembrane domain, and a cytoplasmic region containing single or multiple copies of the consensus tetrapeptide NPXY which is important for coated pit localization. The VLDL receptor is closely related to the LDL receptor both in primary sequence as well as in the intron/exon organization of the gene (Sakai et al., 1994). The primary structural difference between these two receptors is an additional exon in the VLDL receptor gene that encodes for the first cysteine rich class A repeat (L1) found at the amino-terminal region of the VLDL receptor. Thus, the VLDL receptor contains eight cysteine-rich class A repeats in contrast to the seven found in the LDL receptor, and like the LDL receptor also contains an O-linked sugar domain in addition to the EGF-like and YWTD repeats. Multiple forms of the VLDL receptor exist (Sakai et al., 1994) resulting from alternate splicing of the pre-mRNA: type I receptors contain the O-linked sugar domains, while type II receptors lack the O-linked sugar domain. VLDL receptor mRNA which lacks exon 4 (encoding for L3) has also been detected in the brain (Christie et al., 1996).

Despite the structural similarity between the LDL and VLDL receptors, their ligand binding properties differ considerably. The LDL receptor binds apoE and apoB containing lipoproteins, whereas the VLDL receptor binds to apoE-containing lipoproteins, but does not bind to LDL and therefore does not recognize apoB100 (Takahashi et al., 1992, 1995). While both receptors bind Lp(a) (Hofmann et al., 1990; Argraves et al., 1997) they recognize distinct regions of this particle; the LDL receptor recognizes a region on apoB100, whereas the VLDL receptor recognizes a region on apolipoprotein(a) (Argraves et al., 1997). Additionally, the VLDL receptor binds to serpin-enzyme complexes (Argraves et al., 1995; Heegaard et al., 1995), lipoprotein lipase (Argraves et al., 1995; Takahashi et al., 1995) and thrombospondin (Mikhailenko et al., 1997), ligands that do not bind to the LDL receptor. Finally, and importantly, the VLDL receptor binds RAP with high affinity (Battey et al., 1994; Simonsen et al., 1994), whereas RAP binds only weakly to the LDL receptor.

RAP was initially discovered when it co-purified with LRP during ligand affinity chromatography (Ashcom et al., 1990; Jensen et al., 1989). This protein binds tightly to LRP, megalin, and the VLDL receptor and modulates their ligand binding activities (Herz et al., 1991; Williams et al., 1992; Kounnas et al., 1992b; Battey et al., 1994). RAP is found within the endoplasmic reticulum (Biemesderfer et al., 1993; Bu et al., 1995), and disruption of the RAP gene in mice (Willnow et al., 1995) indicates that RAP plays an important role in the early processing of these receptors, perhaps in preventing association of the newly synthesized receptors with ligands, or in regulating receptor transport or trafficking to the cell surface.

The goal of the current investigation was to identify functional regions within the VLDL receptor that allow recognition of diverse, structurally unrelated ligands and to gain insight into how RAP functions to block ligand binding. The present studies demonstrate that high affinity RAP binding requires the first four cysteine-rich class A repeats (L1-4) in the VLDL receptor while the second repeat (L2) appears responsible for binding uPA:PAI-1 complexes. These results are in agreement with a recent study demonstrating that the VLDL receptor variant lacking L3 shows impaired RAP, but not uPA:PAI-1 binding (Rettenberger et al., 1999). Further we show that similar to what has been observed for the LDL receptor (Davis et al., 1987), the EGF and the YWTD modules of the VLDL receptor are necessary for uncoupling of ligands within the endosome confirming an allosteric-type mechanism for ligand uncoupling in this class of receptors.

MATERIALS AND METHODS

Proteins

Human RAP was expressed in bacteria as a fusion protein with glutathione S-transferase as described previously (Williams et al., 1992). Cleavage with thrombin and purification of RAP was carried out as described (Williams et al., 1992). Pro-urokinase (pro-uPA) was provided by Jack Henkin (Abbott Laboratories) and was activated by incubation with plasmin-Sepharose. High molecular mass uPA was then purified by benzamidine-Sepharose. Plasminogen activator inhibitor type I (PAI-1) was generously provided by Dan Lawrence, American Red Cross. RAP and uPA were labeled with [^{125}I]iodine to a specific activity ranging from 2-10 μCi/μg protein using Iodogen (Pierce Chemical Co., Rockford, IL). To generate uPA:PAI-1 complexes, uPA or ^{125}I labeled-uPA were incubated with active PAI-1 (1 to 1.5 molar ratio) for 30 minutes at room temperature in TBS, pH 8.0. Bovine serum albumin, fraction V (BSA) was purchased from Sigma Chemical Co. (St Louis, MO). Cells producing the anti-*myc* IgG 9E10 were obtained from the American Type Culture Collection (Rockville) and the IgG was purified by chromatography on Protein G-Sepharose. R777, a rabbit polyclonal IgG against LRP was affinity purified over LRP-Sepharose as described (Kounnas et al., 1992a)

Construction of cDNAs for mutant VLDL receptors and soluble fragments

The human VLDL receptor *type I* cDNA was provided by J. Strauss (University of Pennsylvania, Philadelphia) and was used as a template to generate the various fragments. A deletion of ligand binding repeats from 2 to 8 to generate the ΔL mutant receptor was produced by PCR amplification of two VLDL receptor fragments. The N-terminal fragment contained the signal peptide sequence and the first ligand binding (type A) repeat. The forward primer (GAATTCCCGCGGATGGGCACGTCCGCGCTCTGG) contained an *Eco*RI site and the reverse primer (GGATCCACAGTTCTTTTCATCACTGCC) contained a *Bam*HI site. The C-terminal fragment started with first EGF-type repeat and contained the cDNA encoding the remainder of the molecule. The forward primer used to generate this fragment (GGATCCCATATAAACGAATGCTTGGTA) contained a *Bam*HI site and the reverse primer (GAATTCTCAAGCTAGATCATCATC) contained an *Eco*RI site. Both PCR products were simultaneously subcloned into the *Eco*RI site of pcDNA3.0 and a clone with the correct orientation of the insert was sequenced to confirm the absence of PCR introduced mistakes. The resulting plasmid, termed pVLDLRΔL, was used for transfection experiments. A similar

approach was used to make a mutant VLDL receptor with deleted EGF repeats, YWTD modules and O-lined sugar domain (ΔEGF mutant). In this case, the first PCR product encoded the VLDLR signal peptide and all of the ligand binding repeats (primers GAATTCCCGCGGATGGGCACGTCCGCGCTCTGG and GGATCCACACTCTTTCAGGGGCTC). The second PCR product encoded transmembrane and intracellular domains (primers GGATCCCCCCCAAAAGGGACTTCTGCC and GAATTCTCAAGCTAGATCATCATC). These two cDNA fragments were subcloned into pcDNA3.0 and resulting plasmid pVLDLRΔEGF was used for transfection. To generate expression vectors for the soluble VLDL receptor fragments, a commercial plasmid pSecTagB (Invitrogen) was utilized. The plasmid contains an Ig *k*-chain leader sequence to ensure the secretion of expressed protein. cDNAs encoding the selected ligand binding domains of the VLDL receptor were produced by PCR and subcloned into *Hin*dIII and *Eco*RI sites of pSecTagB. Fragment designated L1-8 encodes amino acids 1 to 328 of the mature VLDL receptor and contains all eight class A repeats. The other fragments used in this study contain the following amino acid sequences: L1-6, 1-246; L1-4, 1-161; L1-3, 1-122; L1-2, 1-81; L2-8, 41-328; L2-4, 41-161; L2-3, 41-122; L3-6, 82-246; L3-4, 82-161; L5-8, 162-328. All soluble VLDL receptor fragments have *myc*-epitope and polyhistidine tag at their C terminus for detection and purification.

Expression of recombinant forms of VLDL receptor

CHO-K1 cells were transfected with plasmids containing the human VLDL receptor cDNA or cDNA encoding the ΔEGF or ΔL mutant receptors using electroporation (Gene Pulser, Bio-Rad Laboratories). Clones were selected based on their resistance to G418. Expression levels of individual cell lines were analyzed by immunoblotting of cell extracts using R2623, a rabbit anti-VLDL receptor IgG that was prepared against a synthetic peptide representing the carboxyl terminus of the human VLDL receptor (Wittmaack et al., 1995). Soluble fragments of the VLDL receptor ligand binding domain were transiently expressed in Cos-1 cells using calcium phosphate precipitation. Briefly, 100 mm dish at ~60% confluence was cotransfected with 10 μg of pSecTagB containing the cDNA for soluble human VLDL receptor fragments and 5 μg of pcDNA3RAP. Cells were washed 18 hours after transfection and kept in serum containing medium for another 24 hours. Then medium was then replaced with DMEM containing 1% Nutridoma. This medium was harvested after 48 hours of incubation, subjected to immunoblot analysis using anti-*myc* antibody to detect recombinant proteins and used in further experiments. To study the effect of RAP on secretion of VLDL receptor fragments, 10 μg of pSecTagL1-8 was cotransfected with 0, 5 μg or 10 μg of pcDNA3RAP. Empty PcDNA3 plasmid was included in every transfection to adjust the total amount of DNA to 30 μg.

Immunofluorescence experiments

Human transferrin (Bodipy FL conjugate) and fluorescein-dextran (10,000 MW, anionic, lysine fixable) were purchased from Molecular Probes (Eugene, OR). RAP was labeled with Cy3 according to enclosed instructions (Amersham Life Science, Inc. Arlington Heights, IL). To label the lysosomal compartment, dextran fluorescein (10 μg/ml) was incubated with the cells for 15 minutes at 37°C, and then chased for 24 hours in medium. After this period, the cells were incubated for Cy3-labeled RAP (25 μg/ml) for 20 minutes at 37°C prior to washing and fixing. To label the endosomal compartment, cells were incubated with Bodipy-transferrin (200 μg/ml) and Cy3-labeled RAP (25 μg/ml) for 20 minutes at 37°C prior to washing and fixing. Cells were fixed in 3.7% paraformaldehyde for 20 minutes. Confocal microscopy was performed on a Bio-Rad MRC 1024 confocal microscope with a krypton/argon laser (Bio-Rad, Richmond). The excitation and the emission filters were 488 nm and 522 nm for Bodipy/fluorescein and 568 nm and 605 nm for Cy3.

Coimmunoprecipitation assay

Medium from Cos-1 cells transfected with soluble VLDL receptors was concentrated 10 times using centriprep-10 concentrators (Amicon Inc.). Radio-labeled RAP (25 nM final concentration) was mixed with 300 μl of concentrated medium and incubated at 4°C overnight. The medium was then incubated with anti-*myc* IgG (5 μg) and Protein-G Sepharose for 2 hours. The Sepharose was washed, samples were eluted by boiling in reducing SDS buffer and loaded on an 18% gel. The gel was dried and exposed to X-ray film for 1 day.

Solid-phase binding assays

Microtiter wells were first coated with anti-*myc* IgG (10 μg/ml, 100 μl) overnight. The plates were then blocked with 3% BSA for 1 hour and incubated with 100 μl of the medium from transfected cells. Incubation was extended overnight at 4°C. The relative amount of soluble VLDL receptor fragments captured on the microtiter wells was determined by measuring the binding of ^{125}I-labeled anti-his IgG to the wells. The binding we observed was consistent with the amount of fragment present in the medium as estimated by immunoblot analysis. The microtiter wells were then incubated with 10 mM EDTA for 1 hour to remove endogenous RAP, and washed with 10 mM $CaCl_2$, TBS pH 8.0. ^{125}I-labeled RAP or ^{125}I-labeled uPA:PAI-1 complexes at various concentrations was added into wells in 3% BSA, TBS, 5 mM $CaCl_2$, 0.05% Tween-20. After 18 hours incubation at 4°C, the plates were washed and the wells counted in a gamma counter. Non-specific binding was measured in presence of excess of unlabeled ligand and subtracted from total binding to give the specific binding. The data were normalized to the fragment which exhibited the highest anti-his binding ability, and were analyzed by nonlinear regression analysis using the following equation:

$$B = B_{\max}\left(\frac{[L]}{K_{D,app}+[L]}\right),$$

where B is the amount of ligand bound, B_{max} is the amount of ligand bound at saturation, $[L]$ is the molar concentration of free ligand (uPA:PAI-1 complexes or RAP), and $K_{D,app}$ is the apparent dissociation constant.

Cellular-mediated ligand internalization and degradation assays

Parental CHO-K1 cells were cultured in HAMs F-12 medium, 5% FBS, 1% pen/strep while transfected CHO-K1 cells were cultured in the same medium containing G418 (200 μg/ml). Prior to the ligand internalization assay, cells were seeded into 12-well dishes (Corning, Corning, NY) at 2×10^5 cells per well and allowed to grow for 24 hours at 37°C, 5% CO_2. Cellular internalization and degradation assays were conducted as previously described (Mikhailenko et al., 1995). Surface binding, internalization, and degradation of ^{125}I-labeled proteins by cells was measured after incubation for indicated time intervals at 37°C in 0.5 ml HAM's F-12 medium containing 0.3 mg/ml BSA and 0.1 mg/ml anti-LRP IgG (Rb777). Surface binding and internalization is defined as radioactivity that is sensitive or resistant, respectively, to release from cells by trypsin (50 μg/ml) and proteinase K (50 μg/ml) (Sigma) in buffer containing 5 mM EDTA. Degradation is defined as radioactivity in the medium that is soluble in 10% trichloroacetic acid. In all experiments a control was included in which the amount of degraded product generated in the absence of cells was also measured. In single cycle endosytosis experiments cells were preincubated for 1 hour at 37°C in the medium containing blocking anti LRP antibodies, chilled on ice for 45 minutes and than incubated with ^{125}I-labeled RAP (7 nM) at 4°C for 3 hours. Following incubation, the cells were washed, warmed to 37ºC for indicated time intervals and the amount of ^{125}I-labeled RAP that remained on the cell surface, dissociated, internalized and degraded was measured.

RESULTS

Characterization of VLDL receptor deletion mutants

The ectodomain of the VLDL receptor is composed of eight cysteine class A repeats, an EGF precursor homology region composed of three EGF repeats and six YWTD modules that have been suggested to exists as a β-propeller module (Springer, 1998), and an O-linked sugar domain (Fig. 1A). Previous work with the LDL receptor (Davis et al., 1987; Russell et al., 1989) suggested that the class A repeats are important for ligand recognition, while the EGF precursor homology region is important in ligand recognition, for acid-dependent ligand uncoupling, and is necessary for repeated receptor recycling. To gain insight into the function of these modules within the VLDL receptor, deletion mutants of this receptor were constructed (Fig. 1A) and used to transfect cells to generate stable cell lines. The molecule designated ΔL represents a deletion mutant in which cysteine rich class A repeats L2-8 were deleted, while the molecule designated ΔEGF represents a deletion mutant in which the EGF, YWTD, and O-linked sugar domains were deleted. Each mutant receptor contains the VLDL receptor signal peptide to ensure correct processing. Cell lines expressing the wild-type and mutant VLDL receptors were prepared by transfecting Chinese hamster ovary cells with pVLDLR, pVLDLRΔEGF and pVLDLRΔL, respectively. To analyze the expression of these mutant receptors in the transfected cells, detergent extracts were prepared from the cells and were subjected to SDS-PAGE under non-reducing conditions. Following electrophoresis, the proteins were transferred to nitrocellulose, and immunoblotted with a rabbit antibody prepared against the cytoplasmic tail of the VLDL receptor. Previous studies (Iijima et al., 1998) have shown that the type I VLDL receptor is synthesized as a 135 kDa precursor (reducing conditions) that is converted to a glycosylated 155 kDa mature form. The results, shown in Fig. 1B, demonstrate that cells transfected with pVLDLR express a mature (approximately 120 kDa, non-reducing conditions) and precursor receptor (approximately 100 kDa, non-reducing conditions) that co-migrates with the endogenous VLDL receptor. Further, cells transfected with pVLDLRΔL and pVLDLRΔEGF express molecules that have an apparent molecular mass of 65 and 48 kDa, respectively. These values are close to those of their predicted size. Under reducing conditions, the apparent molecular mass of the ΔEGF mutant increased to 65 kDa, consistent with reduction of the many disulfide bonds present in this molecule.

Cells expressing the ΔEGF mutant bind and internalize ^{125}I-labeled RAP

To investigate the functional properties of mutant receptors lacking the EGF-precursor homology region or the ligand binding domain, cellular uptake experiments were performed. In these experiments, ^{125}I-labeled RAP (7 nM) was incubated with parental or transfected cells at 37°C. Since CHO-K1 cells express both the VLDLR and LRP, the cells were first incubated with antibodies known to block LRP activity (Mikhailenko et al., 1995; Kounnas et al., 1996) prior to and during the course of this experiment to eliminate the contribution of LRP. At the indicated time intervals, the amount of ligand associated with the cell surface (Fig. 2A), internalized (Fig. 2B) and released back into the medium as TCA-soluble radioactivity (Fig. 2C) was measured. When incubated with cells, ^{125}I-labeled RAP associated with the cell surface in a time-dependent manner (Fig. 2A). Considerably more RAP is found on the surface of cells expressing the ΔEGF mutant than the parental cells or cells expressing the ΔL mutant. ^{125}I-labeled RAP was readily internalized by all cells, and reached a steady state level at approximately 40 minutes of incubation (Fig. 2B). The amount

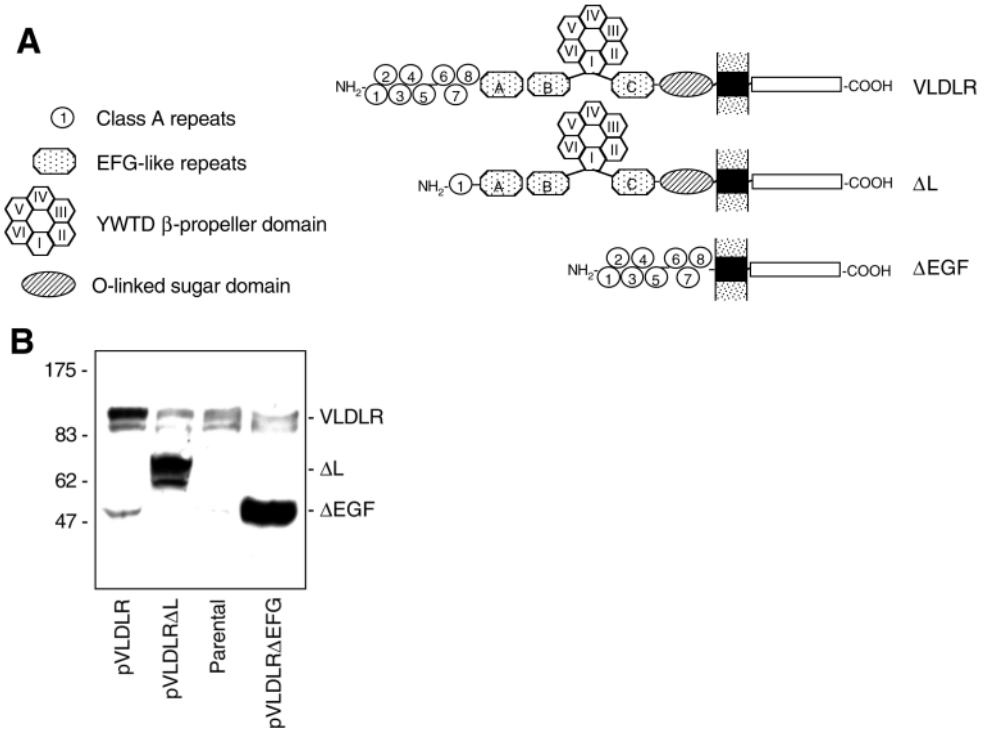

Fig. 1. (A) Schematic model of VLDL receptor and deletion mutants. The VLDL receptor ectodomain contains eight cysteine-rich class A repeats, three EGF-like repeats, six YWTD modules thought to form a β-propeller domain, and an O-linked sugar domain. (B) Immunoblotting analysis of parental and transfected CHO-K1 cells. Cell extracts were prepared from parental CHO-K1 cells and cells transfected with human VLDL receptor, ΔEGF or ΔL mutant VLDL receptors, and an aliquot subjected to SDS-PAGE under non-reducing conditions on 4-12% gradient gels. Following transfer to nitrocellulose, the VLDL receptor and deletion mutants were detected with R2623.

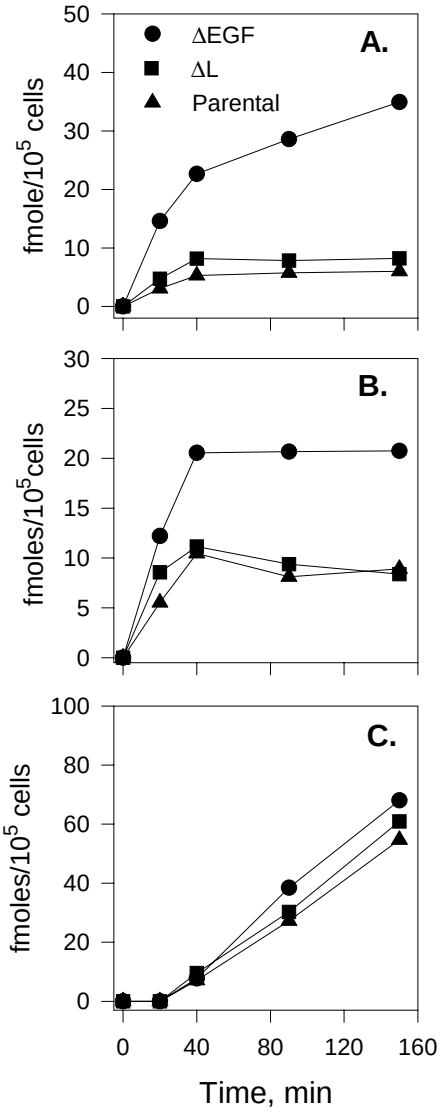

Fig. 2. Cells expressing the ΔEGF mutant bind and internalize ^{125}I-labeled RAP, but fail to degrade it. Parental CHO-K1 cells and cells stably transfected with ΔEGF or ΔL mutant VLDL receptors were plated into 12 well plates (2x10^5 cells/well) one day prior to the assay. Cells were incubated for 1 hour in medium containing anti-LRP IgG (R777) to block LRP activity, then ^{125}I-RAP (7 nM final concentration) was added to each well in the absence or presence of unlabeled RAP (800 nM). At indicated times, the amount of radioactivity that was bound to the surface (A), internalized (B) or degraded (C) was measured as described in Materials and Methods. Nonspecific uptake and degradation were measured in the presence of excess unlabeled RAP was subtracted from the data. Each data point represents the average of duplicate determinations. Circles, CHO-K1 cells expressing ΔEGF mutant receptor; squares, CHO-K1cells expressing ΔL mutant receptor; triangles, parental CHO-K1 cells.

of RAP internalized was considerably greater in cells expressing the ΔEGF mutant when compared with parental cells or cells transfected with the ΔL mutant (Fig. 2B). Following a lag period, TCA soluble degradation products were detected in the medium of all cells; however the cells were indistinguishable in their ability to degrade RAP (Fig. 2C). Thus, the increased internalization of ^{125}I-RAP observed in cells expressing the ΔEGF mutant did not lead to increased degradation of RAP.

The EGF and YWTD repeats appear necessary for acid-dependent ligand dissociation of ^{125}I-labeled RAP

The experiments depicted in Fig. 2 revealed that while cells expressing the ΔEGF mutant receptor demonstrated an increased capacity to internalize ^{125}I-labeled RAP when compared with parental cells expressing the wild-type receptor, no increase in their capacity to degrade RAP was noted. To investigate this further, we designed experiments to examine a single cycle of endocytosis. Parental CHO-K1 cells and CHO-K1 cells transfected with the ΔEGF receptor mutant were then incubated with ^{125}I-labeled RAP at 4°C for 3 hours to allow surface binding. Following incubation, the cells were washed, warmed to 37°C for various time intervals, and the amount of ^{125}I-labeled RAP that remained on the cell surface was measured by its susceptibility to proteinase digestion. In the same experiment, the radioactivity that was resistant to proteinase treatment (internalized), and that which was present in the medium as trichloroacetic acid insoluble (dissociated) and soluble (degraded) was also measured. In the case of parental cells expressing the VLDL receptor (Fig. 3A), ^{125}I-labeled RAP rapidly disappeared from the cell surface with time. Disappearance from the cell surface was associated with an increase in the amount of radiolabeled ligand that was resistant to proteinase treatment, consistent with transport of the ligand into the cell. This internalized radioactivity reached a maximum and declined with increasing time. The decrease was associated with an increase in trichloroacetic acid-soluble material detected in the medium. These results, which measure a single cycle of endocytosis, are consistent with what has been observed for several endocytic receptors (Brown and Goldstein, 1979; Schwartz et al., 1982).

A similar experiment was conducted using the cells transfected with the ΔEGF mutant receptor (Fig. 3B). As expected, based on expression levels (see Fig. 1B), the transfected cells bind more ^{125}I-labeled RAP than the parental cells. Significantly, the fate of the bound RAP was strikingly different when compared with the parental cells. In this case it was noted that the ^{125}I-labeled RAP initially bound to the cell surface decreased, but reached a steady state level with time. This was accompanied by an increase in the intracellular level of radioactivity, which also appeared to reach a steady state level. In further contrast to the wild-type receptor, very little trichloroacetic acid-soluble material was detected in the medium indicating that the ligand was not efficiently degraded nor was it dissociated from the mutant receptor during the course of the experiment.

We interpret the data of Figs 2 and 3 to indicate that the wild-type VLDL receptor binds RAP, mediates its cellular internalization and releases it in the low pH environment of the endosomes, where it is transported to the lysosomes for degradation. In contrast, the ΔEGF mutant appears capable of binding RAP and mediating its endocytosis, but seems unable to release it in the low pH environment of the endosomes. The RAP bound to the ΔEGF mutant receptor is therefore not targeted for degradation, but rather appears to recycle along with the mutant receptor.

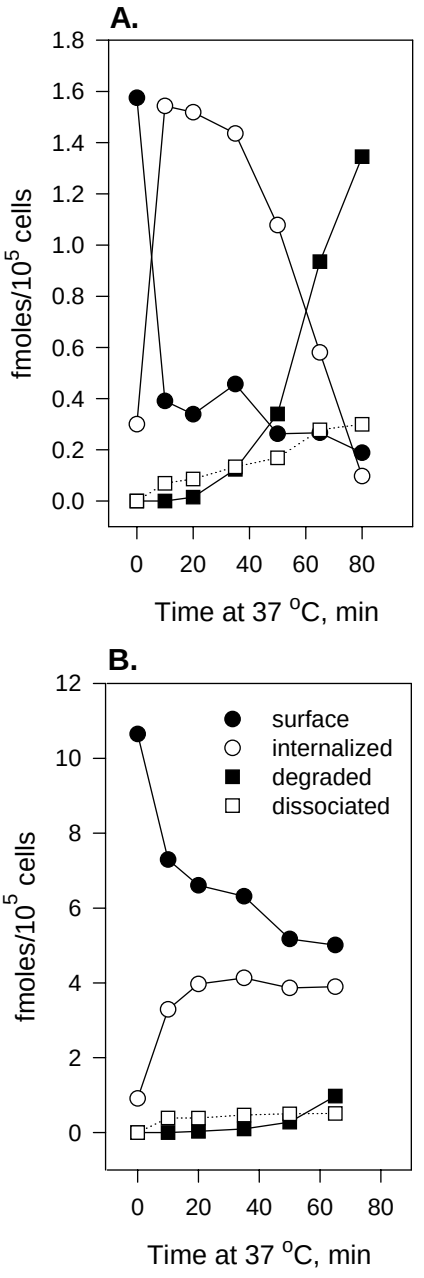

Fig. 3. ^{125}I-labeled RAP remains associated with the ΔEGF mutant receptor during one cycle of endocytosis. Parental CHO-K1 cells (A) and CHO-K1 cells stably transfected with ΔEGF mutant receptor (B) were plated into 12-well plates (2×10^5 cells/well) one day prior to the assay. Cells were preincubated for 1 hour at 37°C with medium containing blocking anti-LRP IgG (R777), chilled on ice for 45 minutes and then incubated with ^{125}I-RAP (7 nM) at 4°C for 3 hours. Following incubation, the cells were washed, warmed to 37°C for indicated time intervals and the amount of ^{125}I-RAP that remained on the cell surface (filled circles), that dissociated into the medium (open squares), that was internalized (open circles) and degraded (filled squares) was measured as described in Materials and Methods. Each data point represents the average of duplicate determinations.

RAP co-localizes with transferrin in cells expressing the ΔEGF receptor mutant

To confirm that RAP is not efficiently released from the ΔEGF receptor mutant within endosomal compartments and subsequently transported into lysosomal compartments, immunofluorescence experiments were performed. In this experiment CHO-K1cells transfected with the human VLDL receptor cDNA (Fig. 4A,B,C and D,E,F) or CHO-K1cells transfected with ΔEGF receptor mutant cDNA (Fig. 4G,H,I and J,K,L) were utilized. To label the endosomal compartments, Bodipy transferrin (Tf) was employed (Fig. 4,A,G), while the lysosomal compartment was labeled with fluorescein dextran (Fig. 4D,J), a well known marker for these compartments (Geisow et al., 1981; Hoock et al., 1997). Following a 20 minutes incubation at 37°C with CHO-K1cells expressing the wild-type VLDL receptor, very little Cy3-RAP fluorescence was observed to co-localize with Bodipy Tf (Fig. 4A,B,C), while noticeable RAP fluorescence co-localized with fluorescein-dextran (Fig. 4D,E,F). This data is consistent with the suggested mechanism of rapid separation of ligand and receptor in endosomes and further recycling of receptors to the cell surface, while the ligand is delivered to lysosomes. In further contrast to these results, when cells expressing the ΔEGF-mutant receptor were incubated with RAP under identical conditions, RAP fluorescence was found to colocalize with Bodipy-Tf, indicating that RAP is localized within endosomes (Fig. 4G,H,I). At the same time, very little RAP was observed to co-localize with fluorescein-dextran in lysosomal compartments of these cells (Fig. 4J,K,L). These results confirm that a significant portion of RAP remains within the endocytic pathway in cells overexpressing the ΔEGF receptor mutant.

Expression of the VLDL receptor ligand binding domain

The use of deletion mutants of the VLDL receptor confirms that RAP binds to the cysteine rich class A repeat region. To derive additional structural information regarding the modules within this region responsible for ligand recognition, we constructed a vector suitable for expression of a soluble form of the VLDL receptor ligand binding domain and various deletion mutants of this region. To accomplish this, the cDNA encoding the ligand binding domain of the VLDL receptor was cloned into a vector containing the cDNA for the Ig κ-chain leader sequence at the 5′ end for targeting the polypeptide into the secretory pathway, and the cDNA for c-*myc* epitope at the 3′ end which can be recognized by an anti-*myc* IgG to facilitate purification and detection.

Bu and Rennke (1996) previously demonstrated that despite the presence of endogenous RAP, co-expression of RAP was required for secretion of soluble LRP ‘mini-receptors’ when human glioblastoma U87 cells were transiently transfected with LRP constructs. Based on these observations, we reasoned that RAP might be important for secretion of our soluble receptor fragments as well. This was tested by transfecting Cos-1 cells with cDNA encoding the VLDL receptor ligand binding domain containing modules 1-8 (L1-8) of the VLDL receptor and co-transfecting with either a control plasmid, or increasing amounts of a plasmid containing the human RAP cDNA. Following transfection, the medium and a cell extract were analyzed for the presence of the VLDL receptor fragment by immunoblotting. The results of this experiment demonstrate that when a control plasmid was utilized for the co-transfection experiments, very little soluble receptor fragment L1-8 was

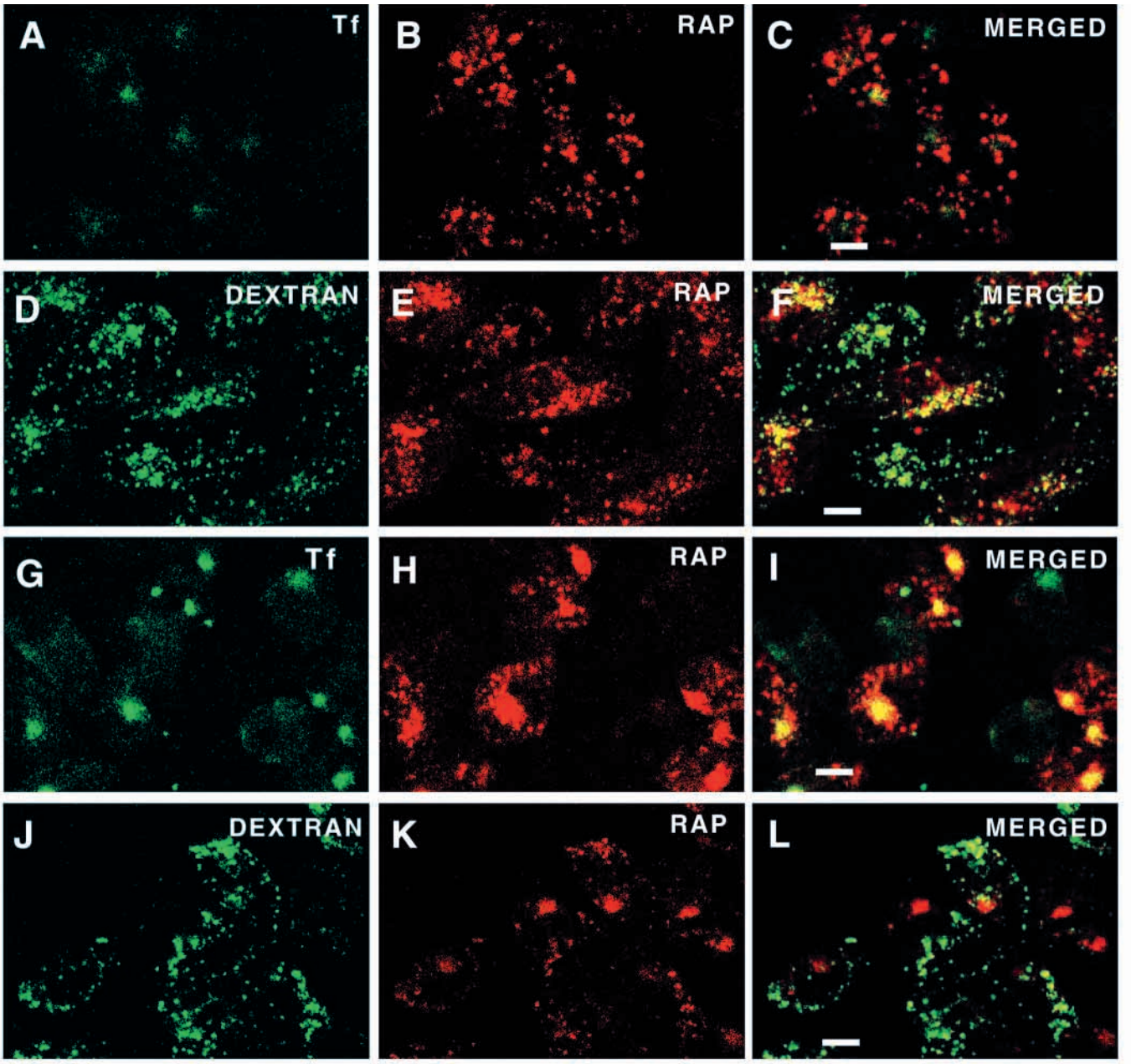

Fig. 4. RAP localizes to lysosomal compartments in CHO-K1 cells transfected with VLDL receptor, and to endosomal compartments in CHO-K1 cells transfected with ΔEGF mutant VLDL receptor. (A-F) CHO-K1 cells transfected with the human VLDL receptor were utilized, while (G-L) CHO-K1 cells transfected with the ΔEGF mutant VLDL receptor were used. Cells were incubated for 20 minutes at 37°C with Bodipy-Tf and Cy3-labeled RAP (A-C; G-I). To label the lysosomal compartments, cells were first preincubated with fluorescein labeled dextran for 15 minutes followed by a 24 hour chase, then incubated with Cy3-labeled RAP for 20 minutes at 37°C (D-F; J-L). Confocal microscopy on the fixed images was performed on a Bio-Rad MRC 1024 confocal microscope. Bar, 10 μm.

detected in the medium (Fig. 5A, lane 1) despite the presence of large amounts of L1-8 in the cell extracts (Fig. 5C, lane 1). However, increasing amounts of plasmid containing human RAP cDNA generated increased amounts of soluble VLDL receptor fragments in the medium (Fig. 5A, lanes 2 and 3) with a concomitant decrease found in the cell extract (Fig. 5C, lanes 2 and 3). Similar results were obtained with a plasmid containing the cDNA encoding L2-8 of the VLDL receptor (not shown). As a control, we noted that transfection of RAP in these cells has little effect on the secretion of endogenous plasminogen activator inhibitor type I (Fig. 5B). We also noticed that medium of cells transfected with the RAP plasmid did contain some RAP (not shown). Overall, these experiments confirm those reported earlier using soluble LRP fragments (Bu and Rennke, 1996) and indicate that excess RAP is required for the efficient secretion of the ligand-binding domain of the VLDL receptor.

RAP binding experiments

To identify the portion of the VLDL receptor ligand binding

domain that is responsible for RAP binding, Cos-1 cells were transfected with constructs encoding various repeats of the VLDL receptor ligand binding domain. Following transfection, the medium was harvested, and an aliquot subjected to immunoblot analysis following SDS-PAGE. Using an anti-*myc* IgG, we were able to detect a polypeptide of the expected molecular mass for each fragment (Fig. 6A), confirming that all fragments were secreted into the medium. We did, however, notice in multiple experiments that less of the fragments containing repeats L1-8, L1-6, and L1-4 were secreted than the other fragments.

To evaluate the potential of various soluble receptor fragments to bind RAP, an aliquot from the medium of transfected Cos-1 cells was incubated with 25 nM ^{125}I-labeled RAP. The recombinant receptor fragments in complex with RAP were then subjected to immunoprecipitation using anti-*myc* IgG. The immunoprecipitates were washed, dissolved in SDS-gel buffer, and subjected to SDS-PAGE followed by autoradiography. The results, shown in Fig. 6B, demonstrate that the medium from transfected Cos-1 cells expressing VLDL receptor fragments L1-4, L1-6, and L1-8 appear capable of binding ^{125}I-labeled RAP as evidenced by its co-precipitation. In contrast, very little ^{125}I-labeled RAP is co-precipitated with medium from transfected Cos-1 cells expressing recombinant receptor fragments L1-2, L1-3, L2-3, L2-4, and L2-8, indicating that these fragments do not appear to bind RAP. When the immunoprecipitation experiment was conducted in the presence of excess unlabeled RAP (1 μM), the amount of co-precipitated radioactivity was significantly reduced (Fig. 6C), confirming that the binding is specific. The results of the co-immunoprecipitation experiments suggest that the RAP binding site of the VLDL receptor is located within

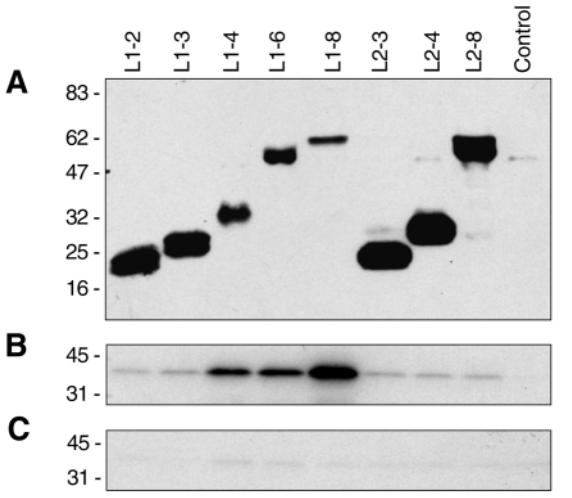

Fig. 6. Expression of soluble fragments from the VLDL receptor ligand binding domain (A), and assessment of their ability to bind ^{125}I-labeled RAP by co-immunoprecipitation experiments (B and C). (A) An aliquot from 10× concentrated medium of Cos-1 cells transiently transfected with plasmids containing inserts encoding for soluble fragments of the VLDL receptor ligand binding domain was subjected to SDS-PAGE on 8-16% gradient gels under reducing conditions. Following transfer to nitrocellulose, the sheets were immunoblotted with anti-*myc* IgG and detected using chemiluminescent reagents. Medium from mock-transfected cells were used as a control. (B and C) An aliquot from 10X concentrated medium from the transfected cells was mixed with 25 nM of ^{125}I labeled RAP in the absence (B) or presence (C) of 1 μM unlabeled RAP. Following incubation, the receptor fragment/RAP complex was immunoprecipitated using the anti-*myc* IgG and analyzed by SDS-PAGE on 8% gels. The gel was dried and exposed to film overnight.

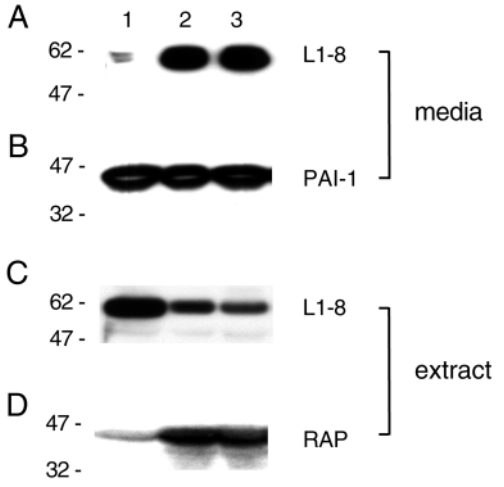

Fig. 5. Co-transfection of RAP facilitates secretion of the recombinant VLDL receptor ligand binding domains. A plasmid containing the human VLDL receptor ligand-binding domain (L1-8) was used to transfect Cos-1 cells. A control plasmid (lane 1) or a plasmid containing the cDNA of human RAP (5 μg, lane 2 and 10 μg, lane 3) was utilized to co-transfect the cells. Following transfection samples of the medium from the transfected cells were immunoblotted for VLDL receptor fragment L1-8 (A) and PAI-1 (B). Cell extracts were prepared and immunoblotted for VLDL receptor fragment L1-8 (C) and RAP (D).

the first four class A repeats, and also suggests that L1 appears important for RAP binding, since deletion of this module to generate L2-8 is associated with a loss of RAP binding.

To derive quantitative information regarding the binding of RAP to soluble VLDL receptor fragments, a solid phase assay was performed. Medium of Cos-1 cells transfected with cDNAs coding for the indicated regions of the VLDL receptor ligand binding domain was utilized as a source of soluble receptor fragment. Microtiter wells, coated with anti-*myc* IgG, were incubated with medium containing the soluble receptor fragments. Binding of ^{125}I-labeled anti-his IgG confirmed that approximately equivalent amounts of each fragment was captured to the microtiter wells (Fig. 7A). Small amounts of RAP are secreted into the medium by the transfected cells, and although this RAP does not appear to prevent binding of ^{125}I-labeled RAP, nonetheless to ensure that this is the case, the receptor fragments captured on the microtiter wells were first washed with EDTA to remove any endogenous RAP present. We observed that RAP did not bind to any soluble VLDL receptor fragments when EDTA was included in the buffer, indicating a requirement for metal ions. The wells were then incubated with increasing concentrations of ^{125}I-labeled RAP. Non-specific binding was determined by measuring the binding in the presence of excess unlabeled RAP, and was subtracted from the total binding to give specific binding. In all cases, the non-specific binding was less than 5% of total binding. The results of this assay (Fig. 7B) demonstrate that

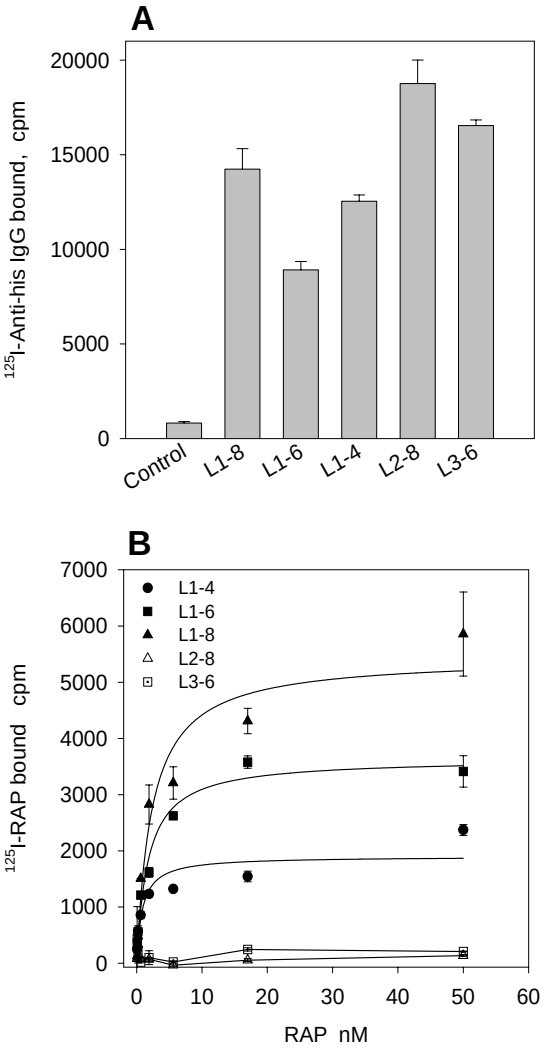

Fig. 7. Binding of ^{125}I-labeled RAP to the VLDL receptor fragments. (A) Solid phase assay measuring binding of anti-his IgG to quantify relative amounts of VLDL receptor fragments captured on microtiter wells. ^{125}I-anti-his antibody was added to the coated wells at a concentration of 20 μg/ml, and incubation was carried out for 18 hours at 4°C. Control wells were coated with the medium from mock transfected cells. (B) Solid phase assay to measure binding of RAP to VLDL receptor fragments captured on microtiter wells. Increasing concentrations of ^{125}I-RAP (500 cpm/fmole) were added to the coated wells, and incubation was carried out for 18 hours at 4°C. Non-specific binding was measured by including an excess of unlabeled RAP, and has been subtracted. The data were normalized to the fragment that exhibited the highest anti-his binding. The curves represent the best fit of the data to equation 1 using non-linear regression analysis, and gave $K_{D,app}$ values of 2.3, 1.7, and 0.9 nM, for L1-8, L1-6, and L1-4, respectively. Filled circles, L1-4; filled squares, L1-6; filled triangles, L1-4; open triangles, L2-8; open square, L3-6.

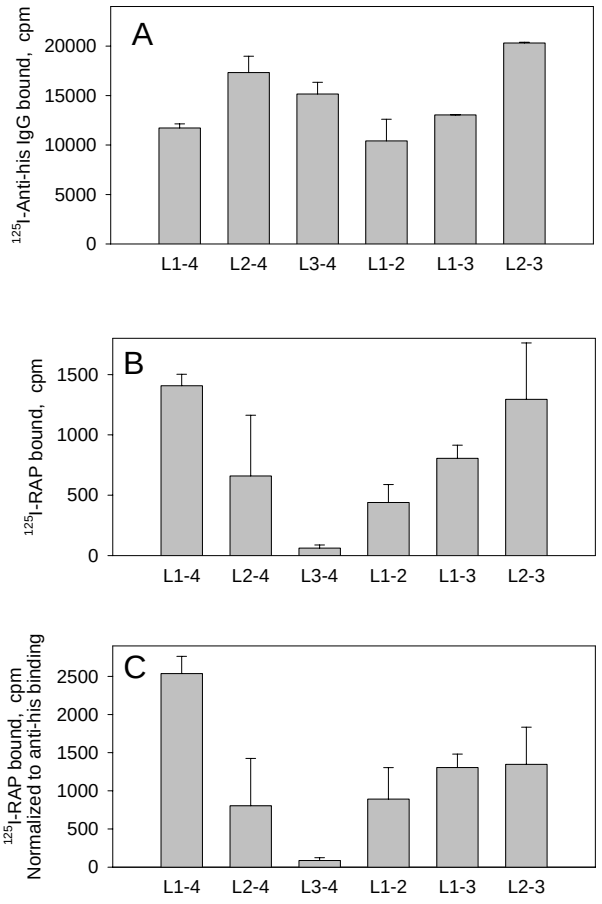

Fig. 8. Mapping the RAP binding domain of the VLDL receptor within L1-4. **(**A) Solid phase assay measuring binding of anti-his IgG to quantify relative amounts of VLDL receptor fragments captured on microtiter wells. ^{125}I-anti-his IgG was added to the coated wells at a concentration of 20 μg/ml, and incubation was carried out for 18 hours at 4°C. Non-specific binding was determined as binding to the wells coated with the medium from mock transfected cells and has been subtracted. (B) Solid phase assay to measure the binding of ^{125}I-labeled RAP to various VLDL receptor fragments captured on microtiter wells. Coated wells were incubated with 50 nM of ^{125}I-labeled RAP (500 cpm/fmole) at 4°C for 18 hours. Non-specific binding was measured by including an excess of unlabeled RAP, and has been subtracted. (C) Normalized binding of RAP to various VLDL receptor fragments. The figure represents the binding of RAP normalized to amount of fragment coated on the microtiter plate as quantified by measuring anti-his IgG binding. The data are an average of duplicate determination.

soluble VLDL receptor fragments encompassing modules L1-8, L1-6, and L1-4 bind RAP with $K_{D,app}$ values of 2.3, 1.7, and 0.9 nM, respectively. Thus RAP interacts with soluble receptors containing only class A repeats with a $K_{D,app}$ similar to that of 0.7 nM determined for the full length receptor (Battey et al., 1994). In agreement with the immunoprecipitation assay, very little binding of ^{125}I-labeled RAP to other soluble receptor fragments was noted. Although not shown in Fig. 7B, fragments L5-6, L5-8, and L7-8 were also utilized in this solid phase assay, and no specific interaction of RAP with these fragments was detected.

To further define the modules within L1-4 responsible for RAP binding, additional soluble VLDL receptor fragments were utilized (Fig. 8). For these experiments, 50 nM of ^{125}I-labeled RAP were added to microtiter wells coated with VLDL receptor fragments. Binding of ^{125}I-labeled anti-his IgG to the wells confirmed that similar amounts of VLDL receptor

fragment were captured (Fig. 8A). The RAP data was normalized to the amount of VLDL receptor fragment captured in the well (Fig. 8C). Consistent with the observations in Figs 2 and 3 suggesting an important role for L1 in binding RAP, when L1 was removed from L1-4 to generate L2-4, a significant reduction in the amount of RAP bound was noted suggesting a reduction in the binding capacity. Further deletion of L2 from L2-4 to generate L3-4 completely abolishes RAP binding. On the other hand, truncation from the C-terminal end also resulted in a loss of affinity, and fragments L1-3 and L1-2 did not bind RAP as well as fragment L1-4. The results of these experiments confirm that high affinity binding of RAP to the VLDL receptor requires the first four class A modules, and removal of any of these modules impacts the ability of RAP to bind to the soluble receptor fragment.

Identification of the regions responsible for binding uPA:PAI-1 complexes

The VLDL receptor also binds uPA:PAI-1 complexes (Argraves et al., 1995; Heegaard et al., 1995) and thus it was of interest to identify the modules responsible for their binding. Initially, an assay was performed to determine if uPA:PAI-1 complexes are able to bind to the ligand binding domain of the VLDL receptor. The results of our solid phase assay, shown in Fig. 9, demonstrate that these complexes bind to soluble VLDL receptor fragments L1-8 and L2-8 with $K_{D,app}$ values of 12 and 14 nM, respectively. These values are similar to the value of 15 nM that Argraves et al. (1995) measured for binding of uPA:PAI-1 to the full length receptor. Thus, unlike the requirements for RAP binding, little effect was noted when L1 was deleted, and uPA:PAI-1 complexes bound to L2-8 as well as they bound to L1-8.

To map out the uPA:PAI-1 binding site, binding of these complexes to several soluble VLDL receptor fragments was measured (Fig. 10). These results demonstrate that the uPA:PAI-1 binding site appears to be located within L1-4, since ^{125}I-labeled uPA:PAI-1 complexes bound to L1-4, but did not bind to L5-8. Deletion of L1 from L1-4 had little impact on the binding; however, further deletion of L2 to generate L3-4 greatly diminished the binding, suggesting that L2 is important in binding uPA:PAI-1 complexes. In support of this, uPA:PAI-1 bound well to all fragments that contained L2 (e.g. L1-2, L1-

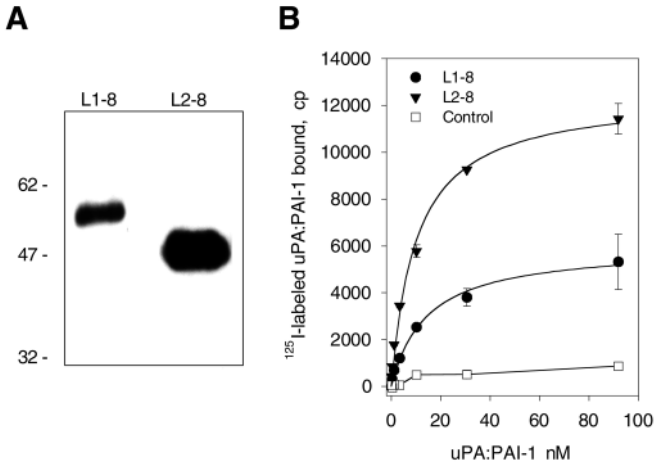

Fig. 9. Solid phase binding of ^{125}I-labeled uPA:PAI-1 complexes to the VLDL receptor ligand binding domain. (A) An aliquot of medium from transiently transfected Cos-1 cells was subjected to immunoblot analysis using anti-myc IgG to demonstrate levels of expression. (B) Solid phase assay to measuring the binding of ^{125}I-labeled uPA:PAI-1 complexes to L1-8 and L2-8 captured on microtiter wells. Coated wells were incubated with increasing concentrations of ^{125}I-labeled uPA:PAI-1 complexes (500 cpm/fmole) at 4°C for 18 hours. Non-specific binding was measured by including an excess of unlabeled uPA:PAI-1 complexes, and has been subtracted. The data are an average of duplicate determination. Solid curves represent the best fit of the data to equation 1 using non-linear regression analysis, and gave $K_{D,app}$ values of 12 and 14 nM for L1-8 and L2-8, respectively. Filled circles, L1-8; filled triangles, L2-8; open squares, medium from mock transfection.

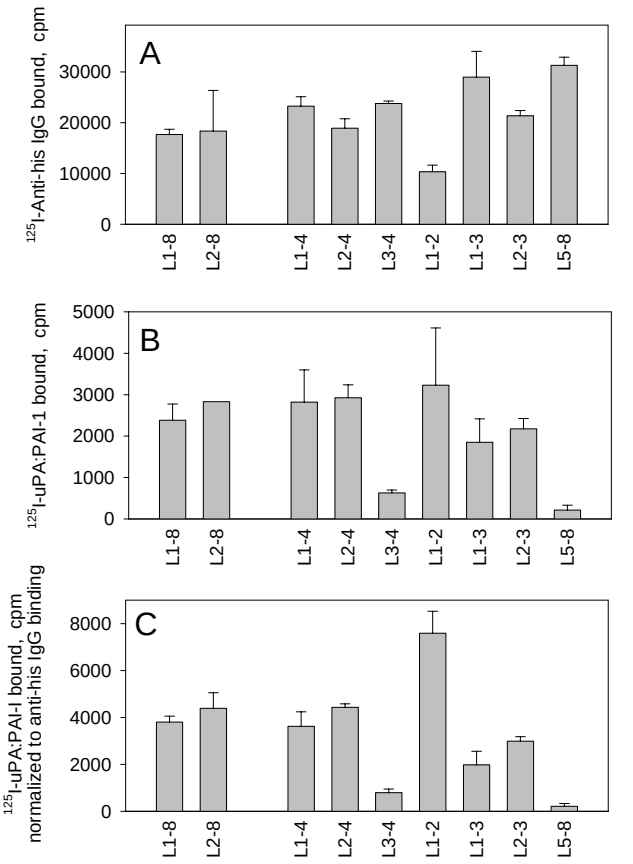

Fig. 10. Binding of ^{125}I-uPA:PAI-1 fragments of the VLDL receptor. (A) Solid phase assay measuring binding of anti-his IgG to quantify relative amounts of soluble VLDL receptor fragments captured on microtiter wells. ^{125}I-anti-his IgG was added to the coated wells at a concentration of 20 μg/ml, and incubation was carried out for 18 hours at 4°C. Non-specific binding was determined as binding to the wells coated with the medium from mock transfected cells and has been subtracted. (B) Solid phase assay to measure the binding of ^{125}I-uPA:PAI-1 to various fragments captured on microtiter wells. The assay was carried out as described for Fig. 8, except that 18 nM ^{125}I-uPA:PAI-1 (1000 cpm/fmole) complexes were added. Non-specific binding was measured by including an excess of unlabeled uPA:PAI-1 complexes and has been subtracted. (C) Binding of ^{125}I-labeled uPA:PAI-1 normalized to the amount of VLDL receptor fragment coated on the microtiter plate as quantified by measuring anti-his IgG binding. The data are an average of duplicate determination.

3, L2-3), and we conclude from these studies, that L2 is an important module for recognition of uPA:PAI-1 complexes. Thus the uPA:PAI-1 binding site overlaps the RAP binding site, but it appears that different modules determine the high affinity interaction for each ligand.

DISCUSSION

An important property that members of the LDL receptor family share is their ability to deliver ligands to endosomes and release them in the low pH environment of these compartments so that the ligands can be transported to lysosomes for degradation. The biochemical mechanisms involved in ligand uncoupling are not completely understood, but clearly involve a significant reduction in affinity of the receptor for its ligand as a consequence of the pH change. Using RAP as a ligand, the current studies demonstrate that when the EGF, YWTD and O-linked sugar domains are deleted from the VLDL receptor, a molecule is generated that is capable of binding and internalizing ligands, but is unable to release them in the endosomal compartments. Very likely this defect arises from deletion of the EGF and/or YWTD repeats, since alternatively spliced variants of the VLDL receptor exist in which the O-linked sugar domain is deleted (Sakai et al., 1994). In the current studies, we determined that the RAP binding site is restricted to the first four cysteine-rich modules located at the amino terminus of the VLDL receptor, and thus our results confirm that ligand uncoupling occurs via an allosteric-type mechanism in which pH induced changes in the EGF and/or YWTD repeats must alter the ligand binding properties at the amino-terminal portion of the molecule.

In the case of the structurally related LDL receptor, deletion of the EGF and YWTD domains also generates a molecule that is unable to release ligands at the endosomal pH (Davis et al., 1987). Thus, the presence of these domains is required for efficient ligand uncoupling in both of these receptors, and this suggests a universal function for the EGF/YWTD repeats in this class of receptors. It is not clear at present whether the EGF module or YWTD repeats, or both, are necessary for this activity. Using a variety of arguments including computational techniques, Springer (1998) suggested that the YWTD repeats do not exist in random coil conformation, but rather group into six repeat folds which form a compact β-propeller structure. One significant consequence of the compact folding pattern is that neighboring EGF modules are brought into close proximity with each other. This might have functional significance, and it is possible that a pH induced conformational change in the β-propeller domain may alter the close proximity of these EGF modules which in turn could affect interdomain interactions in the ligand binding region.

Deletion of the EGF/YWTD repeat region in the LDL receptor also generated a receptor that displayed reduced capacity to bind LDL, but not β-VLDL, and a receptor that was rapidly degraded after ligand binding (Davis et al., 1987). With our ΔEGF mutant receptor, the ligand (or receptor-ligand complex) does not appear to be delivered into lysosomal compartments for degradation, and we conclude that the mutant VLDL receptor does appear to recycle normally.

Despite significant structural similarities between the LDL receptor and the VLDL receptor, their ligand binding properties are significantly different. Notably, the VLDL receptor binds RAP very tightly (Battey et al., 1994). RAP has the unusual property of binding tightly to certain members of this receptor family and antagonizing ligand binding. In order to gain insight into the mechanism of how RAP functions, we mapped out the RAP binding site on the VLDL receptor and the region responsible for binding uPA:PAI-1 complexes. We noted that co-transfection of RAP was required for successful secretion of the soluble fragments into the medium, despite the presence of endogenous RAP in Cos-1 cells. The results are somewhat surprising since both L1-8 and L2-8 seemed to require co-expression of RAP for their efficient secretion, despite the fact that they exhibit large differences in their capacity to bind RAP. Possibly, sufficiently high concentrations of RAP are achieved following transfection to allow interaction between fragment L2-8 and RAP. The results in the present investigation are similar to what was found for secretion of soluble fragments of LRP (Bu and Rennke, 1996), and highlight that an important function of RAP may be to accelerate processing of these receptors.

Expression of the ligand binding domain of the VLDL receptor as a soluble fragment generated a molecule that bound RAP with an affinity comparable to the native molecule. Since we demonstrate that metal ions are required for RAP binding, this indicates that RAP binds to the folded repeats, and not to partially folded intermediates. By generating a series of deletion mutants, we determined that the smallest fragment capable of binding RAP with high affinity was a molecule containing repeats L1-4. Deletion of any additional modules from this fragment greatly reduced their ability to bind RAP. We conclude from our studies that multiple regions within L1-4 are required to generate a high affinity RAP site in the VLDL receptor. Structural studies indicate that, like the VLDL receptor, multiple repeats within LRP seem to be required for RAP binding. Using soluble recombinant receptor fragments, Horn et al. (1997) found that L5-7 in the second cluster of class A modules in LRP are responsible for binding RAP, although the affinity for RAP was reduced somewhat when compared to the soluble fragment containing all eight repeats.

The uPA:PAI-1 binding site is also localized to the first four modules (L1-4) of the VLDL receptor. However, in contrast to the requirements for RAP binding, the uPA:PAI-1 binding site does not seem to require L1 to be present, but rather seems to be located within L2. Thus, RAP and uPA:PAI-1 bind to overlapping regions on the VLDL receptor but different modules seem to play a dominant role in ligand recognition. The fact that these sites overlap explains why RAP is such an effective competitor for the binding of uPA:PAI-1 complexes to the VLDL receptor. While the current studies focused on identifying the region responsible for binding RAP and uPA:PAI-1 complexes, in addition to these molecules, the VLDL receptor also binds many other ligands such as lipoprotein lipase, thrombospondin, apoE-containing lipoproteins, and Lp(a), and it will be important to identify modules that recognize these ligands as well to fully understand the structural requirements for ligand binding. During the preparation of this manuscript, Rettenberger et al. (1999) reported that a VLDL receptor variant lacking L3 showed impared RAP binding, while the binding of uPA:PAI-1 complexes remained unaltered. These results are in good agreement with those of the current study.

The structure of single class A repeats of the LDL receptor have been solved by NMR (Daly et al., 1995) and X-ray crystallography (Fass et al., 1997) approaches. The three dimensional structure of module L5 of the LDL receptor (Fass et al., 1997) revealed a calcium ion coordinated by conserved acidic residues that lie at the carboxyl-terminal end of the domain. Many of these conserved acidic residues are present in the cysteine rich modules of the VLDL receptor as well, and the structural requirement for calcium explains why RAP and uPA:PAI-1 binding to soluble receptor fragments was abolished when EDTA was added in the current studies. While the structure of a single repeat has been solved, it is still not understood which portion of the repeats are involved in ligand recognition, and to identify these regions will require a determination of the structure of the ligand-receptor complex. Since we demonstrate in the current study that modules L1-4 of the VLDL receptor bind RAP with affinity comparable to the native receptor, expression of this fragment may be useful for additional structural studies to resolve these questions.

It should also be pointed out that at present the topology of the repeats within the ligand binding domain are not known. Each of the modules is separated by a short segment of 4-5 amino acids, and thus it seems likely that they may exist in an extended conformation. However, the exception to this is a nine amino acid spacer between repeats L5 and L6 of the VLDL receptor (and between repeats L4 and L5 of the LDL receptor) which may impart some flexibility and allow repeats L1-5 to fold back and interact with repeats L6-8. If so, this might offer an explanation of how RAP binding at the amino-terminal repeats may alter ligand binding to modules 5-8. Further, it may offer an explanation of how conformational changes within the distant EGF/YWTD domains might affect ligand binding.

In summary, by using deletion mutants we have shown that the EGF and/or YWTD repeats of the VLDL receptor play an important role in ligand uncoupling, and have demonstrated that a fragment consisting of the entire ligand binding domain of the VLDL receptor, and one containing just the first four modules of this region, bind RAP with an affinity comparable to that found for the native receptor. These data indicate that the soluble fragments we expressed are biologically active, and with the recent advances in producing large amounts of soluble receptor fragments in *Escherichia coli* (Simmons et al., 1997), it should be possible to produce sufficient quantities of the VLDL receptor ligand binding domain to begin to address important structural questions.

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