INTERACTION OF LIPOPROTEIN LIPASE AND RECEPTOR ASSOCIATED PROTEIN
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Receptor-associated protein (RAP) is a recognized chaperone/escort protein for members of the LDL receptor family. In this report we show that RAP binds to lipoprotein lipase (LPL) and may play a role in the maturation of LPL. Binding of highly purified RAP to LPL was demonstrated in vitro by solid phase assays, surface plasmon resonance and rate zonal centrifugation. The dissociation constant for this interaction measured by the first two techniques ranged between 2.4 and 13 nM, values similar to those reported for the binding of RAP to LRP or gp330. The specificity of the interaction was demonstrated by competition with a panel of LPL monoclonal antibodies. Rate zonal centrifugation demonstrated the presence of a stable complex with an apparent Mr consistent with the formation of a complex between monomeric LPL and RAP. RAP-LPL complexes were co-immunoprecipitated in adipocyte lysates or from solutions of purified LPL and RAP. The interaction was also demonstrated in whole cells by cross-linking experiments. RAP-deficient adipocytes secreted LPL with a specific activity 2.5 fold lower than the lipase secreted by control cells. Heparin addition to cultured RAP-deficient adipocytes failed to stimulate LPL secretion in the medium, suggesting defective binding of the lipase to the plasma membrane. These studies demonstrate that RAP binds to LPL with high affinity both in purified systems and cell extracts, and that RAP-deficient adipocytes secrete poorly assembled LPL. A function of RAP may be to prevent premature interaction of LPL with binding partners in the secretory pathway, namely LRP and HSPG.

INTRODUCTION
Circulating lipoproteins are metabolized in a two-step process. First, the lipoproteins are bound in the capillary endothelium and the triglycerides in their cores are hydrolyzed to release free fatty acids and monoacylglycerol. Second, the resulting lipoprotein remnants are taken up by specific peripheral receptors, including the low density lipoprotein receptor and the low density lipoprotein receptor-related protein (LRP)1 (1;2). Lipoprotein lipase (LPL) is a major enzyme in the hydrolysis of triglycerides from very low density lipoproteins and chylomicrons, enabling the subsequent uptake of free fatty acids by peripheral tissues (3;4). LPL also possesses the ability to enhance the cellular uptake of lipoprotein constituents independent of its enzymatic activity (5-8). LPL is synthesized by the parenchymal cells of extrahepatic tissues, especially myocytes and adipocytes. The lipase must then cross endothelial cells (9) where it associates with heparan sulfate proteoglycans (HSPGs) in the capillary bed (10). Enzymatically active LPL exists as a homodimer, with a head-to-tail orientation (11). Dissociation to monomeric form results in a loss of enzymatic activity (12-14) and a propensity of monomers to aggregate rather than re-associate (12). This dissociation occurs rapidly even under physiological conditions (15;16) in the absence of stabilizing molecules such as...
heparan sulfate (17) or heparan sulfate proteoglycans (14). The LRP receptor-associated protein (RAP) was first discovered as a 39-kDa protein that co-purified with LRP (18-20). RAP has since been found to bind other low density lipoprotein receptor family members and other molecules (21-23). RAP blocks the binding function of LRP in vitro and in vivo; in fact, overexpression of RAP has been successfully used to transiently inactivate LRP in adult mice, leading to remnant lipoprotein accumulation (24). Physiologically, RAP acts as a molecular chaperone necessary for the proper secretion of several cell surface molecules (21;25-27). Specifically, RAP has been shown to facilitate LRP folding and/or to prevent premature ligand binding in the secretory pathway (26-28). Thus, transgenic mice engineered to lack RAP expression show a 75% decrease in functional LRP (29). RAP is an ER-resident protein that is recycled by the KDEL receptor (30). The half-life for its turnover is 13-18 hours (26). Thus, its effects as a molecular chaperone are long-lived, but largely confined to the ER.

van Vlijmen and colleagues examined the effect of hepatic RAP overexpression in mice whose LDL receptor (LDLR) and/or LRP were knocked-out (31). The RAP overexpression in mice deficient in both LDLR and LRP resulted in a defect in the conversion of chylomicrons into smaller remnant particles, a conversion associated with LPL action. In addition, there was a striking hypertriglyceridemia. This defect correlated with the appearance of an increase in the concentration of inactive LPL in plasma. The accumulation of plasma lipids was not due to inhibition of a novel liver lipoprotein receptor since no RAP binding could be demonstrated in liver cells of mice that were LDLR and LRP deficient. These studies suggested the existence of an extra-hepatic RAP sensitive site. High RAP expression may have affected the maturation of LPL. In this study, we endeavored to examine the possibility of a direct binding interaction between LPL and RAP.

Our results show that RAP and LPL are capable of tight binding interactions both in vitro and in vivo. Compared to control adipocytes, cultured adipocytes derived from the somato-vascular fraction of RAP-deficient mice secrete LPL with a lower specific activity and lower affinity for the adipocyte cell surface. These results suggest that RAP may play a role as a chaperone/escort protein of LPL.

METHODS
Materials-The pGEX expression plasmid, pGEX-39-kD, containing the sequence for the rat RAP/glutathione-S-transferase fusion (GST) protein was kindly provided by Dr. Joachim Herz (University of Texas Southwestern Medical Center, Dallas).

RAP ELISA- RAP was expressed as a GST fusion protein in BL21 E. coli and purified by glutathione-agarose chromatography after the method of Iodanato et al. (32). The GST moiety was cleaved by incubating the fusion protein with 1.9U thrombin/mg protein at 37°C for 4-6 hours, and quenching with 0.5 mM phenylmethylsulfonic acid, whereupon the RAP was subjected to a second glutathione-agarose chromatography. To prepare RAP free of degradation products, the protein preparation was further purified by SDS-PAGE and the band corresponding to the full length RAP was excised and electroeluted with a Schleicher and Schuell Elutrap System in 192 mM glycine, 25 mM Tris-HCl, 0.1 % Hecameg, pH 8.3 (Fig.1A). Antibodies were generated in rabbits and immunoglobulins prepared by affinity chromatography using Affi-Prep 10 resin coupled to electroeluted RAP. A sandwich ELISA was designed as described previously (33). The standard curve was linear in the range from 0.05 to 6 ng with an r² of 0.99. At a level of 1 ng the net OD₄₀₀ was 0.170.
Mouse LPL ELISA- The mouse LPL cDNA (mL5 in pGEM-2) was obtained from Dr M. Schotz (UCLA). The 1.43 kb insert was excised with EcoR I and cloned into pBluescript II KS+ to give pB-LPL. pB-LPL was then cut with EcoRV and PstI and subcloned into pQE-32 between the Pst I and Smal sites for overexpression as a six-His fusion protein in E. coli. The six-His-LPL recombinant protein was purified first by a Ni$_2$ chelate affinity step followed by preparative SDS-PAGE. Bands corresponding to the fusion protein were excised and electroeluted. This protein was used for immunization of a goat and construction of an affinity column consisting of LPL-6His protein coupled to Affi-Prep resin (Bio-Rad) for immunoglobulin purification. A sandwich ELISA similar to that used for avian LPL (33) was set up with the following exceptions. Samples were pre-incubated in 1.2 M guanidium hydrochloride for one hour at 4 °C and then diluted five fold in the assay. The initial incubations of samples with the capture antibodies coated on microtiter plates were conducted at 4 °C in 0.8 M NaCl, 1% bovine serum albumin, 0.05% Tween 20, 10 mM sodium phosphate, pH 7.4. To enhance sensitivity the detecting immunoglobulins were coupled to biotin used in conjunction with strepavidin coupled to horseradish peroxidase. The standard curve was linear from 0.01 to 1.5 ng. At 0.1 ng the net OD$_{490}$ was 0.43.

Solid phase interaction of RAP and LPL- To preserve the integrity of the lipase, all steps were conducted at 4 °C. Microtiter plates (Corning) were coated (10 ng/well) with highly purified LPL overnight. Control wells were coated with nothing or an irrelevant protein (carbonic anhydrase (Sigma)). After washing with PBS/0.05% Tween® 20 (Sigma), plates were blocked overnight with 3% bovine serum albumin/PBS/0.05% Tween® 20. After three washes, 200 µl aliquots containing 0-500 ng of GST-RAP or RAP in 1% bovine serum albumin/PBS/0.05% Tween® 20 were added to each well in triplicate, and incubated overnight. All subsequent steps were performed as previously described (34). After washing, a horseradish peroxidase-conjugated rabbit anti-rat-RAP immunoglobulin was then added to the wells for four hours or overnight at 4 °C. After washing, binding was detected by reaction of horseradish peroxidase with o-phenylenediamine substrate solution. The OD$_{490}$ was measured after a 30-minute incubation in the dark.

Surface plasmon resonance- SPR experiments were conducted using a BIAcoreX apparatus (BIAcore Inc.). B1 chips were utilized for immobilization because they possess a low dextran surface density, thereby decreasing nonspecific LPL interaction. Affinity purified polyclonal antibodies to RAP were immobilized using amine coupling, according to manufacturer’s instructions (BIAcore Inc.), to a level of approximately 2500 resonance units (RU). RU have been shown to be proportional to total binding mass for a wide range of proteins (35). Recombinant rat RAP was then injected at concentrations of 300-400 nM to saturate the surface with RAP. The chip was blocked with 3% bovine serum albumin (Sigma) in running buffer. LPL, freshly diluted in running buffer, was injected. After 450 sec., glycine-HCl, pH 2.7 was injected to strip the RAP-LPL complexes from the sensor chip. After equilibration, the process was repeated, using a range of LPL concentrations.

For each curve analyzed, nonspecific binding was measured as LPL binding to an anti-RAP surface in the absence of RAP and subtracted from the experimental data. Binding interaction was measured at various flow rates to verify that mass transport was not limiting. In addition, data were fitted to a model utilizing mass transfer rates as an additional variable. Inclusion of mass transfer rates into the fitting did not lead to a significant increase in fit of the binding curves, indicating that the kinetics were not mass transport-limited.

Data analysis- Solid-phase binding data were plotted using SigmaPlot (Jandel Scientific). Curves were fitted using a rectangular hyperbolic saturation curve: $y=$
\[ a \times / (K_D + x) \] where \( y \) represents the bound ligand, \( x \) the free ligand, \( a \) the maximum amount of bound ligand, and \( K_D \) the dissociation constant for binding.

SPR data was analyzed using BIAevaluation 3.0 (BIAcore Inc.). This program allows for simultaneous global fitting of an entire concentration series of injected ligand. Global fitting provides a robust and stable fitting and reflects physical interaction systems more faithfully (36-38).

**Animal Use**—RAP knockout mice and control mice of the same background strain (C57BL/6J) were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were housed in sterile cages and given free access to autoclaved food and water. Procedures were approved by Cornell University IRB under protocol #97-98.

**Cell Culture**—Primary murine preadipocytes were harvested after the procedure of Litthauer & Serrero (39). Wild-type or RAP knockout mice less than 4 weeks old were sacrificed under CO\(_2\). The mice were quickly immersed in 95% ethanol and dissected. Inguinal and abdominal fat pads were removed and placed into Hank’s balanced salt solution that contained 0.2 mg/ml gentamycin. The tissue was rinsed through two more 50 ml tubes of Hank’s balanced salt solution containing gentamycin. The fat pads were weighed and then placed in pre-warmed digestion buffer (4% BSA, 0.3% collagenase in Hank’s balanced salt solution). With scissors, the fat was minced in the digestion buffer tube. The tissue/digestion buffer mixture was then incubated for one hour at 37°C with shaking. The digested fat was filtered through nylon filters (250 microns and 150 microns) and then centrifuged at 1500 rpm for 10 minutes. The floating adipocytes were aspirated and discarded. Growth medium was then added to the pre-adipocytes (10% FBS, 2 mM L-glutamine, PNS Antibiotic Mixture (Gibco/BRL), Dulbecco’s Modified Eagle’s Medium MCDB 153 (1:1), 0.5% methyl cellulose). The cells were spun, the media was removed by aspiration and the cells were resuspended in growth medium by gentle repeated pipetting. Cells were counted using Trypan blue to assess viability. Cells were plated in 6-well plates at a density of 150,000 cells/well. Upon reaching 80% confluence, the medium was changed to differentiation medium (1 µM dexamethasone, 0.5 mM IBMX, 10 µg/ml insulin, in growth medium). After 2-3 days, the differentiation medium was changed to triglyceride medium (0.02% Intralipid triglyceride solution, 10µg/ml insulin, in growth medium). After 10-14 days, cells were about 90% confluent and no further differentiation was noticeable.

The 3T3-F442A cells were obtained from Dr. Howard Green at Harvard Medical School. 3T3-L1 cells were obtained from ATCC. They were maintained and differentiated as described (40).

**Co-Immunoprecipitation of purified RAP and LPL proteins**—RAP (500 ng) and LPL (500 ng) were pre-incubated for 15 min at room temperature in PBS supplemented with 1% bovine serum albumin in a final volume of 100 µl. The solution was pre-incubated with 12 µg of control IgG coupled to IMMUNOBEADS (Irvine Scientific) for 1 h at 4 ºC and the beads separated by centrifugation. The RAP-LPL complexes in the supernatant were precipitated with approximately 12 µg anti-RAP immunoglobulins coupled to IMMUNOBEADS. This immunoprecipitation was carried out for 90 min at 4°C in a total volume of 225 µl with the following final concentrations: 1% Triton X-100, 1 mM PMSF, 0.05% SDS, 1% bovine serum albumin in PBS supplemented with an anti-protease mix. The immunobeads were separated by centrifugation, washed 3 times with 0.1% N-lauryl sarcosine in PBS and the bound proteins separated by SDS-PAGE. The separated proteins were transferred to PVDF membranes, probed with horseradish peroxidase-coupled anti chicken LPL immunoglobulins. The blots were visualized with the Pierce West Pico Substrate according to manufacturer’s instructions.

**Co-immunoprecipitation of LPL-RAP complexes in cell lysates**—3T3-F442A cells...
were grown to confluency and differentiated to adipocytes as described previously (40). Cells were concentrated by scraping cells in PBS followed by low speed centrifugation. Cell pellets from four 100 mm dishes were lysed and sonicated twice for 30 sec in 1% Triton X-100, 1 mM PMSF, in PBS supplemented with anti-protease cocktails and when indicated, 0.05 % SDS. Co-immunoprecipitation was then carried out as described for purified proteins in the previous section.

Co-immunoprecipitation of RAP-LPL complexes in glioblastoma U87 cells- U87 cells were grown to confluency in 60 mm dishes in MEM medium supplemented with NaHCO₃, HEPES, 10% fetal bovine serum, 1 mM pyruvate, 0.1 mM non-essential amino acids and 2 mM glutamine. Cells were transfected with plasmids (4 µg DNA/plasmid/60 mm dish) expressing either RAP(pCDNA3-RAP), avian LPL(pRcCMV-LPL) (41) or with both plasmids using the FuGENE™ Transfection Reagent (Roche). The RAP expression vector was generated by excising the RAP coding sequence from pGEX-KG-RAP with BamH I and Xho I and cloning into the same restriction sites of pcDNA3 (Invitrogen # V790-20). Total DNA employed was normalized to 8 µg / dish with empty vector. Forty eight hours after transfection cells were lysed (1% Triton X100, 1mM PMSF supplemented with an anti-protease cocktail). The lysate was sonicated (twice at 100W for 20 sec) and cleared by centrifugation at 20,000 g for 30 min. Lysates from 10 dishes were immunoprecipitated with 5 µg of anti-RAP immunoglobulins for 10 min on a shaker at room temperature and the complexes captured with 40 µl of a 50% slurry of Protein G Sepharose™. The matrix was washed six times with 0.1 % NP-40 in PBS, and the bound proteins released with 2X Laemmli’s buffer at 65 ºC for 30 min. Following removal of the beads by centrifugation, 2-mercaptoethanol was added to the supernatant to a concentration of 5% and proteins were resolved by SDS-PAGE prior to transfer to PVDF membranes and Western blotting with anti-avian LPL immunoglobulins as described above.

Cross-linking- 3T3-L1 adipocytes were grown to confluency in 500 cm² dishes. Cross-linking was carried out at 37°C for 20 min with 1 % formaldehyde essentially as described by Hall and Struhl (42). Each dish was washed with TBS (0.15 M NaCl, 20 mM Tris-HCl pH 7.4) and lysed in 7 ml of lysis buffer (50mM HEPES-KOH, pH 7.5, 0.15 M NaCl, 1% Triton X-100, 10 mM EDTA, 5 mM EGTA, 1 mM PMSF, and protease inhibitor mixture). The lysate was centrifuged at 20,000 g for 32 min. The cleared lysate was then incubated for 3 h at 4°C with 108 µg of control or anti-LPL immunoglobulins coupled to immunobeads. The beads were washed twice with each of the following buffer 1 to 4, in sequence. Buffer 1: Lysis buffer supplemented with 0.1% sodium deoxycholate and 0.1 % SDS and adjusted to pH 7.5. Buffer 2: Buffer 1 supplemented with 0.35 M NaCl. Buffer 3: 10 mM Tris-base pH 8.0, 0.25 M LiCl, 1 mM EDTA, 0.5% NP-40 and 0.5 % sodium deoxycholate. Buffer 4: 10 mM Tris-base pH 8.0, 1 mM EDTA. The bound proteins were eluted with 50 mM Tris-HCl pH 7.5, 10 mM EDTA, 1% SDS at 60 ºC, the beads were separated by centrifugation and 10X Laemmli and 2-mercaptoethanol added to the eluted material. After reversing the cross-linking by heating at 95°C for 20 min, aliquots of the eluate were employed for SDS-PAGE and Western blotting for RAP and LPL.

Sucrose gradient ultracentrifugation- RAP and/or LPL were pre-incubated for 2 hours at 4°C in BSA/TBS (20 mM Tris-HCl pH 7.5, 0.15 M NaCl, 0.1% BSA). The various RAP/LPL mixtures were then layered upon sucrose gradients. Sucrose gradients were prepared as follows: Sucrose was added to BSA/TBS to create 5, 7, 9, 11, 13 and 15% sucrose solutions. Two ml of each solution were layered in 14 X 95 mm Ultraclear Ultracentrifugation tubes (Beckman or Seton Scientific) at 4°C. After adding RAP/LPL mixtures to the top of the gradient, the tubes were centrifuged in an SW41 rotor for 18 hr at 38,000 r.p.m. at 4°C. Fractions were...
removed from the tubes by aspiration and placed on ice. RAP and LPL protein in each fraction were assayed by ELISA, as described previously. The refractive index of each fraction was measured by a hand-held refractometer (American Optical). Molecular weight estimation was attained by subjecting molecular weight standards to sucrose gradient ultracentrifugation under identical conditions as above. The molecular weight standards utilized were alcohol dehydrogenase (150,000 daltons), BSA (66,000 and 132,000), carbonic anhydrase (29,000), myosin or beta-amylase (200,000 and 205,000, respectively), and ovalbumin (45,000). One mg of each molecular weight standard was layered upon the sucrose gradient in the same volume of TBS as the RAP/LPL mixtures. After ultracentrifugation, fractions were aspirated as above. Fractions were assayed for protein by measuring OD 280 using a Beckman DU-65 spectrophotometer. The refractive index for each fraction was measured as above. The plot of log Mr against refractive index was fitted to a quadratic equation, \( y = -15,910 + 23580x – 8734x^2 \) (\( r^2 = 0.982 \)), where \( y \) is the log Mr and \( x \) the refractive index.

**Other methods**- The LPL activity assay was performed according to previously published methods (43). Avian LPL was purified from acetone powders of chicken fat (44). Avian LPL ELISA was carried out as described previously (33). The EndoH digestion was performed according to Cisar et al.(45), following acetone precipitation in the presence of 10 \( \mu \)g BSA.

**RESULTS**

**Binding of LPL to RAP by ELISA**

The binding interaction between RAP and LPL was examined first by enzyme-linked immunosorbent assay (ELISA). Binding of GST-RAP to immobilized LPL was saturable, with a \( K_D \) of 11 \( \pm \) 2.5 nM (Fig. 2). The dissociation constant for the binding of LPL to immobilized RAP was not significantly different (\( K_D \) of 9 \( \pm \) 2 nM). In addition, more gentle thrombin cleavage of GST-RAP, such that only a subset of RAP was cleaved, followed by repurification did not alter this result (data not shown).

With the protocol employed the anti-RAP immunoglobulins gave very low signals when microtiter plates were coated with carbonic anhydrase, albumin or GST. For these proteins the OD\(_{490}\) varied between 0 and 0.054 as the GST-RAP concentration was increased from 0 to 2500ng/ml. In contrast, the corrected OD for wells coated with10ng LPL/well ranged between 0 and 0.699.

To establish molecular specificity in the binding interaction between RAP and LPL, monoclonal antibodies against avian LPL were utilized. After pre-incubation with monoclonal antibodies, avian LPL/antibody pre-incubation solutions were added to microtiter plates coated with RAP. An array of 13 anti-LPL monoclonal antibodies was utilized as competitor. Four representative binding curves are shown (Fig 3). Some monoclonal antibodies showed inhibition of LPL binding to immobilized RAP at low concentrations, while others showed little inhibition up to 20 \( \mu \)g anti-LPL per well. Other monoclonal antibodies showed intermediate inhibition. The results suggest that there are specific epitopes that are necessary for the interaction between RAP and LPL. The epitope of XCAL-3-6a has been identified previously (41). Binding to LPL occurs in the C-terminus, requiring amino acid residues 310-465. XCAL 3-6a showed intermediate inhibition of binding, leveling off at about 20% of maximal binding. The data suggest that the LPL/RAP binding may take place in the C-terminus of LPL. The binding of RAP to LPL was inhibited by NaCl concentrations above 0.15 M. At a concentration of 0.5 M NaCl or greater, binding was negligible suggesting that charge interactions may be significant in the binding of RAP to LPL (data not shown).

**Binding of LPL to immobilized RAP by surface plasmon resonance**

To analyze the interaction in an independent experimental system, the binding was examined by surface plasmon resonance. Antibodies to RAP were immobilized by
amine coupling in both the experimental and control flow cell. The polyclonal antibody to RAP was immobilized at 2284 RU in one flow cell and at 2344 RU in the control flow cell. The experimental flow cell only was loaded with RAP. Injections with LPL at various concentrations were done in both flow cells at 5 µl/min. The difference between the resulting sensorgrams was taken as specific binding. The LPL was diluted just before injection, since the dissociation of dimers is increased with decreased LPL concentration (12). The corrected curves were then overlaid, and fit as described (Fig. 4). A representative overlay of the curves with the theoretical global fit is shown. The fitting was done with a 1:1 Langmuir fitting, with $R_{\text{max}}$ being locally fit. The justification for fitting $R_{\text{max}}$ locally is that the RAP was not immobilized but captured on antibody. As a result, there is some variation between injections in the amount of LPL that can bind, i.e. the $R_{\text{max}}$. The kinetic data derived from the individual fitting showed very faithful replication and a low error (Table 1). The calculated $K_D$ of 2.4 (± 2.1) nM is consistent with the $K_D$ estimated by ELISA. Determination of binding constants was also performed at much higher flow rate (75µl/min), to assess possible mass transport-limited binding. The resulting curves showed no change in kinetic parameters. In addition, the error was still small (Chi²=1.02) (data not shown).

To further demonstrate specificity, a series of injections were performed that included competing RAP (0-200 nM) with 100 nM LPL in the injection buffer. The inclusion of RAP led to a linear decrease in the amount of LPL binding the immobilized RAP, demonstrating specificity of binding (data not shown).  

Co-immunoprecipitation of RAP-LPL complexes in solution

The interaction of RAP and LPL was also studied in solution phase with highly purified LPL and recombinant RAP. The two proteins were incubated for one hour at 4°C (PBS, 1% bovine serum albumin, 0.05% SDS, protease inhibitors) and the complexes captured with anti-RAP immunoglobulins coupled to polyclaylamide beads. The bound proteins were released and separated by SDS-PAGE. Following transfer to PVDF membranes the LPL protein was detected with anti-LPL immunoglobulins coupled to horseradish peroxidase. Figure 5 demonstrates that even under stringent conditions, i.e., inclusion of 0.05% SDS in the incubation buffer, a RAP-LPL complex can be co-immunoprecipitated. However, LPL is not detected when RAP is not included in the pre-incubation.  

Distribution of RAP following sucrose gradient centrifugation in the absence or presence of LPL

Since the interaction between RAP and LPL would be predicted to occur in the ER, where both monomeric and dimeric forms of LPL are known to be present, it was desirable to establish the oligomeric state of LPL in the RAP/LPL complex. To this end, solution-phase binding of RAP and LPL was also tested using sucrose gradient ultracentrifugation. RAP and LPL were pre-incubated at 4°C and the mixtures were layered on top of 5-15% sucrose gradients. Following centrifugation, the fractions were assayed for LPL and RAP mass by ELISA. The mass of RAP or LPL in each fraction was plotted versus the refractive index of the fractions. In the experiment illustrated in Figure 6, 10 µg of RAP and 50 µg of LPL were employed. In the presence of LPL there was a dramatic shift in the position of the RAP peak that migrated deeper in the gradient demonstrating, in solution, the formation of a stable RAP-LPL complex (Fig 6). The position of the apex of the RAP protein peak corresponds to a molecular weight of 81 kD compatible with interaction of RAP with monomeric LPL. The LPL protein peak is wide due to denaturation of LPL with concomitant formation of monomer and aggregated forms of LPL (12). When the same experiments were repeated under conditions where RAP was in excess, a free RAP and bound RAP peak could be resolved (data not shown).
data from the sucrose gradient ultracentrifugation experiments thus clearly indicate the formation of a complex between RAP and monomeric LPL, but do not rule out the presence of a complex between RAP and LPL dimer.

Co-immunoprecipitation of RAP-LPL complexes in glioblastoma U87 cells

Since purified RAP showed high affinity interaction for purified LPL in solution we examined whether the interaction could be demonstrated in whole cell extracts. U87 glioblastoma cells were transfected with a plasmid expressing either LPL, RAP or both proteins (Fig 7). In the absence of LPL expression immunoprecipitation of lysates from cells expressing only RAP yielded no signal (lane 1). Expression of LPL alone yielded a very faint band that may be due to a low level of endogenous RAP expression. The signal was dramatically enhanced when both RAP and LPL were expressed in the same cells (lane 3). No signal was observed after the same film exposure time (3 min) when the first antibody (anti-RAP immunoglobulin) was omitted (data not shown).

Co-immunoprecipitation of RAP-LPL complexes in 3T3-F442A cell lysates

The experiments with U87 demonstrated clearly that RAP and LPL associated when the proteins are over-expressed. To explore whether the interaction could be detected with physiologically relevant levels of the two protein partners we turned to differentiated cultured adipocytes. 3T3-F442A adipocytes were lysed with a non-ionic detergent containing lysis buffer and co-immunoprecipitation was carried out as described above for isolation of complexes formed in solution. In this instance also endogenous complexes could be co-immunoprecipitated even under stringent buffer composition when 0.05% SDS was included in the lysis buffer (Fig 8).

Cross-linking experiments

To exclude the possibility that LPL/RAP complexes were formed during cell lysis, adipocytes were crosslinked in vivo while still attached to cell culture dishes with 1% formaldehyde. Following lysis and co-immunoprecipitation with affinity-purified anti-mouse LPL immunoglobulins coupled to immunobeads, the bound proteins were released, cross-linking reversed. The proteins were separated by SDS-PAGE and probed with antibodies to LPL or RAP or control immunoglobulins (Fig 9). When co-immunoprecipitation was carried out with anti-LPL antibodies a single band corresponding to RAP was detected by Western blotting. Since cross-linking was done in whole cells the presence of immunoprecipitated RAP provided direct evidence for in vivo RAP/LPL interaction.

LPL secretion by adipocytes from control mice and RAP deficient mice

To ascertain whether RAP had a physiological function in the secretion and processing of LPL, in vivo, the secretion of LPL in cultured adipocytes derived from rat+/+ and rap-/− mice was determined. The stromal-vascular fraction from adipose tissue was isolated and pre-adipocytes differentiated as described in Methods. The adipocytes derived from RAP deficient mice secreted 3.6 fold less LPL activity and the decrease in secreted enzyme activity could be accounted for mainly by a major decrease in enzyme specific activity (Table 2). The significant decrease in enzyme specific activity supports the notion that RAP expression is necessary for the expression and secretion of native, properly assembled LPL.

High affinity binding of LPL to heparin and heparan sulfate is a diagnostic property of native catalytically active LPL (46); therefore similar secretion measurements were conducted in the absence and presence of 100 units heparin/ml of medium with another cell batch. This treatment showed that heparin failed to stimulate LPL secretion in RAP deficient adipocytes. For control cells (n=6), the secretion rates were 4.47 ± 1.20 and 6.76 ± 2.03 ng/hr/μg DNA (P<0.02) in the absence and presence of heparin, respectively. For the rap−/− cells (n=6), the corresponding values were 3.02 ± 0.53 and 2.98 ± 1.77. These major differences in the secretion of LPL in control and RAP deficient cells suggest that
LPL secreted by rap/- cells fails to bind to the plasma membrane proteoglycans. **Binding of RAP to Endoglycosidase H-sensitive LPL**

Normally, RAP is confined to the ER and early Golgi. Therefore, it was of interest to see if RAP was able to bind LPL before it reaches the medial Golgi, at which point the LPL becomes resistant to Endoglycosidase H (EndoH). To examine this possibility, 3T3-F442A cells (mouse pre-adipocytes) were grown and induced to differentiate into adipocytes. When maximally differentiated, the cells were harvested. Co-immunoprecipitation was then performed as described earlier. Half of the precipitate were treated with EndoH, while the other half were used as control. Following SDS-PAGE, Western blots were carried out, using antibodies to LPL. The resulting blots demonstrated that a large fraction of the co-immunoprecipitated LPL was endoglycosidase H sensitive (Fig 10). This result is consistent with the notion that RAP is capable of binding LPL in the ER.

**DISCUSSION**

In the present study we have characterized the binding interaction between RAP and LPL and shown that RAP-deficient adipocytes secrete LPL with low specific activity thereby demonstrating a novel function of RAP in LPL assembly or secretion. High affinity binding was demonstrated by ELISA, by surface plasmon resonance, by co-immunoprecipitation with highly purified proteins. The coimmunoprecipitation established binding in lysates from overexpression systems, as well as endogenous protein from multiple cell types. Specificity for binding interaction was established by competition experiments with monoclonal antibodies to LPL or in surface plasma resonance experiments where soluble RAP competed with immobilized RAP. Finally, cross-linking studies demonstrated binding between RAP and LPL in vivo.

Catalytically active LPL is a homodimeric protein (12) with high affinity for heparin. In buffer solutions, in the absence of stabilizing agents like detergents or heparin, LPL is strikingly unstable and dissociates into monomers that are in equilibrium with the dimeric form. The monomeric species can associate irreversibly into high molecular aggregates that are catalytically inactive (12). In whole cells, acquisition of enzymatic activity, a process often referred to as “maturation”, occurs following glucose trimming of glycan chains (47). Recent experiments (46) have convincingly shown that assembly of active dimers occurs in the ER. There is direct evidence that calnexin (48) and calreticulin (49) promote the folding of the enzyme and act as chaperones for LPL.

RAP has been described as a “specialized” chaperone/escort protein for members of the LDL receptor family (26-30). Experimental data suggest that one of the major functions of RAP is to prevent the premature binding of ligands to lipoprotein receptors early in the secretory pathway. The C-terminal region of LPL contains the domain interacting with LRP between residues 378 and 423 of human LPL (50). Similarly, the basic residues constituting the major heparin binding domain of avian LPL, lys 321, arg 405, arg 407, lys 409 and lys 416, are also located in the distal carboxyl-terminal region (51). Monoclonal antibody XCAL 3-6a, which inhibits LPL binding to heparin and heparan sulfate (41), inhibited over 75% of the binding of LPL to RAP. Furthermore, our data showing endoglycosidase H-sensitivity of RAP-bound LPL from cell extracts suggests that at least a subset of the LPL/RAP binding is also early in the secretory pathway. The information at hand is compatible with the hypothesis that RAP may have, in maturation of LPL, a function similar to its role with members of the LDL family, i.e., inhibition of interaction with binding partners like LRP or heparan sulfate early in the secretory pathway.

The sucrose density gradient analyses demonstrated the formation of a RAP/LPL complex that migrated with an apparent Mr of 81 kD, most compatible with the binding of RAP to monomeric LPL. If this is also the case in vivo, RAP may play a role in
stabilizing the properly folded monomers prior to their assembly as dimers. RAP-deficient adipocytes secreted LPL with a significantly lower specific activity than control cells. This finding demonstrates, in vivo, that RAP plays a role in the assembly of properly folded native LPL. In control adipocytes, a significant fraction of the newly secreted lipase binds to heparan sulfate on the plasma membrane. From this surface-associated pool a fraction is released and the balance is internalized (33). The internalized LPL is either degraded or recycled to the plasma membrane (33). High affinity binding to heparin and heparan sulfate is a hallmark of native active LPL (46). When heparin is added to adipocytes in culture, LPL binding to the cell surface is inhibited and the apparent secretion rate in the medium is increased and degradation greatly inhibited. However, when RAP-deficient adipocytes were exposed to heparin the expected stimulation of LPL secretion was not observed. This was probably due to ineffective binding of the poorly folded lipase secreted by RAP-deficient cells.

In summary, it was demonstrated that RAP binds LPL with high affinity and interacts with the enzyme in the C-terminal region. Sucrose density experiments suggest that RAP binds to monomeric forms of the enzyme. LPL secreted by RAP-deficient adipocytes exhibits a low specific activity that may be indicative of defective folding. Based on this information the following hypothesis is proposed: Following trimming of glucose residues on glycan chains, RAP binds to folded native monomers, stabilizing LPL transiently while dimeric LPL species are formed. As LPL transits through the secretory pathway, the gradual decrease in RAP concentration and decrease in pH would favor LPL dimer assembly. RAP is a well recognized chaperone/escort protein for members of the LDL receptor family (26). The data presented in this report suggest a novel function of RAP as a molecular chaperone/escort of LPL.

Acknowledgements
We wish to acknowledge the invaluable technical assistance of L. Barry Hughes. In addition, the RAP ELISA was optimized and validated by A. Alexander Hofling. Clément Chappuis assisted with the SPR competition binding. Dr. Joachim Herz is thanked for providing the RAP expression vector and Dr. Mark Doolittle is thanked for kindly providing technical information regarding the sucrose gradient ultracentrifugation of LPL. This work was supported by grants from the National Institutes of Health Grant #HL14990, #DK7158 and internal grant funds from Nutritional Sciences, Cornell University.
FIGURE LEGENDS

**Figure 1- Characterization of the anti-RAP immunoglobulins**

A. Recombinant GST- RAP protein was purified as described in Methods by glutathione-Agarose chromatography, followed by thrombin cleavage, second glutathione-Agarose chromatography and electrophoresis. Six µg of RAP were run on a 12.5% denaturing gel. B. Western Blot of adipose tissue, lane 2; liver extract, lane 3; and 50 ng of recombinant RAP, lane 1. C. McA-RH7777 rat hepatoma cells were labeled for 2 hours with 100 µCi of TRAP-<sup>35</sup>S per 60 mm dish. RAP was immunoprecipitated with anti-RAP immunoglobulin / protein A-Sepharose (lane 1). In lane 2, the anti-RAP immunoglobulins were pre-absorbed with 100 µg of recombinant purified RAP.

**Figure 2- Solid-phase binding of GST-RAP to LPL**

RAP binding to LPL was assayed by ELISA. LPL (10 ng/well ) was immobilized in microtiter plates, as described in Methods. The plate was blocked with 3% bovine serum albumin, 0.05 % Tween® 20 in PBS. GST-RAP at different levels was then added to the wells and incubated overnight at 4°C. Following washing of the wells and addition of horseradish peroxidase-conjugated rabbit anti-RAP immunoglobulins, and development with o-phenylenediamine substrate, OD<sub>490</sub> was measured. Values are mean ± standard deviations of triplicate measurements. With 2500 ng /ml of GST the OD<sub>490</sub> was less than 0.015.

**Figure 3- Epitope-specific inhibition of LPL/RAP solid-phase binding.**

RAP (10 ng/well) was immobilized in microtiter plates as described in Methods. LPL was pre-incubated with various monoclonal anti-LPL antibodies for 2 hours at 4°C. Binding of LPL, assayed as in Figure 2, was normalized to LPL binding to RAP after incubation in the absence of RAP.

**Figure 4- Binding of LPL to immobilized RAP by surface plasmon resonance.**

Sensorgram (squares) illustrating association and dissociation of LPL to RAP at concentrations ranging from 20 to 170 nM. Conditions and controls are described in Methods. Anti-RAP was immobilized on a B1 sensor chip. 400 nM RAP was injected over a single flow-cell with immobilized anti-RAP immunoglobulins. Various concentrations of LPL (13, 33, 49, 65 and 85 nM) were injected over both flow-cells. The signal from the control flow-cell was subtracted from the experimental flow-cell for each concentration of LPL. The curves were overlaid and fit to a 1:1 Langmuir fit individually and using Global fitting (BIACalculator 3.1) (continuous line), with identical results.

**Figure 5- Assembly and co-immunoprecipitation of RAP-LPL complexes in solution.**

Highly purified avian LPL (500 ng) and recombinant RAP (500 ng) were pre-incubated for 90 minutes at 4°C in PBS supplemented with 1% bovine serum albumin in a volume of 100 µl. The mixture was first pre-cleared with control IgG coupled to beads. The RAP-LPL complexes were immunoprecipitated with 12 µg anti-RAP immunoglobulins coupled to IMMUNOBEADS (Irvine Scientific). Co-immunoprecipitation was carried out for 1h at 4°C (1% Triton X-100, 1 mM PMSF, 0.05 % SDS, 1% bovine serum albumin in PBS supplemented with protease inhibitors). Co-immunoprecipitated proteins were separated by SDS-PAGE, transferred to a PVDF membrane and probed with anti-LPL immunoglobulins as described in Methods. Lane 1 was loaded with 5 ng of LPL; In lane 2, co-immunoprecipitation was carried out in the absence of RAP; In lane 3, co-immunoprecipitation was conducted with both RAP and LPL.

**Figure 6- Sucrose density gradient separation of RAP in the presence and absence of LPL.**

Ten µg of RAP and 50 µg of LPL were pre-incubated for 2 hours at 4°C in 20 mM Tris-HCl pH 7.5, 0.15 M NaCl, 0.1 % bovine serum albumin either singly or together. These mixtures were layered on top of a 5-15% sucrose gradient. The samples were subjected to centrifugation for 18 hr. at 38,000 r.p.m in an SW41 Beckman rotor. LPL and RAP were quantified in each fraction by ELISA and the refractive index was measured with a refractometer.
Figure 7- Co-immunoprecipitation of RAP/LPL complexes in U87 cells. Glioblastoma U87 cells were grown in 60 mm dishes and transfected with plasmids expressing RAP (lane 1), LPL (lane 2) or RAP and LPL (lane 3). 50 ng of purified LPL was loaded directly in lane 4. Cell lysate corresponding to ten 60 mm dish was employed for immunoprecipitation with anti-RAP antibodies and Western blotting with anti-chicken LPL immunoglobulins. Blots were visualized with the Pierce SuperSignal West Pico Chemiluminescent Substrate according to the manufacturer’s instructions.

Figure 8- Co-immunoprecipitation of RAP-LPL complexes from 3T3-F442A cell lysates. 3T3-F442A cells were grown to confluency and differentiated to adipocytes. Cells were lysed with 1% Triton X-100, 1 mM PMSF, in PBS supplemented with protease inhibitors. Co-immunoprecipitation was carried out as described in legend to Figure 5. Lanes 1 and 2, cell lysate (1 µl) loaded directly, before immunoprecipitation; lane 3, 25 ng recombinant mouse LPL (6His-LPL). Lane 4 and 5, co-immunoprecipitation of RAP-LPL from 950µl cell lysate. In lane 5, the lysis buffer was supplemented with 0.05% SDS during co-immunoprecipitation.

Figure 9- In vivo cross-linking of RAP and LPL. 3T3-L1 cells were differentiated to adipocytes and cross-linked with 1% formaldehyde as described in Experimental Procedures. Cell extracts from 10 dishes (500 cm²) were immunoprecipitated with immobilized affinity purified anti-LPL immunoglobulins or control immunoglobulins. Following release of bound proteins and high temperature release of cross-linking in a total volume of 222 µl, Western blotting was carried out with anti-RAP immunoglobulins (A) or anti-LPL immunoglobulins (B). In panel A: Lane 1, 28 µl of eluate from control immunoglobin beads; Lane 2, 28 µl of eluate from anti-LPL immunoglobin beads; Lane 3, lane with no protein loaded. Lane 4, 10 ng of recombinant RAP. In panel B: Lane 1, 10 µl of eluate from control immunoglobin beads; Lane 2, 10 µl from the eluate of anti-LPL immunoglobin beads. Lane 3, 25 ng of mouse LPL-6His recombinant protein.

Figure 10- Co-immunoprecipitation of RAP and endoglycosidase H-sensitive LPL. 3T3-F442A cells were differentiated to adipocytes. RAP-LPL complexes were immunoprecipitated as described in legend to Figure 5 except that the complexes were captured with protein G-Sepharose. After washing the beads, the proteins were released from the beads, denatured and subjected to digestion with endoglycosidase H as described in methods. Proteins were then visualized by Western blotting with anti-LPL immunoglobulins. Results are representative of two independent experiments. Lane 1: Cell lysate precipitated with control mouse IgG; Lane 2: immunoprecipitated proteins treated with endoglycosidase H; Lane 3: Immunoprecipitated proteins loaded directly on SDS-PAGE with no endoglycosidase H treatment.

REFERENCES

**FOOTNOTES**

1-The abbreviations used are: ELISA, enzyme-linked immunosorbent assay; EndoH, endoglycosidase H; GST, glutathione-S-transferase; HSPG, heparan sulfate proteoglycan; LDL, low density lipoprotein; LDLR, LDL receptor; LRP, LDLR-related protein; RAP, LRP receptor-associated protein; LPL, lipoprotein lipase; RU, resonance units;

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**KEYWORDS**

LDL receptor, LDL receptor-related protein, LRP receptor-associated protein, lipoprotein lipase.
Table 1: Kinetic binding constants for LPL binding to RAP measured by surface plasmon resonance. Kinetic binding constants were derived as described in Methods. Anti-RAP immunoglobulins were immobilized on both flow cells of a B1 sensor chip. 400 nM of recombinant RAP was injected over a single flow cell. Various concentrations of LPL (13-85nM) were injected over both flow cells. The signal from the control flow cell was subtracted from the experimental flow cell for each concentration of LPL. The resulting sensorgrams were overlaid and fit globally using BIAevaluation 3.0 (BIAcore Inc.).

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<th>Parameter</th>
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<th>$k_{off}(s^{-1})$</th>
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Table 2: Lipoprotein lipase secretion by adipocytes from rap+/+ and rap-/- mice. Somato-vascular fractions were prepared from rap+/+ and rap-/- mice. The cells were differentiated into adipocytes as described in Methods and LPL secretion measured over a 5 hour period. The values represent means ± standard deviations of 6 observations.

<table>
<thead>
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<th></th>
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<th>LPL protein</th>
<th>LPL spec. activity</th>
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<td></td>
<td>nmolFA/h/µg DNA</td>
<td>ng/h/µg DNA</td>
<td>nmolFA/h/µg DNA</td>
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<tr>
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<tr>
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a, b, c, P<0.0001
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
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Figure 10
Interaction of lipoprotein lipase and receptor associated protein
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J. Biol. Chem. published online March 3, 2006

Access the most updated version of this article at doi: 10.1074/jbc.M600995200

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