Analysis of Ligand Binding to the \( \alpha_2 \)-Macroglobulin Receptor/Low Density Lipoprotein Receptor-related Protein

EVIDENCE THAT LIPOPROTEIN LIPASE AND THE CARBOXYL-TERMINAL DOMAIN OF THE RECEPTOR-ASSOCIATED PROTEIN BIND TO THE SAME SITE*

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The endocytic \( \alpha_2 \)-macroglobulin receptor/lipoprotein receptor-related protein (\( \alpha_2 \)MR/LRP) binds several classes of extracellular ligands at independent sites. In addition, \( \alpha_2 \)MR/LRP can bind multiple copies of the 39–40-kDa receptor-associated protein (RAP). Both amino-terminal and carboxyl-terminal fragments of RAP exhibit affinity, and the fragments apparently bind to different sites on the receptor. RAP completely inhibits the binding of all presently known extracellular ligands, whereas several ligands such as \( \alpha_2 \)-macroglobulin and tissue-type plasminogen activator are poor inhibitors of RAP binding. Since RAP is largely an intracellular molecule that normally does not occupy \( \alpha_2 \)MR/LRP at the cell surface, we hypothesized that an established extracellular ligand might bind to those sites on the receptor capable of binding the RAP fragments. We found complete cross-competition between carboxyl-terminal RAP fragments and fragments of lipoprotein lipase containing the recently identified binding domain for \( \alpha_2 \)MR/LRP (Nykjaer, A., Nielsen, M., Lookene, A., Meyer, N., Reigaard, H., Etzerodt, M., Beisiegel, U., Olivcrona, G., and Gliemann, J. (1994) J. Biol. Chem. 269, 31747–31755). Moreover, the lipoprotein lipase fragment completely inhibited the binding of several \( \alpha_2 \)MR/LRP ligands in a pattern similar to that of carboxyl-terminal RAP fragments. On the other hand, the amino-terminal RAP fragment was a poor competitor of binding of the lipoprotein lipase fragment, whereas it competed effectively with pro-uPA for binding to the receptor. The results provide evidence that lipoprotein lipase binds to the site on \( \alpha_2 \)MR/LRP also available for binding of the carboxyl-terminal domain of RAP and suggest that pro-uPA may bind to or overlap the site available for the amino-terminal domain of RAP.

The endocytic \( \alpha_2 \)-macroglobulin receptor/lipoprotein receptor-related protein (\( \alpha_2 \)MR/LRP) is a 60-kDa endocytic receptor that is translated as a single polypeptide and cleaved in the Golgi network into an 85-kDa membrane-spanning chain and a large extracellular chain that remains attached to the 85-kDa chain via noncovalent linkages. The extracellular chain contains multiple copies of two types of cysteine-rich repeats, the growth factor repeats and the complement-type repeats, that provide binding sites for several structurally unrelated ligands. The complement-type repeats are clustered in four regions containing two, eight, 10, and 11 repeats. The ligands include the receptor-binding form of \( \alpha_2 \)-macroglobulin (\( \alpha_2 \)M*), apolipoprotein E-containing lipoproteins, lactoferrin, tissue-type plasminogen activator (tPA), pro-urokinase (pro-uPA), complexes of the type-1 plasminogen activator inhibitor (PAI-1) and tPA as well as uPA, and lipoprotein lipase (LpL), both free and associated with lipoproteins (for reviews, see Refs. 1–4). Several ligands do not compete with one another for binding to \( \alpha_2 \)MR/LRP. For example, \( \alpha_2 \)M* does not compete with tPA or lactoferrin for binding to \( \alpha_2 \)MR/LRP (5, 6), and \( \alpha_2 \)M* is a poor inhibitor of binding of apolipoprotein E-containing lipoproteins (6–8). However, lactoferrin is able to inhibit the binding of apolipoprotein E-containing lipoproteins to \( \alpha_2 \)MR/LRP, suggesting that these ligands may bind to the same site (6).

The 39–40-kDa receptor-associated protein (RAP) was first identified as a component copurifying with \( \alpha_2 \)MR/LRP prepared from human placenta (9–11). Subsequent cloning and sequencing (12) revealed that it is the human homologue of mouse heparin binding protein 44 (13) and of rat Heymann nephritis target protein (14). RAP was first shown to inhibit the binding of \( \alpha_2 \)M* to the receptor (15), and several subsequent studies have demonstrated that RAP, surprisingly, is capable of inhibiting the binding and uptake of all known ligands to \( \alpha_2 \)MR/LRP in vitro and in vivo (1–4, 16). On the other hand, most ligands have been reported to compete poorly for binding of RAP to \( \alpha_2 \)MR/LRP. For instance, tPA and \( \alpha_2 \)M* at high concentrations cause little inhibition of binding of labeled RAP to the receptor (2, 5). This lack of reciprocal cross-inhibition is presumably due to the complex mode of binding of RAP to the receptor.

RAP binds with high affinity to \( \alpha_2 \)MR/LRP, and the capacity of the receptor has been reported to be two (17) or up to six (18) RAP molecules per receptor molecule. Previous studies have identified strong binding of labeled RAP to fragments of \( \alpha_2 \)MR/LRP associated with low density lipoprotein receptor-related protein; RAP, \( \alpha_2 \)MR/LRP-associated protein; LpL, lipoprotein lipase; \( \alpha_2 \)M*, receptor-binding form of \( \alpha_2 \)-macroglobulin; uPA, urokinase-type plasminogen activator; tPA, tissue-type plasminogen activator; PAI-1, type-1 plasminogen activator inhibitor; LDL, low density lipoprotein; VLDL, very low density lipoprotein; GST, glutathione S-transferase.
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LRP containing the cluster of eight complement-type repeats (19, 20). In addition, most of the above cited extracellular ligands are also bound in this region. Binding of RAP, although weak, has also been observed to a domain containing the cluster of 11 complement-type repeats (20). It is uncertain whether the stronger binding to the former domain is simply due to a higher affinity or to multiple binding sites within that domain. In addition to α2MR/LRP, recent results have shown that RAP also binds to the other known members of the LDL receptor family, i.e. gp330/megalin (21, 22), VLDL receptor (23, 24), and LDL receptor (25).

The finding that RAP may function as a universal antagonist has led to the hypothesis that it may modulate the function of LDL receptor family members, particularly α2MR/LRP. However, whereas α2MR/LRP is primarily confined to clathrin-coated pits in the plasma membrane and within endosomal vesicles (26, 27), RAP is largely an intracellular protein located in the endoplasmic reticulum and the Golgi complex (27–29). The function of RAP in those compartments is presently unknown, although it has been hypothesized that RAP may prevent binding of other ligands to the multifunctional receptors before they reach the plasma membrane (29–31). Consistent with this, RAP is only associated with α2MR/LRP at the plasma membrane to a small extent, if at all (27, 32). Furthermore, attempts to detect RAP in conditioned media of cell incubations and in the blood have repeatedly been negative. Since α2MR/LRP is normally unoccupied by RAP at the cell surface, we considered the possibility that the RAP sites might bind one of the established extracellular ligands. Thus, we decided to investigate whether LpL might bind to those sites since we have previously demonstrated that LpL, like RAP, binds to multiple sites on the receptor (33), and since it was recently reported that LpL and the carboxy-terminal folding domain of LpL inhibit binding of RAP to α2MR/LRP (34). In the present analysis, we made use of a peptide containing the receptor binding site of LpL (35). Since amino-terminal and carboxy-terminal RAP fragments can interact with separate sites on α2MR/LRP (36), it became important to analyze the competition of the receptor-binding LpL fragment with both amino-terminal and carboxy-terminal RAP fragments (37). We find that the LpL fragment and the carboxy-terminal RAP fragment exhibit complete reciprocal cross-inhibition and that the LpL fragment can inhibit the binding of several established ligands for α2MR/LRP.

MATERIALS AND METHODS

α2MR/LRP and Natural Ligands—Human α2MR/LRP was prepared from solubilized placental membranes by affinity chromatography using immobilized α2M*. Elution was in 150 mM NaCl, 5 mM EDTA, 10 mM sodium phosphate, 0.6% 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid, pH 6.0, and RAP was removed from the receptor by incubation with heparin-Sepharose at pH 8.0 (15). Human α2M was converted to the receptor-binding form (α2M*) by incubation with 200 mM methylamine for 2 h followed by dialysis (38). Bovine LpL, prepared as described previously (39), was a gift from Dr. G. Olivercrona, Department of Biochemistry and Biophysics, University of Umeå, Sweden. Recombinant human pro-uPA was a gift from Dr. J. Henkin (Abbott Company, Abbott Park, IL) (40). Two-chain active uPA was from Sepragen, Switzerland, and TPA was from Boehringer-Ingelheim, Germany. Recombinant PAI-1 was produced in Escherichia coli and purified essentially as described by Relly et al. (41). Complexes of labeled uPA or TPA and PAI-1 were obtained by incubation with a 10-fold molar excess of PAI-1 for 2 h at 20°C, and SDS-polyacrylamide gel electrophoresis showed >80% of the tracer in the complex form. Exposure and Expression of Reombinant Proteins—The construct HFX-LpL (378–448), containing the hexahistidine-factor X substrate sequence MGSH5/8EGR and amino acids 378–448 of human LpL, was prepared as described previously (39). It was expressed in E. coli DH1 cells and purified on a Ni2+–nitrilotriacetic acid column (44). In some experiments we used the previously described (35) CII_MLCH_FX-LpL (378–448) construct containing a ~150-aminio acid fusion partner (i.e. the NH2-terminal 30 amino acids of the clC1, phage protein plus the NH2-terminal amino acids of chicken myosin light chain). Separate experiments showed that the fusion proteins containing LpL (378–448) and LpL (347–448) had the same inhibitory potencies on the binding of LpL to α2MR/LRP (35).

Fusion proteins of glutathione S-transferase with RAP or RAP fragments (GST-RAP constructs) were expressed and purified as described previously (37). Briefly, polymerase chain reaction was used to amplify specific sequences of the RAP cDNA, which were ligated into the pGEX-4T-2 expression vector. Proteins expressed in E. coli were isolated and purified via affinity chromatography on glutathione-agarose. RAP (1–114) and RAP (115–319) were purified from the thrombin-treated GST constructs as described previously (37). Detailed characterization of all constructs has been described (36, 37, 45). The purity of all recombinant proteins was confirmed by SDS-polyacylamide gel electrophoresis and staining with Coomassie Brilliant Blue.

In some experiments, we used recombinant human RAP with the fusion tail (HFX) cleaved off by factor Xa (44) as the labeled species. Separate experiments showed that the affinities for binding of human RAP and rat RAP without fusion proteins to purified α2MR/LRP were indistinguishable. In addition, the concentrations of rat RAP and GST-RAP required to cause half-maximal inhibition of binding of 125I-labeled human RAP were similar, with EC50 values ranging between 0.1 nM and 0.4 nM.

Protein Iodinations—All proteins were 125I-labeled to specific activities of approximately 5 × 104 Bq/nmol as described previously (33, 35, 40) using chloramine T as the oxidizing agent.

Incubations—Incubation of labeled α2MR/LRP with GST-RAP constructs immobilized onto polyvinylidene fluoride membranes using a Bio-Rad vacuum slot blotter, was performed as described (33) using 140 mM NaCl, 10 mM Hapes, 2 mM CaCl2, 1 mM MgCl2, 1% bovine serum albumin (buffer A), pH 7.8. Following autoradiography, the relative amount of bound tracer was measured by laser scanning densitometry using a 2202 Ultrascan instrument (LKB, Sweden). α2MR/LRP was immobilized in Maxisorp, and LpL was immobilized in Polysorb microtiter wells (NUNC, Denmark) as described (35), and the LpL fragment was immobilized using Covalink microwells (NUNC, Denmark). After wash and blocking with 5% bovine serum albumin, the solid phase assays were performed by incubation for 16 h at 4°C in buffer A. Finally, following a wash with >2 × 200 μl of incubation buffer, radioactivity bound to the well was removed by the addition of 2 × 200 μl of 10% SDS and counted. COS-1 cells (American Type Culture Collection CRL 1650) were incubated in monolayers (about 3.5 × 105 cells/well) essentially as described previously for adherent choriocarcinoma cells (46). Degradation of labeled ligand was assessed by measuring radioactivity in the medium released in 12% trichloroacetic acid. After washes, the cells were lysed in 1 mM NaOH and assayed for cell-associated radioactivity.

RESULTS

The Receptor-binding Fragment of LpL Inhibits Binding of RAP to α2MR/LRP—Since ligands that bind to the same site must cross-compete and since it is established that RAP can inhibit the binding of LpL (33, 47, 48) and its receptor-binding fragment (35) to α2MR/LRP, we first examined whether the LpL fragment could inhibit the binding of RAP to the purified receptor. As shown in Fig. 1A, the LpL fragment (amino acids 378–448) completely inhibited the binding of 125I-labeled RAP to purified α2MR/LRP immobilized in microtiter wells. The inhibitory potency of dimeric LpL was higher than that of the
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The Pattern of Inhibition Is Similar to That of Carboxy-terminal RAP Fragments—Since previous results have demonstrated independent receptor binding sites within the amino-terminal (amino acids 1–114) and the carboxy-terminal (amino acids 115–319) regions of the RAP molecule (37), the question arose as to whether LpL might compete with binding of either the amino-terminal or the carboxy-terminal RAP fragments.

As shown in Fig. 3, the LpL fragment in fact blocked the binding of 125I-labeled α₂MR/LRP to immobilized fusion proteins (GST-RAP constructs) containing RAP-(1–114) or RAP-(115–319) and to constructs containing truncated variants of the carboxy-terminal RAP fragment. GST-RAP-(1–100) was included as a negative control since this fragment has been reported not to bind to the receptor (37). These experiments might suggest that the LpL fragment interacts directly with all sites on α₂MR/LRP available for binding of different domains in RAP. However, since the amino- and carboxy-terminal RAP fragments compete with one another for binding to the different sites on cellular α₂MR/LRP (36), it seemed possible that LpL might bind predominantly to one of the sites on the receptor molecule.

To answer this question, we first analyzed the abilities of the GST constructs containing the amino-terminal and the carboxy-terminal RAP fragments, and their truncated variants, to compete with binding of 125I-labeled full-length RAP to immobilized α₂MR/LRP. As shown in Table I, unlabeled GST-RAP-(115–319) was nearly as effective a competitor as unlabeled GST-RAP-(1–319). The truncated variants of the carboxy-terminal RAP fragment, e.g. RAP-(187–319), also competed effectively, with EC_{50} values of approximately 2 nM. On the other hand, the NH₂-terminal fragment and its truncated variants, which bound to the receptor approximately as effectively as GST-RAP-(187–319), were poor competitors for binding of 125I-labeled full-length RAP, with EC_{50} values of 200 nM or higher. Since the LpL fragment and the carboxy-terminal RAP fragment both competed effectively with full-length RAP for binding to α₂MR/LRP, the possibility arose that they might bind to the same site on the receptor.

FIG. 1. Competition by LpL and the receptor-binding fragment of LpL for binding of RAP to immobilized α₂MR/LRP. A, purified α₂MR/LRP (about 100 fmol/well) was immobilized in microtiter wells, and incubations (100 μl) were performed for 16 h at 4 °C with about 20 pm 125I-RAP for 16 h at 4 °C, in the absence of unlabeled competitor, and this maximal binding was set at 100%. The blank value (<1% bound tracer) was subtracted from all other values. B, bovine LpL (about 10 fmol/well) was immobilized in microtiter wells and incubated (36 h, 4 °C) with about 10 pm 125I-α₂MR/LRP and varying concentrations of LpL-(378–448). Fifty-five percent of the added 125I-RAP was bound to the immobilized receptor in the absence of unlabeled competitor, and this maximal binding was set at 100%. The blank value (<1% bound tracer) was subtracted from all other values. The results show the mean values of triplicates ± 1 S.D.

FIG. 2. Competition by the receptor-binding LpL fragment for binding and degradation of RAP in cells. Upper panel, COS-1 cells (3.5 × 1 0⁶ cells/well) were incubated with about 20 pm 125I-RAP for 16 h at 4 °C without additions, with 400 nM unlabeled RAP, or with 2 μM LpL-(347–448). The results show the cell-bound 125I-RAP in percentage of the amount of tracer added to the incubations. Lower panel, COS-1 cells (3.5 × 10⁶ cells/ml) were incubated for 4 h at 37 °C with additions as described in the upper panel, followed by measurement of the cell-bound (open bars), trichloroacetic acid-precipitable (hatched bars), and trichloroacetic acid-soluble radioactivity (filled bars) in the incubation medium. The results are the mean values of triplicate incubations ± 1 S.D.
To further test this hypothesis, the amino- and carboxy-terminal GST-RAP constructs were labeled with a fluorescent dye and incubated with LpL in the presence or absence of unlabeled GST-RAP constructs. As shown in Fig. 4, the inhibitory potencies of GST-RAP-(1–114) and GST-RAP-(115–319) toward binding of GST-RAP-(1–114) on the one hand, and GST-RAP-(115–319) on the other, were of similar magnitudes. By contrast, GST-RAP-(1–114) was a poor inhibitor of binding of the carboxy-terminal RAP fragments. Thus, the receptor-binding fragment of LpL showed a pattern similar to the carboxy-terminal RAP fragments in the sense that the inhibitory potencies were similar toward binding of the labeled amino- and carboxy-terminal fragments. In addition, the LpL fragment was immobilized in microtiter wells and incubated with labeled amino- and carboxy-terminal RAP fragments produced by thrombin cleavage of the GST constructs. As shown in Fig. 4, the inhibitory potencies of GST-RAP-(1–114) and GST-RAP-(115–319) were similar to that of RAP, although the GST moiety appeared to cause a slight increase in the inhibitory potency of the carboxy-terminal domain. The potency of GST-RAP-(1–114) was lower than that of GST-RAP-(115–319), and RAP-(1–114) caused inhibitions only at very high concentrations (i.e., its inhibitory potency was much lower than that of RAP-(115–319)). We conclude from the combined data that the receptor-binding domain of LpL and the carboxy-terminal RAP fragment cross-compete, indicating that they bind to the same or strongly overlapping sites on LpL. In addition, like the carboxy-terminal RAP fragment (36), the LpL fragment is an effective competitor for binding of the amino-terminal RAP fragment to the receptor, whereas the amino-terminal RAP fragment is a poor competitor for binding of both the carboxy-terminal RAP fragment and the LpL fragment.

The Amino-terminal RAP Fragment May Bind Near the Site for pro-uPA in LpL—In the course of these analyses we examined the ability of the amino- and carboxy-terminal GST-RAP constructs to inhibit the binding of other ligands, and we chose pro-uPA, since the pattern of inhibition has already been established for α2M* and tPA. As shown in Table I, GST-RAP-(1–114) and its truncated variants were effective inhibitors of radioactivity binding to immobilized α2M* or LpL. In fact, similar EC50 values were found for inhibition of binding of α2M* by GST-RAP-(1–114) and GST-RAP-(115–319) toward binding of α2M* and LpL. Furthermore, the carboxy-terminal RAP fragments were potent inhibitors of α2M*-RAP binding to α2M* or LpL. Surprisingly, this included RAP-(187–319), which is noninhibitory to binding of α2M* or tPA (35). In other experiments (not shown) we found that RAP-(187–319) was also able to completely inhibit the binding of the uPA-PAI-1 complex.

The LpL Fragment Inhibits Binding of Other Established α2M/LP Ligands—Since RAP inhibits the binding of all known ligands, and since LpL competes effectively for binding of both the carboxy- and amino-terminal RAP fragments, it was expected that LpL should inhibit the binding of several ligands. Fig. 5A shows that the LpL fragment containing the receptor-binding domain was capable of blocking the binding of labeled pro-uPA, the uPA-PAI-1 complex, and α2M*. As shown in Fig. 5B, the LpL fragment did not bind the binding of uPA. Interestingly, the LpL fragment inhibited the binding of the tPA-PAI-1 complex, suggesting that the major epitope for binding to α2M* or LpL is in the PAI-1 moiety of the complex (Fig. 5B). On the other hand, full-length LpL did inhibit tPA binding (Fig. 5B), and a similar result (not shown) was obtained when the LpL fragment was coupled to a large fusion partner consisting of the NH2-terminal 30 amino acids of the α2 antiplasmin (35). This inhibition is therefore most likely caused by steric hindrance. This notion was further supported by the finding (not shown) that tPA inhibited binding of 125I-labeled α2M* to the immobilized LpL fragment by up to 80%, although with a very low potency (EC50 > 500 nM), consistent with a steric hindrance when compared with a Kd of 12 nM for tPA binding to the purified receptor (50).

**DISCUSSION**

The present results show that the binding of RAP can be completely inhibited by peptides containing the receptor-binding domain of LpL. This strongly suggests interaction of the LpL fragment with an important site on the receptor also available for binding of RAP. In addition, the peptide blocked the α2M*/LpL-mediated uptake of RAP in cells. Furthermore, it became important to determine the site specificity in view of the separate sites on the receptor (34) for binding of amino and carboxy-terminal RAP fragments (36). Since amino-terminal RAP fragments were poor competitors for binding of both carboxy-terminal RAP fragments and the LpL fragment, whereas carboxy-terminal RAP fragments and the LpL fragment cross-competed completely, the data indicate binding of LpL and the carboxy-terminal RAP fragment to the same site or to strongly overlapping sites.

The COOH-terminal regions of LpL and RAP exhibit about 29% sequence identity when allowing for several gaps (34), but sequence alignment provides no immediate clue as to where interaction with the receptor may occur. Positively charged residues have been shown to be important for binding of several ligands to α2M* or LpL (2, 31), and both the LpL fragment and the carboxy-terminal RAP fragment contain clusters of basic residues. We suggest that both fragments achieve affinity...
by interacting with the receptor via two patches containing such clusters. For the LpL fragment, these may include residues 379–383 (K LKWK in human LpL) and residues 403–407 (KIRVK in human LpL), the former being supported by the observation that amino acids 378–391 are necessary for binding (35) and the latter by mutational studies (34, 35). For binding of the RAP fragment, it is likely that amino acids 200–203 (RLRR in rat and human RAP) (37) participate in the binding together with several more COOH-terminally located basic residues (e.g. Lys202, Lys205, Lys209, Lys294, Lys299, Lys301, Lys302 in the rat sequence). The COOH-terminal residues 312–319 are important determinants for the inhibitory pattern since RAP (187–311) is noninhibitory toward α2M* and tPA binding (37) even though it binds to α2M/LRP and inhibits pro-uPA binding with about the same affinity as RAP (187–311) (Table I). Further COOH-terminal truncation including residue 305 results in loss of binding affinity (Ref. 37, Table I). This may be due to a disruption of the structure of the carboxy-terminal domain, although the possibility of direct participation of residues 305–311 in the binding cannot be excluded.

RAP binds strongly to the domain on α2M/LRP containing a cluster of eight complement-type repeats (cluster 2) and more weakly to the cluster containing 11 complement-type repeats (cluster 4) (20). Unfortunately, it is not known whether the

**TABLE I**

| Unlabeled GST constructs | Binding of 125I-RAP to α2M/LRP (percent of maximum) | Inhibition of 125I-RAP binding to α2M/LRP (EC50) | Inhibition of 125I-pro-uPA binding to α2M/LRP (EC50) |
|--------------------------|-----------------------------------------------------|-------------------------------------------------|---------------------------------------------------|
| RAP-(1–1319)             | 100                                                 | 0.1–0.4                                         | 0.3–0.4                                           |
| RAP-(1–114)(18–114)(12–107) | 34–45                                               | 200–500                                          | 1.2–3.0                                           |
| RAP-(24–114)(1–100)      | 0–1                                                  | >500                                            | >500                                              |
| RAP-(151–319)            | 60–63                                                | 0.25–0.5                                         | 0.6–0.7                                           |
| RAP-(187–319)(187–311)(200–319) | 27–41                                               | 1.2–2.4                                          | 1.6–1.8                                           |
| RAP-(151–304)            | 0–1                                                  | >500                                            | >500                                              |

* Binding of 125I-RAP reduced by 80% when using 2 μM competitor.

**TABLE II**

| Unlabeled competitor (GST constructs) | Inhibition of 125I-RAP (1–114) binding (EC50) | Inhibition of 125I-RAP (187–311) binding (EC50) | Inhibition of 125I-pro-uPA (115–319) binding (EC50) |
|--------------------------------------|-----------------------------------------------|-------------------------------------------------|---------------------------------------------------|
| RAP-(1–114)                          | 1.01–1.8                                      | 53.3–62.5                                       | >1000                                              |
| RAP-(115–319)                        | 0.20–0.24                                     | 0.40–0.74                                       | 0.19–0.28                                         |
| RAP-(187–319)                        | 1.04–1.21                                     | 1.04–1.26                                       | 1.02–1.49                                         |
| LpL-(347–448)                        | 23.0–24.5                                     | 16.8–33.6                                       | 23.2–35.0                                         |

**FIG. 4.** Competition by carboxy- and amino-terminal RAP fragments for binding of α2M/LRP to the LpL fragment. LpL-(347–448) was immobilized in microtiter wells (about 500 fmol/well) and incubated for 16 h at 4 °C with about 30 pm 125I-α2M/LRP (100,000 cpm/mi) in the absence or presence of the indicated concentrations of unlabeled RAP (left panel), RAP-(115–319) (middle panel), and RAP-(1–114) (right panel) or the GST-constructs of the RAP fragments (filled bars). The binding of 125I-α2M/LRP in the absence of unlabeled competitor (maximal binding) was set at 100% and ranged in individual experiments from 11 to 25% of the added tracer. The bars represent the mean values of triplicates.
strong binding to the cluster 2 domain is due to high affinity or to a large number of sites. The finding of Iadonato et al. (18) that the calculated number of RAP sites on α2MR/LRP in hepatoma cells is about 6 times higher than the number of tPA sites argues for the presence of multiple RAP sites in the cluster 2 domain. Within the RAP sites, nonreciprocal inhibition occurs between RAP fragments since RAP-(115–319) inhibits binding of RAP-(1–114), whereas the reverse inhibition is incomplete and only occurs with a very low inhibitory potency. This pattern may be explained by a close juxtaposition of the sites for binding of the carboxyl- and amino-terminal RAP domains, with steric hindrance for binding of the latter provided by residues in the former. Such a model may also explain the ability of the carboxyl-terminal RAP fragment to inhibit the binding of pro-uPA as a putative ligand for the site on α2MR/LRP that binds the amino-terminal RAP domain. Interestingly, the bulky GST moiety can induce or strengthen the inhibitory properties of the amino-terminal RAP domain (Ref. 37, Fig. 4), probably due to steric hindrance of ligand binding to neighboring sites.

It is established that α2M*, tPA, pro-uPA, and complexes of PAI-1 and uPA or tPA bind in the region containing the cluster 2 domain (19, 20, 52). The inhibition of α2M* and tPA by carboxyl-terminal RAP fragments is most likely due to steric hindrance. Residues 312–319 appear particularly important for the pattern of inhibition since RAP-(187–311) is noninhibitory toward binding of α2M* and tPA and since the removal of even a single residue (leucine 319) alters the inhibitory pattern without changing the binding affinity (45). Residues 312–319 should therefore be important for the putative function of RAP in preventing binding of coexpressed ligands in the endoplasmic reticulum of e.g. hepatocytes (29–31). The LpL fragment appears to inhibit ligand binding in a manner similar to that of the carboxy-terminal RAP fragment with deleted leucine 319, i.e. inhibition of α2M* binding but not tPA binding (45). It is remarkable that LpL itself (Fig. 5B) and the LpL fragment combined with a large fusion partner inhibit tPA binding as well.

Although not proven, the present data suggest that pro-uPA may bind to the sites available for the amino-terminal RAP domain. In addition, complexes such as uPA-PAI-1 with binding patches both in the uPA and the PAI-1 moieties (40) may bind in a mixed fashion involving multiple sites on α2MR/LRP that can also interact with amino- or carboxyl-terminal RAP fragments. Thus, α2MR/LRP deserves designation as a "molecular flypaper" even though this term was first coined for another important endocytic receptor, the macrophage scavenger receptor, that binds a multitude of negatively charged ligands (1).

RAP also binds to the homologous giant receptor gp330/megalin (21, 22), also known to bind lipoprotein lipase (6, 53). It is therefore anticipated that the LpL fragment should block the binding of RAP to gp330, and this result has in fact been obtained in experiments analogous to that shown in Fig. 1A. In addition, RAP has recently been shown to bind to the smaller members of the LDL receptor family, the VLDL receptor that binds RAP with high affinity (23, 24), and the LDL receptor that exhibits somewhat lower binding affinity (25). It is therefore probable that these receptors bind LpL as well.

Since RAP normally occupies these receptors expressed at the cell surface to at most only a small extent (27, 32), we suggest that LpL is one of the natural extracellular ligands for the multiple sites on α2MR/LRP that also bind carboxyl-terminal RAP fragments. Physiologically, this may imply that binding to the receptor is strengthened when multiple copies of a ligand, which binds with moderate affinity as a single entity, are assembled on a particle. This may apply to LpL-lipoprotein complexes assembled on cell surface proteoglycans (35, 54). Since efficient apolipoprotein E-mediated binding of lipoproteins to α2MR/LRP appears to require multiple copies of the apolipoprotein on the particle (6, 55), it will be important to determine whether apolipoprotein E may also bind to multiple RAP sites in α2MR/LRP. In addition, complexes of molecules (e.g. protease-inhibitor complexes) can achieve high affinity for binding to α2MR/LRP even though the individual components bind with low affinities. As an example, uPA binds with low affinity (via at least two binding patches) as does PAI-1, whereas the affinity of the uPA-PAI-1 complex is much higher whether bound to the cell surface urokinase receptor or free in

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**Fig. 5. Inhibition by the LpL fragment and LpL of the binding of established α2MR/LRP ligands.** A, α2MR/LRP immobilized in microtiter wells was incubated for 16 h at 4°C with 125I-pro-uPA (A— ● A), 125I-uPA-PAI-1 (● ● ● ●), and 125I-α2M* (□ — □), all at approximately 10 pm, and varying concentrations of LpL-(378–448). The amount of immobilized receptor was about 150 fmol/well when using 125I-pro-uPA and 125I-α2M*, and about 15 fmol/well when using 125I-uPA-PAI-1. The amount of tracer bound in the absence of unlabeled competitor was 12% for 125I-α2M*, 15% for 125I-uPA-PAI-1, and 5% for 125I-pro-uPA. The binding of each of the tracers in the absence of the LpL fragment was set at 100%. The calculated EC50 values for the LpL fragment were as follows: 125I-uPA, 54 nM; 125I-uPA-PAI-1, 35 nM; 125I-α2M*, 214 nM. B, the experiment was performed as in panel A using 125I-TPA (● ● ● ●) or the 125I-TPA-PAI-1 complex (● — ● — ●). The amount of immobilized receptor was about 150 fmol/well when using tPA and about 15 fmol/well when using the TPAs-PAI-1 complex. The amount of tracer bound in the absence of competitor was 5% for 125I-tPA and 19% for 125I-TPA-PAI-1. The competitor was either bovine LpL (open symbols) or LpL-(378–448) (closed symbols). The results are the means of triplicate values.

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2 M. S. Nielsen and J. Gliemann, unpublished observation.
solution (40). Binding of uPA-PAI-1 complexes may in part occur at sites that also bind RAP, a hypothesis further supported by the finding that uPA-PAI-1 complexes can inhibit binding of RAP by at least 80%.

In conclusion, we have provided evidence that LpL and the carboxyl-terminal RAP fragment bind to the same sites on α2MR/LRP, whereas pro-uPA and the amino-terminal RAP fragment may bind to the same or strongly overlapping sites.

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