The low density lipoprotein receptor-related protein (LRP) is a large endocytic receptor that recognizes more than 30 different ligands and plays important roles in protease and lipoprotein catabolism. Ligand binding to newly synthesized LRP is modulated by the receptor-associated protein (RAP), an endoplasmic reticulum-resident protein that functions as a molecular chaperone and prevents ligands from associating with LRP via an allosteric-type mechanism. RAP is a multidomain protein that contains two independent LRP binding sites, one located at the amino-terminal portion of the molecule and the other at the carboxyl-terminal portion of the molecule. The objective of the present investigation was to gain insight into how these two regions of RAP interact with LRP and function to modulate its ligand binding properties. These objectives were accomplished by random mutagenesis of RAP, which identified two critical lysine residues, Lys-256 and Lys-270, within the carboxyl-terminal domain that are necessary for binding of this region of RAP to LRP and to heparin. RAP molecules in which either of these two lysine residues was mutated still bound LRP but with reduced affinity. Furthermore, the mutant RAPs were significantly impaired in their ability to inhibit αM* binding to LRP via allosteric mechanisms. In contrast, the mutant RAP molecules were still effective at inhibiting uPA-PAI-1 binding to LRP. These results confirm that both LRP binding sites within RAP cooperate to inhibit ligand binding via an allosteric mechanism.

The low density lipoprotein receptor-related protein (LRP) is one of twelve or more receptors that make up the LDL receptor superfamily (for reviews, see Refs. 1 and 2) and is essential for embryonic development in mice (3). LRP recognizes more than 30 different ligands and plays important roles in protease and lipoprotein catabolism. The deduced amino acid sequence of LRP reveals that it is composed of a cytoplasmic domain containing two copies of an NPXY consensus sequence, a transmembrane domain, and a large extracellular region containing a total of 22 growth factor repeats and 31 complement-type repeats that are arranged into four clusters (clusters I–IV). Most ligands seem to bind to repeats located within clusters II and IV (4–6). One ligand, the activated form of αM-macroglobulin (αM*), requires repeats from both cluster I and cluster II for binding (7).

While purifying LRP by ligand affinity chromatography, a 39-kDa protein, termed the receptor-associated protein (RAP) was identified (8–10). Analysis of the primary sequence of RAP revealed a possible internal triple repeat structure (11, 12) leading to the suggestion that this molecule may contain three domains, termed D1, D2, and D3, that roughly correspond to thirds of the molecule. The independent nature of the domains was confirmed by expressing individual fragments and performing structural analysis by 3H NMR spectroscopy (12) and differential scanning calorimetry (DSC) (13). Interestingly, the DSC measurements suggested that D2 might be composed of two subdomains (13). The structure of the amino-terminal domain (residues 18–112, termed D1) of RAP has been solved by NMR spectroscopy (14) and contains three helices composed of residues 23–34, 39–65, and 75–88 that are arranged in an anti-parallel topology.

Within the cell, RAP is localized primarily to the endoplasmic reticulum due to the presence of an HNEL sequence at its carboxyl terminus (9, 15–17). Here RAP appears to function as a molecular chaperone for LRP and other LDL receptor family members by binding to the newly synthesized receptors and preventing them from associating with ligands also present within the ER (15–19). The mechanism by which RAP antagonizes the binding of all known ligands to LRP is not fully understood. LRP contains at least three independent binding sites for RAP, one each in cluster II, cluster III, and cluster IV. By preparing soluble fragments encompassing portions of cluster II, Vash et al. (20) demonstrated that RAP binds complement repeats 5–7 within this repeat. Repeats 5–7 are also responsible for binding two LRP ligands, uPA-PAI-1 complexes and lactoferrin. Thus, one mechanism by which RAP antagonizes ligand binding is by direct competition for their LRP binding site. However, other ligands, such as αM*, bind to a different set of complement-type repeats found in clusters I and II, and thus the αM* binding site does not strictly overlap with the RAP binding site (7). These results indicate that RAP also inhibits ligand binding to LRP by allosteric-type mechanisms.
Further evidence for an allosteric inhibition mechanism is also derived from studies showing that, although RAP binds tightly to a soluble fragment spanning the second cluster of complement-type repeats (C3-C10) and the amino-terminal flanking epidermal growth factor repeat of LRP (amino acids 787–1165), it is not a very effective inhibitor of ligand binding to this LRP fragment (21).

Increasing the TBS of RAP to LRP is complicated by the fact that two independent binding regions within RAP bind LRP (11). One of these binding sites is localized at the amino-terminal region of RAP containing D1 and a portion of D2 (and includes residues 1–164) that binds to LRP with a KD of 9 nM (13). In addition, the carboxy-terminal domain of RAP, which encompasses amino acid residues 216–323, termed D3, binds to LRP with a KD of 6 nM (13, 22). The objective of the present investigation was to gain insight into how these two regions of RAP interact with LRP and function to modulate its ligand binding properties.

EXPERIMENTAL PROCEDURES

Proteins and Antibodies—LRP was isolated from human placenta as described by Ashcom et al. (8). Human RAP was expressed in bacteria as a fusion protein with glutathione S-transferase and was purified as described previously (19). Monoclonal antibody S1G has been described previously (23). Rabbit anti-RAP IgG (R438) was prepared against recombinant human RAP, and the IgG fraction was purified. RAP mutants were prepared using the ExSiteTM PCRam-based site-directed mutagenesis kit from Stratagene (24). Full-length RAP in the pGEX-2T vector was used as the template for the PCR. The D4 RAP mutant (206–323) inserts were prepared by PCR using the following primers: 5′-GACCcGCAATCCAGGTCAGCACC-3′ and 5′-CA-GAATTCACGGTGCTGGCCGACG-3′. A denaturing temperature of 94 °C was used with an annealing temperature of 74 °C. The inserts were cleaved with the restriction enzymes BamHI and EcoRI and ligated into the pGEX-2T vector. All plasmids were sequenced in entirety. Protein concentrations in all experiments were determined spectrophotometrically using the following absorption coefficients (ε280,1%): 0.29 for RAP, 0.29 for the D4 mutant of RAP (206–323), and 0.29 for the D4 mutant of RAP (206–323). Protein solutions were prepared at concentrations of 1.5–2.0 mg/ml in phosphate buffer (10 mM, pH 8.65). A 1-mm path length cell was used, and CD spectra data points were recorded every 0.5 nm for 10 scans. The spectra were analyzed using the same software.

Circular dichroism (CD) spectra were recorded on a Jasco J-715 spectropolarimeter with a Peltier PFD-350S unit for temperature control. Proteins were dissolved at a concentration of 0.28 mg/ml in phosphate buffer (10 mM, pH 8.65). A 1-mm path length cell was used, and CD spectra data points were recorded every 0.5 nm for the wavelength range 300–180 nm at 25 °C with 20 nm/min scans and a 2-s response time. Four scans were accumulated per spectrum.

Cell Uptake Assays—Cellular internalization assays were generally conducted as described previously (27). Human WI-38 fibroblasts were seeded into 12-well culture dishes (5 × 104 cells/well) and grown in Dulbecco's modified Eagle's medium supplemented with 10% bovine calf serum and penicillin/streptomycin for 2 days. Cells were washed and incubated in assay media (Dulbecco's modified Eagle's medium containing 1.5% BSA, 1% nutradoma, and 20 mM HEPES, pH 7.5). Assay media containing 3 μg/ml RAP was added to the corresponding wells and incubated for 3 h at 37 °C. Following incubation the cells were washed with phosphate-buffered saline and detached from plastic using trypsin (0.5 mg/ml), protease K (0.5 mg/ml), and EDTA (5 mM) containing buffer. Internalized [125I]-labeled RAP was defined as radioactivity associated with the cell pellet. The cell numbers for each experimental condition were measured in parallel wells that did not contain radioactive.
RESULTS

Random Mutagenesis of the D3—To identify amino acid residues within D3 that are critical for its binding to LRP, a library of random D3 mutants was constructed. This library was screened for deficiency in LRP binding using a receptor ligand blotting protocol. Those clones with impaired binding in this assay were selected and sequenced. As expected from the mutation frequency of the library, most clones had multiple mutations. Of interest, we found that the mutation frequency was significantly higher at three lysine residues located within D3: Lys-256, Lys-270, and Lys-306. One mutant that displayed impaired LRP binding had a single mutation in which Lys-270 was converted to glutamic acid. D3 is known to contain an important LRP binding site (13, 28), and because prior studies have implicated charged residues in LRP binding (29), the current results implicate a role for these residues in binding to LRP. To test this hypothesis, we introduced the individual K256A, K270E, and K306A mutations into RAP and subjected the purified mutant molecules to further analysis.

Binding Analysis of RAP Point Mutants to LRP—Fig. 1 shows a receptor blot analysis measuring the binding of LRP to wild-type RAP and the three mutant RAP molecules following SDS-PAGE and transfer to nitrocellulose. It is apparent that both the K256A mutant and the K270E mutant have impaired LRP binding in this assay. Although the protein load on the gel for the K256A mutant was slightly lower in this experiment than the other proteins, in several repeats of this experiment, the K256A mutant binding was always deficient in binding LRP. In contrast, to the K256A and K270E mutants, the K306A mutant appeared normal in its binding to LRP. Thus, for the remainder of this study, experiments focused on the K256A and K270E mutants.

The direct binding of RAP and the K256A and K270E RAP mutants to purified LRP was also measured by ligand blotting approaches in which various concentrations of RAP were incubated with LRP following SDS-PAGE and transfer to nitrocellulose. The results (Fig. 2A) demonstrate that even at low RAP concentrations (1 nM) wild-type RAP binds to LRP, whereas neither of the two mutant RAP molecules bind very effectively to LRP at this concentration. The mutant RAP molecules do bind to LRP, but even at higher concentrations (10 nM) the extent of binding is somewhat less than that of wild-type RAP (Fig. 2B).

We next measured the ability of RAP molecules containing these individual mutations to inhibit the binding of 125I-RAP to purified LRP using a solid-phase binding assay in which LRP was coated to the surface of microtiter wells. The results of this experiment (Fig. 3) reveal that wild-type RAP competes for 125I-labeled RAP binding with a $K_{\text{app}}$ of 3.3 nM. Both the K256A and K270E mutants showed a significant defect in their ability to inhibit the binding of 125I-labeled RAP to LRP, with $K_{\text{app}}$ values of 86 and 94 nM, respectively. These results indicate that RAP molecules containing point mutations at residues 256 and 270 are defective in competing for the binding of 125I-RAP to LRP.

Analysis of D3 Point Mutants—To determine if mutations at Lys-256 and Lys-270 alter the binding of D3 to LRP, we expressed and purified recombinant D3 containing the K256A and K270E point mutations. The binding of the wild-type and mutant D3 to purified LRP was assessed by solid-phase assays. The results (Fig. 4A) demonstrated that in contrast to wild-type D3, mutant D3 molecules containing the K270E mutation failed to bind to LRP, whereas only weak binding of the K256A mutant to LRP is apparent. Together, these results reveal that mutation at Lys-256 and Lys-270 impact binding of the carboxyl-terminal domain of RAP to LRP, implying that these residues contribute significantly to this interaction. D3 is also known to bind to heparin (29), and thus we performed titrations to measure the effect of these mutations on heparin binding. Wild-type D3 bound heparin with a $K_d$ of 15.4 $\mu$m, whereas the K256A mutant showed a diminished affinity for heparin and the K270E mutants failed to bind (Fig. 4B). To further examine this, these mutant molecules were subjected to affinity chromatography on heparin-Sepharose (Fig. 4C). Wild-type D3 bound to the column and was eluted at 0.4 m NaCl. In contrast, the K256A mutant had markedly reduced affinity for

Fig. 1. LRP receptor blot of RAP and various RAP point mutants. 5 $\mu$g of RAP, and the K256A, K270E, and K306A mutants were subjected to SDS-PAGE on 4–20% gradient gels under non-reducing conditions. Following electrophoresis, the proteins were transferred to nitrocellulose. Left panel, Ponceau stain of nitrocellulose following transfer of proteins; right panel, the nitrocellulose sheet was incubated with 14 nM human LRP, and bound LRP was detected with monoclonal antibody 8G1 (1 $\mu$g/ml).

Fig. 2. Ligand blotting with RAP and RAP point mutants. LRP (10 ng) was subjected to SDS-PAGE on 4–20% gradient gels under non-reducing conditions and then transferred to nitrocellulose. Following blocking with BSA, 1 nM (A) or 10 nM (B) RAP, RAP K256A, or RAP K270E was incubated with immobilized LRP, and, following washing, the amount of binding was detected with anti-RAP polyclonal IgG R438 (1 $\mu$g/ml).

Fig. 3. Inhibition of 125I-RAP binding to LRP by wild-type RAP and RAP point mutants. 125I-RAP (2 nM) was incubated with immobilized LRP (100 $\mu$l, 4 $\mu$g/ml) in the presence of increasing concentrations of unlabeled RAP (circles), RAP K256A (open squares), or RAP K270E (closed squares). Each plotted value represents the average of duplicates with the range indicated by bars.
RAP Inhibits LRP by an Allosteric Mechanism

Characterization of the Folding of D3 Point Mutants—The loss of binding activity in D3 associated with the Lys-256 → Ala or Lys-270 → Glu mutations could result from a general unfolding of this domain induced by the amino acid changes, because this region is known to be relatively unstable (13). Thus, we compared the unfolding properties of these two mutant proteins with wild-type D3 by differential scanning calorimetry (Fig. 5A). As reported earlier (13), D3 unfolds as a two-state transition with an enthalpy of 45.3 kcal/mol and a transition temperature of 41.5 °C. The endotherm of the K256A mutant was virtually identical to wild-type D3 and revealed that the K256A mutation unfolded with an enthalpy of 43.2 kcal/mol and a transition temperature of 43.1 °C, confirming that this mutation does not alter the stability or unfolding properties of the domain. Interestingly, the K270E mutant was actually stabilized when compared with wild-type D3 and unfolded with an enthalpy of 59.5 kcal/mol and a transition temperature of 49.1 °C. Lys-270 is predicted to occur in a large α-helix, and it is possible that changing lysine to glutamic acid replaces a charge repulsion that occurs between two helical regions with a charge attraction leading to stabilization. Regardless of the mechanism, these two mutations in the RAP carboxyl-terminal domain do not lead to destabilization or unfolding of this region.

Previous studies have shown that D3 exhibits a CD spectrum typical for α-helical proteins that is abolished when the temperature is raised (13). We therefore compared the CD-spectra of wild-type D3 domain with the mutant molecules and the results, shown in Fig. 5B, reveal that both mutant D3 molecules contain extensive α-helical content, confirming that the secondary structure of these mutant molecules is not significantly altered. Together with the data of Fig. 5A, the results indicate that these mutations in the carboxyl-terminal domain of RAP do not lead to general unfolding of the structure nor to a decrease in the helical content of the molecule.

The K256A and K270E Mutations in RAP Effect Its Ability to Inhibit αMβ2 Binding to LRP—A major function of RAP is to inhibit binding of all known ligands to LRP. We therefore measured the ability of the mutant RAP molecules to inhibit two ligands whose binding sites on LRP have been mapped: uPA-PAI-1 complexes and uPA-M* complexes. Like wild-type RAP, both the K256A and K270E mutants were potent inhibitors of 125I-labeled uPA-PAI-1 binding to LRP (Fig. 6A). Although differences in the dose-response curves were noted, importantly, at saturating amounts of competitor, uPA-PAI-1 binding was completely inhibited by all RAP molecules.
RAP Inhibits LRP by an Allosteric Mechanism

In contrast to the data obtained for inhibition of uPA-PAI-1 binding, both the K256A and K270E mutants were unable to completely inhibit the binding of α2M* to LRP and only reduced the binding to about 50% at saturating concentrations (Fig. 6B), indicating that mutations at residues 256 and 270 generate a RAP molecule altered in its ability to antagonize α2M* binding to LRP.

We also examined the effect of these mutants on the ability of WI-38 fibroblasts to internalize 125I-labeled α2M* (Fig. 7). At a concentration of 200 nM, RAP completely blocked the internalization of 3 nM 125I-labeled α2M*. In contrast, both the K256A and K270E mutants reduced the amount of 125I-labeled α2M* internalized but were unable to completely block it, even at saturating concentrations of inhibitor. The magnitude of the effect was similar to that seen with saturating amounts of a fragment of RAP, which contains the amino-terminal LRP binding site (RAP1–164) or with D1–2D (data not shown). Curiously, D3 consistently increased the amount of 125I-labeled α2M* that was internalized, suggesting that its binding to LRP alters the conformation of the receptor, resulting in increased affinity for α2M*.

Inhibition of Mutant RAP Binding to LRP by the Amino- and Carboxy-terminal Domains of RAP—To gain insight into the relationship between the amino- and carboxy-terminal LRP binding sites on RAP, competition experiments were performed to determine if these two RAP fragments are capable of competing with one another for binding to LRP. The results indicate that RAP and both RAP fragments were effective competitors for the binding of 125I-labeled RAP1–164 to LRP (Fig. 8A). In contrast, only RAP and D3 were able to compete for the binding of 125I-labeled D3 to LRP (Fig. 8B). The inability of RAP1–164 to block the binding of D3 to LRP reveals that these two regions of RAP bind to distinct sites on LRP. Thus, the binding of D3 to LRP must induce a conformational change in LRP that blocks binding of the amino-terminal RAP domain to its site on LRP but at the same time increases binding of α2M* to LRP.

Mutation of either Lys-256 or Lys-270 impaired binding of the carboxy-terminal region of RAP to LRP. Thus RAP molecules containing these mutations are likely to interact with...

***DISCUSSION***

RAP is an endoplasmic-resident protein that associates tightly to newly synthesized LRP, gp330/megalin, and very low density lipoprotein receptor and prevents them from binding endogenously produced ligands (15–17). Recent studies reveal that RAP also acts in concert with MESD, another ER resident protein, to facilitate the export of LRP5 and LRP6 from the ER (18). The importance of RAP was revealed when the gene was deleted in mice (31), and it was found that the functional levels of LRP in the liver and brain were significantly reduced. Al-
though the reason for this is not entirely clear, it appears that RAP is required to prevent receptor aggregation suggesting that it may assist in protein folding (16). Furthermore, association of LRP with certain ligands in the ER, which occurs in the absence of RAP, leads to degradation of the receptor, thereby reducing the amount of LRP on the cell surface (31). Mechanisms by which RAP prevents ligands from binding to LDL receptor family members are not fully understood at this time.

In the current investigation, we employed a random mutagenesis approach to identify critical residues in the carboxy-terminal domain of RAP (D3) that are important for its interaction with LRP. This domain contains one of the two sites on RAP that bind to LRP (12, 13, 32, 33) and was chosen because the isolated domain can completely inhibit RAP binding to LRP (12) and can prevent some, but not all, ligands from binding to LRP (33). Furthermore, transfected U87 cells are unable to secrete soluble LRP mini-receptors unless RAP or D3 is co-expressed (28). A second RAP binding site on RAP is located at the amino-terminal portion of RAP within D1–D2. In contrast to D3, a fragment containing this site is unable to compete for RAP binding to LRP, and it does not promote secretion of soluble fragments of LRP but can partially inhibit ligands like RAP that bind to LRP (34). A more rigid structure within RAP D3 may not accommodate these necessary conformational changes upon association with LRP.

When we introduced these mutations into full-length RAP, mutant RAP molecules were generated that are defective in their ability to compete for RAP binding and for $\alpha_4M^+$ binding. Interestingly, the mutant RAP molecules were still effective in inhibiting the binding of uPA-PAI-1 complexes to LRP. Together, these data give insight into the mechanisms by which RAP modulates ligand binding by LRP and suggest that both D1–D2 and D3 domains are required for inducing conformational changes in LRP that reduce ligand binding (Fig. 10). The conformation change induced in LRP as a result of RAP binding must be initiated by D3. This is based on evidence in the current study indicating that the binding of isolated D3 to LRP actually increases the binding of $\alpha_4M^+$ to LRP. It seems likely that the conformational change in LRP upon D3 binding alters the relationship between ligand binding clusters in LRP. Increased binding of $\alpha_4M^+$ in the presence of D3 is consistent with a rearrangement of cluster I and cluster II to facilitate $\alpha_4M^+$ binding (Fig. 10C).

The results in the current study reveal that mutation of lysine residues in D3 impact not only LRP binding properties but also the heparin binding properties of D3. Previous studies have noted that mutations in RAP that lead to a loss of heparin binding also lead to a loss of LRP binding (29). These investigators found that mutation of basic amino acids within two
clusters of basic residues (Arg-203 to Arg-206 along with Arg-282 to Lys-289) reduced binding of RAP to both LRP and heparin, suggesting that overlapping motifs within RAP are required for both LRP binding and heparin binding. Together with our study, it is apparent that basic residues within the carboxy-terminal domain of RAP seem critical for binding of this portion of the molecule to LRP. A complete understanding of how RAP interacts with this receptor, however, will require solving the three-dimensional structure of the receptor-ligand complex.

In summary, we have identified critical residues on the carboxy-terminal domain of RAP important for the binding of this region to LRP. By introducing these mutations into full-length RAP, we have generated a molecule that is defective in preventing ligands, such as RAP, from binding to LRP but is still very effective in antagonizing the binding of uPA-PAI-1. The data reveal that interaction of multiple regions of RAP with LRP are important for the potent effect this molecule has on ligand binding to LRP.

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