The 39-kDa receptor-associated protein (RAP) is a specialized chaperone for members of the low density lipoprotein receptor gene family, which also binds heparin. Previous studies have identified a triplicate repeat sequence within RAP that appears to exhibit differential functions. Here we generated a series of truncated and site-directed RAP mutants in order to define the sites within RAP that are important for interacting with heparin and low density lipoprotein receptor-related protein (LRP). We found that high affinity binding of RAP to heparin is mediated by the carboxyl-terminal repeat of RAP, whereas both the carboxyl-terminal repeat and a combination of amino and central repeats exhibit high affinity binding to LRP. Several motifs were found to mediate the binding of RAP to heparin, and each contained a cluster of basic amino acids; among them, an intact R\textsuperscript{282}VSR\textsuperscript{285}SR\textsuperscript{287}EK\textsuperscript{289} motif is required for high affinity binding of RAP to heparin, whereas two other motifs, R\textsuperscript{203}LR\textsuperscript{205}R\textsuperscript{206} and R\textsuperscript{314}ISR\textsuperscript{317}AR\textsuperscript{319}, also contribute to this interaction. We also found that intact motifs of both R\textsuperscript{289}LR\textsuperscript{205}R\textsuperscript{206} and R\textsuperscript{289}VSR\textsuperscript{287}SR\textsuperscript{289}EK\textsuperscript{289} are required for high affinity binding of RAP to LRP, with the third motif, R\textsuperscript{314}ISR\textsuperscript{317}AR\textsuperscript{319}, contributing little to RAP-LRP interaction. We conclude that electrostatic interactions likely contribute significantly in the binding of RAP to both heparin and LRP and that high affinity interaction with both heparin and LRP appears to require mostly overlapping sequence motifs within RAP.

The 39-kDa receptor-associated protein (RAP)\textsuperscript{1} is a 323-amino acid ER chaperone for members of the LDL receptor gene family, which are cysteine-rich endocytic receptors (1, 2). Two unique features differentiate RAP from other general ER chaperones. First, whereas most other ER chaperones function primarily in substrate folding, RAP functions both in receptor folding (3, 4) and subsequent trafficking (5, 6). Second, RAP is a specialized chaperone that functions primarily with members of the LDL receptor gene family, whereas other ER chaperones interact with a variety of structurally and functionally divergent proteins that are synthesized and folded in the ER. Although the chaperone function of RAP was defined primarily with the LDL receptor-related protein (LRP) (3, 5–7), evidence accumulated to date suggests that RAP is likely to function as a chaperone for other members of the LDL receptor gene family (8–10).

The function of RAP during folding may be primarily to inhibit indiscriminate disulfide bond formation, in particular inter-molecularly between different LRP molecules during and after their translation (3). The function of RAP during the trafficking of receptors within the early secretory pathway is to prevent premature ligand interaction with the receptors (2, 5, 6). This function of RAP is consistent with the fact that RAP universally antagonizes ligand interaction with all members of the LDL receptor gene family. In this respect, it resembles the function of the invariant chain in regulating the peptide binding activity of major histocompatibility complex class II molecules within the secretory pathway (11). Largely because of its ability to inhibit the binding of ligands, recombinant RAP has been used extensively in the study of the biological properties and functions of members of the LDL receptor gene family (2).

Several groups of investigators have studied the structure of RAP. In those works, the primary structure of RAP has been shown to comprise of a sequence of about 100 amino acids, repeated three times (5, 12–14). The boundaries of these repeats have been similarly offered as either 1–100, 101–200, and 201–323 (5, 14) or 18–112, 113–218, and 219–323 (13), based upon sequence alignment and biophysical characterization. Although the three repeats of RAP share a high degree of homology to each other (5, 13, 14), they appear to exhibit differential functions. For example, repeat 1, but not repeat 3, can inhibit interaction of activated α\textsubscript{v}-macroglobulin to LRP, whereas repeat 3, but not repeat 1, can promote proper folding of LRP (14).

The three-dimensional structure of full-length RAP has not yet been solved, but the solution structure of the first repeat has revealed that portion of the structure to consist of three helices that are oriented in an anti-parallel bundle (15). It is highly likely that these three helices in repeat 1 are complemented by a fourth helix in the structure of full-length RAP. This proposition was supported by denaturation studies which showed that repeat 1 apparently interacts with some other portion of
the RAP structure, and we suggested that a putative helical segment in repeat 2 (residues 134–159) was a possible candidate for completing the four-helix bundle (16). The notion of interaction between repeats 1 and 2 is now also supported by functional data, as we report in this manuscript.

Previous studies find that RAP is a heparin-binding protein (17–19), although the biological significance of this interaction is presently unknown. The carboxyl-terminal region of RAP has been implicated in heparin binding (17, 18), but the exact motifs that are important for designating high affinity for heparin are unknown. It is additionally not clear whether such heparin binding motifs are also those in RAP that mediate its binding to LRP. In this study we have generated multiple RAP constructs coding for RAP peptides of different lengths from throughout its structure as well as site-directed mutants of full-length RAP. We utilized these RAP mutants to investigate the interaction of each with both heparin and LRP in order to study whether these two functions utilize overlapping or distinct sequences within RAP. We identified three basic amino acid sequence motifs within repeat 3 of RAP that contribute both high affinity binding to RAP and high affinity binding to LRP.

**EXPERIMENTAL PROCEDURES**

**Materials**—Human recombinant apolipoprotein E3 (apoE3) was kindly provided by Dr. Karl Weisgraber (The Gladstone Institute of Cardiovascular Disease). Heparin-Sepharose was prepared by coupling porcine intestinal mucosal heparin (Grampian Enzymes, Arbuthnott, UK) to Sepharose CL-6B (Amersham Pharmacia Biotech) that had been activated with cyanogen bromide as previously described (20). Human antithrombin (at3) was isolated from fresh frozen plasma using heparin-Sepharose as previously described (21). Glutathione-agarose beads were from Sigma. Rainbow molecular size markers were from Amersham Pharmacia Biotech, and Complete® protease inhibitor mixture was from Roche Molecular Biochemicals. All other chemicals were reagent grade from Sigma.

**Binding Analysis of RAP, ApoE3, and at3 from heparin-Sepharose**—A column containing 1 ml of heparin-Sepharose was prepared and attached to an AKTA fast protein liquid chromatography unit (Amersham Pharmacia Biotech) and run at a flow rate of 1 ml/min. The resin was equilibrated in 200 ml Tris-HCl, pH 7.4, and then 200 µg of tested protein in the same buffer was loaded, and the column was washed for 10 column volumes. Bound protein was eluted in the same buffer using a linear gradient of up to 1M NaCl over 20 column volumes. The column was then washed with 2 ml NaCl and equilibrated again in 20 ml Tris-HCl, pH 7.4, before the addition of the next sample. Each sample was analyzed at least three times, and standard deviations were calculated.

**Generation of GST/RAP Constructs**—The method for constructing GST/RAP fusion constructs has been described previously (5, 17). Briefly, human RAP cDNA (5) was used as the template for polymerase chain reaction were verified by DNA sequencing.

**Purification of GST/RAP Fusion Proteins**—Purification of GST/RAP constructs was performed essentially according to the manufacturer’s instructions (Amersham Pharmacia Biotech) and as described previously (17), except with the addition of Complete® protease inhibitor mixture in the lysis buffer. All the purified proteins were dialyzed against 50 mM Tris-HCl, pH 8.0, before performing experiments and/or storage. For some experiments, RAP was released from the GST/RAP fusion protein by thrombin cleavage, after which it was re-purified using heparin-Sepharose.

**Binding of GST/RAP Constructs to LRP**—MEF-7 cells were derived from RAP knockout mouse embryos (6) and were kindly provided by Dr. Joachim Herz (University of Texas Southwestern Medical Center at Dallas). These cells were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum. Cells were plated the day before the experiments and were ~80% confluent at the time of labeling. Metabolic labeling with [35S]cysteine was performed essentially as described previously (5, 22). Briefly, cells were first depleted of cysteine by using cysteine-free medium and then labeled with 0.2 µCi/ml [35S]cysteine for 4 h at 37 °C. Cells were then lysed with PBS with 1 mM CaCl2 and 0.5 mM MgCl2 containing 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride and Complete® protease inhibitor mixture. After pre-clearing with protein A-agarose, cell lysates were divided into equal parts and incubated separately with 50 nM GST or 50 nM each of the GST/RAP constructs at 4 °C for 16 h. Glutathione-agarose resin was then added to each tube to bind GST/RAP-LRP complexes. The agarose beads were then washed and pelleted, and the attached LRP was analyzed via SDS-polyacylamide gel electrophoresis (5% polyacrylamide) under reducing conditions. The radiolabeled LRP band intensity was quantified using a PhosphorImager (Storm 8400, Molecular Dynamics, Sunnyvale, CA).

**RESULTS**

**RAP Is a High Affinity Heparin-binding Protein**—Previous studies implicate binding of RAP to heparin (17, 18); however, its affinity to heparin relative to other heparin-binding proteins was unknown. Thus, we compared purified RAP to apoE3 and at3 for its binding to heparin-Sepharose. As seen in Fig. 1, RAP was eluted from the heparin-Sepharose column at a salt concentration of 0.55 M, whereas human apoE3 and at3 were eluted at salt concentrations of 0.67 and 0.85 M, respectively. The affinity of apoE3 and at3 to heparin agrees well with those described in previous studies (21, 23–25). Thus, RAP exhibits a high affinity binding to heparin comparable with that of apoE3.

**Characterization of the GST/RAP Constructs**—To identify the region(s) within RAP that is important for heparin and LRP binding, we generated various GST/RAP constructs (Fig. 2). The design of these truncated RAP constructs was influenced by previous structural and functional studies (14–16, 26). As seen in Fig. 2, the GST/RAP-1 fusion contains full-length human RAP. GST/RAP-2 contains RAP that lacks the last four amino acids, HNEL, the sequence in RAP that functions as an ER retention signal (5, 27). GST/RAP-3 contains a truncated RAP with all of repeats 1 and 2 and only the amino-terminal half of repeat 3. GST/RAP-4, GST/RAP-5, and GST/RAP-6 contain RAP sequences representing the first, second, and third repeats of RAP, respectively, with slightly overlapping regions (5). GST/RAP-7 contains RAP in which a classical ER retention signal, KDEL, has replaced the native HNEL sequence. GST/RAP-8 contains that portion from within repeat 1 of RAP that was seen to form a three-helical bundle in a solution structure (15), and GST/RAP-9 and GST/RAP-10 contain RAP residues representing the first two and the last two helices seen in the solution structure (15) of repeat 1, respectively. GST/RAP-11 and GST/RAP-12 are two constructs containing RAP sequences that partially cover repeat 2. GST/RAP-13, GST/RAP-14, GST/
RAP-15, GST/RAP-16, GST/RAP-21, and GST/RAP-22 contain RAP sequences representing various portions of repeat 3. Finally, GST/RAP-17, GST/RAP-18, GST/RAP-19, and GST/RAP-20 contain RAP sequences that represent combined regions of repeats 1 and 2.

All GST/RAP fusion proteins were purified with glutathione-agarose resin. Fig. 3A represents Coomassie Blue-stained gels showing the migration and purity of these proteins by SDS-polyacrylamide gel electrophoresis. As seen in the figure, all the purified fusion proteins, except for GST/RAP-6 and GST/RAP-15, are >90% pure and exhibit expected molecular sizes. GST/RAP-6 includes, in addition to the full-length fusion protein, an extra band of ~27 kDa. This band is detected with anti-GST antibody (Fig. 3B), but not anti-RAP antibody (data not shown), and likely represents a degradation product of GST/RAP-6 containing mostly the GST portion of the fusion protein. GST/RAP-15 shows doublet bands, both of which react with anti-GST and anti-RAP antibodies (Fig. 3B), suggesting that the lower band is likely a degradation product of the full-length fusion protein. The integrity of the purified GST/RAP fusion proteins was also determined by Western blot analyses using anti-GST antibody as shown in Fig. 3B and anti-RAP antibody (data not shown).

Binding of GST/RAP Constructs to Heparin—We next examined the binding of each GST/RAP construct to heparin-Sepharose using fast protein liquid chromatography analysis. As shown in Table I (also Fig. 5A), all but five GST/RAP constructs bind to heparin, although with various affinities. The full-length RAP fusion protein (GST/RAP-1) was eluted at a salt concentration of 0.55 M, the same as that of RAP alone (see Fig. 1), suggesting that the GST tag within the GST/RAP fusion protein neither contributes to nor interferes with RAP binding to heparin; neither did GST on its own bind to the heparin-Sepharose column under the chromatographic conditions used in these studies (data not shown). Other GST/RAP constructs that exhibit high affinity binding to heparin include GST/RAP-2, GST/RAP-6, GST/RAP-7, and GST/RAP-13. Relative to GST/RAP-13, significant loss of heparin affinity was seen in GST/RAP-14, GST/RAP-16, GST/RAP-21, and GST/RAP-22, suggesting that residues 311–319 in RAP are required for high affinity binding to heparin. It is important to note that although high affinity binding of RAP to heparin requires this sequence, it alone is not sufficient to constitute a high affinity heparin-binding site. This is demonstrated by GST/RAP-15, a fusion containing a shorter RAP sequence than GST/RAP-13 but also with the 311–319 sequence and yet a lower affinity binding to heparin (0.37 M). The difference between GST/RAP-13, with high heparin affinity, and GST/RAP-15, with lower affinity, is residues 221–275, which themselves did not bind heparin (see GST/RAP-14). When an additional 15 residues was added to GST/RAP-14, however, giving GST/RAP-16 (residues 221–290), low affinity binding to heparin was seen, implicating the basic sequence R282VSR285SR287EK289 as contributing to the high affinity binding of GST/RAP-13. Thus, it

![Fig. 2. Schematic representation of the GST/RAP constructs employed in this study.](http://www.jbc.org/)

### Table I

| GST/RAP constructs | Affinity to heparin-Sepharose (M[NaCl] ± S.D.) |
|--------------------|---------------------------------|
| GST/RAP-1          | 0.55 ± 0.007                    |
| GST/RAP-2          | 0.59 ± 0.010                    |
| GST/RAP-3          | 0.39 ± 0.002                    |
| GST/RAP-4          | 0.33 ± 0.002                    |
| GST/RAP-5          | 0.27 ± 0.005                    |
| GST/RAP-6          | 0.54 ± 0.004                    |
| GST/RAP-7          | 0.56 ± 0.002                    |
| GST/RAP-8          | 0                               |
| GST/RAP-9          | 0                               |
| GST/RAP-10         | 0.33 ± 0.006                    |
| GST/RAP-11         | 0.54 ± 0.010                    |
| GST/RAP-12         | 0.24 ± 0.005                    |
| GST/RAP-13         | 0.21 ± 0.003                    |
| GST/RAP-14         | 0.29 ± 0.002                    |
| GST/RAP-15         | 0.39 ± 0.005                    |
| GST/RAP-16         | 0.28 ± 0.002                    |
| GST/RAP-21         | 0.32 ± 0.002                    |

![Diagram](http://www.jbc.org/)
appears that high affinity binding of RAP to heparin requires residues 310–319 as well as the more minor heparin-binding site located between residues 280 and 290. The ER retention signal (HNEL, residues 320–323) at the extreme carboxyl terminus is not required for high affinity heparin binding since neither deletion (GST/RAP-2) nor replacement of this signal with the classical ER retention signal KDEL (GST/RAP-7) altered the high affinity binding of RAP to heparin. Examination of the binding patterns of all GST/RAP constructs to heparin allowed us to identify several other low affinity heparin-binding sites, each of which contains at least three basic amino acid residues. These sites along with the site within RAP required for high affinity binding to heparin are listed in Table II.

Binding of GST/RAP Constructs to Native LRP—Several studies indicate an involvement of electrostatic interactions between LRP and its ligands (28, 29). To examine whether such regions that are important for heparin binding also participate in LRP binding, we analyzed the binding affinity of each GST/RAP construct to native LRP obtained from the membranes of mouse MEF-7 cells (6). For these studies, MEF-7 cells were metabolically labeled with [35S]cysteine for 4 h. The lysates were then divided into equal parts for incubation with either excess GST alone or various GST/RAP constructs. The potential GST/RAP/LRP complexes were then pelleted with glutathione beads to which the GST moiety of the GST fusion protein bound, and the attached proteins were analyzed by SDS-polyacrylamide gel electrophoresis. The use of cell lysates in these analyses avoided denaturation of LRP, which occurs in ligand-blottting assays using SDS-polyacrylamide gel electrophoresis. Shown in Fig. 4 are the results of a representative experiment. As seen in the figure, GST/RAP-1, but not GST, brought down the 35S-labeled LRP, with both LRP subunits (LRP-515 kDa and LRP-85 kDa) detected. The other GST/RAP constructs were employed in the same assay and demonstrated a range of binding affinities to LRP. The band intensity from each interaction was quantified and plotted as a percentage of that of GST/RAP-1 (Fig. 5B). In this way we determined that the GST/RAP fusion proteins with high affinity binding to LRP included GST/RAP-1, GST/RAP-2, GST/RAP-6, GST/RAP-7, and GST/RAP-20. Comparison of these fusions with other GST/RAP fusions that exhibited either no or low affinity binding to LRP indicated that amino acid residues 201–210 of RAP were required for high affinity LRP-binding. A note was taken that although GST/RAP-3 (residues 1–250) contained all the sequence elements of GST/RAP-20 including residues 201–210 and was actually longer, it showed lower affinity to LRP. This is possibly due to the fact that the region important for high affinity LRP binding (residues 201–210) had a different conformation in GST/RAP-3 relative to GST/RAP-20 because GST-RAP-3 terminated halfway through a structural domain of RAP (16, 26). Finally, binding of RAP to LRP was slightly enhanced by either deletion of the ER retention signal (GST/RAP-2) or replacement of this signal with the classical ER retention signal KDEL (GST/RAP-7), suggesting that the native HNEL sequence may negatively influence the RAP-LRP interaction.

**TABLE II**  
| Basic sequence cluster | Putative structure | Heparin binding | Putative heparin-binding site | LRP binding |
|------------------------|--------------------|----------------|-------------------------------|------------|
| R203LR205R206          | Loop               | ++            | A                             | +         |
| R282VSR285SR287EK289   | Helical (loop)     | +++           | B                             | +++        |
| R74ISR117R119          | Loop               | ++            | C                             | ++         |
| KPSFR113R14            | Loop               | ++            | d                             | +         |
| R116LKR11LWHK123AK125PGK129 | Helical   | ++            | e                             |           |

**FIG. 3.** Expression of GST/RAP proteins. A, GST/RAP fusion proteins were purified as described under “Experimental Procedures.” Five µg of each GST/RAP protein were electrophoresed in 12.5% polyacrylamide gels in the presence of SDS and detected by Coomassie Blue staining. B, each GST/RAP protein (100 ng) was transferred from 12.5% polyacrylamide gels containing SDS onto nitrocellulose and Western-blotted using polyclonal anti-GST antibody. The molecular size markers are indicated in kDa.
MEF-7 cells were metabolically labeled with [35S]cysteine for 4 h. Cell lysates were then divided into equal parts and incubated with 50 nM GST/RAP-LRP complexes. LRP that had pelleted with the glutathione beads was analyzed by 5% polyacrylamide gels run in the presence of SDS and under reducing conditions. The positions of the LRP-515-kDa and LRP-85-kDa subunits are indicated. The solid and open arrowheads indicate the top of the stacking and separating gels, respectively. The numbers on top of each lane indicate the corresponding GST/RAP protein (see Fig. 2). The molecular size markers are indicated in kDa.

**Fig. 4. Interaction of GST/RAP proteins with native LRP.**

**Site-directed Mutagenesis of Clustered Basic Residues in RAP Important for Heparin and LRP Binding**—Having identified regions within RAP that are important for its interaction with heparin and/or LRP, we next performed mutagenesis analysis of these sequence motifs within full-length RAP. Based on our findings from the truncated RAP proteins, we chose three sequence motifs for mutagenesis analysis, R203LR205R206 (site A), R282VSR285SR287EK289 (site B), and R314ISR317AR319 (site C). All basic residues within each site were substituted by alanine, simultaneously yielding GST/RAP mutants A, B, and C (Table III). GST/RAP mutants AB, BC, AC, and ABC were then created by combining the mutants of individual sites as shown in Table III. The integrity and immunoreactivity to GST and RAP antibodies of each mutant protein were tested in a similar fashion as described for the data in Fig. 3 (data not shown).

The ability of each of these site-directed mutants to bind heparin and LRP was compared with that of wild type RAP, as for Fig. 5. Of the three sites, the most significant reduction in RAP binding to heparin was seen when site B was mutated (Fig. 7A), although this only caused an ~25% reduction. A further decrease in binding was seen in the AB mutant in which the binding activity to heparin was ~58% of wild type RAP. Including site C in the mutagenesis only caused an additional 4% reduction in heparin binding (mutant ABC, Fig. 7A), suggesting site C contributed little to heparin binding. Further indication that site C mutagenesis had very little effect on the binding of RAP to heparin can also be seen by comparing C to wild type (wt), AC to A, and BC to B (Fig. 7A). Overall, mutagenesis of site B caused the greatest reduction in the heparin binding activity of RAP (compare AB to AC and BC to AC), with site A having an effect intermediate between that of site B and site C.

The effects of basic cluster mutagenesis within RAP showed similar trends on LRP binding activity as for heparin binding activity, although they were generally more severe in magnitude. Substituting the positively charged residues at single sites by alanine had only small effects for the A and B mutants (~13–15% reduction in binding) and no effect for the C mutant (Fig. 7B). The double AB mutant, however, had significantly reduced LRP binding (~39% of normal), even more reduced than for heparin binding. No further reduction was achieved by additionally mutating site C (compare AB with ABC in Fig. 7B). As for heparin, the overall effect of mutation at site B in RAP appeared to reduce its LRP binding activity the most (compare AB to AC and BC to AC in Fig. 7B). Site A also contributed a significant effect when in combination with site B (AB and ABC mutants) but not when mutated alone (A mutant) or in combination with site C (AC mutant).

The differences between the binding of basic cluster mutants to either heparin or LRP were 1) site B mutagenesis alone (B mutant) had more effect on heparin binding than LRP binding (25 versus 13% reduction in binding, respectively), 2) the loss of basic residues in sites A and B simultaneously (AB mutant) resulted in maximal reduction in LRP binding (no further loss in binding was seen by including site C mutagenesis), whereas further small reduction in heparin binding was achieved by also mutating site C (ABC mutant), and 3) the magnitude of the maximum binding reduction caused by basic cluster mutagenesis was greater for LRP binding (61% reduction in the AB mutant) than for heparin binding (46% reduction in the ABC mutant).

**DISCUSSION**

Previous studies show that RAP consists of a triplicate repeat sequence and that each of the three individual repeats makes different contributions to RAP function (5, 12–14). Un-
ing a GST/RAP fusion system, we have investigated the roles of these repeats in the heparin and LRP binding activities of RAP. In addition, we made various combinations of these repeats as well as other truncated RAP sequences from which we have identified three sites rich in basic amino acids that appear to be important for heparin and LRP binding. The contributions of these specific clusters of basic residues to heparin and LRP binding were further investigated by site-directed substitution of the positively charged amino acids within these clusters by alanine. Fig. 8 illustrates the locations of these three sites.
within a three-dimensional computer-generated model of RAP. The RAP model was derived from a combination of NMR analysis of repeat 1 (15), secondary structure prediction, and limited proteolysis and guanidine-HCl denaturation studies (16). The locations of the three important sites for LRP and/or heparin binding are indicated in both the sequence and the model. Note that both sites A and C are localized within putative loop regions of RAP, whereas site B is largely located within a putative helical region.

Fig. 7. The heparin and LRP binding activities of site-directed GST/RAP mutants. A, the heparin binding affinities of the indicated GST/RAP mutants are presented as percentages of the NaCl concentration at which they eluted from the heparin-Sepharose column relative to GST/RAP-1 (n = 3). Error bars represent S.D. B, the amount of [35S]LRP that bound to each of the indicated GST/RAP mutants was quantified using phosphorimage analysis and plotted as a percentage of the amount of [35S]LRP that had bound to GST/RAP-1 (n = 3). The sequence of RAP contains multiple clusters of positively charged amino acids. The mutants represented here contain the substitution by alanine of the basic residues within three such clusters within RAP according to the following: A, R203LR205R206 → A203LA205A206; B, R282VSR285SR287EK289 → A282VSA285SA-287EA289; C, R314ISR317AR319 → A314ISA-317AA319 (also see Table III). Error bars represent S.D.

Fig. 8. The sequence and computer-generated three-dimensional model of human RAP. The RAP sequence is presented with each of the three repeats in different colors which correspond to the colors in the three-dimensional model. The model of RAP is derived from a combination of NMR analysis of repeat 1 (15), secondary structure prediction, and limited proteolysis and guanidine-HCl denaturation studies (16). The locations of the three important sites for LRP and/or heparin binding are indicated in both the sequence and the model. Note that both sites A and C are localized within putative loop regions of RAP, whereas site B is largely located within a putative helical region.
was deduced by comparing the heparin binding properties of GST/RAP-4 and GST/RAP-8 from within repeat 1, and site e (R^{116}ELE^{119}WK^{123}K^{125}TSK^{129}) was deduced by comparing the heparin binding properties of GST/RAP-11 and GST/RAP-12 from within repeat 2. Two of the constructs that bound heparin with medium affinity, GST/RAP-11 (containing site e) and GST/RAP-15 (containing sites B and C), were relatively short, 56 and 48 residues in length, respectively. Such short constructs would not be expected to promote extensive buried surface area during the formation of the RAP-heparin complex, supporting the notion that electrostatic interactions are likely to be important in mediating the binding of RAP to heparin as they are for at least two other proteins that bind heparin, thrombin (32) and antithrombin (33).

Although our current and previous studies have clearly defined RAP as a heparin-binding protein, the physiological function of this binding is not clear. Under normal biological conditions, RAP is an ER chaperone that functions within the early compartments of the secretory pathway. This has been shown by immunoelectron localization of RAP in human glioblastoma U87 cells, which indicated that it distributed primarily within the ER (70%) and early Golgi compartments (24%), with little found either on the cell surface or within compartments of the endocytic pathway (5, 34). RAP contains an ER retention signal, HNEL, at its carboxyl terminus, which binds to the KDEL receptors/ERD2 proteins for its retrieval from the Golgi back to the ER (5, 27). Overexpression of RAP results in the saturation of the retrieval system and the secretion of RAP into the extracellular space (35). Thus, it is possible that RAP could be secreted when its expression is up-regulated under certain physiological or pathophysiological conditions. In fact, several studies detect cell surface localization of RAP. For example, immuno-staining analyses have found RAP on the apical surface of kidney proximal tubule cells (36, 37) and on the surface of rat yolk sac carcinoma cells (38). In addition, RAP was detected on the surface of gingival fibroblasts via cell surface iodination (39). Finally, using flow cytometry analysis, Li et al. (40) find significant amounts of RAP on the cell surface of two melanoma cell lines. Since RAP is a potent antagonist for all ligand interactions with members of the LDL receptor family, it might be important for cells to sequester those RAP molecules that have escaped the intracellular retrieval system. Thus, binding of RAP to the large pool of cell surface heparan sulfate proteoglycan may serve as a mechanism for efficient trapping of secreted RAP and subsequent degradation by members of the LDL receptor family. Such a “safety” mechanism can prevent a nonproductive paracrine function of RAP in inhibiting ligand binding to members of the LDL receptor family on the cell surface, either in systemic or microextracellular environments.

It has been shown previously that RAP contains multiple LRP-binding sites and also that each LRP molecule possesses multiple RAP-binding sites that may utilize different mechanisms for RAP binding (14, 41–43). Results from the current study confirm the presence of multiple LRP-binding sites within RAP (see Table II). For example, each of the three repeats, represented by GST/RAP-4, GST/RAP-5, and GST/RAP-6, can independently bind to native LRP (Fig. 5B). The series of truncated proteins we utilized in this study indicated that clusters of basic residues in the sequence of RAP might be important for mediating its binding to LRP as well as to heparin. The design of the truncated fragments, however, preceded this finding and was therefore not optimized for a systematic analysis of all such clusters of positively charged amino acids. The fragments of RAP we did have indicated that the clusters of basic residues, referred to as sites A and C, contributed to LRP binding. This was deduced for site A by comparing the binding affinities between GST/RAP-19 (residues 1–200) and GST/RAP-20 (residues 1–210) especially, but also between GST/RAP-6 (residues 191–323) and GST/RAP-13 (residues 221–323), which indicated that the sequence between residues 201–210 contributed to the high affinity binding of RAP to LRP. Although GST/RAP-5 (residues 91–210) also contains this site, it has only intermediate affinity for LRP. This could be because functional expression of this site for high affinity LRP binding either requires an interaction of RAP repeat 2 with repeat 1, as shown functionally here by GST/RAP-20 and also suggested previously by biophysical analyses (16, 26), or it requires the presence of repeat 3 (GST/RAP-6). The importance of site C was deduced by comparing the binding of GST/RAP-13 (residues 221–323) with GST/RAP-22 (residues 221–310), which indicated that residues 311–323 contributed at least to medium affinity binding of RAP to LRP.

The actual importance of the clusters of basic residues at sites A and C of RAP to LRP binding was further confirmed by mutagenesis within full-length RAP. A further basic cluster between residues 282–289 (site B) was also mutated because 1) it occurred within repeat 3, which had the highest LRP binding activity of the three repeats, being almost as high as full-length RAP (Fig. 5B), and 2) a portion of it was contained within a region (residues 287–306) proposed by Orlando and Farquhar (18) as the most likely heparin-binding site in RAP, and the investigations reported here had shown us by the time we designed the basic cluster mutants that RAP used similar sequences to bind both heparin and LRP. These cluster mutagenesis studies indicated that site B contributed the most significant effect to the LRP binding activity of RAP with site A close behind and site C making a more minor contribution (Fig. 7B).

Heparin competition studies (Fig. 6) support the notion that site B (R^{282}VSR^{285}SK^{287}EK^{289}) of RAP may provide the principal binding site for both heparin and LRP under normal physiological conditions. When heparin was used as a competitor of the RAP-LRP interaction, it was found to significantly block the binding of full-length RAP to LRP, whereas its effect was much less on GST/RAP-20 (residues 1–210). In addition, heparin significantly inhibited the interaction of GST/RAP-6 (residues 191–323) with LRP. Although all three of these RAP constructs contained site A (R^{203}LR^{205}R^{206}), only the two constructs (GST/RAP-1 and GST/RAP-13) containing site B (R^{282}VSR^{285}SK^{287}EK^{289}) were significantly inhibited by heparin, whereas the least affected by heparin, GST/RAP-20, does not contain site B. These results would be consistent with the hypothesis that site B of RAP may be the most accessible to the receptor under normal circumstances and that site A may require some rearrangement of the RAP structure to be fully expressed.

It is tempting to speculate that a primarily electrostatic mode of interaction utilizing multiple sites within its structure is what facilitates the ability of RAP to inhibit the binding of all known ligands to LRP. Evidence is accumulating that the various other ligands of LRP have discrete binding sites on this receptor that are different from one another (1, 2). A relatively nonspecific electrostatic binding mechanism would promote the ability of RAP to bind more promiscuously to all these various sites on LRP, thereby facilitating its function of preventing premature ligand interaction during the translation of LRP. The validity of this hypothesis can be tested in future biophysical and structural studies. Because of its ability to interact at multiple sites on LRP, utilizing multiple sites within its own structure, it is likely that RAP is a flexible molecule undergoing...
conformational changes readily during its normal physiological function. Such changes, perhaps in response to various environmental conditions, would determine which site in RAP it utilized in any given binding interaction.

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