The 39-kDa receptor-associated protein (RAP), a specialized chaperone for endocytic receptors of the low density lipoprotein receptor gene family, is a triplicate repeat sequence (residues 1–100, 101–200, and 201–323, respectively), with the three repeats having different functional roles. The goal of the present study was to use a combination of protease sensitivity and guanidine denaturation analyses to investigate whether human RAP correspondingly contained multiple structural domains. Protease sensitivity analysis using six proteolytic enzymes of varying specificity showed that RAP has two protease-resistant regions contained within repeat 1 (residues 15–94) and repeat 3 (residues 223–323). Guanidine denaturation analysis showed that RAP has two phases in its denaturation, an early denaturation transition at 0.6 M guanidine HCl and a broad second transition between 1.0 and 3.0 M guanidine HCl. Analysis of the denaturation of the individual repeats showed that, despite the similarity in sequence and protease sensitivity between repeats 1 and 3, repeat 1 was a stable structure, with a sharp transition midpoint at 2.4 M guanidine HCl, while repeat 3 was relatively unstable, with a transition midpoint at 0.6 M guanidine HCl. Repeat 2 had a denaturation profile almost identical to that of repeat 3. Denaturation analysis of the contiguous repeats 1 and 2 (residues 1–210) indicated that repeats 1 and 2 probably interact to form one structural domain represented by the broad transition, while repeat 3 constitutes a separate domain represented by the early transition. A two-domain model of RAP three-dimensional structure is proposed that integrates both structural and functional information, in which a helical segment from repeat 2 interacts with the known three-helix bundle of repeat 1 to form a four-helix bundle structural domain, while repeat 3 forms the other structural domain.

The 39-kDa receptor-associated protein (RAP) is a receptor antagonist (1) for endocytic receptors of the low density lipoprotein (LDL) receptor family (2) that currently comprises five members (1, 2). RAP was discovered initially as a protein co-purifying with LDL receptor-related protein (LRP) (3, 4), to which it binds with high affinity. Subsequently, it has been shown that RAP inhibits all known ligands of LRP (5, 6), of which there are at least 15 (1, 2, 7). However, inhibition of ligand binding to LRP on the cell surface does not seem to be the natural function of RAP, since it is not detected extracellularly despite the presence of a putative signal peptide (8). The physiological role of RAP instead appears to be one of an intracellular chaperone. It is localized in the early compartments of the secretory pathway (9), using its COOH-terminal HNEL sequence as a retention signal (7). Within the endoplasmic reticulum, RAP acts as an antagonist by transiently interacting with LRP to prevent premature ligand binding by maintaining LRP in an inactive state (7, 10). In addition, RAP functions as a folding chaperone for LRP by preventing aggregation of newly synthesized receptors (11). These roles for RAP are supported by the finding that RAP-null cells have a 75% reduction of functional LRP (12).

Structurally, RAP is a triplicate repeat sequence (7) where repeat 1 comprises approximately residues 1–100, repeat 2 residues 101–200, and repeat 3 residues 201–323. Recently, an alternative alignment of the three repeats has been proposed (13), with repeat 1 encompassing residues 18–112, repeat 2 residues 113–218, and repeat 3 residues 219–323. There is a high degree of homology between repeats 13, 14, especially between repeats 1 and 2 and 3 and 14. There are 5 or 6 binding sites on LRP for RAP (14, 15) and different regions of RAP differentially inhibit at least some of the ligands (14, 16) and differentially perform the folding chaperone functions (14). For example, repeat 1, but not repeat 3, can inhibit the interaction of α2-macroglobulin with LRP, while repeat 3, but not repeat 1, can promote the folding and subsequent secretion of LRP (14). In addition, RAP has been shown to be a major pathogenic epitope in Heymann nephritis; residues 31–53 in repeat 1 are sufficient to induce immune deposits in passive Heymann nephritis (17).

RAP has about 38% predicted α-helical structure, based on circular dichroic analysis (8). The solution structural studies of Nielsen et al. (18) demonstrated that repeat 1 (residues 18–112) consists mostly of an up-down-up three-helix bundle comprising residues 23–88. The NMR analyses of Ellgaard et al. (13) further indicated that repeats 1 and 2 were highly ordered but that repeat 3 was not, despite the similarity in primary structure between repeats 1 and 3.

The goal of the present study was to ascertain whether the repeats of RAP constitute separate structural domains that might coincide with the established functional differences of these regions (1, 7, 11, 13, 14). Using a combination of protease sensitivity and guanidine denaturation analyses, we have found that RAP has two protease-resistant domains, contained within repeats 1 and 3. However, repeats 1 and 3 are consid-
erably different in their resistance to guanidine denaturation, while repeat 2 is extremely sensitive to both proteases and guanidine denaturation. Our studies suggest that RAP has two structural domains of different characteristics that may relate to the functional differences of the RAP repeats.

**EXPERIMENTAL PROCEDURES**

**RAP Expression and Purification**—A full-length clone for human RAP (6) fused to glutathione S-transferase was expressed in bacteria and purified by a modification of the method of Warshawsky et al. (19). After lysis of the cells by sonication in the presence of Triton X-100, the lysate was bound to glutathione-agarose to enrich for the RAP fusion protein, which was released from the resin with 20 mM reduced glutathione in 50 mM Tris-HCl, pH 8.0. After dialysis into 20 mM Tris-HCl, pH 8.0, and digestion with α-thrombin at E:S = 1:6000 for 6 h at 4 °C, RAP was purified to homogeneity on heparin-Sepharose in a gradient of 0 to 0.75 M NaCl in 50 mM Tris-HCl, pH 8.0. The purified RAP was dialyzed, lyophilized, and reconstituted in water (which was exposed briefly to ammonia vapors to aid the solubilization process) at a concentration of 1–2 mg/ml and stored frozen at −80 °C until use.

Initial isolation of RAP was performed in the absence of protease inhibitors. Upon storage at later stages of purification, degradation from endogenous proteolysis occurred. Therefore, a mixture of inhibitors (Complete, Boehringer Mannheim GmbH, Mannheim, Germany; 1 tablet per 50 ml) was included in the lysis, glutathione-agarose, and heparin-Sepharose steps, but excluded in the dialysis steps (before thrombin cleavage of glutathione S-transferase-RAP and before lyophilization). The cDNA construction, expression, and isolation of the various individual repeats of RAP have been described previously (7, 14).

**Limited Proteolysis Analyses**—Eight enzymes of varying specificities were screened as possible probes of RAP structure. All enzymes were dissolved in water at 1–2 mg/ml and stored in aliquots at −80 °C before use. Of these eight enzymes, six were found to be informative: porcine pancreatic elastase (protease type IV, Sigma), Staphyloccocus aureus V8 protease (protease type VII-B, Sigma), α-thrombin (generous gift of Dr. David Agard, University of California, San Francisco), chymotrypsin (type VII, 1-chloro-3-tosylamido-7-amino-2-heptanone-treated, Sigma), trypsin (type XIII, tosophenylalanyl chloromethyl ketone-treated, Sigma), and thermolysin (protease type X, Sigma). α-Thrombin, prepared by a modification of the procedure of Miletich et al. (20), did not digest RAP appreciably at either high E:S ratios (up to 1:10) or at elevated temperatures (up to 37 °C). Subtilisin (protease type VIII, Sigma) digested RAP rapidly at all ratios tested into fragments too small to be informative.

RAP (40 μg) was digested at a concentration of 1.0 mg/ml in 5 mM NH4HCO3, pH 7.8 (except subtilisin digests, which were carried out in 50 mM Tris-HCl, pH 7.5, 50 mM CaCl2) at ambient temperature (−22 °C) for various times and various E:S ratios to establish optimal conditions for analysis. We were seeking conditions that would allow identification of the most rapid initial cleavages and reasonably large fragments that were relatively stable with longer times of digestion. Times in the range of 0–7 h and the following E:S ratios were selected: elastase, 1:100; V8 protease 1:100; α-thrombin 1:2000; chymotrypsin, 1:640; trypsin, 1:1000; thermolysin, 1:200.

After digestion at the optimal conditions, reactions were terminated by addition of SDS sample buffer and snap freezing in liquid N2, then electrophoresed on 10–20% SDS-polyacrylamide gels (25 mA until the sample penetrated the running gel, then 40 mA for 4 h), blotted onto polyvinylidene difluoride membranes using a Bio-Rad trans blot semi-dry transfer cell and then stained briefly with Coomassie Blue. The proteins were subjected to sequence analysis on a Perkin-Elmer/Applied Biosystems (Costa Mesa, CA) Procise 494 to identify the fragment NH2-terminal sequence. In some cases, fragments were isolated from digests of V8 (type VII, 150 mm) in a gradient of 2–54% acetonitrile in 0.1% trifluoroacetic acid, dried, redissolved in 50% methanol, 1% acetic acid, and infused directly into a Finnigan LCQ ion trap mass spectrometer for electrospray ionization. Spectra were collected from 600–2000 m/z and deconvoluted using Finnigan Bioworks v.8.3 software. The peptide sequence and mass spectrometry analyses were performed by personnel at the Protein and Nucleic Acid Core Laboratory at Washington University School of Medicine.

**Guanidine HCl Denaturation and Circular Dichroic Analyses**—Guanidine HCl denaturation was performed at ambient temperature in 20 mM sodium phosphate, pH 7, using a final RAP concentration of 100 μg/ml and various guanidine HCl (Ultrapure, ICN Pharmaceuticals, Costa Mesa, CA) concentrations. After overnight denaturation (22 h), circular dichroic spectra were obtained on a Jasco 600 C spectrometer. A cell with 0.1-cm path length was used, and three repetitions of scans between 250 and 210 nm were averaged for each sample. The molar ellipticity, [θ]222, was calculated at 222 nm from the equation [θ] = MRWθ0/(1–10000) (where MRW is mean residue weight = 117 for RAP, θ0 is the ellipticity at wavelength λ in degrees, l is path length in cm, c is concentration in g/ml, and plotted against the guanidine HCl concentration. The double negative troughs at 208 and 222 nm are often used to estimate secondary structure. For our purposes, the helical content (ψ310) of RAP was estimated at 222 nm (0 M guanidine HCl) from the equation [θ] = −30,300ψ310–2340 (21).

A renaturation experiment was also performed to ascertain whether the denaturation of RAP was reversible. After denaturation for 22 h in 3.0 M guanidine HCl at a RAP concentration of 1.0 mg/ml, the sample was diluted 10-fold with 20 mM sodium phosphate, pH 7, to 0.3 M guanidine HCl, 100 μg/ml RAP, and allowed to renature for 5 h before measurement of the circular dichroic spectra.

**RESULTS**

**Limited Proteolysis**—Fig. 1 shows the time course of digestion of RAP with each of the six informative enzymes. In most cases, initial digestion yielded a large fragment encompassing about two-thirds of RAP that disappeared with time and a smaller fragment encompassing about one-third that was relatively stable. The exception was with V8 protease, where the initial digestion yielded approximately half-molecules.

The major fragments identified from the enzyme digests are given in Table I. For elastase, the first cleavage was at residue 99–100, giving rise to fragments PPE-1 and PPE–4 and contributed to the disappearance of PPE-1. The α-thrombin protease digest was similar: the first cleavage at residue 100–101 or 101–102 yielded a stable NH-terminal fragment, αLP-5, and αLP-1. Another early cleavage at residue 173–174 yielded αLP-2 and αLP-3 (the latter gave way to αLP-4 after cleavage at residue 208–209). αLP-6 repre-

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**Fig. 1. Time course of digestion of RAP with six proteolytic enzymes.** RAP was digested at ambient temperature at 1.0 mg/ml in 5 mM NH4HCO3, pH 7.8, for the times indicated. Upper panel, elastase, 1:100; α-thrombin, 1:2000; S. aureus V8 protease, 1:100. Lower panel, chymotrypsin, 1:640; trypsin, 1:1000; thermolysin, 1:200. The migration of molecular mass markers is indicated on the left. The numbers within each panel correspond to the fragments listed in Table I.
sent an internal fragment (residues 101/102–173) that was not present in the elastase digest.

The half-molecules arising initially in the V8 digest as a result of cleavage at residue 164–165 were both NH$_2$- (V8-1) and COOH-terminal (V8-2) fragments giving way to V8-4, which represents both NH$_2$-terminal (presumed cleavage at residue 134–135) and COOH-terminal (cleavage at residues 189–190 and 192–193) peptides that persist over time. V8-3 was comprised of slightly larger NH$_2$-terminal fragments that also gave way to V8-4. Chymotryptic digestion occurred first at residue 183–184, giving rise to Ch-1 and Ch-2. Each of these gave way to the more stable fragments Ch-3 and Ch-4, respectively, deriving from further cleavages at residues 121–122 and 206–207 (thermolysin) led to the disappearance of Tr-1 or Th-1, and the appearance of Tr-2 or Th-3, respectively.

Proteolytic fragments that resulted from bacterial endopeptidase activity when RAP was isolated in the absence of inhibitors (not shown) were also purified and the cleavage sites identified (Table I). The cleavages at residues 94–95, 101–102, and 176–177 (Fig. 2) were probably the result of more than one enzyme, since the characteristics of the sequences at the cleavage sites were somewhat different from one another.

A summary of the cleavage sites identified is presented in Fig. 2. It is apparent that the protease-sensitive regions are the extreme NH$_2$-terminus (residues 1–15); the boundary between RAP repeats 1 and 2 (residues 94–103); most of repeat 2, especially the region from residues 164–192; and near the boundary between repeats 2 and 3 (residues 204–222). The protease-resistant regions, defining probable structural domains of highly ordered structure, are in repeat 1, residues 15–94, and repeat 3, residues 223–323.

**Guanidine HCl Denaturation**—Fig. 3 shows the guanidine HCl denaturation profile for intact RAP. In the absence of guanidine, the protein had a $[\delta]_{222} = -13,992$ degrees cm$^2$ dmol$^{-1}$, corresponding to a helical content of about 38%, using the relationship described under “Experimental Procedures.” This result agrees with the findings of Strickland et al. (8). The denaturation profile had two distinct features. The first was the rapid denaturation of a portion of the RAP structure at low guanidine HCl concentrations, with a transition midpoint at approximately 0.6 M. The second feature was denaturation of another portion of the RAP structure over a broad range up to $>3$ M guanidine HCl. This result indicates that there may be at least two structural domains within RAP.

We also performed renaturation experiments with intact RAP. After standing 22 h in 3 M guanidine HCl, rapid dilution to 0.3 M guanidine HCl, and 5 h of renaturation before measurement of the circular dichroic spectra, RAP had regained about 94% of its ordered structure, based on $[\delta]_{222}$ at 0.3 M guanidine HCl, comparing with to without prior denaturation at 3 M guanidine HCl. This result indicates that RAP denaturation is almost entirely reversible within 5 h.

In order to ascertain which portions of the RAP structure corresponded to the two phases of the guanidine denaturation profile, the individual repeats of RAP (14) were also subjected to guanidine denaturation and circular dichroic analysis. As shown in Fig. 4, repeats 1 (represented by residues 1–110) and 3 (represented by residues 191–323), despite their similarities in primary structure, had very different denaturation profiles. Repeat 3 denatured rapidly at low guanidine HCl concentrations, with a transition midpoint at about 0.6 M, similar to the
first transition observed in the intact protein (Fig. 3). In contrast, repeat 2 was much more stable to guanidine denaturation, unfolding in a fairly sharp transition with a midpoint at approximately 2.4 M guanidine HCl. This latter profile was dissimilar to either of the transitions of intact RAP. Repeat 2 (represented by residues 91–210) had a denaturation profile almost identical to that of repeat 3 (Fig. 4B), indicating a structure of low stability. Therefore, it appeared that either repeat 2 or 3 represented the early transition in the intact protein, and that repeat 1 interacted with one or the other of repeat 2 or 3 to yield the second, broad transition of the intact protein. In an attempt to differentiate the contribution of repeat 2 from that of repeat 3 to the unfolding of repeat 1, we performed guanidine denaturation on a RAP fragment consisting of contiguous repeats 1–2 (14). Although the denaturation profile of RAP 1–2 (residues 1–210) was not exactly like the second transition of the intact protein, the sharp second transition occurring in the sum of the individual profiles of repeats 1 + 2 had been reduced to a significant extent (Fig. 5).

DISCUSSION

We have demonstrated in this study that human RAP has two highly protease-resistant regions corresponding to a majority of repeats 1 (residues 15–94) and 3 (residues 223–323). In contrast, RAP repeat 2 was found to be very susceptible to limited proteolysis along almost its entire sequence, with the possible exception of the region approximately from residues 123–164 (only two presumed cleavages occur within this re-
After measurement of the circular dichroic spectra, the molar ellipticity, at ambient temperature at the indicated guanidine HCl concentrations.

A consensus of secondary structural prediction algorithms predicts about 50% helical content for RAP, somewhat higher than the 38% determined experimentally by us and Strickland et al. (8). Even so, the protease-resistant regions correspond mostly to predicted helical segments and that, with only a few exceptions, the protease cleavage sites fall within predicted coil regions; the major coil regions are predicted to be residues 1–18, 88–115, 160–184, 197–225, and 294–323 (Fig. 2). Furthermore, the predicted helical segments within repeat 1 correspond almost exactly with the solution structure of repeat 1 determined by Nielsen et al. (18); that is, residues 23–34, 39–65, and 73–88. This helical segment of repeat 1 was completely resistant to limited proteolysis (Fig. 2).

The results of the guanidine HCl denaturation study suggest that repeat 1 is a highly ordered, stable structure while repeats 2 and 3 are relatively unstable (Fig. 4B). This is despite the sequence similarities between repeats, especially between repeats 1 and 3 (13, 14), and despite the similarity between repeats 1 and 3 in their resistance to limited proteolysis. The highly stable nature of repeat 1 is in agreement with the findings of Ellgaard et al. (13) and Nielsen et al. (18), who found that repeat 1 was a highly ordered, stable structure by NMR analysis. Our results with repeat 2 are in contrast with those authors, however, who suggested that repeat 2 was similar in structural properties to repeat 1. We found that repeat 2 was relatively unstable, as assessed by guanidine HCl denaturation, and less ordered as measured by its extreme sensitivity to limited proteolysis. However, the helical content of all three repeats appears to be similar, based on the consensus of secondary structure predictions (see footnote 2) in (Fig. 2) and on $|\theta|_{222}$ at 0 M guanidine HCl (Fig. 4B). We found that repeat 3, while highly structured as determined by resistance to limited proteolysis (in this respect resembling repeat 1), is unstable as measured by guanidine HCl denaturation (in this respect resembling repeat 2). Ellgaard et al. (13) stated that repeat 3 appeared to be an unstable structure but implied that its structural properties might be different in the context of the intact protein. Resolution of these matters will ultimately require the complete three-dimensional structural determination of the intact RAP.

It is intriguing to compare some of the properties we have found for RAP segments with those of the two structural domains of apolipoprotein (apo) E, which is one of the ligands for LRP. Some similarities between RAP and apoE have been noted previously, in both function, such as binding to LRP (2) and heparin (19), and in sequence (8). The two domains of apoE are rather similar in structural properties to those of RAP repeats 1 and 3. The NH$_2$-terminal domain of apoE is a stable and highly ordered helical structure resembling a globular protein (22, 23). It is an antiparallel four-helix bundle domain (24) that is resistant to proteolysis and has a guanidine HCl denaturation transition at about 2.4 M (22). RAP repeat 1, with three helices (18), is nevertheless resistant to proteolysis (Fig. 2) and has a guanidine HCl denaturation transition at 2.4 M (Fig. 4B). Likewise, the COOH-terminal domain of apoE is a highly ordered helical structure that is protease-resistant but relatively unstable, with a guanidine HCl denaturation transition at about 0.7 M (22), very similar to RAP repeat 3 (Figs. 2 and 4B). Another similarity between RAP repeat 3 and the COOH-terminal domain of apoE is that they are both predicted to have extremely long uninterrupted helical segments. However, Strickland et al. (8) have suggested that RAP repeat 3 has some sequence and other structural similarities to the NH$_2$-terminal (rather than the COOH-terminal) domain of apoE. There are functional similarities as well; both RAP repeat 3 and the NH$_3$-terminal domain of apoE bind to members of the LDL receptor gene family and to heparin, for example.

It is also tempting to compare the apoE “hinge” region, a protease-sensitive connection between the two domains (22, 23), with repeat 2 of RAP. The protease sensitivity is similar, but the analysis of RAP repeat 2 does indicate that it has some
degree of secondary structure (Figs. 2 and 4B). It is possible that RAP repeat 2 could function as a hinge between two independent domains represented by RAP repeats 1 and 3. However, arguing against this possibility is the fact that repeat 1 does not appear to fold independently as do the two domains of apoE (22, 23), because the sharp denaturation transition of repeat 1 (Fig. 4B) is not maintained in the intact protein (Fig. 3). Thus, although the repeat 1 structure has been solved and shown to be a three-helix bundle (18), our data indicate that, in the intact protein, repeat 1 is not itself a discrete structural domain.

The fact that repeat 1 apparently interacts with some other portion of the RAP structure is an issue not completely resolved by our study. This supposed interaction somewhat destabilizes that region of the protein, with the sharp denaturation transition of repeat 1 at 2.4 M guanidine HCl (Fig. 4B) replaced by a very broad transition in the intact protein from about 1.0 to 3.0 M guanidine HCl (Fig. 3). Repeat 1 could interact with either repeat 2 or 3 to yield the broad transition of Fig. 3, with the remaining repeat yielding the early transition. Our attempts to identify that interaction showed that repeat 1–2 (residues 1–210) did not exactly reproduce the second transition (Fig. 5). This may indicate that the particular fragment selected (residues 1–210) was not a close enough approximation of the true interacting portions of the molecule. Nevertheless, there was some indication that repeat 1 was influenced by repeat 2. In contrast, for apoE, the approximations of the two domains produced by thrombolytic digestion exactly reproduced the guanidine HCl denaturation transitions of the intact apoE (22).

Among the possibilities for a portion of repeat 2 or 3 contributing to the interaction with repeat 1, a likely one is that a helical segment from repeat 2 or 3 interacts with the up-down helical arrangement of repeat 1 to form a four-helix bundle. Such a possibility is suggested from the analyses of Nielsen et al. (18), who identified a similarity in the repeat 1 topology to that of cytochrome b_{562}, which is itself a four-helix bundle. A strong candidate to serve the role of providing the fourth helix for the three-helix bundle in repeat 1 of RAP might be the predicted helical segment within repeat 2 that is mostly protease-resistant; i.e., residues 134–159. In addition, functional data support the notion of repeats 1 and 2 as a structural domain. Whereas repeats 1 and 2 individually have low affinity for LRP (14), repeat 1–2 (residues 1–210) has high affinity for LRP, suggesting that there is a synergistic effect involving repeat 1 and 2 interaction that contributes to LRP binding. This sort of arrangement would yield a structure that would allow for the functioning of repeats 1 and 2 together as a domain and repeat 3 as a separate domain, which has some basis from previous functional studies (13, 14, 16, 25). In Fig. 6 we present a highly schematized model of this structure, which we emphasize is only one of many possibilities. Whether this will be borne out by the complete structural determination of RAP remains to be seen.

As stated in the Introduction, two versions of the triplicate repeats of RAP have been offered. The version of Bu and co-workers (7, 14), with boundaries of residues 1–100, 101–200, and 201–323, leaves a unique 23-residue segment at the COOH terminus of repeat 3. The version of Ellgaard et al. (13), with boundaries of residues 18–112, 113–218, and 219–323, leaves a 17-residue NH2-terminal extension as a unique portion. Ellgaard et al. (13) argue that their version better fits the functional analysis of RAP repeats. The protease sensitivity map (Fig. 2) seems compatible in some respects with either the boundaries proposed by Bu and co-workers (7, 14) or with those proposed by Ellgaard et al. (13). However, we note that two unique functions of RAP repeat 3 are contained in its extreme COOH terminus and therefore might be expected to be associated with a unique structure, which the Bu and co-workers version would predict. The endoplasmic reticulum retention signal is contained within this segment (7), and a major binding site to heparin occurs in the COOH-terminal region (26) and has now been localized to the 301–323 sequence.

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