Identification of the Minimal Functional Unit in the Low Density Lipoprotein Receptor-related Protein for Binding the Receptor-associated Protein (RAP)

The low density lipoprotein receptor-related protein (LRP), a member of the low density lipoprotein receptor family, mediates the internalization of a diverse set of ligands. The ligand binding sites are located in different regions of clusters consisting of ~40 residues, cysteine-rich complement-type repeats (CRs). The 39–40-kDa receptor-associated protein, a folding chaperone/escort protein required for efficient transport of functional LRP to the cell surface, is an antagonist of all identified ligands. To analyze the multisite inhibition by RAP in ligand binding of LRP, we have used an Escherichia coli expression system to produce fragments of the entire second ligand binding cluster of LRP (CR3–10). By ligand affinity chromatography and surface plasmon resonance analysis, we show that RAP binds to all two-repeat modules except CR910. CR10 differs from other repeats in cluster II by not containing a surface-exposed conserved acidic residue between Cys IV and Cys V. By site-directed mutagenesis and ligand competition analysis, we provide evidence for a crucial importance of this conserved residue for RAP binding. We provide experimental evidence showing that two adjacent complement-type repeats, both containing a conserved acidic residue, represent a minimal unit required for efficient binding to RAP. Bound to LRP-like receptors are internalized by classical endocytosis (2). In humans, the group of known LDLR-like receptors includes the canonical LDLR (3), LRP (4, 5), the very low density lipoprotein receptor (VLDLR) (6), the apoE receptor (apoER2) (7), megalin/gp330 (8), and two recently discovered members, LRP6 (9) and LRP7 (10, 11). The normal processing of LRP and megalin requires the presence of RAP (12), a 39–40-kDa protein (13) that appears to consist of three homologous domains (14–17) of which domain 1 has been shown to consist of a three-helix bundle (18). RAP interacts with all LDLR-like receptors and is a universal antagonist for receptor/ligand interactions. RAP domains 1 and 3 (RAPd3) are both receptor-binding (15, 19), but only domain 3 is sufficient to mimic the chaperone-like functions of RAP in cells (20, 21). RAP domain 2 is a substrate for cAMP-dependent protein kinase (22) but has only a very low affinity for LRP and megalin compared with RAP domains 1 and 3 (23).

The ectodomain of LRP members contains clustered complement-type repeats and epidermal growth factor precursor precursor homology domains, consisting of multiple copies of cysteine-rich epidermal growth factor-like repeats and regions with a 6-fold YWTD consensus sequence, the latter suggested to form a compact β-propeller domain (24). CRs consist of ~40 amino acids of which six are cysteines forming three disulfide bridges identified in all known repeats connecting Cys IV–Cys V, Cys II–Cys V, and Cys IV–Cys VI (25–27). The three-dimensional structure has been solved for three LRP modules (27–29) and one LRP module (30), revealing an octahedral calcium cage that is formed by four conserved acidic residues plus two nearby carboxyl oxygens (27, 31).

A multitude of ligands are binding to these complement-type repeats, some exhibiting cross-competition such as, for example, tissue-type plasminogen activator and transformed α₂-macroglobulin (α₂M*), while others do not. This suggests a variety of individual binding sites. So far, no reports have succeeded in identifying single residues as important for the ligand specificity of any LDLR-like receptor. Recently, Rong et al. (32) were able to substitute the fourth ligand binding repeat (LB4) of LDLR into the avian receptor for subgroup A Rous sarcoma virus (the Tva receptor), instead of the single naturally occurring complement-type repeat. They succeeded with...
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EXPERIMENTAL PROCEDURES

Construction of Expression Plasmids and Site-directed Mutagenesis—A plasmid containing a complete LRP cDNA insert (kindly provided by Dr. J. Herz, University of Texas Southwestern Medical Center, Dallas, Texas) served as template in polymerase chain reactions (PCRs), using the following sets of primers to generate ubiquitin (U)-fused expression constructs encoding the four single-repeat derivatives CR3, CR4, CR5, and CR6 and the seven two-repeat derivatives CR34, CR45, CR56, CR67, CR78, CR89, and CR910 corresponding to CR pairs 3–4, 4–5, 5–6, 6–7, 7–8, 8–9, and 9–10. For U-CR5 (LRP residues Ala-844 to His-983), the primer pairs 5'GGG GGA TCC ATG GAG AGG AAC CCA TTC TAC G-3' and 5'GGC AAG TTG ATG GAT GGC AGA GG-3'; for U-CR4 (LRP residues Gin-892 to Arg-954), the primer pairs 5'GGG GGA TCC ATG GAG AGG CAG CAC ACC TTC GGC G-3' and 5'GGC AAG TTG ATG GAT GGC AGA GG-3'; for U-CR3 (LRP residues Gly-982 to Arg-1023), the primer pairs 5'GGG GGA TCC ATG GAG AGG AAC CCA TTC GAC TGC TTC-3' and 5'GGG AAG TTG ATG GAT GGC AGA GG-3'; and for U-CR6 (LRP residues Tyr-973 to His-1013), the primer pairs 5'GGG GGA TCC ATG GAG AGG AAC CCA TTC GAC ACG G-3' and 5'GGG AAG TTG ATG GAT GGC AGA GG-3'; were used.

For U-CR34 (LRP residues Ala-844 to Arg-934), the primer pairs 5'GGG GGA TCC ATG GAG AGG AAC CCA TTC TAC G-3' and 5'GGC AAG TTG ATG GAT GGC AGA GG-3'; for U-CR45 (LRP residues Gin-892 to Pro-974), the primer pairs 5'GGG GGA TCC ATG GAG AGG CAG CAC ACC TTC GGC G-3' and 5'GGC AAG TTG ATG GAT GGC AGA GG-3'; and for U-CR56 (LRP residues Ser-952 to His-1013), the primer pairs 5'GGG GGA TCC ATG GAG AGG AAC CCA TTC GAC TGC TTC-3' and 5'GGC AAG TTG ATG GAT GGC AGA GG-3'; for U-CR67 (LRP residues Tyr-973 to Tyr-1056), the primer pairs 5'GGG GGA TCC ATG GAG AGG AAC CCA TTC ACC CTC ACC-3' and 5'GGG AAG TTG ATG GAT GGC AGA GG-3'; and for U-CR89 (LRP residues Tyr-1056 to Leu-1143), the primer pairs 5'GGG GGA TCC ATG GAG AGG AAC CCA TTC ACC GTC-3' and 5'GGC AAG TTG ATG GAT GGC AGA GG-3'; were used.

The present study was undertaken to define and characterize the minimal functional unit in LRP binding RAP. Our molecular dissection of the second ligand binding cluster of CR modules of LRP and expression in Escherichia coli now delineate a minimal two-repeat RAP-binding unit and demonstrate the importance of a conserved acidic residue in each RAP-binding CR for the LRP high affinity binding of RAP.
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Identification of a Minimal RAP Binding LRP Unit—Fig. 1 outlines the structural architecture of LRP and the dissection of the second ligand binding cluster of complement-type repeats (cluster II) into single- or double-repeat fragments produced in E. coli cells. An identified high affinity RAP binding site in LRP (41) has previously been located to cluster II (42), but none of the tested single repeats (CR3, CR4, CR5, and CR6) displayed affinity for RAP or RAPd3 affinity columns after in vitro refolding. We subsequently generated a complete set of overlapping two-domain derivatives of cluster II, representing CR pairs CR34, CR45, CR56, CR67, CR78, CR89, and CR90. Except for the last construct, all bound the RAP and RAPd3 affinity matrices used for purification of the constructs. Each RAP affinity-purified derivative was recovered as a pure homogeneous product, producing a well defined single band by SDS-PAGE analysis of nonreduced samples (Fig. 2A). Analysis of crude and affinity-purified receptor fragment preparations by nonreducing SDS-PAGE (Fig. 2B) showed that the well defined single bands corresponding to the affinity-purified species (U-CR34, U-CR45, U-CR56, U-CR67, U-CR78, and U-CR89) were abundant components in the crude refolding mixture. A similar prominent (i.e. probably correctly folded) component was also present in the crude U-CR910 refolding product, suggestive of efficient refolding of this receptor fragment also.

In order to provide further evidence for correct folding we analyzed the disulfide-bridging pattern in one of the refolded, factor Xa-processed, and affinity-purified two-domain products, CR56. A set of disulfide-bridged peptides from digested constructs was isolated by reverse-phase HPLC, characterized by partial sequencing and mass spectrometry, and found compatible with the expected three Cys–Cys bonds, Cys–Cys, and Cys–Cys disulfide bridges of both CR5 and CR6 (a data summary is presented in Table I).

To examine the ligand binding properties of affinity-purified two-repeat receptor fragments by SPR analysis, we prepared biosensor chips with immobilized fusion proteins U-CR34, U-CR45, U-CR56, U-CR67, U-CR78, and U-CR89 on the surface. SPR analysis of the interaction of RAP and RAPd3 with immobilized two-repeat fragments showed that RAP (Fig. 3A) and RAPd3 (Fig. 3B) both bound strongly to U-CR34, U-CR45, U-CR56, U-CR67, U-CR78, and U-CR89. Experiments made with two separately prepared sensor chips coated with different surface densities of CR pairs produced virtually identical results. For RAP binding, fitting of the recorded sensorgrams to a simple one-site model generated estimated K values in the range of 1–5 nM for each two-repeat protein.

Given that U-CR34, U-CR45, U-CR56, U-CR67, U-CR78, and U-CR89 were all able to bind RAP when immobilized on biosensor chips, we conducted solid-phase competition analysis to examine whether they showed any difference in ability to compete with the binding between immobilized LRP and 125I-RAP. As seen from the results shown in Fig. 4A, U-CR34, U-CR45, U-CR56, U-CR67, U-CR78, and U-CR89 were all able to compete the binding between native LRP and 125I-RAP.

Tentative Identification from a Sequence Alignment of a Conserved Acidic Residue in LRP—An alignment of the sequences
of the eight repeats from LRP cluster II (CR3–10) and of those of the seven repeats of the ligand binding domain of LDLR (LB1–7) is shown in Fig. 5A. A striking difference is the conservation of a surface-exposed acidic residue located at the center position between CysIV and CysV. This acidic residue is conserved in most repeats of LRP cluster II, but only in few repeats of the LDLR domain. An acidic residue at this position was identified in the study by Rong et al (32) for ligand binding (32). Notably, the only repeat in the LRP cluster that lacks the negative charged residue is CR10, which was the only repeat found not to be involved in binding to RAP. Accordingly, we directed our experimental efforts toward investigations of the contribution of this conserved aspartic acid residue to high affinity RAP binding.

Since U-CR34, U-CR45, and U-CR56 were slightly better inhibitors of RAP binding to LRP than either of U-CR67, U-CR78, and U-CR89 (Fig. 4A), we restricted the further analysis to these three fragments. The conserved aspartic acid residues in CR3 to CR6 (Asp-876, Asp-917, Asp-958, and Asp-999) were then replaced with asparagine residues to obtain U-CR34D876N, U-CR34D917N, U-CR56D958N, U-CR56D999N, and U-CR56D958N,D999N.

Isolation, Refolding, and RAP Binding of Asp-mutated Derivatives—The expression levels of mutant proteins were virtually identical to those obtained for the wild-type two-domain fragments. However, the purification step employing RAP affinity chromatography, which was very efficient in isolating refolded U-CR34, U-CR45, and U-CR56, was less useful for the mutant proteins. U-CR34D876N, U-CR34D917N, U-CR56D958N, and U-CR56D999N exhibited decreased affinity for RAP and RAPd3 immobilized on Sepharose, and U-CR45D917N and U-CR45D958N did not bind at all. Instead of using RAP affinity chromatography, the U-CR45 mutant derivatives were purified by gel filtration. A data summary is given in Table II.

The decreased RAP affinity was apparently not a result of
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misfolding, since nonreducing SDS-PAGE analysis (Fig. 6A) revealed similar migration patterns for mutants and native two-domain proteins, suggesting the presence of fully oxidized mutant proteins with the authentic disulfide-bridge pattern. Furthermore, all mutant protein products were found to bind Ca\(^{2+}\) in 45-calcium blots with the same efficiency as the non-mutated protein (Fig. 6B, lane 10). Here both conserved acidic residues (Asp-958 and Asp-999) in U-CR56 were replaced, each located at the critical position in the individual repeats, and calcium binding was found to be as strong as for native U-CR56. The U-CR45 derivatives showed weaker calcium binding relative to U-CR34 and U-CR56 (lanes 4–6 versus lanes 1–3 and 7–10). This was also true for wild-type U-CR45 and was therefore not indicative of improper folding of the mutant proteins.

The decreased RAP affinity was further confirmed by solid-state competition analysis of the mutant derivatives (Fig. 4B). When 1 \(\mu\)M U-CR34 was used as competitor, 33% of added \(^{125}\)I-RAP bound to immobilized LRP as compared with wells without any competitor. In contrast, 55 or 85% binding was achieved in the presence of 1 \(\mu\)M U-CR34D876N or U-CR34D917N, respectively. A similar effect was observed for U-CR45 and U-CR56 where RAP binding to LRP was also inhibited less efficiently by Asp→Asn mutant derivatives thereof (U-CR34D876N, U-CR34D917N, U-CR45D917N, U-CR45D958N, U-CR56D958N, U-CR56D999N, and U-CR56D958N,D999N) each at a concentration of 1 \(\mu\)M.

The present study defines two complement-type repeats as the minimal unit of LRP required for the high affinity binding to RAP. Instead of only one site responsible for RAP recognition, as previously suggested (43, 44), we demonstrate multisite binding of RAP to LRP cluster II. Furthermore, simultaneous binding of RAP to two adjacent CR modules is suggested, since impaired RAP binding is not located to only one repeat but is dependent of residues in both repeats in the two-repeat proteins.

From these results, it is tempting to suggest a model of RAP binding to members of the LDLR family, where binding is mediated by the interaction between one RAP domain and one two-CR unit. This model implies the existence of a high number of binding sites on LRP in agreement with previous reports.
TABLE II

| U-CR\textsuperscript{x}y derivatives | Location in LRP | RAP binding\textsuperscript{a} (affinity score) | \(\%\) inhibition of LRP binding\textsuperscript{b} |
|----------------------------------|-----------------|---------------------------------|---------------------------------|
| U-CR34                           | 3(4)56789(10)   | +++                             | 67%                             |
| U-CR34D876N                      | UOD             | +                              | 45%                             |
| U-CR34D8917N                     | UOD             | +                              | 17%                             |
| U-CR34D917N                      | UOD             | +                              | 7%                              |
| U-CR34D958N,D999N,               | UOD             | -                              | 50%                             |
| U-CR34D958N                      | UOD             | -                              | 3%                              |
| U-CR36                           | UOD             | +                              | 81%                             |
| U-CR36D958N                      | UOD             | +                              | 48%                             |
| U-CR36D999N                      | UOD             | +                              | 40%                             |
| U-CR36D999N,D999N,              | UOD             | -                              | 10%                             |
| U-CR37                           | UOD             | +                              | 46%                             |
| U-CR37D958N                      | UOD             | +                              | 41%                             |
| U-CR37D999N                      | UOD             | +                              | 36%                             |

\textsuperscript{a} Affinity score of RAP\textsuperscript{3} binding evaluated from RAP\textsuperscript{3} affinity chromatographic analysis. Equal amounts of protein were loaded on the column, and the amount of bound protein was classified ranging from effective purification (+ + +) to no detectable interaction (−).

\textsuperscript{b} Determined as the fraction of the \(\textsuperscript{125}\text{I}\)-RAP binding to LRP (Fig. 4) inhibited by 1 \(\mu\text{M}\) U-CR\textsuperscript{x}y (derivative).

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We also describe a preference for an acidic residue in the second cluster of ligand binding modules from LRP, identified by the alignment of the repeats with those from LDLR. The acidic residue is not buried inside the module as are other acidic residues, previously suggested to be involved in ligand recognition but now known to coordinate calcium binding. The present work considers only residues Asp-876, Asp-917, Asp-958, and Asp-999 present in the four amino-terminal repeats (CR3–CR6) of cluster II, but in view of the equivalent binding of RAP also to tandem repeats comprising CR7, CR8, and CR9, we predict a similar role of the conserved aspartic acid residues in these repeats (Asp-1037, Asp-1085, and Asp-1128).

Furthermore, because RAP contains at least two high affinity domains, RAP may cross-link regions of LRP in various ways. This gives a plausible explanation of why RAP is co-purifying with LRP when RAP affinity chromatography is used (1). We have previously shown that in addition to RAP\textsuperscript{3}, also RAP domains 1 and 2 bind RAP, although with weaker affinity than RAP\textsuperscript{3}. This also supports the present hypothesis, since only one repeat in the carboxyl-terminal part of cluster III showed affinity for RAP (20).

According to an alignment of sequences of repeats from other human members of the LDLR family (not shown) a negative charged residue at the center position between Cys\textsuperscript{IV} and Cys\textsuperscript{V} is present at a high frequency in other RAP binding receptors as well. It is therefore tempting to speculate whether the number of modules containing the conserved acidic residue correlates with efficient binding of RAP in general. In fact, such a tendency seems apparent when comparing the RAP binding properties of various LDLR-like receptors. A schematic representation of the LDLR family is shown in Fig. 8.

Obermoeller et al. (20) demonstrated efficient RAP binding of the second and the fourth cluster and low affinity binding to the third cluster, whereas no binding was observed to the first cluster of CR modules from the LRP. This observation is in good correlation with the number of acidic residues being 1(2), 3(7), 4(10), and 8(11)\textsuperscript{3} (see Fig. 8). Further detailed investigation showed that whereas both the amino- and the carboxyl-terminal halves of cluster II and cluster IV were able to bind immobilized RAP on nitrocellulose membranes, only the carboxyl-terminal part of cluster III showed affinity for RAP (20). This also supports the present hypothesis, since only one repeat in the amino-terminal part contains an acidic residue at the position in contrast to 3(5) repeats in the carboxyl-terminal end.

Furthermore, only low affinity interaction between RAP and LDLR was reported by Medh et al. (45), in agreement with 3(7) CR modules in the LDLR ligand binding domain harboring a negative charge at the center position between Cys\textsuperscript{IV} and Cys\textsuperscript{V}.

Finally, the VLDLR, apoER2, and SorLA-1, having 6(8), 6(7),...
and 5(11) Asp/Glu CR modules, respectively, bind strongly to RAP (46–48). It should be noted that various splice variants of both apoER2 and VLDLR have been identified (47, 49), and the reported RAP binding properties of those show no discrepancies with our hypothesis. The major variant of apoER2 in human brain lacking CR4–6 of the normally seven repeats present shows efficient RAP recognition (47), where the remaining four repeats all contain the negatively charged residue. The binding was found to be weaker than for the seven-repeat protein, in agreement with the loss of two repeats containing Asp.

Several groups have carried out detailed analysis of the RAP binding properties of VLDLR. Savonen et al. (21) reported that RAP interacts as well with CR1–3 as with CR1–5, but not with CR6–8. These results are in agreement with another study by Rettenberger et al. (50) demonstrating impaired RAP binding to a naturally occurring VLDLR variant lacking the third CR module, which contains a negatively charged residue. An analysis by Mikhailenko et al. (51) suggested that the RAP binding site of VLDLR is located within the amino-terminal four class A repeats and also suggested that the most amino-terminal CR is especially important for RAP binding.

It should be noted that the ligand binding repeat also is present in several proteins not exhibiting other homologies with the LDLR family. In these proteins, there is no preference for an acidic residue (e.g. the two novel discovered serine pro tease, Matriptase (52), containing four modules with a tryptophan, a valine, and two lysine residues at the critical position, and TMPRSS2 (53), with a single class A repeat with a valine residue). No RAP binding has been reported for these molecules.

In conclusion, the present molecular dissection of LRP cluster II has resulted in the identification of a range of different CR pairs and RAP domains will provide further information on the molecular interactions.

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Olav Michael Andersen, Lisa Lystbæk Christensen, Peter Astrup Christensen, Esben S. Sørensen, Christian Jacobsen, Søren K. Moestrup, Michael Etzerodt and Hans Christian Thøgersen

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