Receptor-associated Protein Is a Folding Chaperone for Low Density Lipoprotein Receptor-related Protein*

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The 39-kDa receptor-associated protein (RAP) is a receptor antagonist that inhibits ligand interactions with the receptors that belong to the low density lipoprotein receptor gene family. Our previous studies have demonstrated that RAP interacts with the low density lipoprotein receptor-related protein (LRP) within the endoplasmic reticulum and prevents premature interaction of ligands with the receptor. To analyze whether RAP is also involved in the folding of LRP during receptor biosynthesis, we generated anchor-free, soluble minireceptors that represent each of the four putative ligand-binding domains of LRP (SLRP1, -2, -3, and -4, corresponding to the clusters with 2, 8, 10, and 11 cysteine-rich complement-type repeats, respectively). When these SLRPs were overexpressed by cell transfection, only SLRP1 was secreted. Little or no secretion was observed for SLRP2, -3, and -4. However, when RAP cDNA was cotransfected with SLRP2, -3, and -4 cDNAs, each of these SLRPs was secreted. The cellular retention of SLRPs in the absence of RAP coexpression appeared to be a result of the formation of SDS-resistant, oligomeric aggregates observed under nonreducing conditions. Such oligomers of the SLRPs likely resulted from formation of intermolecular disulfide bonds since they were reduced to monomers when analyzed under reducing conditions. The oligomers were formed not only among molecules of a given SLRP, but also between different SLRPs. The role of RAP in the process of LRP folding was shown by the reduction in aggregated SLRP oligomers upon RAP coexpression. A similar role of RAP in preventing the aggregation of newly synthesized receptor was also observed using membrane-containing minireceptor of LRP. Comunoprecipitation and ligand binding studies demonstrated that RAP binds avidly to SLRP2, -3, and -4, but not to SLRP1. These results suggest that these interactions may be important for proper folding of LRP by ensuring the formation of proper intradomain, but not intermolecular or interdomain, disulfide bonds. Thus, our results strongly suggest that, in addition to the prevention of premature binding of ligands to LRP, RAP also plays an important role in receptor folding.

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† The abbreviations used are: RAP, receptor-associated protein; LRP, low density lipoprotein receptor-related protein; SLRP, soluble LRP; MLRP, membrane-containing LRP; ER, endoplasmic reticulum; PCR, polymerase chain reaction; HA, hemagglutinin; PAGE, polyacrylamide gel electrophoresis.
trans-Golgi compartment into two subunits (Herz et al., 1990; Willnow et al., 1996). The 515-kDa subunit contains all the putative ligand-binding domains and remains noncovalently associated with the 85-kDa subunit, which includes the transmembrane domain and the cytoplasmic tail. LRP contains 31 copies of complement-type ligand-binding repeats arranged in four clusters with 2, 8, 10, and 11 repeats, respectively (see Fig. 1) (Herz et al., 1988; Krieger and Herz, 1994). Also present in its structure are 22 copies of cysteine-rich epidermal growth factor precursor-type repeats that flank the ligand-binding clusters. The complement-type repeats in LRP are similar to those in the low density lipoprotein receptor, in which the 40-residue-long cysteine-rich repeats exhibit a highly conserved spacing pattern of 6 cysteine residues that form three intramolecular disulfide bonds (Goldstein et al., 1985). The disulfide bonds are believed to be important for the stability of the ligand-binding sites on the receptor, particularly when the receptor traffics through the endosomes, where the acidic environment uncouples ligand from receptor.

The complexity of LRP structure, largely due to the extensive intradomain disulfide bonds, presents a challenging task for proper folding during its biosynthesis. This process may well be assisted by molecular chaperone(s) within the ER. Since RAP is the only known protein that interacts with LRP within the ER, we examined the hypothesis that RAP may be involved in LRP folding in addition to its role in preventing premature binding of ligands to LRP. Thus, by constructing anchor-free, soluble mini-receptors that represent each of the four putative ligand-binding domains of LRP (SLRPs), as well as membrane-containing mini-receptors of LRP, we developed a system with which receptor folding and subsequent trafficking may be examined. Herein, we demonstrate that misfolded receptors that result from the formation of intramolecular disulfide bonds are retained within the ER and that coexpression of RAP is both necessary and sufficient to prevent such misfolding.

MATERIALS AND METHODS

Construction of cDNAs for SLRP, MLRP, and RAP—A PCR fragment encoding the LRP signal peptide and the first 5 amino acids was generated and cloned into HindIII and BamHI sites of the expression vector pcDNA3 (in vitro). The 3′-primer for this PCR fragment includes a built-in sequence encoding the amino acid sequence of the hemagglutinin (HA) tag (YPYDVPDYA) (Handley-Gearhart et al., 1994) and a linker restriction site (BamHI) encoding 2 extra amino acids (GS) (see Fig. 1B). The resulting plasmid, designated pcDNASLRP5, was used as the base plasmid for the construction of cDNAs for each of the four SLRPs. The cDNA for each SLRP was generated by PCR and subcloned into BamHI and XhoI sites of pcDNASLRP5. All the restriction sites were included in the PCR primer sequences. The regions represented in the four SLRPs are marked in Fig. 1A and contain the following amino acid sequence: SLRP1, 6–171; SLRP2, 787–1244; SLRP3, 2462–3004; and SLRP4, 4401–4425. The entire region of the cytoplasmic tail (amino acids 4426–4525). The PCR primers for the anchoring region of LRP were 5′-GATGAAATTCATGGCGGCAGC-3′ and 5′-GATGATCCAGCTATGCAAGG-GTGCCCCTCTCTC-3′. The construction of cDNA for RAP (pcDNA-RAP) has been described previously (Bu et al., 1995).

Cell Culture and Transfection—Human glioblastoma U87 cells were cultured in Earle’s minimum essential medium supplemented with 10% fetal calf serum, 2 mM l-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, and 1 mM sodium pyruvate and maintained at 37 °C in humidified air containing 5% CO2 (Bu et al., 1994). For transient transfection, U87 cells were transfected with various plasmids at 40–60% confluence using a calcium phosphate precipitation method (Chen and Okayama, 1987). For each well of 6-well dishes (3.5 cm in diameter), 10 µg of DNA were used in a total volume of 4 ml of medium. Sixteen h after the start of transfection, cells were washed with medium and cultured continuously for an additional 24 h before use in experiments. The efficiency of transfection in these studies was consistently ~20% as assessed by immunofluorescent staining of expressed proteins.

Metabolic and Pulse-Chase Labeling—Metabolic labeling with [35S]cysteine was performed essentially as described previously (Bu et al., 1995). For pulse-chase experiments, cells were pulse-labeled and chased with serum-containing medium for different periods of time as specified in each experiment.

Expression of Recombinant SLRP2 in Bacteria—A PCR fragment encoding the complement-type cysteine-rich repeats of the second ligand-binding cluster of LRP (LRP2, amino acids 826–1165) was generated and cloned into BamHI and EcoRI sites of the fusion protein expression vector pGEX-2T (Pharmacia Biotech Inc.). Restriction sites for subcloning were included in PCR primer sequences. The two dige- nuotides used for PCR amplification were 5′-CCGGTTGGAATC-CAACACATCTAAGGTCTCA-3′ and 5′-TCAATGATCCCGTATCTGCT-3′. The glutathione S-transferase (GST) fusion protein was expressed in Escherichia coli and purified using glutathione-agarose beads and standard techniques. The fusion protein was then cleaved with thrombin, and recombinant RAP was repurified by removing glutathione S-transferase using glutathione-agarose beads.

Antibodies, Immunoprecipitation, and SDS-PAGE—Polyclonal anti-RAP and anti-LRP antibodies have been described previously (Bu et al., 1995). Monoclonal anti-HA antibody was obtained from Babco (12CA5). Polyclonal anti-LRP antibody (specific for domain 2 of LRP) was generated in rabbit using recombinant LRP2 as described above. Immunoprecipitations were carried out essentially as described pre- viously (Bu et al., 1993), except the washing buffer for monoclonal anti-HA antibody contained 0.1% SDS instead of 1% SDS. In the case of immunoprecipitation using anti-RAP antibody, SDS was omitted from the washing buffer. A peripheral antibody (LAMMlI) was used to ensure that the primary antibody used in each immunoprecipitation was in excess. Protein A-agarose beads were used to precipitate protein-Lg complexes. The immunoprecipitated material was released from the beads by boiling each sample for 5 min in Laemmli sample buffer (62.5 mM Tris–HCl, pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol) (Laemmli, 1970). If the immunoprecipitated material was analyzed under reducing conditions, 5% (v/v) β-mercaptoethanol was included in the Laemmli sample buffer. The percentage of SDS-polyacrylamide gels is indicated in each figure legend. Rainbow molecular mass markers (Bio-Rad) were used as the molecular mass standards.

Interaction of 125I-RAP with SLRPs—Lodination of RAP has been described previously (Bu et al., 1993). Media from either control– or SLRP-transfected cells were collected and diluted with 125I-RAP (3 mCi) in the absence or presence of excess unlabeled RAP (1 µM). SLRPs with or without the interaction with 125I-RAP were then immunoprecipitated with anti-HA antibody in the absence of SDS and analyzed by SDS-PAGE.

RESULTS

Construction and Expression of SLRPs—The putative ligand-binding domains of LRP are cysteine-rich with extensive disulfide bonds. To analyze the folding and subsequent trafficking of LRP, we constructed soluble receptor molecules corresponding to each of the four putative ligand-binding domains (designated SLRP1, –2, –3, and –4) using PCR techniques (Fig. 1A). Each of these SLRPs consists of the signal peptide, the first 5 amino acids after the signal peptide cleavage site, a 9-amino acid HA tag, and the designated ligand-binding do-
Secretion of SLRPs Requires Coexpression of RAP—To analyze the expression of these SLRPs, cDNAs for these constructs were transiently transfected into human glioblastoma U87 cells (Bu et al., 1994). After transfection, cells were metabolically labeled with [35S]cysteine for 4 h, and cell lysates were immunoprecipitated with either polyclonal anti-LRP antibody or monoclonal anti-HA antibody and analyzed on 10% SDS-polyacrylamide gel under reducing conditions. The positions of SLRPs and endogenous LRP are marked. The molecular mass standards are given in kilodaltons.

Fig. 1. Construction and expression of SLRPs. A, domains of LRP that are included in SLRP constructs. Each SLRP consists of the entire continuous complement-type repeats and those epidermal growth factor (EGF) precursor-type repeats that immediately flank the complement-type repeats. B, structure of cDNA constructs for SLRPs. Each cDNA construct contains sequences that encode the signal peptide (SP), the first 5 amino acids after the signal peptide cleavage site, a 9-amino acid HA tag, and the designated ligand-binding cluster. C, expression of SLRPs via cell transfection in U87 cells. U87 cells were transiently transfected with cDNAs for each of the four SLRPs. After transfection, cells were metabolically labeled with [35S]cysteine for 4 h, and cell lysates were immunoprecipitated with either polyclonal anti-LRP antibody or monoclonal anti-HA antibody and analyzed on 10% SDS-polyacrylamide gel under reducing conditions. The positions of SLRPs and endogenous LRP are marked. The molecular mass standards are given in kilodaltons.

To analyze the expression of these SLRPs, cDNAs for these constructs were transiently transfected into human glioblastoma U87 cells (Bu et al., 1994). After transfection, cells were metabolically labeled with [35S]cysteine for 4 h, and cell lysates were immunoprecipitated with either polyclonal anti-LRP antibody or monoclonal anti-HA antibody (Fig. 1C). As shown in Fig. 1C, in addition to endogenous full-length LRP, anti-LRP antibody effectively immunoprecipitated SLRP2, -3, and -4 (lanes 2–4), but not SLRP1 (lane 1), indicating that our polyclonal anti-LRP antibody recognizes regions that reside within putative ligand-binding domains 2, 3, and 4, but not domain 1. The expression of SLRP1 in U87 cells was confirmed by immunoprecipitation with anti-HA antibody (lane 5), which also immunoprecipitated SLRP2, -3, and -4 (lanes 6–8), but not endogenous LRP. The molecular sizes of each of the four SLRPs correlate with those predicted from the number of amino acid residues and the number of putative N-linked glycosylation sites. Of note, although it consists of fewer amino acid residues, SLRP3 (559 amino acids) exhibited a higher apparent molecular mass compared with SLRP4 (566 amino acids), presumably due to the contribution of additional N-linked glycosylation (seven putative sites in SLRP3 versus five in SLRP4).

Secretion of SLRPs Requires Coexpression of RAP—To analyze whether SLRPs are secreted when overexpressed in U87 cells, we performed pulse-chase metabolic labeling and immunoprecipitation analysis. After transient transfection with cDNAs for each of the four SLRPs, U87 cells were metabolically pulse-labeled with [35S]cysteine for 1 h and chased for 3 h. Both media and cell lysates were quantitatively immunoprecipitated with anti-HA antibody and analyzed by SDS-PAGE (Fig. 2A). As shown in Fig. 2A, except for SLRP1, little or no secretion of SLRP2, -3, and -4 was detected, indicating that folding and/or trafficking of these SLRPs was impaired when overexpressed. Similar results were obtained when these cDNAs were transfected into several other cell lines (e.g., HepG2 and COS cells) (data not shown). To analyze whether coexpression of RAP can rescue the cellular retention of SLRPs, we performed cotransfection of cDNAs for SLRPs and RAP, followed with pulse-chase metabolic labeling and immunoprecipitation. When RAP was coexpressed, SLRP2, -3, and -4 all exhibited significant secretion (Fig. 2B), whereas no significant effect was observed for the secretion of SLRP1. The secreted SLRPs exhibited lower mobility on SDS-polyacrylamide gel when compared with their corresponding species in cell lysates. This was likely due to the addition of complex carbohydrates as these proteins trafficked through the trans-Golgi compartment en route to secretion. Since RAP was not labeled with [35S]cysteine (the RAP sequence does not contain cysteine), the expression of RAP in transfected cells was analyzed by Western blotting. We found that the expression of RAP was typically ~50-fold higher than that of endogenous RAP in cotransfected U87 cells when compared with untransfected U87 cells. Shown in Fig. 2C is an
example of an anti-RAP Western blot of cells cotransfected with cDNAs for SLRP2 and either vector pcDNA (first lane) or pcDNA-RAP (second lane) (Bu et al., 1995). Thus, endogenous RAP in U87 cells may well have been limiting when SLRPs were overexpressed.

To confirm the requirement for coexpression of RAP for SLRP secretion, we focused on SLRP2 and performed kinetic analyses of its secretion. SLRP2 cDNA was cotransfected into U87 cells with either vector pcDNA (Fig. 3A, -RAP) or pcDNA-RAP (Fig. 3B, +RAP). The transfected cells were pulse-labeled with [35S]cysteine for 1 h and chased for 1, 3, or 5 h. After each chase, media and cell lysates were immunoprecipitated with anti-HA antibody and analyzed by SDS-PAGE. As shown in Fig. 3A, little secretion of SLRP2 was seen in the absence of RAP even after 5 h of chase, whereas SLRP2 was readily secreted in RAP-coexpressed cells with kinetics typical of a secretory protein. By the end of 5 h of chase, >70% of radiolabeled SLRP2 had been secreted into the medium.

RAP Prevents Formation of Intermolecular Disulfide Bonds and Aggregation of SLRPs—To explore the potential mechanisms by which RAP coexpression ensures the secretion of SLRPs, we analyzed the cellularly retained SLRPs via metabolic labeling and SDS-PAGE. Interestingly, in the absence of RAP coexpression, we observed high molecular mass, SDS-resistant oligomers for SLRP2, -3, and -4, but not for SLRP1, when analyzed under nonreducing conditions. Shown in Fig. 4A are SLRP2 and SLRP3 analyzed under either nonreducing or reducing conditions after their expression via transfection and metabolic labeling. In these experiments, U87 cells were transfected with cDNAs for SLRP2 (lanes 1), SLRP3 (lanes 2), or both (lanes 3) and metabolically labeled with [35S]cysteine for 4 h. Cell lysates were then immunoprecipitated with either anti-HA antibody (Fig. 4, A and B) or anti-LRP2 antibody (Fig. 4, C and D) and analyzed by SDS-PAGE under either nonreducing (Fig. 4, A and C) or reducing (Fig. 4, B and D) conditions. As shown in Fig. 4A, when analyzed under nonreducing conditions, both SLRP2 and SLRP3 appeared, in addition to monomers, as various oligomers (i.e. dimer, trimer, and multimers) and migrated as slowly as those observed on top of the stacking gel. These oligomers appeared to be SDS-resistant since they were not diminished after immunoprecipitation and SDS-PAGE analysis in the presence of SDS (1–2%). However, when these oligomers were analyzed under reducing conditions, all species collapsed to monomer form (Fig. 4B). This suggests that all oligomer forms likely resulted from intermolecular disulfide linkages. To determine whether disulfide bonds can be formed between different SLRPs, we employed an antibody to LRP that was generated using recombinant domain 2 of LRP. As shown in Fig. 4 (C and D), anti-LRP2 antibody specifically immunoprecipitated SLRP2 (lanes 1), but not SLRP3 (lanes 2). However, SLRP3 was communoprecipitated with anti-SLRP2 antibody from cells that had been cotransfected with cDNAs for both SLRP2 and SLRP3. This observation suggests that these SLRP3 molecules were associated with the coexpressed SLRP2 molecules via interdomain disulfide linkages. Thus, misfolding of LRP may include intermolecular disulfide bonds between identical domains or between different domains as well as interdomain disulfide bonds within a given LRP molecule.

To analyze the potential role of RAP in the folding process of LRP, we examined the effects of RAP coexpression on oligomerization of the SLRPs. Shown in Fig. 5A is metabolically labeled SLRP2 in the absence or presence of RAP coexpression and analyzed by SDS-PAGE under nonreducing conditions. As shown in Fig. 5A, oligomers of SLRP2 in the absence of RAP coexpression (lane 1) were similar to those observed in Fig. 4. However, when RAP was coexpressed (lane 2), SLRP2 migrated predominately as the monomer, with few oligomers seen. This differential appearance of SLRP2 was not due to differential rates of protein synthesis since the level of newly synthesized SLRP2 was similar in the absence and presence of RAP coexpression (Fig. 5B, compare lanes 1 and 2). Similar effects of RAP coexpression on the prevention of oligomerized SLRPs were also observed for SLRP3 and SLRP4 (data not shown).

RAP Interacts with LRP Domains 2, 3, and 4, but Not with
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Domain 1—The involvement of RAP in the folding process of LRP requires direct interaction of this chaperone protein with the receptor. Thus, we analyzed the potential interactions of RAP with each of the four putative ligand-binding domains of LRP using SLRPs. Two separate approaches were employed for these analyses. First, we examine the ability of RAP to coimmunoprecipitate each of the four SLRPs following transfection and metabolic labeling. Our previous studies have demonstrated that our affinity-purified anti-RAP IgG is specific for RAP, with no cross-reactivity with other cellular proteins (Bu et al., 1994, 1995). Cells were transfected with cDNAs for each of the four SLRPs in the absence or presence of cotransfection of the cDNA for RAP (Fig. 6). The transfected cells were metabolically labeled with [35S]cysteine (200 μCi/ml) for 4 h, and cell lysates were immunoprecipitated with anti-HA antibody. The immunoprecipitated material was analyzed on 6.5% SDS-polyacrylamide gel under either nonreducing (A) or reducing (B) conditions. The position of the monomer and the regions for oligomers are marked. The top of the stacking gel and the separating gel are indicated with closed and open arrowheads, respectively. The molecular mass standards are given in kilodaltons.

![Fig. 5. Coexpression of RAP prevents formation of intermolecular disulfide bonds and oligomerization of SLRP2. U87 cells were transiently transfected with cDNA for SLRP2 with cotransfection of either vector pcDNA (−RAP) or pcDNA-RAP (+RAP). Cells were then metabolically labeled with [35S]cysteine (200 μCi/ml) for 4 h, and cell lysates were immunoprecipitated with anti-HA antibody. The immunoprecipitated material was analyzed on 6.5% SDS-polyacrylamide gel under either nonreducing (A) or reducing (B) conditions. The position of the monomer and the regions for oligomers are marked. The top of the stacking gel and the separating gel are indicated with closed and open arrowheads, respectively. The molecular mass standards are given in kilodaltons.](image)

As shown in Fig. 7, SLRP2, -3, and -4 (lanes 1, 2, and 4), were coimmunoprecipitated with RAP. Coexpression of RAP resulted in a significant increase in the amounts of coimmunoprecipitated SLRPs (compare lanes 4 and 3, 6 and 5, and 8 and 7), indicating that endogenous RAP in U87 cells was limiting in the presence of overexpressed SLRPs. In addition, more SLRP2 and -4 molecules were coimmunoprecipitated with RAP compared with SLRP3, suggesting that these regions of LRP may exhibit higher affinity and/or contain multiple binding sites for RAP.

The second approach involved in vitro analysis of 125I-RAP interaction with secreted SLRPs. U87 cells were cotransfected with cDNAs for each of the four SLRPs and for RAP. Media containing the secreted SLRPs were collected and incubated with exogenously added 125I-RAP. Medium from vector-transfected U87 cells was used as a negative control. Potential 125I-RAP-SLRP complexes were immunoprecipitated with anti-HA antibody, and the ability of each of the four SLRPs to coprecipitate 125I-RAP was assessed by SDS-PAGE analysis. As shown in Fig. 7, SLRP2, -3, and -4, but not SLRP1, interacted with 125I-RAP. These interactions appeared to be specific since 125I-RAP was not precipitated in the presence of excess unlabeled RAP. Consistent with experiments shown in Fig. 6, SLRP2 and -4 bound more RAP (i.e. exhibited higher affinities and/or multiple binding sites) compared with SLRP3. Thus, these results clearly demonstrate that multiple domains of LRP can independently interact with its chaperone protein, RAP.

RAP Prevents Aggregation of Membrane-containing LRP Mini-receptors—To analyze whether RAP plays the same role in receptor folding for membrane-containing minireceptors of LRP (MLRP), we constructed a MLRP that corresponds to SLRP2 with the inclusion of the immediate extracellular domain, the transmembrane domain, and the cytoplasmic tail of LRP (MLRP2) (see “Material and Methods” for the construction of cDNA). To analyze whether MLRP2 can interact with RAP, we transfected U87 cells with cDNAs for MLRP2 and RAP. The transfected cells were metabolically labeled with [35S]cysteine for 4 h, and the cell lysates were immunoprecipitated with either anti-HA antibody or anti-RAP antibody (Fig. 8A). As
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Fig. 8. Coexpression of RAP prevents formation of intermolecular disulfide bonds and oligomerization of MLRP2. A, U87 cells were transfected with cDNAs for MLRP2 and RAP. The transfected cells were then metabolically labeled with [35S]cysteine (200 μCi/ml) for 4 h, and cell lysates were immunoprecipitated with either anti-HA antibody or anti-RAP antibody. The positions of endogenous LRP and expressed MLRP2 are labeled. B, U87 cells were transfected with cDNA for MLRP2 with cotransfection of either vector pcDNA (lane 1) or pcDNA-RAP (lane 2). Cells were then metabolically labeled with [35S]cysteine (200 μCi/ml) for 4 h, and cell lysates were immunoprecipitated with anti-HA antibody. The immunoprecipitated material was analyzed on 6% SDS-polyacrylamide gel under either nonreducing or reducing conditions. The position of the monomer and the regions for oligomers were marked. The top of the stacking gel and the separating gel are indicated with closed and open arrowheads, respectively. The molecular mass standards are given in kilodaltons.

shown in Fig. 8A, anti-HA antibody effectively immunoprecipitated MLRP2, which exhibited the expected molecular mass (~160 kDa). Although the construction of MLRP2 included the authentic proteolytic site (Herz et al., 1990), little processing of this mini-receptor was seen. This was likely due to the limitation of available protease for processing when MLRP2 was overexpressed. Similar to SLRP2 (Fig. 6), both endogenous LRP and MLRP2 were communoprecipitated with anti-RAP antibody. To examine the effects of RAP coexpression on the potential aggregation of MLRP2, we transfected U87 cells with the cDNA for MLRP2 with the cotransfection of cDNA for either vector pcDNA (Fig. 8B, lane 1) or pcDNA-RAP (lane 2). The transfected cells were then metabolically labeled with [35S]cysteine for 4 h. Cell lysates were immunoprecipitated with anti-HA antibody and analyzed by SDS-PAGE under either nonreducing or reducing conditions. Similar to the role of RAP for SLRP2, oligomers of MLRP2 (mostly migrating on top of the gel) were seen in the absence of RAP coexpression under nonreducing conditions (lane 1). However, when RAP was coexpressed (lane 2), MLRP2 migrated predominantly as the monomer, with significantly reduced oligomers. When analyzed under reducing conditions, MLRP2 migrated slightly slower on SDS gel in the presence of RAP coexpression, likely due to differential glycosylations within the ER. Thus, RAP appears to be capable of preventing the aggregation of both soluble and membrane-containing mini-receptors of LRP.

DISCUSSION

The important roles of RAP in the post-translational folding and/or trafficking of LRP have been recently reported in two independent studies. Our previous studies have shown that RAP interacts with LRP early within the secretory pathway and inhibits premature interaction of its ligands with LRP (Bu et al., 1995). Studies by Willnow et al. (1995) have shown that disruption of the gene for RAP results in a significant reduction of functional LRP in liver and brain. However, the mechanisms responsible for the requirements of RAP in the functional expression of LRP have not been established. Given the present results, it is likely that, in the absence of RAP, the proper folding of LRP is partially impaired due to the formation of intermolecular disulfide bonds. Such covalent cross-linking of multiple molecules can in turn result in the oligomerization and aggregation of newly synthesized receptors. In the case of SLRPs, aggregation of these mini-receptors prevents them from being secreted, likely due to the quality control systems within the ER. Indeed, when we analyzed the effects of dithiothreitol on the folding and secretion of SLRPs, we found that dithiothreitol completely eliminated the formation of disulfide bonds, but exhibited little effect on the secretion of SLRPs (data not shown). In addition, such misfolding events may be significant only in cells where LRP is highly expressed. In this regard, it is interesting to note that significant reduction of LRP in RAP-deficient mice was observed mainly in tissues and cells that express high levels of LRP (e.g. liver and brain). In tissue and cells that express low levels of LRP, such abnormal intermolecular disulfide bonds may be minimal in the absence of RAP due to lower amounts of receptor along the secretory pathway. Thus, it will be of interest to examine the nature of molecular aggregations of LRP in various tissues and cell types from RAP knockout mice.

The mechanisms responsible for the involvement of RAP in intradomain disulfide bond formation of LRP are not clear at present. However, the observation that RAP may interact with multiple domains of LRP suggests that RAP may serve as a chaperone by maintaining regions of the receptor in a given state so as to prevent incorrect disulfide bond formation and/or to guide the proper alignment of correct disulfide bonds. Thus, the formation of intermolecular disulfide bonds in the absence of RAP may be a result of misrecognition of cysteine residues located within different molecules by protein-disulfide isomerase. In addition, the challenge for the formation of correct disulfide bonds during folding also lies within the LRP molecule, as inappropriate bond formation may occur both between LRP molecules as well as between domains of the same molecule. In addition to the role of RAP in LRP folding, the timing of folding for each domain may also be crucial.

Although membrane anchoring may affect the trafficking of proteins due to bulk membrane flow (Burgess and Kelly, 1989; Klausner, 1989), soluble and membrane-bound proteins generally undergo similar post-translational folding and modifications along the secretory pathway. Since the folding of newly synthesized proteins often occurs concomitantly with translation, the role of the membrane in the folding process may be minimal. Thus, soluble recombinant receptors such as those used in this study may be useful in analysis of the folding process of membrane-bound proteins. Indeed, our results indicated that RAP exhibits similar roles in preventing the aggregation of both soluble and membrane-containing mini-receptors of LRP. Similar types of analyses using soluble recombinant protein in the folding and trafficking of other membrane-bound glycoproteins have been performed (Gething and Sambrook, 1982; Singh et al., 1990). Misfolded proteins often result in molecular aggregation and subsequent ER retention. A delay in the secretion of soluble proteins is in turn often a result of misfolding. In this regard, the soluble receptor systems described in this study should provide a useful strategy to dissect the structural elements within RAP and/or LRP that are im-

2 T. E. Willnow, personal communication.
our laboratory (Iadonato et al., 1993). Using cell-surface saturation binding analyses, we have shown that there are approximately six to seven RAP-binding sites for each LRP molecule. Thus, domains 2 and 4 of LRP may well contain multiple RAP-binding sites. Furthermore, this notion is consistent with the fact that LRP domain 2 contains multiple ligand-binding sites, including those for tissue plasminogen activator-plasminogen activator inhibitor complexes (Willnow et al., 1994) and cry-macroglobulin (Moestrup et al., 1993). Each of these ligand-binding sites within domain 2 of LRP may overlap with an independent RAP-binding site (Warshawsky et al., 1994).

The precise stoichiometry of RAP-binding sites within each domain of LRP awaits future studies. Due to the nature of transient transfection, the effects of overexpression of SLRPs on endogenous LRP are not clear (the majority of LRP seen in our experiments was derived from untransfected cells). In future studies, the affinity of RAP for endogenous LRP versus the single domain of LRP should be directly compared using stably SLRP-transfected cell lines.

The extremely large size and complicated domain structure of LRP suggest that the folding process of this receptor may be a much more complicated process than our current understanding allows. In this regard, although RAP is the only known protein currently identified to interact with LRP within the ER, other chaperones (e.g. immunoglobulin heavy chain binding protein and calnexin) may well be involved in the folding process of the receptor. Identification of such chaperones should significantly aid in understanding the folding process both of LRP and of proteins in general.

In summary, we have demonstrated that RAP interacts with multiple domains of LRP. Such interactions appear to be important in the prevention of formation of intermolecular disulfide bonds during receptor folding and subsequently in the premature binding of ligands to the receptor during trafficking along the secretory pathway. The soluble receptor systems described in this study should be useful not only for the study of receptor folding, but also for the mapping of ligand-binding sites within LRP.

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