Ca\(^{2+}\) and Receptor-associated Protein Are Independently Required for Proper Folding and Disulfide Bond Formation of the Low Density Lipoprotein Receptor-related Protein*

(Received for publication, May 4, 1998, and in revised form, June 24, 1998)

Lynn M. Obermoeller‡, Zhou Chen‡, Alan L. Schwartz§, and Guojun Bu¶

From the Departments of Pediatrics, Molecular Biology and Pharmacology, and Cell Biology and Physiology, Washington University School of Medicine, St. Louis, Missouri 63110

The low density lipoprotein receptor-related protein (LRP) is a cysteine-rich, multifunctional receptor that binds and endocytoses a diverse array of ligands. Recent studies have shown that a 39-kDa receptor-associated protein (RAP) facilitates the proper folding and subsequent trafficking of LRP within the early secretory pathway. In the current study, we have examined the potential role of Ca\(^{2+}\) and its relationship to RAP during LRP folding. We found that depletion of Ca\(^{2+}\) following either ionomycin or thapsigargin treatment significantly disrupts the folding process of LRP. The misfolded LRP molecules migrate as high molecular weight aggregates under nonreducing SDS-polyacrylamide gel electrophoresis, suggesting the formation of intermolecular disulfide bonds. This misfolding is reversible because misfolded LRP can be re-folded into functional receptor molecules upon Ca\(^{2+}\) restoration. Using an LRP minireceptor representing the fourth ligand binding domain of LRP, we also observed significant variation in the conformation of monomeric receptor upon Ca\(^{2+}\) depletion. The role of Ca\(^{2+}\) in LRP folding is independent from that of RAP because RAP remains bound to LRP and its minireceptor following Ca\(^{2+}\) depletion. Furthermore, Ca\(^{2+}\) depletion-induced LRP misfolding occurs in RAP-deficient cells. Taken together, these results clearly demonstrate that Ca\(^{2+}\) and RAP independently participate in LRP folding.

The low density lipoprotein (LDL) receptor-related protein (LRP) is an endocytic receptor that belongs to the LDL receptor gene family (1, 2). Recent biological studies on LRP have been facilitated by the identification of an array of structurally and functionally distinct LRP ligands that include various circulating lipoproteins and protease/protease inhibitor complexes. LRP is a widely expressed, extremely large glycoprotein of 4525 amino acids, the extracellular domain of which structurally roughly resembles four combined LDL receptor molecules (1–3). Studies by Herz et al. (4) and Willnow et al. (5) have shown that LRP is synthesized as a single polypeptide chain of approximately 600 kDa and in the trans-Golgi network is cleaved by furin into two subunits of a 515-kDa ligand binding domain and an 85-kDa transmembrane domain, which remain associated with one another as they travel to the cell surface. The 515-kDa NH\(_2\)-terminal subunit binds ligands and remains attached to the membrane through noncovalent association with the 85-kDa transmembrane subunit (4). The primary structure of LRP is remarkable for its high content of cysteine residues. Most of these cysteine residues are found within clusters of tandemly arranged complement-type or EGF-type repeats within the extracellular domain. Each of these repeats contains ~40 amino acid residues, including six cysteine residues, which form three disulfide bonds (6, 7). Thus, a single LRP molecule may contain at least 159 disulfide bonds. This extensive degree of posttranslational modification presents a challenging task for the cell to correctly fold the receptor within the endoplasmic reticulum (ER). Thus, the folding of LRP, including the formation of disulfide bonds, is likely assisted by enzymes and molecular chaperones.

Indeed, recent studies on the 39-kDa receptor-associated protein (RAP) have defined the participation of this protein in receptor biogenesis, including its proper folding and trafficking along the early secretory pathway. Using anchor-free, soluble minireceptors that represent each of the four putative ligand binding domains of LRP (sLRPs), our previous studies (8) showed that co-expression of RAP is both necessary and sufficient for the correct folding and subsequent secretion of the sLRPs. In the absence of RAP co-expression, sLRPs misfolded as a result of formation of intermolecular disulfide bonds and are retained within the ER with little secretion. In addition to assisting receptor folding, continuous interaction between RAP and LRP along the early secretory pathway is also important for preventing premature ligand binding to LRP. This latter function of RAP correlates its ability to universally antagonize ligand interactions with the receptor (9). The role of RAP in the maturation and trafficking of LRP is also supported by gene knockout studies (10, 11), which demonstrate that cells lacking RAP exhibit ER-retention of aggregated LRP and a 75% reduction in functional LRP.

RAP contains three internal repeats (12, 13). Our recent studies (14) showed that the carboxyl-terminal repeat of RAP functions similarly to the full-length RAP in terms of assisting the receptor to fold. However, this repeat of RAP did not emulate full-length RAP in the inhibition of \(a_2\)-macroglobulin, a ligand for LRP. In contrast, the amino-terminal and central repeats of RAP, which were unable to assist receptor to fold, were found to inhibit \(a_2\)-macroglobulin binding to LRP (14). These differential roles of the RAP repeats suggest that the effects of RAP in receptor folding and inhibition of ligand interaction are independent functions.
Ca\(^{2+}\) and RAP Are Independently Required for LRP Folding

It is well known that Ca\(^{2+}\) binds to the LDL receptor (15) and LRP (1) and that binding of ligands to members of the LDL receptor gene family is Ca\(^{2+}\)-dependent (2, 3). However, the molecular basis of Ca\(^{2+}\) interaction with these receptors was not clear until recently. Examination of the crystal structure of a ligand binding repeat from the LDL receptor revealed that each repeat contains a single Ca\(^{2+}\) ion trapped in an octahedral cage formed primarily by four conserved acidic residues (7). Interactions between Ca\(^{2+}\) and these acidic residues appear to be important for stabilizing and maintaining the receptor in its native conformation. In the current study, we examined the potential role for Ca\(^{2+}\) in the folding process of LRP. We found that Ca\(^{2+}\) and RAP are independently required for LRP folding, including the formation of correct disulfide bonds.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Human hepatoma HepG2 cells were cultured in minimum essential medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 \(\mu\)g/ml streptomycin and maintained at 37°C in humidified air containing 5% CO\(_2\) (16). Mouse embryonic fibroblast (MEF)-1 and MEF-7 cells were cultured under the same conditions, except that Dulbecco’s minimum essential medium was used in place of minimum essential medium.

**Metabolic Pulse-Chase Labeling**—Metabolic labeling with \(^{35}\)S-cysteine was performed as described before (16). For pulse-chase experiments, cells were generally pulse-labeled for 30 min with 10 mM \(^{35}\)S-cysteine and analyzed under the same conditions, except that Dulbecco’s minimum essential medium was used in place of minimum essential medium.

**Antibodies, Immunoprecipitation, and SDS-PAGE**—Rabbit polyclonal anti-LRP (generated against purified human LRP) and anti-RAP (generated against recombinant human RAP) antibodies have been described before (13). Monoclonal anti-HA antibody was obtained from Babco (12CA5). Immunoprecipitations were carried out essentially as described before (16), except that the washing buffer for monoclonal anti-HA antibody contained 0.1% SDS instead of 1% SDS. Preliminary experiments were performed to ensure that the primary antibody used in each immunoprecipitation was in excess. Protein A-agarose beads were used to precipitate protein-IgG complexes. The immunoprecipitated material was analyzed by SDS-PAGE under either nonreducing (Fig. 1A) or reducing (Fig. 1B) conditions. As seen in Fig. 1A, the presence of ionomycin (Fig. 1A, lanes 6–8), thapsigargin (Fig. 1A, lanes 10–12), or both (lanes 14–16), most of the LRP migrated as intermolecular disulfide bond-linked aggregates, which disappeared upon reduction (Fig. 1B). In each case, the increase in LRP aggregates resulted in a corresponding decrease in monomeric LRP. These data suggest that Ca\(^{2+}\) is required for LRP folding and, in particular, the formation of correct intramolecular disulfide bonds.

To further examine whether Ca\(^{2+}\) depletion retards the intramolecular disulfide bond formation in addition to promoting the formation of intermolecular disulfide bonds, we compared LRP migration patterns in the presence or absence of NEM, which protects free -SH groups during cell lysis. HepG2 cells were pulse-labeled with \(^{35}\)S-cysteine for 30 min and chased for 0, 30, or 60 min with complete medium or complete medium containing 5 \(\mu\)M ionomycin. After each chase period, cells were lysed in either the presence or absence of NEM, immunoprecipitated with anti-LRP antibody, and analyzed by SDS-PAGE under either nonreducing (Fig. 1A) or reducing (Fig. 1B) conditions. In the absence of NEM during cell lysis, exposed free sulfhydryl groups within cysteine residues can link randomly to other free sulfhydryl groups, including those in other LRP molecules forming intermolecular disulfide bonds. Thus, by comparing the extent of aggregated LRP in the presence or absence of NEM, the free sulfhydryl groups at the time of cell lysis can be compared under various conditions. As seen in Fig. 2A, and similar to the results shown in Fig. 1A, LRP aggregation due to Ca\(^{2+}\) depletion was seen when ionomycin was present during the chase (lanes 5 and 6). In the absence of NEM during cell lysis, exposed free sulfhydryl groups within cysteine residues can link randomly to other free sulfhydryl groups, including those in other LRP molecules forming intermolecular disulfide bonds. Thus, by comparing the extent of aggregated LRP in the presence or absence of NEM, the free sulfhydryl groups at the time of cell lysis can be compared under various conditions. As seen in Fig. 2A, and similar to the results shown in Fig. 1A, LRP aggregation due to Ca\(^{2+}\) depletion was seen when ionomycin was present during the chase (lanes 5 and 6). In the absence of NEM, most aggregated LRP was seen (lanes 10, 11, 12). Most notably, after 30 or 60 min of ionomycin treatment, almost all of the monomeric LRP became aggregated into intermolecular disulfide bond-linked aggregates when cells were lysed in the absence of NEM, whereas without ionomycin treatment, LRP remained predominantly monomeric with little aggregation (lanes 8 and 9). These results suggest that depletion of Ca\(^{2+}\) during LRP folding results in the exposure of free sulfhydryl groups, which probably then form nonnative disulfide bonds with thiols in neighboring molecules.

**RESULTS**

**Depletion of Ca\(^{2+}\) Results in LRP Misfolding**—To examine whether Ca\(^{2+}\) is required for LRP folding, we analyzed the states of LRP folding upon Ca\(^{2+}\) depletion. We used two approaches to deplete ER Ca\(^{2+}\): ionomycin (A23187, a Ca\(^{2+}\) ionophore) and thapsigargin (an ATPase inhibitor). Human hepatoma HepG2 cells, which express abundant LRP (16), were metabolically pulse-labeled with \(^{35}\)S-cysteine for 30 min and chased for 0, 30, 60, or 120 min with complete medium or complete medium containing either 5 \(\mu\)M ionomycin, 100 \(\mu\)M thapsigargin, or both at the given concentrations. After each chase period, cell lysates were immunoprecipitated with anti-LRP antibody and analyzed by SDS-PAGE under either nonreducing (Fig. 1A) or reducing (Fig. 1B) conditions. As seen in Fig. 1A, at the beginning of the chase, small amounts of LRP existed in aggregated forms (lanes 1, 5, 9, and 13). These LRP aggregates are likely intermolecular disulfide bond-linked LRP molecules because they disappeared when analyzed under reducing conditions (Fig. 1B). When cells were chased in the presence of ionomycin (Fig. 1A, lanes 6–8), thapsigargin (Fig. 1A, lanes 10–12), or both (lanes 14–16), most of the LRP migrated as intermolecular disulfide bond-linked aggregates, which disappeared upon reduction (Fig. 1B). In each case, the increase in LRP aggregates resulted in a corresponding decrease in monomeric LRP. These data suggest that Ca\(^{2+}\) is required for LRP folding and, in particular, the formation of correct intramolecular disulfide bonds.

**Misfolded LRP Can Be Refolded upon Ca\(^{2+}\) Restoration**—To examine whether misfolded LRP molecules can be rescued upon Ca\(^{2+}\) restoration, we examined the states of LRP folding following ionomycin removal. HepG2 cells were metabolically labeled with \(^{35}\)S-cysteine for 30 min, chased with complete medium containing 5 \(\mu\)M ionomycin for 60 min, and continuously chased with complete medium without ionomycin for an additional 0, 60, and 120 min. After each chase, cell lysates were either immunoprecipitated with anti-LRP antibody or incubated with activated \(\alpha\)-macroglobulin-Sepharose, and analyzed under either nonreducing (Fig. 3A) or reducing (Fig. 3B) conditions. As seen in Fig. 3A, LRP was refolded from aggreg-
gated forms into monomeric form upon Ca\(^{2+}\) restoration. The refolded LRP appears to be functional because it is capable of binding its ligand α2-macroglobulin.

Ca\(^{2+}\) Depletion Results in Both Intermolecular Disulfide Bonds and Mislinked Intramolecular Disulfide Bonds—The very large size of LRP (≈600 kDa) precludes the separation by SDS-PAGE.

**Fig. 1. Depletion of Ca\(^{2+}\) results in LRP misfolding.** HepG2 cells were metabolically pulse-labeled with \[^{35}S\]cysteine for 30 min and chased for 0, 30, 60, or 120 min with complete medium or complete medium containing either 5 μM ionomycin, 100 nM thapsigargin, or both at the indicated concentrations. After each chase, cell lysates were immunoprecipitated with anti-LRP antibody and analyzed via 5% SDS gels under either nonreducing (A) or reducing (B) conditions. In this figure and subsequent figures, the positions of monomeric LRP and aggregated LRP in the nonreducing gels are marked. The 200-kDa molecular size markers are also indicated. The band below the full-length LRP (B, and in Figs. 2, 3, and 8) may represent either incompletely translated or partially degraded LRP species.

**Fig. 2. Ca\(^{2+}\) depletion results in exposed sulfhydryl groups.** HepG2 cells were pulse-labeled with \[^{35}S\]cysteine for 30 min and chased for 0, 30, or 60 min with complete medium or complete medium containing 5 μM ionomycin. After each chase, cells were lysed either in the presence or absence of NEM (10 mM), immunoprecipitated with anti-LRP antibody, and analyzed via 5% SDS gels under either nonreducing (A) or reducing (B) conditions. Note that the majority of the monomeric LRP after 30 or 60 min of ionomycin treatment was aggregated into intermolecular disulfide bond-linked aggregates when lysed in the absence of NEM, whereas without ionomycin treatment, monomeric LRP showed little aggregation.
of misfolded monomeric LRP forms with nonnative intramolecular disulfide bonds into discrete species. Therefore, we generated an LRP minireceptor that could mimic the folding process of LRP. This LRP minireceptor encodes residues 2462–4525 (1) and represents from the beginning of the fourth cluster of ligand binding repeats (2) through the carboxyl terminus of the receptor (designated mLRP4, with “m” denoting that this minireceptor contains repeats (2) through the carboxyl terminus of the receptor (designated mLRP4, with “m” denoting that this minireceptor contains the membrane-spanning sequence; see Fig. 4). To facilitate immunoprecipitation following metabolic labeling, an HA epitope was included near the NH2-terminal end of mLRP4.

Our previous studies using soluble LRP minireceptor 4 (sLRP4, see Ref. 8) have shown that proper folding and secretion of sLRP4 requires the co-expression of RAP. Thus, we transiently transfected cDNA for mLRP4 into HepG2 cells with co-transfection of either empty vector (pcDNA3) or vector containing RAP cDNA (pcDNA-RAP; see Ref. 13). The transfected cells were metabolically pulse-labeled with [35S]cysteine for 30 min and chased for 0, 30, 60 or 120 min. Cell lysates were then immunoprecipitated with anti-HA antibody and analyzed by SDS-PAGE under either nonreducing (Fig. 6A) or reducing (Fig. 6B) conditions. As seen in Fig. 5, mLRP4 is folded and transported from the ER to the Golgi faster following co-transfection with either reducing or nonreducing conditions. For example, after 60 min of chase, 66% of mLRP4 had been converted to post-ER forms (including the Golgi form and the processed forms) when co-transfected with RAP, compared with only 29% in the absence of RAP co-transfection (Fig. 5A). These results are consistent with our previous conclusion that RAP facilitates LRP folding and trafficking through the early secretory pathway. It is interesting to note that in the absence of RAP co-transfection, significant amounts of mLRP4 migrated at the top of the stacking gel under nonreducing conditions (Fig. 5A), suggesting the formation of excessive intermolecular disulfide bonds and a concomitant delay for their folding and trafficking.

We next examined the effects of ionomycin on the folding and processing of mLRP4. As in the experiment shown in Fig. 5, HepG2 cells were transiently transfected with cDNA for mLRP4, with co-transfection of either pcDNA3 (-RAP) or pcDNA-RAP (+RAP). The transfected cells were metabolically pulse-labeled with [35S]cysteine for 30 min and chased in the presence of ionomycin for 0, 30, 60, or 120 min. Cell lysates were then immunoprecipitated with anti-HA antibody and analyzed by SDS-PAGE under either nonreducing (Fig. 6A) or reducing (Fig. 6B) conditions. As shown in Fig. 6, the depletion of Ca2+ caused misfolding of mLRP4 in both the absence and the presence of RAP co-expression, consistent with the notion that RAP and Ca2+ are independently required for LRP folding (see below). More noticeably, misfolding of mLRP4 can be seen not only as intermolecular-linked aggregates but also as a broad band indicative of heterogeneously folded minireceptor monomers (Fig. 6A, lanes 3, 4, 7, and 8), which likely arise because of mislinked intramolecular disulfide bonds.

**Ca2+ and RAP Are Independently Required for LRP Folding**—The interaction between RAP and LRP is known to be Ca2+-dependent (9, 13). Ca2+ depletion with ionomycin decreases the Ca2+ concentration within the ER but does not completely eliminate Ca2+ (18). To analyze whether RAP still interacts with LRP and its minireceptor under the conditions of ionomycin treatment, we performed chemical cross-linking experiments using a membrane-permeable cross-linker, DSP (13). HepG2 cells were transiently co-transfected with cDNAs for mLRP4 and RAP. The transfected cells were then metabolically pulse-labeled with [35S]cysteine for 30 min, either followed or not followed by a 30-min chase of complete medium containing ionomycin. Cells were then incubated in the absence or presence of DSP cross-linker, immunoprecipitated with anti-RAP antibody (13), and analyzed by SDS-PAGE under either nonreducing (Fig. 7A) or reducing (Fig. 7B) conditions. As seen in Fig. 7, following chemical cross-linking with DSP, LRP and mLRP4 were co-immunoprecipitated with anti-RAP antibody both without (lane 2) and with (lane 4) ionomycin treatment. Thus, under the conditions of Ca2+ depletion with ionomycin, RAP remains associated with LRP. These results indicate that even when RAP remains associated with LRP, depletion of Ca2+ results in LRP misfolding.

To further analyze whether Ca2+ and RAP are independ-
ently required for LRP folding, we compared the effects of Ca^{2+} depletion on LRP folding in RAP-expressing MEF-1 cells and RAP-deficient MEF-7 cells (11). Previous studies by Willnow et al. (11) have shown that folding of LRP and its trafficking in RAP-deficient MEF-7 cells is not significantly impaired. In the present study, MEF-1 and MEF-7 cells were metabolically pulse-labeled with [35S]cysteine for 30 min and chased for 0, 30, or 60 min in the absence or presence of ionomycin. Cell lysates were then immunoprecipitated with anti-LRP antibody and analyzed by SDS-PAGE under either nonreducing (Fig. 8A) or reducing (Fig. 8B) conditions. As seen in Fig. 8A, in the presence of ionomycin during chase, aggregation of LRP was seen in both MEF-1 (lanes 5 and 6) and MEF-7 (lanes 11 and 12) cells, suggesting that irrespective of whether RAP is present, Ca^{2+} depletion results in significant LRP misfolding.

**DISCUSSION**

Ligand binding to members of the LDL receptor gene family requires Ca^{2+}. The underlying mechanism has recently been defined; Ca^{2+} binds to conserved acidic residues at each of the cysteine-rich repeats and stabilizes the receptors in their native conformation (7). In the present report, we analyzed the potential role of Ca^{2+} in LRP folding and examined its relationship to the function of RAP as a folding chaperone. We found that Ca^{2+} and RAP are independently required for LRP folding. When Ca^{2+} and RAP are limited during receptor folding, LRP becomes misfolded with the formation of intermolecular disulfide bonds. Intermolecular (also termed interchain) disulfide bonds have been observed in other cases of glycoprotein misfolding, e.g. influenza hemagglutinin (19, 20). In addition to intermolecular disulfide bond-linked molecules, heterogeneously migrating monomeric LRP minireceptors were also observed upon Ca^{2+} depletion. This suggests that binding of Ca^{2+} to LRP is important not only in preventing the formation of intermolecular disulfide bonds, but also in facilitating the formation of correctly linked intramolecular disulfide bonds. Because ER Ca^{2+} is also required for the folding process of at least one other endocytic receptor, the asialoglycoprotein receptor (21), Ca^{2+} may serve as an essential folding chaperone for some of the endocytic receptors within the ER.

The ER is an oxidative environment in which disulfide bond-containing proteins may fold correctly (22). The oxidative redox state of the ER maintained in part by the ratio of oxidized: reduced glutathione (GSH:GSSH) at 1:1–3:1 is favorable toward disulfide bond formation and rearrangement (23). Such an oxidative environment within the ER would also facilitate the spontaneous formation of mislinked disulfide bonds, especially during folding of cysteine-rich proteins. For example, in the pulse-chase experiments performed in the present study (see Figs. 1 and 2), we consistently observed intermolecular disulfide bond-linked LRP aggregates immediately following pulse labeling. Binding of Ca^{2+} and RAP may reduce the amounts of mislinked disulfide bonds, perhaps by protecting certain cysteine residues against oxidation at early stages of LRP folding. In addition, because mislinked disulfide bonds are present during the normal folding process, it is possible that Ca^{2+} and RAP facilitate the disulfide bond reshuffling process catalyzed by protein disulfide isomerase (PDI, 24). Thus, the process of folding for proteins with extensive disulfide bonds is likely dynamic and may involve constant trial and error, especially in the formation of correct disulfide bonds. Rearrange-
ment of disulfide bonds during the normal folding process is also suggested by our observation that misfolded LRP containing extensive intermolecular disulfide bonds can be refolded into functional monomeric LRP (Fig. 3). These results suggest that the ER possesses all the factors and machinery necessary to convert LRP molecules from misfolded states to correctly folded states. Such functions not only support the existence of the trial and error theory of protein folding but also provide a means for cells to recover protein functions following various stress conditions.

It is interesting to note that the structure of the fifth complement-type ligand binding repeat of the LDL receptor is organized around a calcium ion (7). Six residues, including four acidic residues, contribute to the octahedral coordination geometry around the Ca$^{2+}$ ion. It is possible that the formation of the Ca$^{2+}$ coordination within each repeat juxtaposes at least two pairs of cysteine residues to form native disulfide bonds. Thus, Ca$^{2+}$ ions may play critical roles in the initial structure organization during receptor folding, which precedes the formation of disulfide bonds. This hypothesis is supported by in vitro folding studies on the fifth repeat of the LDL receptor (25).

In an experiment not shown here, we found that depletion of Ca$^{2+}$ following the completion of LRP folding (i.e. 30-min pulse labeling and 60- or 120-min chase) did not induce further misfolding of LRP, suggesting that Ca$^{2+}$ is not required for maintaining correctly disulfide-bonded LRP structure. However, because Ca$^{2+}$ is required for ligand binding to LRP (2), this metal ion may play a certain role in maintaining the receptor in a conformation that is competent for ligand binding (7).

The present study demonstrates that Ca$^{2+}$ and RAP are independently required for LRP folding. However, whether these factors function in similar ways remains to be determined. It appears from the current study that ER Ca$^{2+}$ is

**Fig. 5. RAP facilitates folding of mLRP4.** HepG2 cells were transiently transfected with cDNAs for mLRP4, with the co-transfection of either vector pcDNA3 (-RAP), or pcDNA-RAP (+RAP). The transfected cells were metabolically pulse-labeled with $[^{35}\text{S}]$cysteine for 30 min and chased for 0, 30, 60, 90 or 120 min. Cell lysates were then immunoprecipitated with anti-HA antibody and analyzed via 6% SDS gels under either non-reducing (A) or reducing (B) conditions. The ER and Golgi forms of unprocessed mLRP4 and processed forms (see Fig. 4) are indicated. Molecular size markers are given at the left in kDa. The percentage of post ER-forms (including Golgi and processed forms) in the absence or presence of RAP co-transfection is plotted against chase time and is shown beneath B.

**Fig. 6. Ca$^{2+}$ depletion results in mislinked intermolecular and mislinked intramolecular disulfide bonds for mLRP4.** HepG2 cells were transfected as described in Fig. 5. The transfected cells were then metabolically labeled with $[^{35}\text{S}]$cysteine for 30 min and chased in the presence of 5 μM ionomycin for 0, 30, 60, or 120 min. Cell lysates were then immunoprecipitated with anti-HA antibody and analyzed via 6% SDS gels under either non-reducing (A) or reducing (B) conditions. The top of the stacking and separating gels are marked with closed and open arrows, respectively. Areas showing the aggregated mLRP4 and misfolded monomeric mLRP4 are indicated.
essential for LRP folding. Depletion of Ca\(^{2+}\) results in the eventual total misfolding of LRP. In contrast, participation of RAP in LRP folding appears to be important but not essential. For example, deletion of the RAP gene by homologous recombination resulted in a 75% reduction but not total elimination of functional LRP molecules (10, 11). In addition, when LRP minireceptors are expressed in the absence of RAP co-expression, some of them appear able to fold and be processed correctly (see Fig. 5 and Ref. 26). The presence of less efficacious redundant factors or chaperones remains to be defined. Thus, RAP likely functions as a facilitator during LRP folding. The fact that RAP functions in both LRP folding and subsequent trafficking along the early secretory pathway (11, 13) emphasizes the specialized chaperone function of RAP during the biogenesis of LRP, and likely other members of the LDL receptor gene family (27).

**Fig. 7. RAP remains associated with LRP after Ca\(^{2+}\) depletion.** HepG2 cells were transiently co-transfected with cDNAs for mLRP4 and RAP. The transfected cells were then metabolically pulse-labeled with \(^{35}\)S-cysteine for 30 min and then either not followed by (lanes 1 and 2) or followed by (lanes 3 and 4) a 30-min chase with complete medium containing 5 \(\mu\)M ionomycin. Cells were then incubated in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of cross-linker DSP, followed by immunoprecipitation using anti-RAP antibody and analysis via 6% SDS gels under either nonreducing (A) or reducing (B) conditions. The top of the stacking and separating gels are marked with closed and open arrows, respectively. Areas showing the cross-linked LRP-RAP and mLRP4-RAP complexes are indicated. The positions of LRP and mLRP4 in B are also marked.

**Fig. 8. LRP is also misfolded in RAP-deficient cells upon Ca\(^{2+}\) depletion.** MEF-1 or MEF-7 (RAP-deficient) cells were metabolically pulse-labeled with \(^{35}\)S-cysteine for 30 min and chased for 0, 30, or 60 min, in the absence or presence of 5 \(\mu\)M ionomycin. Cell lysates were then immunoprecipitated with anti-LRP antibody, and analyzed via 5% SDS gels under either nonreducing (A) or reducing (B) conditions.

Ca\(^{2+}\) and RAP Are Independently Required for LRP Folding

22380
bond content, should provide us with additional insight as to how such a protein can fold correctly and efficiently within the ER.

Acknowledgments—We thank Mark Wardell for helpful discussion and reading of the manuscript and Joachim Herz for providing LRP cDNA. We also thank Peter van Kirkhof and Ger Strous for their contribution to the characterization of the LRP minireceptors.

REFERENCES
1. Herz, J., Hamann, U., Rogne, S., Myklebost, O., Gausepohl, H., and Stanley, K. K. (1988) EMBO J. 7, 4119–4127
2. Krieger, M., and Herz, J. (1994) Annu. Rev. Biochem. 63, 601–637
3. Goldstein, J. L., Brown, M. S., Anderson, R. G. W., Russell, D. W., and Schneider, W. J. (1985) Annu. Rev. Cell Biol. 1, 1–39
4. Herz, J., Kowal, R. C., Goldstein, J. L., and Brown, M. S. (1990) EMBO J. 9, 1769–1776
5. Willnow, T. E., Moehring, J. M., Inocencio, N. M., Moehring, T. J., and Herz, J. (1996) Biochem. J. 313, 71–76
6. Bieri, S., Djordjevic, J. T., Daly, N. L., Smith, R., and Kroon, P. A. (1995) Biochemistry 34, 13059–13065
7. Fass, D., Blacklow, S., Kim, P. S., and Berger, J. M. (1997) Nature 388, 691–693
8. Bu, G., and Renke, S. (1996) J. Biol. Chem. 271, 22218–22224
9. Herz, J., Goldstein, J. L., Strickland, D. K., Ho, Y. K., and Brown, M. S. (1991) J. Biol. Chem. 266, 21232–21238
10. Willnow, T. E., Armstrong, S. A., Hammer, R. E., and Herz, J. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 4537–4541
11. Willnow, T. E., Rohlmann, A., Horton, J., Otani, H., Braun, J. R., Hammer, R. E., and Herz, J. (1996) EMBO J. 15, 2632–2639
12. Warschawsky, I., Bu, G., and Schwartz, A. L. (1995) Biochemistry 34, 3404–3415
13. Bu, G., Geuze, H. J., Strous, G. J., and Schwartz, A. L. (1995) EMBO J. 14, 2269–2280
14. Obermoller, L. M., Warschawsky, I., Wardell, M. R., and Bu, G. (1997) J. Biol. Chem. 272, 10761–10768
15. van Driel, I. R., Goldstein, J. L., Sudhof, T. C., and Brown, M. S. (1987) J. Biol. Chem. 262, 17443–17449
16. Bu, G., Makysmovitch, E. A., and Schwartz, A. L. (1993) J. Biol. Chem. 268, 15002–15009
17. Chen, C., and Okayama, H. (1987) Mol. Cell. Biol. 7, 2745–2752
18. Fasolato, C., Zottini, M., Clementi, E., Zaccetti, D., Meldolesi, J., and Pozzan, T. (1991) J. Biol. Chem. 266, 20159–20167
19. Braakman, I., Helenius, J., and Helenius, A. (1992) EMBO J. 11, 1717–1722
20. Braakman, I., Helenius, J., and Helenius, A. (1992) Nature 356, 260–262
21. Lodish, H. F., Kong, N., and Wikstrom, L. (1992) J. Biol. Chem. 267, 12753–12760
22. Gething, M.-J., and Sambrook, J. (1992) Nature 355, 33–45
23. Hwang, C., Sinskey, A. J., and Lodish, H. F. (1992) Science 257, 1496–1502
24. Gilbert, H. F. (1997) J. Biol. Chem. 272, 29399–29402
25. Blacklow, S. C., and Kim, P. S. (1996) Nat. Struct. Biol. 3, 756–762
26. Willnow, T. E., Orth, K., and Herz, J. (1994) J. Biol. Chem. 269, 15827–15832
27. Bu, G., and Schwartz, A. L. (1998) Trends Cell Biol. 8, 272–276