Unfolding of the RAP-D3 Helical Bundle Facilitates Dissociation of RAP−Receptor Complexes†

Kristine Estrada, Carl Fisher, and Stephen C. Blacklow*

Department of Pathology, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts 02115

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ABSTRACT: The receptor-associated protein (RAP) functions as an escort protein for receptors of the low-density lipoprotein receptor (LDLR) family by preventing premature intracellular binding of ligands and assisting with delivery of mature receptors to the cell surface. The modulation of affinity by pH is believed to play an important role in the escort function of RAP, because RAP binds tightly to proteins of the LDLR family at near-neutral pH early in the secretory pathway where its high affinity precludes premature binding of ligands but then dissociates from bound receptors at the lower pH of the Golgi compartment. The third domain of RAP (RAP-D3), which forms a three-helix bundle, is sufficient to reconstitute the escort activity. Here, we test the hypothesis that low-pH induced unfolding of the RAP-D3 helical bundle facilitates dissociation of RAP−receptor complexes. First, variants of RAP-D3 resistant to low pH-induced unfolding were constructed by replacing interior histidine residues with phenylalanines. In contrast to native RAP-D3, which exhibits an unfolding pK_a of 6.3 and a T_m of 42 °C, the most hyperstable variant of RAP-D3, in which four histidine residues are replaced with phenylalanine, has an unfolding pK_a of 4.8, and a T_m of 58 °C. The phenylalanine substitutions in RAP-D3 confer increased stability to pH-induced dissociation of complexes formed between RAP-D3 and a two-repeat fragment of the LDLR (LA3-4). When introduced into full-length RAP, the four mutations that confer hyperstability on RAP-D3 interfere with transport of endogenous LRP-1 to the cell surface in a dominant negative fashion under conditions where expression of normal RAP has no effect on LRP-1 transport. Our studies support a model in which low pH-dependent unfolding of RAP-D3 facilitates dissociation of RAP from the LA repeats of LDLR family proteins in the mildly acidic pH of the Golgi.

The 39 kD receptor-associated protein (RAP) is an ER-resident protein that was originally identified because it co-purified with the low-density lipoprotein related protein LRP-1 (*1*−*3*). Subsequent functional studies then established that RAP plays a role in ensuring efficient delivery of LRP-1 and other transmembrane proteins of the low-density lipoprotein receptor (LDLR) family to the cell surface (*4*, *5*).

RAP itself consists of three domains of approximately 100 residues each (*6*, *7*). As deduced from NMR structures solved for each domain in isolation (*6*−*10*) and a high-resolution (1.26 Å) X-ray structure of a complex between the third domain of RAP (RAP-D3[1]) and a two-repeat fragment of the LDLR (*11*), each of these domains adopts a three-helix bundle fold, in which a short, N-terminal helix buttresses an elongated helical hairpin.

The LDL receptor type-A (LA) repeats constitute the RAP-binding sites on LRP-1 and related proteins of the LDL receptor family. Biochemical studies have shown that each of the three domains of RAP can independently bind to two-repeat fragments of LRP-1, with D3 binding with higher affinity than either domains one (D1) or two (D2) in isolation (*12*, *13*). In the complex between RAP-D3 and the LA3-4 repeat pair from the LDLR, RAP-D3 contacts the receptor fragment at two docking sites, one per module (*11*). The interface at each docking site is dominated by electrostatic interactions between conserved acidic residues on the LA module and a lysine residue projecting from helix 2 of the RAP helical bundle, with the mode of recognition nearly identical at the two sites. The LA repeats are found in clusters on the receptors, and like the three domains of RAP, are typically connected by flexible linkers (*14*−*17*), suggesting that the full-length proteins bind tightly to each other in part as a result of avidity effects (*18*).

RAP binds tightly to proteins of the LDLR family at near-neutral pH early in the secretory pathway but then dissociates from bound receptors at the lower pH of the Golgi compartment (*4*, *9*). The modulation of affinity by pH is believed to play an important role in the function of RAP as an escort protein for members of the LDLR family by preventing premature intracellular binding of ligands to the receptors during the routing stage of receptor transport, thus enabling delivery of mature receptors to the cell surface (see ref *19* for a recent review).

The strong avidity of RAP for LDLR family proteins at neutral pH thus raises the interesting mechanistic question

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* To whom correspondence should be addressed. Telephone: 617-525-4415. Fax: 617-525-4414. E-mail: sblacklow@rics.bwh.harvard.edu.

[1] Abbreviations: D3, domain three; ER, endoplasmic reticulum; LA, LDL receptor type A repeat; LDL, low-density lipoprotein; LDLR, LDL receptor; LRP-1, LDL receptor-related protein 1; NMR, nuclear magnetic resonance; RAP, receptor-associated protein; RAP-D3, domain three of RAP; RAP-D3-quad, RAP-D3 with H257, H259, H268, and H290 all replaced by phenylalanines; WT, wild-type.

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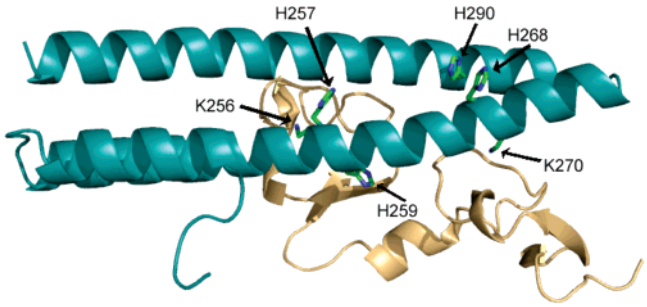

FIGURE 1: Overall structure of LA3-4−RAP-D3 complex (PDB ID code 2FCW) indicating histidine residues of RAP-D3 buried in the interhelical interface. The LA3-4 (tan) and RAP-D3 (blue) polypeptide chains are illustrated as ribbons. Key interfacial residues, histidines and lysines, are highlighted as sticks.

of how binding affinity is reduced to allow dissociation of RAP−receptor complexes in response to the reduced pH of the Golgi compartment. The X-ray crystal structure of the RAP-D3/LA3-4 complex revealed that several histidine residues are adjacent to or buried in the intrahelical interface of the RAP-D3 helical bundle (Figure 1), suggesting that titration of these histidine residues might trigger unfolding of the helical bundle at the reduced pH of the Golgi, driving dissociation of RAP−lipoprotein receptor complexes. Indeed, RAP-D3 does appear to undergo an unfolding transition at a pH of ~6.3, and forms of RAP-D3 in which all histidine residues are replaced by alanine appear to remain associated with soluble receptor fragments in the Golgi, pointing to an important role for the histidine residues as a pH sensor for dissociation of complexes (*9*).

Here, we directly test the hypothesis that low-pH induced unfolding of the RAP-D3 helical bundle drives dissociation by constructing variants of RAP-D3 resistant to low pH-induced unfolding, examining the effects of these mutations on complex stability as a function of pH in biochemical assays, and monitoring the effect of these mutations on the influence of RAP on LRP-1 export in cell-based assays. Our studies support a model in which dissociation of RAP-D3 from the LA repeats of LDLR family proteins in response to mildly acidic pH depends on low pH-dependent unfolding of RAP-D3.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification. The LA3-4 module pair was expressed as described previously (*11*). RAP-D3 (residues 216−323) was cloned into the donor vector, pDONR201 (Gateway Cloning System, Life Technologies), and then into the expression vector, pDEST15, for expression as a GST-fusion protein with an N-terminal tobacco etch virus (TEV) protease cleavage site (ENLYFQG). Mutations in RAP-D3 were introduced via site-directed mutagenesis with a Quickchange mutagenesis kit (Stratagene) and were verified by DNA sequencing.

Expression of RAP-D3 variants was induced by the addition of 0.5 mM IPTG during log phase growth (A_{595} = 0.6−0.8) in BL21(DE3) cells. Bacteria were harvested by centrifugation and lysed by probe sonication in lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 20% (w/v) sucrose, and 1 mM EDTA). Cell debris was removed by centrifugation, and the soluble GST-{TEV site}-RAP-D3 variant in the supernatant was captured on Glutathione Sepharose 4B beads (GE Healthcare Biosciences Corp.). After two washes with TBS (Tris-buffered saline; 150 mM NaCl, 20 mM Tris, pH 8.0), the fusion protein was cleaved with TEV protease (6XHis-tagged) in TBS supplemented with 0.5 mM EDTA and 1 mM DTT for 1 h at 20 °C and then overnight at 4 °C to release the desired RAP-D3 variant from the beads. The TEV protease was then removed from the solution by capture onto Ni-NTA beads (Qiagen) followed by filtration through fritted glass. Monomeric RAP-D3 was subsequently isolated via size-exclusion chromatography on a Superdex-S75 column (Pharmacia).

Circular Dichroism (CD). CD scans were acquired on an AVIV 62DS spectropolarimeter, equipped with a Peltier effect temperature controller. All protein samples (~1.5 μM) were dialyzed against 10 mM sodium phosphate pH 7.3, 100 mM NaCl. Scans were conducted at 4 °C and were acquired from 196 to 260 nm, with a bandwidth of 1.5 nm, a scan step of 1 nm, an averaging time of 3 s, and three repeats per scan in a 1 cm path length cuvette. For thermal denaturation experiments, the temperature dependence of the molar ellipticity was followed at 222 nm, where the temperature was raised 1 °C/min, using a bandwidth of 1.5 nm, an averaging time of 30 s, and an equilibration time of 2 min. For pH stability studies, the pH was altered by diluting a stock into a buffer of defined pH and then adjusting the pH to the desired value with 0.1 M HCl or NaOH, if needed. CD collection conditions were as described for thermal denaturation studies.

Isothermal Titration Calorimetry (ITC). RAP-D3 titrations were performed in 20 mM HEPES, pH 7.3, 50−150 mM NaCl, 5 mM $CaCl_2$ at 25 °C using a VP-ITC calorimeter (MicroCal Inc., Northampton, MA) in a 1.3 mL reaction vessel. A RAP-D3 stock solution (132−178 μM) was added in 6−8 μL increments to LA3-4 (20−25 μM). Calorimetry data were fitted to a single-site binding model using the program Origin 5.0 (OriginLab Corp., Northampton, MA).

Gel Filtration Studies. Equimolar quantities (140 μM) of LA3-4 and wild-type or mutated RAP-D3 were incubated in 10 mM HEPES buffer containing 100 mM NaCl and 2.5 mM $CaCl_2$ at pH 7.3 for 1 h at 4 °C to form complexes. Aliquots of these complexes were then diluted to 20 μM final concentration into buffers with final pH values ranging from 7.3 to 5.0. Samples were incubated for an additional hour at 4 °C prior to injection (200 μL) onto a pre-equilibrated Superdex 75 HiLoad 16/60 gel filtration column (Pharmacia) on an FPLC system with a UV-2 detector at 25 °C at a flow rate of 0.8 mL/min.

Cell Surface LRP-1 Expression Analysis by FACS. HepG2 cells (kind gift of Chinweike Ukomadu) cultured in EMEM (Eagle's Minimum Essential Medium supplemented with 10% fetal bovine serum (Cambrex) and 100 U penicillin/mL (Invitrogen) and 100 μg streptomycin/mL (Invitrogen)) were split into 10 cm dishes at a confluency of 33%. When cells were 90% confluent, they were transfected using Lipofectamine 2000 reagent (Invitrogen) with 10 μg each of plasmids expressing GFP and empty vector, wild-type full-length RAP or full-length RAP harboring the H257,259,-268,290F mutations (RAP-quad H:F). All plasmids were of the pcDNA5/FRT/TO backbone (Invitrogen). For the RAP variants, the constructs were built to include the RAP signal

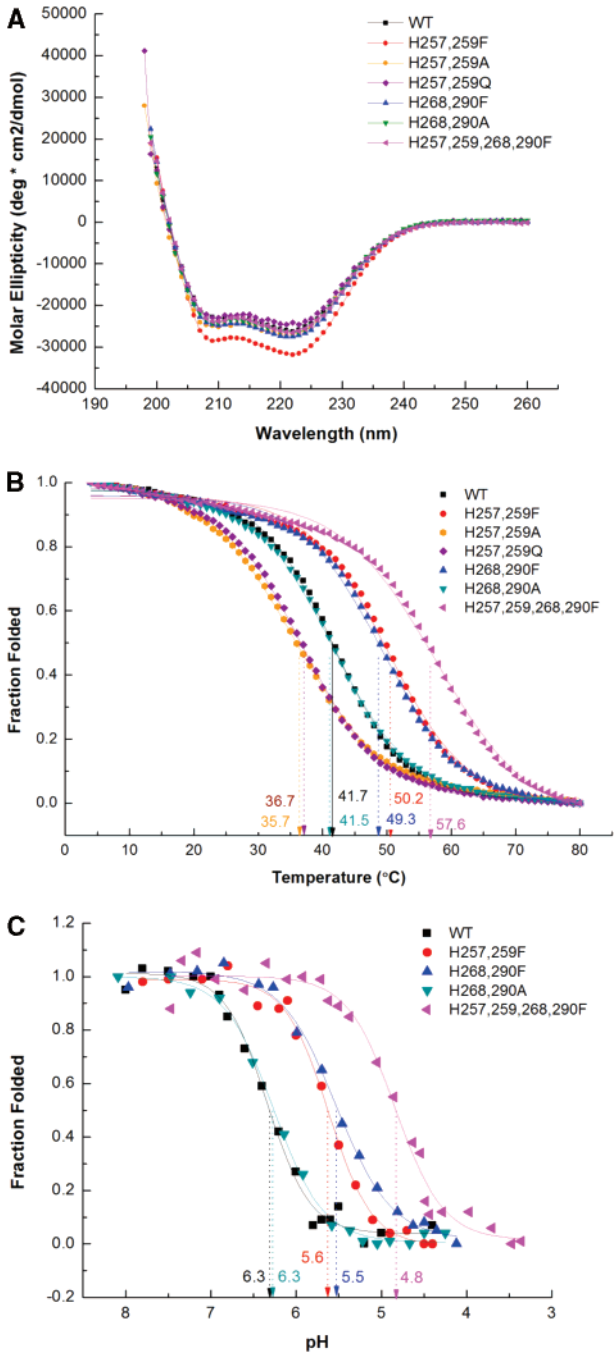

FIGURE 2: Circular dichroism (CD) studies of WT and mutated forms of RAP-D3. A. Far-UV CD spectra. Each of the RAP-D3 mutants tested is at least as helical as WT. B. Temperature-induced unfolding of RAP-D3 WT and mutants monitored by changes in molar ellipticity at 222 nm. The reported T_m values correspond to the inflection points of the unfolding curves. C. pH-induced unfolding of RAP-D3 WT and mutants monitored by changes in molar ellipticity at 222 nm, 30 °C. The unfolding pK_a value for both WT RAP-D3 and H268,290A mutant is 6.3, whereas for the two phenylalanine double mutants and RAP-D3-quad, the values are 5.5 and 4.8, respectively.

peptide plus four amino acids (YSRE) followed by a hemagglutinin (HA) tag upstream of the insert.

Twenty-four hours post-transfection, each 10 cm dish of transfected cells was split into 10 cm dishes at 50% confluency. This procedure yields three groups of cells, two dishes per group: a mock-treated pool and two pools of cells treated with wild-type full-length RAP or full-length RAP-Quad H:F, respectively.

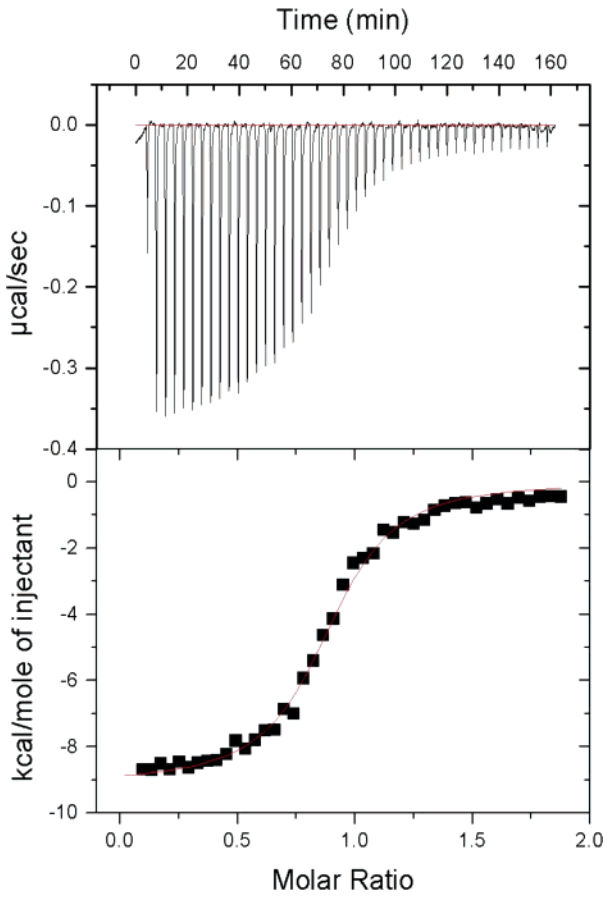

FIGURE 3: Measurement of the affinity of RAP-D3 WT for LA3-4 by isothermal titration calorimetry. Curve fitting to a single site-binding model (bottom panel) yields a value for K_D of 480 nM. The titration was performed by adding 6 μL aliquots of RAP-D3 WT (180 μM) into a solution of LA3-4 (20 μM) in 20 mM HEPES, pH 7.4, containing 50 mM NaCl and 5 mM $CaCl_2$. The titration was performed at 25 °C.

Forty-eight hours post-transfection, cells were harvested by detaching them from each dish with warm EMEM and were then transferred into an Eppendorf tube. Cells were washed with ice-cold PBS. Cells for analysis of cell-surface expression of endogenous LRP-1 were pelleted at 2000 rpm for 3 min using a table-top microfuge and then resuspended in 500 μL of primary anti-LRP-1 antibody solution and incubated on a rotator at 4 °C for 30 min. Cells were then recovered by centrifugation, washed once in PBS, and resuspended in 500 μL of APC-conjugated secondary antibody solution and incubated on a rotator in the dark at 4 °C for 30 min. Primary antibody solution was a monoclonal mouse anti-LRP-1 antibody (Abcam) diluted 1:250 in PBS with 0.1% sodium azide. Secondary antibody solution was an APC-conjugated goat anti-mouse antibody (Molecular Probes) diluted 1:100 in PBS with 0.1% sodium azide. Cells for analysis of total expression of LRP-1 were fixed and semipermeabilized (BD Cytofix/Cytoperm, BD Biosciences) and then blocked with 10% goat serum (Jackson ImmunoResearch) prior to incubation with primary antibody solution.

For flow cytometric analysis, pelleted cells were washed in ice-cold PBS, transferred into 5 mL culture tubes (Falcon 2093) and placed on ice. To restrict analysis to live, transfected cells, a double gating strategy was set up as follows: Scattering properties were used to gate on live cells,

Table 1: Thermodynamic Parameters Derived from the Microcalorimetric Titration of LA3-4 with RAP-D3 Protein Variants[a,b]

ligand	N	ΔH (kcal/mol·K)	K_D (= 1/K) (μM)	[NaCl] (mM)
RAPD3 (WT)	0.89 ± 0.01	−9.1 ± 0.1	0.48 ± 0.04	50
RAPD3 (WT)	0.85 ± 0.01	−6.8 ± 0.1	1.17 ± 0.08	150
RAPD3 H257F/H259F	0.89 ± 0.02	−8.6 ± 0.2	0.85 ± 0.16	50
RAPD3 H268F/H290F	0.98 ± 0.02	−9.3 ± 0.3	1.16 ± 0.27	50
RAP-D3-quad	0.89 ± 0.02	−5.4 ± 0.2	0.85 ± 0.16	100

[a] N = number of binding sites. [b] Estimate of run-to-run error ~25%.

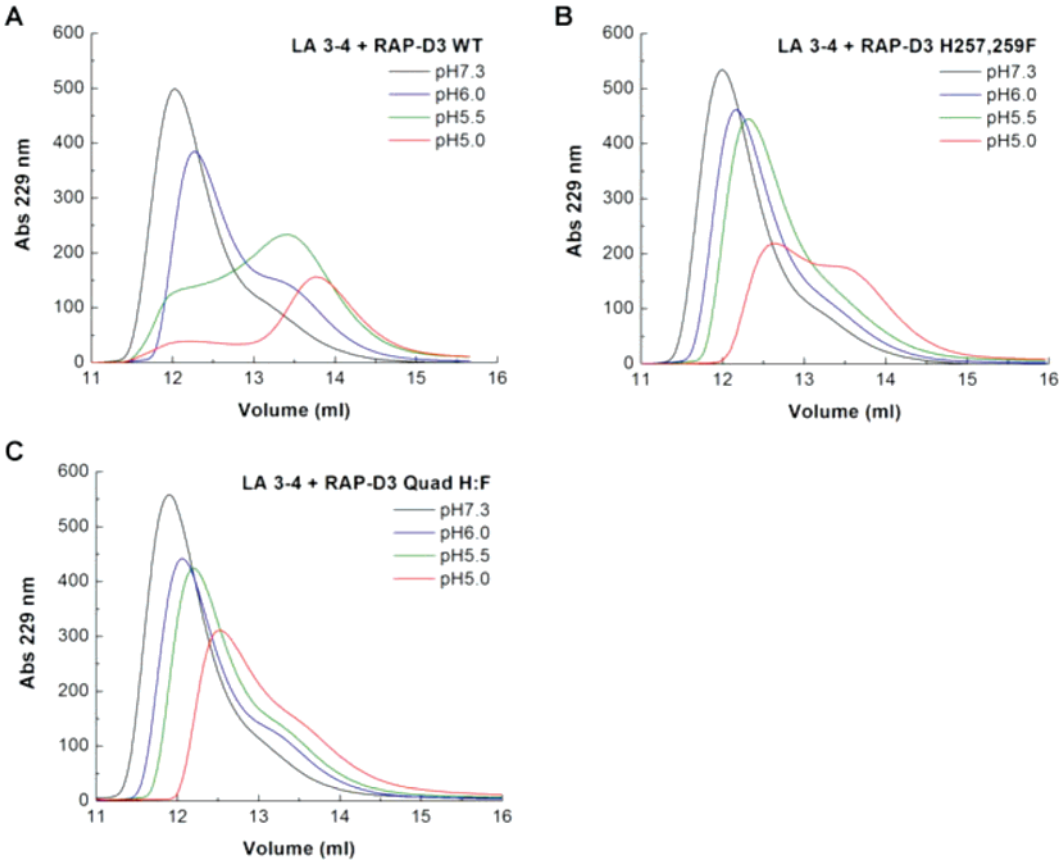

FIGURE 4: pH-dependent dissociation of RAP-D3 protein variants from LA3-4. Analytical size-exclusion chromatograms for (A) RAP-D3 WT, (B) RAP-D3 H257,259F, and (C) RAP-D3 H257,259,268,290F (quad H:F) in complex with LA3-4. Dissociation of wild-type RAP-D3 from LA3-4 is evident at pH 6.0, while that of the H257,259F mutant is evident at pH 5.0. Dissociation of RAP-D3 quad H:F is not apparent, even at pH 5.0.

followed by careful selection of GFP-positive cells to enrich for cells transfected with RAP. Using 5000 cells, a histogram plot was generated for each experimental condition. All data were acquired on a FACScalibur apparatus (BD Biosciences) and analyzed using the CellQuest Pro software (version 5.1.1).

RESULTS

Wild-type RAP-D3 is highly helical at neutral pH (Figure 2A), undergoes a cooperative thermal unfolding transition at 42 °C (Figure 2B), and exhibits a pH-induced unfolding transition with a pK_a of 6.3 (Figure 2C). These observations are all consistent with previously reported studies (*7*, *9*). To test the hypothesis that low pH-induced bundle unfolding drives dissociation of RAP−receptor complexes, we sought to construct variants of the RAP-D3 helical bundle that were thermostable, retained near-native affinity for LA repeat pairs, and exhibited increased resistance to unfolding induced by low pH.

To create such altered forms of RAP-D3, we focused attention on four histidine residues at or near the interface between helices two and three of the RAP-D3 helical bundle: H257, H259, H268, and H290 (Figure 1). We designed mutated forms of RAP-D3 in which either two or four of these histidines were replaced by alanines, glutamines, or phenylalanines, three different nontitratable amino acids, in order to create a form of RAP-D3 resistant to low-pH induced unfolding.

First, we replaced pairs of histidines with alanines or glutamines to create the H257,259A, H257,259Q, and H268,-290A mutants of RAP-D3. Each RAP-D3 exhibits essentially the same helical content as the wild-type protein at 4 °C (Figure 2A), as judged by circular dichroism spectroscopy (CD). However, the T_m values for the H257,259A and H257,-259Q mutants are 36.7 and 35.7 °C, respectively (Figure 2B), indicating that each protein would be at least 50% unfolded at physiologic temperature and not suitable for further functional studies. The other alanine variant, H268,290A

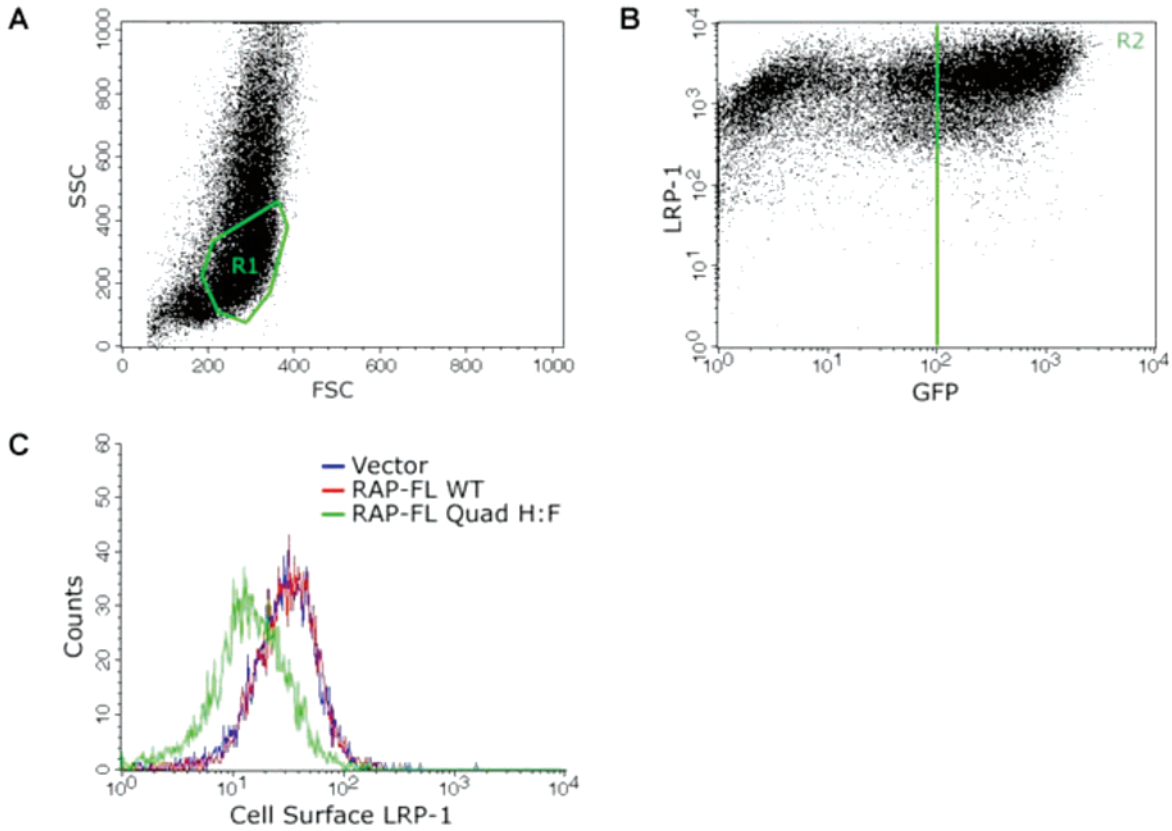

FIGURE 5: Response of endogenous LRP-1 to treatment with RAP quadruple H:F mutant in HepG2 cells. (A) Flow cytometry forward (*X*-axis) and side scatter (*Y*-axis) dot plot illustrating cell population selected for subsequent analysis (R1). (B) Gating for GFP-positive cells (R2). (C) Flow cytometry plot illustrating endogenous LRP-1 cell surface expression after gating on live, GFP-positive cells. HepG2 cells transfected with plasmids expressing GFP and wild-type, full-length RAP (red) have similar expression levels of LRP-1 as those transfected with plasmids expressing GFP and empty vector (blue), while cells transfected with plasmids expressing GFP and full-length RAP harboring H257,259,268,290F mutations (green; quad H:F) have reduced expression levels.

exhibits a melting temperature (T_m) and low-pH-induced unfolding transition indistinguishable from those of wild-type RAP-D3, making it also unsuitable as a probe to test the pH-induced unfolding model for dissociation of complexes.

Because the alanine and glutamine substitutions tested did not lead to increased thermal stability or resistance to low pH-induced unfolding, we next tried substituting these histidine residues with phenylalanine, which is nontitratable, hydrophobic, and nearly isosteric to histidine. In all, three proteins were designed, H257,259F and H268,290F, and a four-site mutant H257,259,268,290F (also referred to as RAP-D3-quad). Each of these phenylalanine variants is as helical as the wild-type protein, as judged by CD (Figure 2A). Unlike the alanine and glutamine mutants, however, all three phenylalanine variants are more resistant to thermal denaturation and to low pH-induced unfolding, with RAP-D3-quad being the most resistant to both acid and heat (Figures 2B,C): the T_m values for the three proteins range from 49.3 (H268,290F) to 57.6 °C (RAP-D3-quad), and the midpoints for low pH-induced unfolding range from pH 5.6 (H257,259F) to pH 4.8 (RAP-D3-quad).

We next investigated whether the acid- and heat-resistant forms of RAP-D3 still bound to the LA3-4 module pair of the LDLR, measuring the binding affinity of each RAP protein for LA3-4 by isothermal titration calorimetry (ITC) at neutral pH (Figure 3 and Table 1). The stoichiometry of binding for all proteins tested is 1:1, as predicted from the X-ray structure of the complex between wild-type RAP-D3 and LA3-4, and the affinities of the mutant proteins for LA3-4 are all within a factor of 2 when compared with wild-type RAP-D3. At low salt concentrations (50 mM NaCl), the measured dissociation constant for LA3-4 with wild-type RAP-D3 is 480 ± 40 nM, a value that rises to 1.2 ± 0.1 μM at a NaCl concentration of 150 mM, consistent with the electrostatic nature of the protein−protein interface seen in the X-ray structure (*11*). The K_d values for H257,259F and H268,290F, both determined at 50 mM NaCl are 850 ± 160 nM and 1.2 ± 0.3 μM, respectively. The value of K_d for RAP-D3-quad, determined at 100 mM NaCl because titrations performed at other concentrations did not exhibit good-quality fits to a single-site binding model (data not shown), is 850 ± 160 nM.

With stable, high-affinity forms of RAP-D3 in hand, we then examined whether the sensitivity of the different forms of RAP-D3 to pH-induced unfolding correlated with the sensitivity of RAP-D3−LA3-4 complexes to low pH-induced dissociation, as judged using a size exclusion chromatography assay (Figure 4). For wild-type RAP-D3, complexes exhibit evidence of dissociation by pH 6.0, and dissociation is nearly complete by pH 5.5 (Figure S1). In contrast, complexes formed with the H257,259F form of RAP do not exhibit evidence of dissociation until pH 5.0, and complexes with RAP-D3-quad do not undergo dissociation even at pH 5.0. The dissociation data correlate with the pH-induced unfolding studies of the different RAP-D3 proteins, consistent with a

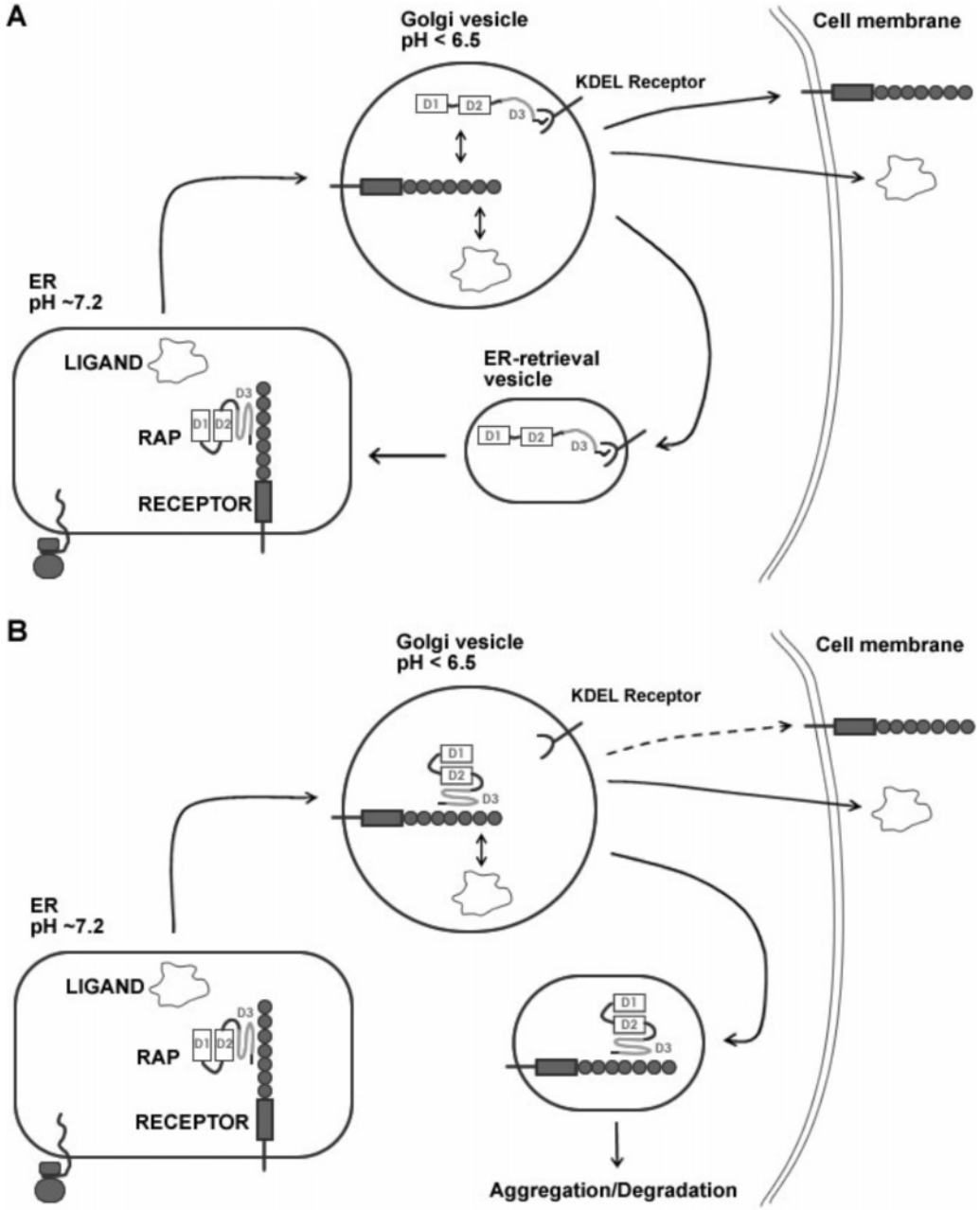

FIGURE 6: Model for the escort function of RAP (adapted from Herz; *19*). (A) Wild-type RAP forms a complex with proteins of the LDL receptor family in the neutral pH environment of the ER, thus preventing premature ligand association. The complex is transported to the Golgi and low-pH induced unfolding of RAP-D3 results in complex dissociation and exposes the C-terminal HNEL sequence for retrieval by the KDEL receptor, while receptor and ligands are exported to the cell surface. (B) RAP-D3 harboring histidine to phenylalanine mutations does not unfold and therefore does not dissociate from the receptor in the Golgi. This may result in either aggregation or degradation of the complex and reduced export of receptors to the cell surface.

model in which dissociation of complexes is normally driven by unfolding of the helical bundle at the mildly acidic pH of the Golgi.

If low-pH induced dissociation of RAP−LRP-1 complexes is functionally important in cells, then a RAP mutant with the H257,259,268,290F substitutions (also referred to as RAP-quad H:F) would be expected to remain bound to LRP-1 at the pH of the Golgi, and interfere with transport of LRP-1 to the cell surface in a dominant negative fashion. Therefore, we compared the endogenous LRP-1 reaching the cell surface in HepG2 cells transfected with either wild-type RAP or the full length RAP-quad H:F mutant (Figure 5). Transfection of RAP into HepG2 cells does not detectably alter the amount of endogenous LRP-1 at the cell surface in this assay when compared with vector alone (Figure 5, compare red and blue traces). In contrast, transfection with RAP-quad H:F results in a dramatic reduction in cell surface LRP-1 levels, as predicted by the pH-induced unfolding model.

DISCUSSION

The studies reported here investigate the question of how changes in pH modulate the stability of complexes between RAP and LDLR family proteins. Previous studies implicated the histidine residues in RAP-D3 as key components of the sensor involved in mediating low pH-induced dissociation of RAP-LRP-1 complexes (*9*). In those experiments, a protein in which all histidines in RAP-D3 were replaced with

alanines exhibited reduced pH sensitivity in binding to LRP-1 than normal RAP. The mutated receptor also exhibited decreased activity in an assay for secretion of soluble LRP-1-derived "minireceptors" into the conditioned medium of COS-1 cells cotransfected with the mutated RAP and the LRP-derived soluble minireceptor (*9*), consistent with the interpretation that the presence of the histidines are needed to allow for low pH-induced dissociation of RAP−LRP-1 complexes.

Although those studies identified a requirement for the native histidines in encoding the observed sensitivity of RAP−LRP-1 complexes to a low pH environment, they were not able to determine whether dissociation was induced by destabilization of the protein−protein interface or secondary to unfolding of the RAP-D3 helical bundle induced by low pH. The all-alanine mutant of RAP used in the previous studies exhibits a lower affinity for LRP-1 even at neutral pH (*9*). In addition, although the RAP-D3 variant with all histidine residues replaced by alanine was not evaluated here, our observation that H257,259A is more than half unfolded at neutral pH and physiologic temperature (37 °C) suggests that replacement of all histidines with alanines may substantially destabilize the folded conformation of RAP-D3.

In the X-ray structure of the RAP-D3 complex with the LA3-4 domain pair from the LDLR, several histidine residues are buried in the hydrophobic interior of the RAP-D3 helical bundle, where burial of a positive charge would be energetically unfavorable (*11*). This observation, combined with the observed marginal stability of RAP-D3 at neutral pH and its unfolding pK_a of ~6.3, suggested a model for pH-induced dissociation in which the reduced pH of the Golgi titrates one or more of the histidine residues in the interior of the RAP-D3 helical bundle, which in turn results in bundle unfolding and dissociation of complexes. The unfolded form of RAP-D3 would also confer increased accessibility of the HNEL retention signal at the C-terminal tail of RAP to allow it to be readily returned to the ER.

Here, we tested the low-pH induced unfolding model by employing a protein design approach, constructing forms of the RAP-D3 helical bundle resistant to thermal and low-pH induced unfolding, while maintaining the ability of these proteins to bind a representative pair of LA repeats, the LA3-4 domain pair from the LDL receptor, at neutral pH. Strikingly, the pH-sensitivity of complexes to dissociation correlated tightly with the resistance of the RAP-D3 variant to low-pH induced unfolding, as predicted by the bundle-unfolding model. In addition, when these stabilizing mutations are introduced into full-length RAP and the stabilized form of RAP is transfected into HepG2 cells, the transfected cells exhibit reduced surface expression of endogenous LRP-1, consistent with the interpretation that the stabilized form of RAP is leading to intracellular retention of LRP-1. Together, our findings are consistent with a refined version of the model recently proposed by Herz (*19*) for RAP-assisted export of LDLR family proteins (Figure 6).

The thermostable and low-pH resistant forms of RAP and RAP-D3 developed in these studies may also have practical value as potential starting points for the development of agents protective against neuronal injury. Previous work has shown that vasogenic edema induced by tissue plasminogen activator (tPA) in cases of neuronal injury or certain types of embolic stroke depends on interactions between tPA and LRP-1, and that this interaction can be blocked by anti-LRP antibodies or by soluble RAP, which is likely to be competing for tPA binding in this context (*20*). The more thermo- and low-pH resistant forms of RAP-D3 developed in these studies, expected to exhibit increased resistance to degradation *in vivo*, might be able to more effectively mitigate the vascular permeability effects of tPA while still allowing tPA to exert its protective antithrombotic effects in ischemic stroke or other thombosis-prone conditions.

ACKNOWLEDGMENT

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SUPPORTING INFORMATION AVAILABLE

Gel filtration analysis of LA3-4−RAP-D3 complexes at various pH values (Figure S1). This material is available free of charge via the Internet at http://pubs.acs.org.

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