Generation of a Potent Low Density Lipoprotein Receptor-related Protein 1 (LRP1) Antagonist by Engineering a Stable Form of the Receptor-associated Protein (RAP) D3 Domain*

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Background: RAP is a chaperone for LRP1 and dissociates at low pH when the D3 domain unfolds.

Results: Stabilizing the D3 domain by introducing a disulfide bond allowed high affinity binding at pH 5.5.

Conclusion: Stabilized RAP is an effective inhibitor of LRP1.

Significance: Stable RAP may be useful in a number of pathological settings for LRP1 blockade.

The low density lipoprotein receptor-related protein 1 (LRP1) is a member of the low density lipoprotein receptor family and plays important roles in a number of physiological and pathological processes. Expression of LRP1 requires the receptor-associated protein (RAP), a molecular chaperone that binds LRP1 and other low density lipoprotein receptor family members in the endoplasmic reticulum and traffics with them to the Golgi where the acidic environment causes its dissociation. Exogenously added RAP is a potent LRP1 antagonist and binds to LRP1 on the cell surface, preventing ligands from binding. Following endocytosis, RAP dissociates in the acidic endosome, allowing LRP1 to recycle back to the cell surface. The acid-induced dissociation of RAP is mediated by its D3 domain, a relatively unstable three-helical bundle that denatures at pH <6.2 due to protonation of key histidine residues on helices 2 and 3. To develop an LRP1 inhibitor that does not dissociate at low pH, we introduced a disulfide bond between the second and third helices in the RAP D3 domain. By combining this disulfide bond with elimination of key histidine residues, we generated a stable RAP molecule that is resistant to both pH- and heat-induced denaturation. This molecule bound to LRP1 with high affinity at both neutral and acidic pH and proved to be a potent inhibitor of LRP1 function both in vitro and in vivo, suggesting that our stable RAP molecule may be useful in multiple pathological settings where LRP1 blockade has been shown to be effective.

The low density lipoprotein receptor-related protein 1 (LRP1) and other members of the low density lipoprotein receptor (LDLr) family play important roles in a number of biological processes, including development, lipoprotein metabolism, cell signaling, modulation of blood-brain barrier integrity, and blood coagulation and fibrinolysis (1, 2). LRP1 mediates these processes via its role as an endocytic or signaling receptor and binds numerous ligands that include certain lipoproteins, proteases, protease-inhibitor complexes, and certain growth factors (2).

The efficient delivery of LRP1 and other LDLr family members to the cell surface requires a 39-kDa receptor-associated protein (RAP) (3). RAP was initially discovered when it co-purified with LRP1 (4, 5) and is an endoplasmic reticulum (ER)-resident protein that binds tightly to most LDLr family members and prevents them from associating with ligands in the ER (6, 7). As a molecular chaperone, RAP traffics with newly synthesized LDLr family members to the Golgi where the mildly acidic environment leads to its dissociation (6, 8, 9) and subsequent retrieval back to the ER. The pH-induced dissociation of RAP from LRP1 is mediated by the third domain of RAP (D3) (9), a three-helical bundle that is relatively unstable (10). Lowering of the pH activates a histidine switch in which protonation of histidine residues on the second and third helices of RAP D3 destabilizes this domain, leading to its denaturation (10, 11). Mutation of key histidine residues generates a molecule with increased stability at low pH that does not dissociate from LRP1 and fails to release from LRP1 in the Golgi, resulting in ineffective delivery of LRP1 to the cell surface due to retrieval of the receptor-RAP D3 complex from the Golgi back to the ER (10, 11).

RAP is a potent inhibitor of ligand binding to LRP1 (6), LRP2 (12), VLDL receptor (13), and apolipoprotein E receptor 2 (14) and has been extensively used to inhibit the functional activity of these LDLr family members both in vitro and in vivo. These studies have identified potential therapeutic uses for RAP. Thus, intraventricular administration of RAP in mice following occlusion of the middle cerebral artery resulted in a significantly faster recovery of motor activity and a reduction in
blood-brain barrier leakage (15), suggesting that LRP1 antagonism might be effective during stroke. In the liver, LRP1 functions to remove a number of molecules from the plasma, including blood coagulation protein VIII, which is deficient in the bleeding disorder hemophilia A. Studies in mice have revealed that co-injection of RAP delays the hematically mediated clearance of 125I-labeled FVIII (16). These studies may have clinical significance as purified FVIII is administered to hemophilia A patients. Prophylactic treatment can be highly effective in preventing bleeding episodes and minimizing the secondary effects associated with this disease (17); however, the short plasma half-life of FVIII dictates that the molecule be injected 3 times a week to be effective. Thus, a highly effective LRP1 antagonist that delays FVIII clearance might also be of therapeutic use in conjunction with FVIII therapy.

The effectiveness of RAP as an antagonist is limited due to the fact that it dissociates from these receptors as they cycle through the low pH environment of the endosomal compartments and is subsequently transported to lysosomal compartments where RAP is degraded. The objective of the current studies was to develop a potent inhibitor of LDLr family members by engineering a RAP molecule containing a D3 domain that is resistant to pH-induced denaturation. We rationalized that this mutant would not dissociate from LRP1 at reduced pH and would therefore be a more effective inhibitor of LDLr family members. To accomplish this, we introduced a disulfide bond between the second and third helices of the RAP D3 domain to stabilize this domain. Our results reveal that this stabilized RAP is highly effective at inhibiting LRP1 function.

**Experimental Procedures**

*Site-directed Mutagenesis, Protein Expression, and Purification of RAP, RAP D3, and Their Mutants—*Mutagenesis of RAP and RAP D3 was performed using the GENERAT Site-directed Mutagenesis System (Invitrogen) using RAP pGex2T and RAP D3 pGex2T (7, 18) as templates. Primer pairs were designed to introduce the desired mutation(s) of interest. All mutations were confirmed by forward and reverse sequencing. Proteins were expressed in *Escherichia coli* and purified as described previously (7, 18).

*5,5'-Dithiobis(2-nitrobenzoic acid) (DTNB) Titration—*Proteins in 0.1 M Tris-HCl, pH 8.0 buffer were treated with 10 mM dithiothreitol (DTT) for 2 h at room temperature. DTT was removed from the proteins by centrifugation using Vivaspin 500 5-kDa molecular mass-cutoff ultrafiltration devices (GE Healthcare). Briefly, proteins were concentrated by centrifugation at 15,000 × g for 15 min in Vivaspin 500 ultrafiltration units and then diluted ~10-fold in Tris-HCl. This process was repeated four times, resulting in a decrease of the DTT concentration by ~99%. The proteins were then diluted to 1 mg/ml and treated with 0.1 mM DTNB. Absorbance of all samples was measured at 410 nm. A molar extinction coefficient of 14,140 M⁻¹ cm⁻¹ was used for the nitrothiophenolate anion (19).

*Circular Dichroism—*Circular dichroism (CD) spectra were recorded on a Jasco-715 spectropolarimeter with a Peltier PFD-350S unit for temperature control. For melting curves, proteins were dialyzed in 10 mM phosphate buffer, pH 7.5, and thermal stability was measured at a constant wavelength of 222 nm from 5 to 90 °C in steps of 0.1 °C using a 1-mm-path length cell. For pH-dependent stability, the proteins were dialyzed in 10 mM phosphate buffer at the indicated pH, and the pH was confirmed by measurement using a pH meter. Spectra were collected at 20 °C from 260 to 190 nm in a 1-mm cell with data recorded every 0.1 nm.

*Surface Plasmon Resonance—*Purified LRP1 was immobilized onto a CM5 sensor chip surface to the level of 10,000 response units using a working solution of 20 μg/ml LRP1 in 10 mM sodium acetate, pH 4 according to the manufacturer’s instructions (BIAcore AB). An additional flow cell was activated and blocked with 1 mM ethanolamine without protein to act as a control surface. All pH 7.4 binding experiments were performed in Hepes-buffered saline (HBS)-P buffer (0.01 M Hepes, 0.15 M NaCl, 0.005% Surfactant P, 1 mM CaCl₂). The pH 5.5 binding experiments were performed in MES buffer (0.01 M MES, 0.15 M NaCl, 0.005% Surfactant P, 1 mM CaCl₂). All experiments were performed on a BIAcore 3000 instrument using a flow rate of 20 μl/min at 25 °C with binding and dissociation occurring for 5 min each using RAP concentrations from 200 to 0.78 nm. Sensor chip surfaces were regenerated by 15-s injections of 100 mM phosphoric acid at a flow rate of 100 μl/min. The data were fit for a pseudo-first order process to determine response units at equilibrium that were plotted versus ligand concentration. The *Kₐ* was determined by fitting the data to a single class of sites using non-linear regression analysis using GraphPad Prism 6.0 software.

*Proteolytic Cleavage of RAP D3—*240 μg of WT or stable RAP D3 at a concentration of 1 mg/ml was digested with 1:100 trypsin or 1:1000 chymotrypsin in HBS, pH 7.4 at 37 °C for the indicated amount of time. At each time point, 40 μg of protein was removed, 115 mM PMSF was added to inhibit enzyme activity, and the samples were snap frozen in liquid nitrogen. Samples were thawed and run under reducing conditions on 4–12% Bis-Tris gels (Novex Life Technologies) in MES buffer. Gels were Coomassie-stained to visualize protein.

*α₂-Macroglobulin Internalization—*Cellular internalization assays were conducted generally as described previously (20). Briefly, WI-38 fibroblasts were seeded into 12-well culture dishes (1 × 10⁵ cells/well) and grown in Dulbecco’s modified Eagle’s medium with 10% bovine calf serum and penicillin/streptomycin overnight. Cells were treated with HBS vehicle control, 1 μM RAP, or 1 μM stable RAP for 5.5 h at 37 °C. After washing with phosphate-buffered saline (PBS), assay medium (1.5% bovine serum albumin, 20 mM Hepes, pH 7.5, 1 mM Ca²⁺) containing 2 nm 125I-labeled activated α₂-macroglobulin (α₂-M*) or 20 nm 125I-labeled 5A6 was added and incubated for 1 h at 37 °C. After incubation, wells were washed with PBS, and cells were detached from the plate using 0.05% trypsin-Versene (Lonza) plus 0.5 mg/ml proteinase K. Radioactivity associated with the cell pellet was defined as the internalized protein. Nonspecific uptake was determined by measuring the uptake in the presence of 200 nM RAP (for α₂-M*) or 1 μM unlabeled 5A6 (for 5A6).

*In Vivo Clearance of α₂-M—*C57Bl/6 mice were anesthetized with ketamine/xylazine and then injected with 50 μM endotoxin-free RAP or stable RAP or with HBS vehicle control intrave-
Stable RAP as an LRP1 Antagonist

Results

Generation of Stable RAP Molecules—The introduction of engineered disulfide bonds into proteins has been widely used to stabilize them (21, 22), and computational methods have been developed that analyze the three-dimensional structure of a protein to choose optimal sites for the creation of disulfide bonds. To engineer a RAP molecule with a stable D3 domain, we rationalized that introducing a disulfide bond between helices 2 and 3 of this domain would lead to its stabilization. The solution structure of the RAP D3 domain has been solved (10, 23), and we used these coordinates with MODIP (MOdeling DIsulphide bonds in Proteins) (24) to identify sites within this domain that are optimal for generation of a disulfide bridge with dihedral angles that are within an ideal range of values. The program identified only one pair of possible residues, Tyr-260 (in helix 2) and Thr-297 (in helix 3), that we mutated into cysteines to generate RAP D3 disulfide (Table 1 and Fig. 1A). To serve as a control for this mutant, we also altered four of the histidine residues (His-257, His-259, His-268, and His-290) into phenylalanines (RAP D3 Quad H:F) to eliminate the “histidine switch” that has previously been demonstrated (10, 11) to increase the stability of the RAP D3 domain in low pH (Table 1 and Fig. 1B). Finally, we combined all of these mutations into a single molecule, which we termed stable RAP (Table 1). Each of these mutations were prepared in full-length RAP as well as in the isolated RAP D3 domain.

Confirmation of Appropriate Disulfide Bond Formation in the RAP D3 Domain—To confirm that the cysteine residues introduced in the RAP disulfide and stable RAP molecules formed a disulfide bond, we titrated the molecules with DTNB. As expected given the lack of cysteine residues, wild type RAP did not react with DTNB. Furthermore, neither RAP disulfide nor stable RAP reacted with DTNB (Fig. 2), revealing the absence of free thiols in these molecules and consistent with the presence of a disulfide bond between the introduced cysteine residues. To further confirm this, we treated the RAP molecules with DTT. Following removal of the DTT, RAP disulfide and stable RAP, but not WT RAP, reacted with the DTNB, confirming the presence of free thiol groups following reduction of the disulfide bond. Using the known molar extinction coefficient for the nitrothiophenolate anion at this pH (19) revealed 1.9 and 2.1 mol of thiol/mol of RAP disulfide and stable RAP, respectively. These values are close to the expected value of two cysteines per molecule. These findings were consistent regardless of whether the DTNB titration was performed on full-length RAP or D3 domain mutants and confirm the appropriate formation of disulfide bonds. Furthermore, analysis of the purified proteins by SDS-PAGE under non-reducing conditions revealed that stable RAP and RAP disulfide do not form intermolecular disulfide-linked forms. The data confirm appropriate formation of a disulfide bond in RAP disulfide and stable RAP.

Thermal Stability of RAP D3 Mutants—Structural studies have revealed that RAP D3 is composed of a three-helical bundle that is relatively unstable with a melting temperature of 43 °C (18). We investigated the stability of each of our RAP D3 mutants by measuring the thermally induced unfolding using CD spectroscopy measurements at a wavelength of 222 nm, a wavelength that measures changes in α-helical structure. These experiments revealed that WT RAP D3 has a melting temperature of 43 °C, a value in close agreement with the published accounts (Fig. 3, A and B). The RAP D3 Quad H:F mutant

| Mutant name       | Residues | Mutations                           |
|-------------------|----------|-------------------------------------|
| RAP WT RAP        | 1–323    | None                                |
| RAP disulfide     | 1–323    | Y260C, T297C                        |
| RAP Quad H:F      | 1–323    | H257F, H259F, H268F, H290F          |
| Stable RAP        | 1–323    | H257F, H259F, Y260C, H268F, H290F, T297C |
| RAP D3            |          |                                     |
| WT RAP D3         | 206–323  | None                                |
| RAP D3 disulfide  | 206–323  | Y260C, T297C                        |
| RAP D3 Quad H:F   | 206–323  | H257F, H259F, H268F, H290F          |
| Stable RAP D3     | 206–323  | H257F, H259F, Y260C, H268F, H290F, T297C |

FIGURE 1. Location of mutated residues in the RAP D3 domain. Structure of helices 2 and 3 in the RAP D3 domain (teal) highlighting mutated residues (yellow) in the RAP disulfide (A) and RAP Quad H:F (B) mutants. In A, the putative introduced disulfide bond is represented with a dashed line.
Introduction of a disulfide bond and/or mutation of key histidine residues improves the thermal stability of the RAP D3 domain. A. CD spectroscopy was used to determine the heat-induced denaturation of WT RAP D3 (black), RAP D3 disulfide (blue), RAP D3 Quad H:F (red), and stable RAP D3 (pink) monitored by changes in molar ellipticity at 222 nm. 8, the reported Tm values were determined as the temperature at which 50% of the molecules were unfolded. C, thermally induced denaturation of WT RAP D3 (black) and RAP D3 disulfide (blue) after 2-h incubation with 10 mM DTT. Note that the increased thermal stability of the disulfide mutant relative to WT (seen in A) is entirely dependent on the formation of the disulfide bond.

Protease Sensitivity of RAP Mutants—RAP is known to be sensitive to proteolysis, and thus we examined the sensitivity of the WT and stable D3 domain to proteolysis mediated by trypsin (Fig. 4A) or chymotrypsin (Fig. 4B). The results reveal that stable RAP D3 is much more resistant to trypsin- or chymotrypsin-mediated proteolysis. Thus, for example, digestion of stable RAP D3 with chymotrypsin revealed little cleavage even after 120 min (Fig. 4B). In contrast, WT RAP was mostly cleaved at this time point.

pH-dependent Unfolding of RAP D3—The unfolding of the RAP D3 domain can be revealed by changes in the circular dichroism spectra where the highly helical nature of RAP D3 can be seen in the negative bands at 222 and 208 nm that are characteristic of α-helices. Consequently, we used circular dichroism measurements to determine whether the conformation of RAP D3 mutants is sensitive to pH changes. At pH 7.5, all of our proteins had CD spectra indicative of helical content, and the spectra from WT RAP D3, RAP D3 disulfide, and RAP D3 Quad H:F overlaid each other (Fig. 5A). Interestingly, stable RAP D3 had a more negative ellipticity at 222 and 208 nm suggestive of increased helical content. This may indicate that the wild type protein is in equilibrium with a partially unfolded form and that the equilibrium is shifted toward the folded form in stable RAP.

At pH 5.5, wild type RAP D3 had substantially less negative ellipticity at 222 and 208 nm, consistent with the unfolding of this domain. In contrast, the RAP D3 disulfide and Quad H:F mutants maintained a substantial fraction of their helical content (Fig. 5B). Remarkably, the spectrum of stable RAP D3 was not altered when the pH was reduced to 5.5, indicating that unlike wild type RAP D3 stable RAP D3 does not undergo unfolding in response to low pH. This is most evident in Fig. 5C where the spectra from stable RAP at pH 7.5 and 5.5 overlap perfectly, whereas the spectra of WT RAP D3 change substantially as the pH is reduced to 5.5, demonstrating a loss of helical content with the decreased pH.

Effects of pH on Binding of RAP to LRP1—We used surface plasmon resonance measurements to test whether the increased stability of our RAP mutants at low pH improved their ability to bind to LRP1 at this pH. For these experiments, LRP1 was coupled onto a surface plasmon resonance chip, and then increasing concentrations of either WT RAP or its mutants were flowed over the coated chip in either pH 7.4 or 5.5 buffer. WT RAP is well known to bind to LRP1 with high affinity near neutral pH, which remained true in our experiments, and we determined a Kd of 0.68 nM for WT RAP binding LRP1. Each of our full-length RAP mutants also bound to LRP1 with high affinity with Kd values of 0.96 nM for stable RAP and 1.67 nM for RAP disulfide (see Fig. 6 for representative sensograms and Table 2). Given the unfolding of WT RAP at low pH, we predicted poor binding to LRP1 at pH 5.5, and indeed, we saw a substantial reduction in affinity (Kd = 181 nM) for WT binding to LRP1 at pH 5.5. In contrast, whereas RAP disulfide and RAP Quad H:F mutants also bound LRP1 with lower affinity at pH.

**Stable RAP as an LRP1 Antagonist**
5.5 than at pH 7.4, the changes were less substantial than WT RAP with the affinity decreasing only 35- and 53-fold, respectively (Table 2). The stable RAP mutant bound LRP1 at low pH with fairly little change in affinity ($K_D = 21.5 \text{ nM}$). Surface plasmon resonance binding studies using WT RAP D3 and its respective mutants had similar results (Table 2). These findings indicate that the improved stability of stable RAP at low pH led to a substantial increase in binding affinity for LRP1 at this low pH, suggesting that our stable RAP mutant might not uncouple from LRP1 in the low pH environments of the Golgi or endosome. If so, this molecule would likely be an improved inhibitor of LRP1 function.

**Effectiveness of Stable RAP as an Inhibitor of LRP1 Function**

Because our data indicate that stable RAP still binds tightly to LRP1 at pH 5.5, we reasoned that this molecule would not dissociate in the acidic environment of the endosome and would therefore be a more effective LRP1 inhibitor than WT RAP. To test this hypothesis, we pretreated LRP1-expressing WI-38 cells with vehicle, WT, or stable RAP at 37 °C. Following incubation and thorough washing, the cells were incubated with $^{125}$I-labeled 5A6 (Fig. 7B), a monoclonal antibody that binds to the LRP1 light chain, which is not
Stable RAP as an LRP1 Antagonist

FIGURE 7. Stable RAP is an inhibitor of LRP1 function in vitro and in vivo. WI-38 human fibroblasts were treated with 1 µM WT RAP, stable RAP, or vehicle control (HBS) for 5.5 h. Cells were thoroughly washed and then treated with 2 nM 125I-labeled activated α2M (A) or 20 nM 125I-labeled SA6 (B) for 1 h at 37 °C. Internalization was defined as the amount of radioactivity associated with the cell pellet. In A, p < 0.05, and in B, p is not significant by one-way analysis of variance. C, 100 µl of 50 µM WT RAP (squares), stable RAP (triangles), or HBS vehicle control (circles) was injected into the tail vein of mice (n = 3 per treatment). 5 min later, 100 µl of 125I-labeled activated α2M (10 nM) was injected. At the indicated times, blood was collected, and a 15-µl aliquot was counted for 125I radioactivity. The 30-s time point was defined as 100% radioactivity. Data are presented as mean ± S.E. (error bars) and are representative of three separate experiments. *p < 0.05 for stable versus WT RAP by Student’s t test and p < 0.03 by two-way analysis of variance.

Blocked by RAP. These results reveal that preincubation of LRP1 with RAP or stable RAP does not lead to receptor degradation but allows effective recycling of this receptor.

We also measured the ability of RAP and stable RAP to block the hepatic uptake of 125I-labeled α2M*. In these experiments, RAP or stable RAP was preinjected into the circulation 5 min prior to injecting 125I-labeled α2M*. The results (Fig. 7C) confirm that stable RAP is more effective than WT RAP at delaying the clearance of α2M* from the circulation in vivo.

Discussion

By using mouse models, studies have demonstrated that LRP1 blockade using RAP results in a potent attenuation of ischemic area following occlusion of the middle cerebral artery (15) and prevents tissue-type plasminogen activator-mediated disruption of the blood-brain barrier (27). These and other studies identify potential therapeutic uses for RAP to block LRP1 function. One objective in the current study was to engineer a RAP molecule with a high affinity for LRP1 at both neutral and acidic pH to prevent dissociation of the antagonist as the receptor cycles through endosomal compartments. To accomplish this, we mutated key histidine residues previously shown to stabilize this domain and introduced a disulfide bond between the second and third helices of D3, which resulted in a dramatic stabilization of the domain with regard to temperature and pH changes. Importantly, its interaction with LRP1 was not dramatically altered at reduced pH values, suggesting that stable RAP might function as a more effective inhibitor of LRP1 function, which was confirmed by examining the effect of this RAP mutant on LRP1 function.

RAP functions as a molecular chaperone that assists in the folding and escort of newly synthesized LDLr family members through the secretory pathway (8, 28). Once bound, RAP traffics with the receptors to the Golgi where the reduced pH environment leads to its dissociation from the receptor and allows the retrograde transport of RAP back to the ER by the ERD2 retrieval receptor (29). This chaperone function of RAP has been ascribed to its third domain (9, 30), a relatively unstable domain that denatures when the pH is reduced below ~6.2 (10). The mechanism by which RAP dissociates from this family of receptors in response to lowering of the pH was uncovered when the solution structure of RAP D3 was solved. This structure revealed a three-helical bundle domain consisting of a short helix of 10 amino acids and two longer helices containing 40 and 38 amino acids, respectively. Importantly, the work identified a histidine switch in which the low pH environment of the Golgi results in protonation of histidine residues, resulting in an unfavorable accumulation of positive charges between helices 2 and 3, causing the domain to unfold and dissociate from LRP1 (10, 11).

This D3 destabilization model in which dissociation occurs due to pH-mediated denaturation of D3 has recently been questioned by Jensen et al. (31). These studies investigated the effect of pH on the binding of the RAP D3 domain to pairs of LDLr repeats from the second ligand binding cluster of LRP1. The studies observed only a 6-fold increase in Kd when the pH was changed from 7.4 to 5.5, and Jensen et al. (31) concluded that this small effect of pH on affinity was not likely to lead to acid-mediated dissociation of RAP from LRP1. Until now, the Kd for RAP binding to full-length LRP1 at pH 5.5 had not been measured, and the present studies found a substantial (175-fold) increase in the Kd for the binding of D3 to full-length LRP1 as the pH was reduced from 7.4 to 5.5. In contrast, stable RAP D3 displayed only a 3-fold increase in the Kd for LRP1 at pH 5.5. These results provide strong support for the D3 destabilization model. Furthermore, the stable form of RAP proved to be a much more potent inhibitor than WT RAP of LRP1 function as measured by α2M* uptake presumably due to its ability to remain bound to LRP1 during endosomal recycling. Together with the results of Jensen et al. (31) the studies further suggest that the binding mode of RAP to full-length LRP1 may differ from its binding to a small fragment containing only two LDLr repeats. This will need to be confirmed by additional studies. Further studies are also needed to test the role of stable RAP on other LRP1 functions, including its role in multiple signaling pathways. Inhibition of these pathways could conceivably lead to both beneficial and detrimental effects, so these studies will be important prerequisites to any potential use of stable RAP in the clinic.

In summary, the current studies have engineered a mutant form of RAP that is resistant to pH-induced denaturation. Because we noted only minor changes in the binding affinity for this mutant molecule to LRP1 as the pH was reduced to 5.5, we provide additional support for the histidine switch that leads to D3 destabilization and subsequent dissociation from LRP1.
Stable RAP as an LRP1 Antagonist

Furthermore, our studies confirm that stable RAP is a potent inhibitor of LRP1 function and might be useful in certain pathological situations when LRP1 blockade is effective.

Author Contributions—J. M. P. and D. K. S. conceived and coordinated the study and wrote the paper. J. M. P., M. M., and R. G. designed, performed, and analyzed the experiments.

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