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RESEARCH ARTICLE

Receptor-associated protein impairs ligand binding to megalin and megalin-dependent endocytic flux in proximal tubule cells

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Abstract

Proximal tubule (PT) cells retrieve albumin and a broad array of other ligands from the glomerular ultrafiltrate. Efficient uptake of albumin requires PT expression of both megalin and cubilin receptors. Although most proteins engage cubilin selectively, megalin is required to maintain robust flux through the apical endocytic pathway. Receptor-associated protein (RAP) is a chaperone that directs megalin to the cell surface, and recombinant RAP dramatically inhibits the uptake of numerous megalin and cubilin ligands. The mechanism by which this occurs has been suggested to involve competitive inhibition of ligand binding and/or conformational changes in megalin that prevent interaction with ligands and/or with cubilin. To discriminate between these possibilities, we determined the effect of RAP on endocytosis of albumin, which binds to cubilin and megalin receptors with high and low affinity, respectively. Uptake was quantified in opossum kidney (OK) cells and in megalin or cubilin (*Cubn*) knockout (KO) clones. Surprisingly, RAP inhibited fluid-phase uptake in addition to receptor-mediated uptake in OK cells and *Cubn* KO cells but had no effect on endocytosis when megalin was absent. The apparent K_i for RAP inhibition of albumin uptake was 10-fold higher in *Cubn* KO cells compared with parental OK cells. We conclude that in addition to its predicted high-affinity competition for ligand binding to megalin, the primary effect of RAP on PT cell endocytosis is to globally dampen megalin-dependent endocytic flux. Our data explain the complex effects of RAP on binding and uptake of filtered proteins and reveal a novel role in modulating endocytosis in PT cells.

NEW & NOTEWORTHY Receptor-associated protein inhibits binding and uptake of all known endogenous ligands by megalin and cubilin receptors via unknown mechanism(s). Here, we took advantage of recently generated knockout cell lines to dissect the effect of this protein on megalin- and cubilin-mediated endocytosis. Our study reveals a novel role for receptor-associated protein in blocking megalin-stimulated endocytic uptake of fluid-phase markers and receptor-bound ligands in proximal tubule cells in addition to its direct effect on ligand binding to megalin receptors.

endocytosis; low-density lipoprotein receptor family; megalin; proximal tubule

INTRODUCTION

Proteins that escape the glomerular filtration barrier are captured from the tubular ultrafiltrate by megalin and cubilin/amnionless (CUBAM) receptors expressed at the apical membrane of proximal tubule (PT) cells (1, 2). Megalin is a >600-kDa member of the low-density lipoprotein receptor (LDL-R) family and contains four clusters of ligand-binding domains separated by epidermal growth factor (EGF)-like and β-propeller domain/YTWD repeats repeats. Recent structural analysis of megalin demonstrated that the receptor forms an intertwined dimer that undergoes large pH-dependent conformational changes (3). CUBAM receptors comprise a trimer of cubilin molecules (>440 kDa) tethered to the membrane via association with a single copy of amnionless (4). Although megalin and cubilin interact, the receptors are differentially expressed in tissues and can function independently. Scavenging efficiency for a given ligand in the PT is dependent on multiple factors, including its affinity for each receptor, the distribution of receptors along the tubule axis, effects of fluid reabsorption on ligand concentration, and the tubular concentrations of other ligands that may compete for uptake (5–8).

The apical endocytic pathway of PT cells is uniquely structured, and elegant studies by Christensen and colleagues have shown that its function in vivo is critically dependent on the expression of high levels of megalin (2, 9, 10). Ligands bound to megalin or cubilin are internalized together with fluid-phase markers in clathrin-coated irregular invaginations that form at the base of microvilli that compose the apical brush border (11–13). After budding, these vesicular compartments are thought to fuse with early endosomes that rapidly acidify and mature into larger apical vacuoles (1, 14). Megalin, cubilin, and other membrane proteins recycle from endosomes and vacuoles via a network of Rab11a-positive dense apical tubules characteristic of PT cells. Remarkably, loss of megalin expression (and, to a lesser extent, cubilin) profoundly reduces the flux of proteins and fluid-phase markers through these compartments without

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apparently altering the physical integrity of the endocytic pathway (13). Acidification-driven conformational changes in megalin may contribute to driving traffic through the pathway to maintain efficient uptake of filtered proteins.

LDL-R-related associated protein 1 (LRPAP1 or RAP) is a small (44 kDa) protein highly expressed in the PT (15, 16). At a steady state, the protein is localized to the endoplasmic reticulum. Analogous to its role in protecting other LDL-R family members from binding intracellular ligands, RAP appears to function as a chaperone that assists megalin folding and surface delivery (17–19). In vitro, very low concentrations of RAP (<10 nM) block ligand binding to megalin and other LDL-R family members, consistent with direct competition, and recombinant full-length RAP or individual domains inhibit PT uptake of a considerable variety of ligands from the PT (20–26). RAP binds to at least two of the complement repeats that comprise the ligand-binding domains of the LDL-R family member LRP, and is readily internalized (27, 28). Once internalized, RAP dissociates from its receptor in acidic compartments and is delivered to lysosomes for degradation (28–30). RAP binding to receptors is believed to compete with the electrostatic interactions of ligands with acidic residues within complement repeats (31, 32). This high-affinity inhibition of ligand binding to megalin is observed even in immunoblots, consistent with a conformation-independent effect. In addition, it has been proposed that RAP may stabilize a structure that inhibits multiple receptor-ligand interactions. This is supported by the finding that not all RAP sites within each receptor need to be occupied to maximally inhibit ligand binding (28). LDL-R and related proteins undergo dramatic pH-dependent conformational changes, and it is tempting to speculate that these could be induced, mimicked, or stabilized by RAP binding. This structural change might also impair megalin association with cubilin receptors with consequences to the uptake of cubilin-specific ligands.

We recently examined the roles of megalin and cubilin in the uptake of albumin and other ligands in a highly differentiated opossum kidney (OK) PT cell culture model that expresses these receptors at levels comparable with the PT in vivo (5, 13, 33). Both receptors have dual roles: direct ligand binding and promotion of endocytic flux. Albumin binds megalin and cubilin with distinct affinities (K_m of 200 and 10 μg/mL, respectively). Accordingly, megalin (*Lrp2*) knockout (KO) reduced low-affinity albumin uptake, and cubilin (*Cubn*) KO reduced high-affinity albumin uptake. Reduced internalization of fluorescent dextran in both KOs provided evidence for effects on apical endocytic flux, with effects larger for megalin KO (−70%) than for cubilin KO (−50%) (13, 33). This endocytic flux effect also largely accounted for reduced cubilin-dependent uptake after megalin KO and reduced megalin-dependent uptake after cubilin KO. Based on these studies and on the differential distribution of megalin and cubilin along the PT axis in rodents, we speculated that cubilin is responsible for the majority of uptake of normally filtered albumin, whereas megalin provides low-affinity reserve capacity for albumin uptake when the glomerular barrier is breached (5, 8, 34–37). Here, we leveraged these tools to investigate the function(s) of RAP in PT apical endocytosis.

METHODS

Cell Culture

All cell culture reagents were from Sigma unless otherwise specified. OK cells (female, OK-P subclone, RRID:CVCL_D040) were cultured at 37°C with 5% CO_2 in DMEM-F-12 supplemented with 5% FBS and GlutaMax (Atlanta Biologicals). For all experiments, 4×10^5 cells were seeded onto 12-mm Transwell permeable supports (No. 3401, Costar) in 12-well dishes. After overnight incubation, the filters were transferred to an orbital platform shaker in the incubator and rotated at 146 rpm for 72 h with daily medium changes. CRISPR/Cas9 *Lrp2* and *Cubn* KO clones in OK cells were generated and characterized as previously described in detail in Refs. (13, 33).

HK-2 cells (RRID:CVCL_0302, *Homo sapiens*, adult male, cortex/PTs, papilloma immortalized) were obtained from American Type Culture Collection and cultured in DMEM-F-12 with 5 μg/mL insulin, 0.02 μg/mL dexamethasone, 0.01 μg/mL selenium, 5 μg/mL transferrin, 2 mM L-glutamine, and 10% FBS as previously described (6).

Quantitation of Albumin and Dextran Uptake

Cells were pretreated for 30 min at 37°C unless otherwise noted with the indicated concentration of apically added human RAP (Innovative Research, IHURAP) in serum-free DMEM-F-12 supplemented with 25 mM HEPES (No. 15630-080, GIBCO). Alexa Fluor-647 albumin [40 μg/mL (600 nM) unless otherwise indicated, A34785, Invitrogen] or Alexa Fluor-568 fixable 10-kDa dextran (1 mg/mL, D22912, ThermoFisher) was added apically in the continued presence or absence of RAP. After 30 min, filters were washed with cold PBS three times (with Mg^{2+} and Ca^{2+}). Cells were solubilized, and cell-associated Alexa Fluor-647 fluorescence intensity was quantified by spectrofluorimetry on a GloMax Multi-Detection System (Promega). For individual experiments performed in duplicate or triplicate, data in each experiment were normalized to the average values of OK cell controls. Replicates in each experiment typically varied by <10%. To visualize cell-associated albumin, filters were incubated with albumin for 15 min, washed twice with PBS/$+Ca^{2+}$/$+Mg^{2+}$ (D8662, Sigma) prewarmed to 37°C, and fixed in 4% paraformaldehyde-100 mM sodium cacodylate for 15 min at ambient temperature. After two washes in PBS, filters were cut from their supports, mounted onto glass slides with ProLong Glass Antifade Mountant (P36981, Molecular Probes), and imaged on a Leica SP8 confocal microscope using a ×63 oil immersion objective.

Surface Binding of Albumin

Parental OK or *Lrp2* KO cells were pretreated in serum-free medium with 0.5 μM RAP for 1 h and then incubated on ice for 30 min with the indicated concentration of Alexa Fluor-647 albumin added apically. Filters were washed rapidly five times with ice-cold PBS and solubilized as described earlier. Surface-bound albumin was quantified by spectrofluorimetry.

Immunofluorescence Staining

Filter-grown OK cells were washed twice in warm PBS/$+Ca^{2+}$/$+Mg^{2+}$ and fixed in warm 4% paraformaldehyde/100 mM sodium cacodylate (3 mm $CaCl_2$, 3 mm $MgCl_2$, and

3 mm KCl, pH 7.4) for 15 min at ambient temperature. After two washes in PBS, the filters were quenched (PBS/20 mM glycine/75 mM ammonium chloride) for 5 min and permeabilized for 5 min in quench solution containing 0.1% Triton X-100. After being rinsed with PBS, the filters were blocked with PBS/1% BSA/0.1% saponin for 30 min and incubated for 1 h with primary antibodies [anti-megalin rabbit polyclonal antibody MC-220, 1:1,000 (38); anti-EEA1 mouse monoclonal antibody, 1:50, sc-365652, Santa Cruz Biotechnology; anti-Rab5 rabbit polyclonal antibody, 1:500, ab218624, Abcam; and anti-Rab7 rabbit polyclonal antibody, 1:100, No. 9367, Cell Signaling] diluted in wash buffer (PBS/0.5% BSA/0.025% saponin). The filters were washed three times in wash buffer and then incubated for 30 min with secondary antibodies (1:500, A-11029 and A-21245, Invitrogen) and Alexa Fluor 488 Fab Fragment Goat Anti-Rabbit (1:500, No. 111-547-003, Jackson Immuno Research) for double labeling using previously described methods (37). The filters were washed in wash buffer three times for 5 min each and then cut and mounted onto glass slides with ProLong Glass Antifade Mountant. Cells were imaged on a Leica Stellaris-8 inverted confocal microscope using a ×63 oil immersion objective. Images were acquired with a voxel size of 45 × 45 × 130 nm (*x*, *y*, *z*). OK cell images were deconvolved with Huygens Essential version 17.04 using the CMLE algorithm (Scientific Volume Imaging, http://svi.nl).

RESULTS

Dose-Dependent Effect of RAP on Endocytic Uptake of Albumin Versus Dextran in OK Cells

We tested the effect of increasing concentrations of RAP on the uptake of fluorescent albumin versus dextran in filter-grown OK cells cultured under continuous orbital shear stress as described in METHODS. Although RAP binds to megalin with nanomolar affinity, most uptake studies have been performed using concentrations up to 1 μM. We measured uptake of albumin (40 μg/mL) or dextran (1 mg/mL) over 30 min after a 30-min preincubation with RAP (Fig. 1). At low concentrations, RAP had comparable effects on both albumin and dextran uptake (−27% at 50 nM RAP compared with untreated cells). At higher concentrations, RAP had a greater effect on the uptake of albumin than of dextran. At the concentration of RAP, we chose for further experiments (0.5 μM), albumin and dextran uptake were reduced by 67% and 51%, respectively. This residual uptake of dextran is very likely an overestimate of the internalized pool, as we found that some polymer remained associated with the cell surface despite repeated washes (not shown). Longer preincubations with this concentration of RAP (up to 6 h) did not further reduce fractional uptake of albumin (Supplemental Fig. S1*A*). Qualitatively, there was no change in the distribution of megalin or markers of early endosomes (EEA1 and Rab5), dense apical tubules (Rab11a), or apical vacuoles (Rab7) in cells incubated for 6 h with RAP (Supplemental Fig. S1*B*). Together, these data suggest that RAP impairs endocytic flux without appreciably affecting the structural organization of the apical endocytic pathway. We observed a similar effect when we silenced or knocked out megalin in OK cells (13, 33).

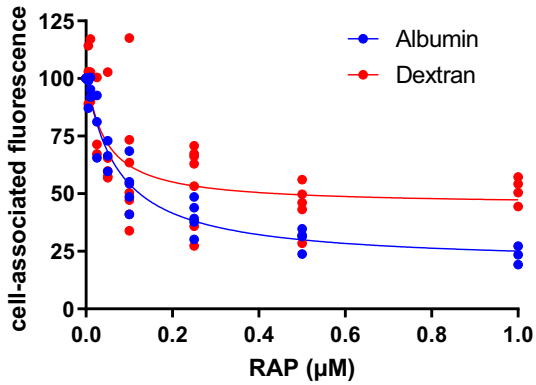

Figure 1. Dose-dependent receptor-associated protein (RAP) inhibition of albumin and dextran in opossum kidney cells. Opossum kidney cells cultured on filter supports were incubated for 30 min with apically added RAP at the indicated concentrations. Alexa Fluor-568 dextran (0.5 mg/mL; red) or Alexa Fluor-648 albumin (40 μg/mL; blue) were added in the continued presence of RAP for 30 min, and cells were washed and solubilized. Cell-associated florescence was quantified by spectrofluorimetry. Data from five independent experiments quantifying albumin and seven independent experiments quantifying dextran uptake were normalized to values in the absence of RAP.

Other commonly used PT cell models, such as human kidney HK-2 cells, express considerably less megalin and internalize far less albumin than OK cells (39). We hypothesized that endocytic flux in these cells would be insensitive to RAP because of their low megalin expression. Indeed, incubation of HK-2 cells for 1 h with albumin and dextran revealed a RAP-dependent reduction in albumin uptake as expected but no discernable effect on dextran endocytosis (Supplemental Fig. S2*A*). In contrast, RAP inhibited both dextran and albumin uptake in OK cells incubated for 3 min with fluorescent conjugates (Supplemental Fig. S2*B*).

RAP Inhibition of Endocytic Flux Is Megalin Dependent

To dissect the effects of RAP on endocytic uptake, we took advantage of our CRISPR/Cas9 KO cells lacking megalin (*Lrp2* KO) or cubilin (*Cubn* KO). Cells were preincubated with or without apically added 0.5 μM RAP for 30 min before the addition of albumin (Fig. 2*A*) or dextran (Fig. 2*B*) as described earlier. At this low concentration of albumin and in the absence of RAP, uptake was more compromised by the absence of cubilin than of megalin due to their relative affinities for albumin (Fig. 2*A*). That order is reversed for dextran because uptake of a fluid-phase marker reflects only changes in endocytic flux, and KO of *Lrp2* has the greater effect. In parental OK cells, RAP inhibited albumin and dextran uptake by ∼73% and 61%, consistent with the results shown in Fig. 1. Similarly, RAP inhibited relative albumin and dextran uptake in *Cubn* KO cells to similar extents (80% and 47%, respectively). Strikingly, RAP did not affect albumin or dextran uptake in *Lrp2* KO cells. Similar megalin-dependent effects of RAP were observed on the uptake of lysozyme (unpublished observations). We conclude that the effect of RAP on endocytic flux requires megalin.

Uptake of dextran is linear over a broad concentration range in OK cells, consistent with its uptake via the fluid phase (33). To further confirm that dextran is not engaging megalin or cubilin receptors, we tested its uptake in the presence of

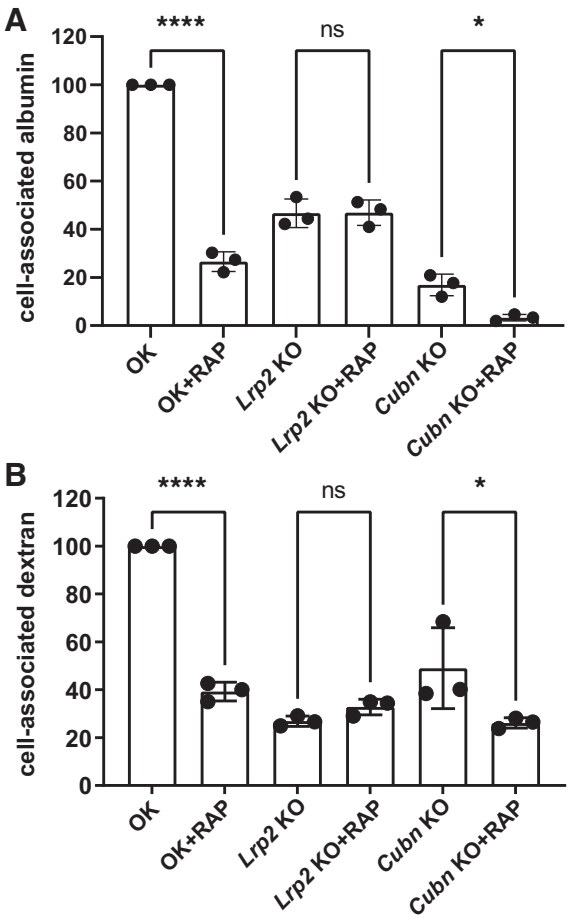

Figure 2. Inhibition of albumin and dextran uptake by receptor-associated protein (RAP) requires expression of megalin. Uptake of 40 μg/mL Alexa Fluor-648 albumin (*A*) or 0.5 mg/mL Alexa Fluor-568 dextran (*B*) in the presence or absence of 0.5 μM RAP was quantified in parental opossum kidney (OK), megalin (*Lrp2*) knockout (KO), or cubilin (*Cubn*) KO cells. Data from three independent experiments performed in duplicate or triplicate were normalized to control values (OK cells in the absence of RAP), and means ± SD are plotted. Significance was assessed by one-way ANOVA with multiple comparisons. ****$P < 0.0001$ and *$P < 0.05$; P values for *Lrp2* KO with or without RAP in *A* and *B* are >0.9999 and 0.9132, respectively. ns, not significant.

supraphysiological concentrations of albumin. In previous studies, we have found that albumin inhibits the uptake of numerous ligands, including hemoglobin, immunoglobulins, and β_2-microglobulin (5, 40). As shown in Fig. 3, dextran internalization in OK cells was not significantly changed by inclusion of up to 1 mg/mL albumin in the uptake medium. Similarly, albumin had little effect on dextran uptake in *Lrp2* KO or *Cubn* KO cells (Fig. 3). Thus, the behavior of dextran is consistent with that of a fluid-phase marker in our model. In addition, we conclude from these data that albumin binding to cubilin or megalin does not appreciably affect endocytic flux in OK cells.

Albumin Binding Affinity for Cubilin Receptors Is Independent of Megalin Expression and Unaffected by RAP

Because megalin and cubilin form coprecipitable complexes, RAP might affect their interaction with consequences to high-affinity binding of albumin to cubilin. Alternatively,

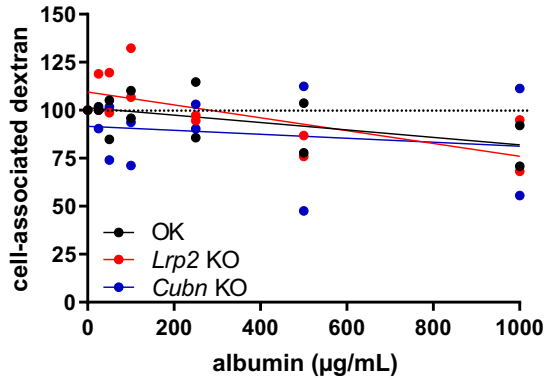

Figure 3. Proximal tubule (PT) endocytic flux is unaffected by ligand binding to PT receptors. Opossum kidney (OK; black), megalin (*Lrp2*) knockout (KO; red), and cubilin (*Cubn*) KO (blue) cells were incubated with 0.5 mg/mL Alexa Fluor-568 dextran in the presence of the indicated concentrations of unlabeled fatty acid-free albumin for 30 min, and cell-associated dextran was quantified by spectrofluorimetry. Normalized data from two independent experiments performed in duplicate are plotted together with the fitted linear regression [slopes/P values (vs. 0 slope)/R^2 values were −0.019/0.06/0.285, −0.033/0.01/0.461, and −0.0104/0.55/0.033 for OK, *Lrp2* KO, and *Cubn* KO cells, respectively]. The absence of any effect of albumin on dextran internalization demonstrates that dextran is an appropriate marker for fluid-phase uptake and confirms that albumin binding to receptors does not influence the endocytic rate of PT cells.

or in addition, the presence of numerous low-affinity albumin-binding sites on megalin might serve as a "sponge" to increase the local concentration of ligand and thus facilitate increased binding to cubilin in a manner inhibitable by RAP. If this were the case, the affinity of cubilin for albumin is predicted to be reduced in the absence of megalin expression or in the presence of RAP. We measured the binding of fluorescent albumin to cell surface receptors to test these possibilities. Alexa Fluor-647 albumin (0–100 μg/mL) was added for 1 h on ice to OK cells preincubated with or without 0.5 μM RAP or to *Lrp2* KO cells. Cells were washed rapidly, and cell-associated fluorescence was quantified. As shown in Fig. 4, albumin bound to the apical surface of OK cells with a K_m of 38.8 μg/

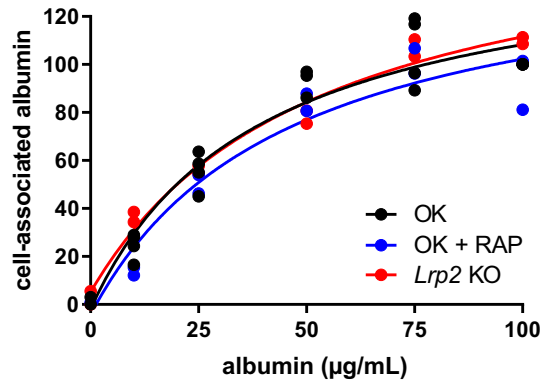

Figure 4. Neither megalin nor receptor-associated protein (RAP) affects albumin binding to cubilin. Albumin binding to cell surface cubilin receptors in parental opossum kidney (OK) cells (black) with or without 0.5 μM RAP (blue) and megalin (*Lrp2*) knockout (KO) cells (red) was assessed on ice as described in METHODS. Data were normalized to the binding of 100 μg/mL albumin in parental OK cells in each experiment. K_m values were 38.8, 45.3, and 52.2 μg/mL, with R^2 values of 0.951, 0,940, and 0.982 for OK, OK + RAP, and *Lrp2* KO, respectively, consistent with our results in previous studies.

mL, similar to previous reports (33, 41). Neither RAP nor the absence of megalin expression affected either the affinity or capacity of albumin binding. Together with previous data, we conclude that cubilin surface expression and engagement of albumin occur independently of megalin expression (13).

RAP Inhibits Albumin Binding to Megalin in Addition to Its Effect on Endocytic Flux

Based on our deconvolution experiments in OK cells, cubilin binding mediates the uptake of ~90% of albumin at the 40 μg/mL concentration we typically used in our endocytosis experiments (13, 33). To detect whether RAP competes for albumin binding to megalin in addition to its effect on endocytic flux, we tested the effect of RAP on albumin uptake in *Cubn* KO cells (Fig. 5). This maneuver allows us to remove the contribution of cubilin-mediated uptake, which is dominant in parental OK cells. If RAP is a competitive inhibitor for albumin binding to megalin, as it is for other ligands binding to LDL-R family members, we would predict higher apparent affinity compared with parental cells and complete inhibition of uptake at high RAP concentrations. Indeed, half-maximal inhibition of albumin uptake in *Cubn* KO cells occurred at 10 nM compared with 94 nM in OK cells. However, inhibition at 1 μM RAP was similar for parental and *Cubn* KO cells, and no further inhibition was observed at concentrations up to 10 μM RAP (not shown). These data demonstrate competitive inhibition of albumin uptake through megalin and also provide evidence for an additional RAP-insensitive albumin uptake pathway.

DISCUSSION

Deciphering the specific mechanism by which RAP impairs PT endocytosis is challenging for several reasons. First, no protein other than RAP itself has been conclusively identified to date that binds solely to megalin receptors (and not to cubilin). In addition, because both megalin and cubilin are required for maximal endocytic flux in PT cells, it is challenging to discern the direct effects of RAP on ligand binding from effects on general endocytic flux (13). In this study, we took advantage of recently generated CRISPR/Cas9 *Lrp2* and *Cubn* KO cell lines in a highly differentiated model of the PT to examine the mechanism of action of RAP. Surprisingly, our data show that RAP impairs uptake of both receptors and fluid-phase markers through the PT endocytic pathway. This effect is completely dependent on megalin. The effect is rapid, as we found no dependence on preincubation times between 0 and 6 h. Consistent with this, prolonged (6 h) incubation with RAP had no discernible effect on the structural integrity of apical endocytic compartments visualized by fluorescence staining of EEA1, Rab5, Rab7, and Rab11. In addition, we confirmed that RAP directly inhibits albumin binding to megalin receptors with high affinity. Moreover, we showed that RAP does not impact direct binding of albumin to cubilin receptors. Finally, our study demonstrated that endocytic flux is not affected by luminal albumin concentration. Together, our data highlight a newly appreciated effect of RAP on cultured PT cells and provide a new explanation for the confounding observation that RAP inhibits uptake of a wide variety of seemingly unrelated ligands. Our study sheds new light on how megalin drives endocytic flux in PT cells and shows that inhibited uptake of a ligand by RAP is insufficient evidence to demonstrate that it binds directly to megalin.

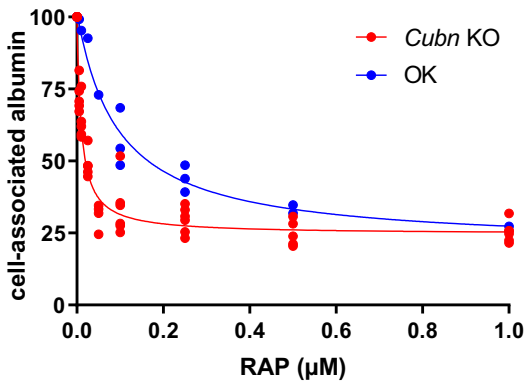

Figure 5. Receptor-associated protein (RAP) inhibits albumin binding to megalin in addition to dampening endocytic flux. The effect of RAP on the uptake of 40 μg/mL albumin was determined in cubilin (*Cubn*) knockout (KO) cells, where uptake is mediated solely by megalin receptors. Data combined from six independent experiments were normalized to uptake in the absence of added RAP (red). For comparison, data points from Fig. 1 showing dose-dependent RAP inhibition of 40 μg/mL albumin in parental opossum kidney (OK) cells (~90% of which is mediated by cubilin receptors) are replotted in blue. Nonlinear regression was used to calculate the apparent K_i of RAP on albumin uptake in each cell type (9.0 nM in *Cubn* KO cells vs. 75 nM in OK cells, R^2 values of 0.954 and 0.949, respectively). The lower K_i for albumin uptake in *Cubn* KO cells concentrations is consistent with a direct effect of RAP on ligand binding to megalin in addition to its global effect on endocytic flux.

Notably, RAP never fully inhibited uptake of albumin or dextran, even in *Cubn* KO cells where uptake via megalin is the only known pathway, and even at RAP concentrations 1,000-fold higher than the affinity we and others measured for RAP binding to megalin. Residual uptake plateaued at 25–30% for albumin in *Cubn* KO and parental OK cells. It is not surprising that endocytosis is not fully inhibited, as RAP itself is known to be internalized (42, 43). However, the absence of complete inhibition of albumin uptake by megalin in *Cubn* KO cells suggests the presence of an additional albumin-binding site on megalin or another receptor that is not inhibited by RAP. Consistent with this idea, a previous study using surface plasmon resonance reported residual transcobalamine-B12 binding to purified megalin after RAP inhibition, which was interpreted to represent ligand binding to a second site on megalin (21). The Fc γ receptor and transporter (FcRN) has been suggested to ferry albumin across the PT monolayer as a method to retrieve filtered protein (44, 45). However, whereas megalin is the highest expressed protein in OK cells cultured under orbital shear stress, these cells express essentially no FcRN (46, 47). Similarly, quantitative proteomic analysis of microdissected rat nephron segments revealed copious megalin and cubilin but no FcRN protein expression in the PT (35).

The effect of RAP on endocytic flux is functionally equivalent to silencing megalin expression and highlights an important nuance about megalin function in the PT. Rather than the common perception that loss of megalin dampens endocytic flux, our data show that expression of megalin is a positive driver of endocytosis above the baseline rate in PT cells. Remarkably, all steps along the endocytic pathway seem to be equivalently impacted by the presence of

megalin, as the steady-state number of surface cubilin receptors was unaffected by either *Lrp2* KO or addition of RAP. Moreover, the effect of RAP on endocytic uptake remained constant over a 6-h pretreatment period, suggesting that a new steady state level of endocytic flux is rapidly attained.

How might RAP function to disrupt endocytic flux? One possibility, similar to its proposed function in megalin biosynthetic delivery, is that RAP binding generates higher-order nonfunctional complexes of megalin that can traffic normally (48). Whether and how RAP binding impacts higher-order megalin assembly along the endocytic pathway and the consequences to membrane traffic remain to be determined. Another possibility is that RAP prevents a conformation-dependent shift in megalin needed to boost endocytic flux. RAP might inhibit the pH-dependent shift in megalin dimer structure that drives ligand release and splays apart the cytoplasmic tails of the protein. This conformational shift in megalin structure might be necessary for proper signaling to maximize endocytic flux. Related to this, RAP binding might alter the pH threshold required for megalin to undergo the conformational change thought to drive ligand release (3). Indeed, dissociation of RAP from LRP requires lower pH compared with other ligands (49). As a consequence, megalin appears unable to recycle via its usual route, as Percoll density gradient fractionation of megalin/RAP complexes internalized from the surface of L2 yolk sac carcinoma cells showed that they did not dissociate until they reached late endosomes, in contrast to lipoprotein lipase/megalin complexes, which dissociated in early endosomes (43). Such a change in megalin trafficking, if it occurs in PT cells, might affect spatial signaling to enhance flux. Deciphering which of these possibilities alone or in combination is responsible for the effects, we observed will require significant effort.

RAP has been suggested as a possible therapeutic reagent to limit PT accumulation of nephrotoxic ligands that bind to megalin. Indeed, Molitoris and colleagues recently reported protection from gentamicin-induced toxicity when rats were predosed with RAP before drug administration (25). This study also demonstrated a reversible effect of RAP on the uptake of fluorescent albumin and dextran. Whereas the effect on dextran uptake was ascribed to inhibition of a separate clathrin-independent pathway in the PT, our data demonstrate a common requirement for megalin expression in the inhibition of albumin and dextran uptake. Consistent with this, our previous studies have shown that dextran uptake occurs concurrently with albumin and presumably enters with fluid internalized in the large irregular invaginations that constitute PT endocytic pits (13, 50). Our finding that RAP globally inhibits apical endocytic uptake and its absolute dependence on megalin expression broadens the potential utility of this protein in selectively limiting PT uptake of harmful molecules.

DATA AVAILABILITY

Data will be made available upon reasonable request.

SUPPLEMENTAL DATA

Supplemental Figs. S1 and S2: https://doi.org/10.6084/m9.figshare.23519145.v1.

GRANTS

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DISCLOSURES

O.A.W. has served as a consultant for and received grant support for investigator-initiated research from Aktis Oncology and Judo Biosciences. None of the other authors has any conflicts of interest, financial or otherwise, to disclose.

AUTHOR CONTRIBUTIONS

O.A.W. conceived and designed research; K.R.L. and Y.R. performed experiments; K.R.L., O.B.K., and O.A.W. analyzed data; O.B.K. and O.A.W. interpreted results of experiments; Y.R. and O.A.W. prepared figures; O.A.W. drafted manuscript; K.R.L., Y.R., O.B.K., and O.A.W. edited and revised manuscript; K.R.L., Y.R., O.B.K., and O.A.W. approved final version of manuscript.

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