Embryos deficient in the morphogen Sonic hedgehog (Shh) or the endocytic receptor megalin exhibit common neurodevelopmental abnormalities. Therefore, we have investigated the possibility that a functional relationship exists between the two proteins. During embryonic development, megalin was found to be expressed along the apical surfaces of neuroepithelial cells and was co-expressed with Shh in the ventral floor plate of the neural tube. Using enzyme-linked immunosorbent assay, homologous ligand displacement, and surface plasmon resonance techniques, it was found that the amino-terminal fragment of Shh (N-Shh) bound to megalin with high affinity. Megalin-expressing cells internalized N-Shh through a mechanism that was inhibited by antagonists of megalin, viz. anti-receptor-associated protein and anti-megalin antibodies. Heparin also inhibited N-Shh endocytosis, implicating proteoglycans in the internalization process, as has been described for other megalin ligands. Use of chloroquine to inhibit lysosomal proteinase activity showed that N-Shh endocytosed via megalin was not efficiently targeted to the lysosomes for degradation. The ability of megalin-internalized N-Shh to bypass lysosomes may relate to the finding that the interaction between N-Shh and megalin was resistant to dissociation with low pH. Together, these findings show that megalin is an efficient endocytic receptor for N-Shh. Furthermore, they implicate megalin as a new regulatory component of the Shh signaling pathway.

Sonic hedgehog (Shh) is a secreted signaling molecule that is expressed in spatially restricted patterns during embryonic development. Shh signaling has been shown to regulate a wide range of developmental patterning events in Drosophila and vertebrates involved in lung (1), nervous system (2), eye (3), midbrain (4), and forebrain and facial (5, 6) morphogenesis. During early vertebrate development, Shh signaling at the midline leads to patterning of the ventral neural tube and adjacent somites. Mice lacking Shh activity have anomalies of midline structures such as the notochord and floor plate of the early brain (7). Later, these mice display an absence of ventral neuronal cells and cranial motor neurons (8). The result of errant Shh signaling in humans has been directly linked to basal cell carcinoma (9, 10) and holoprosencephaly (11).

Post-translational modification of the 45-kDa Shh polypeptide produces an ~19-kDa amino-terminal fragment (designated N-Shh) that has palmitic acid and cholesterol moieties covalently coupled to its amino and carboxyl termini, respectively (12–14). N-Shh is secreted and represents the biologically active form of the protein, capable of initiating signaling. The current model for Shh signaling involves a pair of multiple-pass plasma membrane proteins, Patched (Ptc or Ptc-1) and Smoothened (Smo) (reviewed in Ref. 15). Ptc functions as the Shh-binding subunit/receptor, and Smo as the signal transducing subunit. When bound to Smo, Ptc acts as a repressor of Smo signaling activity. Following N-Shh interaction with Ptc, bound Ptc releases from Smo and de-represses the signaling activity of Smo. The expression of Ptc-1, Gli-2, HNF3β, Nkx2.2, and netrin-1 has been shown to be activated by Shh, and genes including pax-3, gli-3, and ephrin A5 have been shown to be suppressed by Shh (16, 17).

Megalin (also known as gp330 and low density lipoprotein receptor-related protein (LRP)-2) is an endocytic receptor belonging to the low density lipoprotein receptor (LDLR) family (18). The receptor is expressed on apical surfaces of numerous epithelia, where it functions to mediate endocytosis of ligands, targeting them for lysosomal degradation or transcytosis (18). Mice deficient in the expression of megalin demonstrate the critical neurodevelopmental role for this protein (19). These mice display numerous craniofacial abnormalities, including absence of olfactory bulbs, absence of the corpus callosum, and fusion of forebrain hemispheres, collectively an holoprosencephaly phenotype (19). During development, megalin-deficient embryos (9.5 days postcoitus) have pronounced cell death in several structures, including cranial nerves, the neural crest, and the optic vesicle (19). The spectrum of defects that constitute the megalin-deficient phenotype suggests that megalin expression is required for normal viability of the neural epithelium at an early embryonic stage.

The phenotype of megalin-deficient mice suggests a role for megalin in regulating cell fate specification in the patterning of the neural tube and is consistent with phenotypes observed in mice deficient in Shh and the Shh signal transducer, Smo (8, 20). For example, Shh-deficient embryos lack cranial motor neurons (8). Inhibition of Shh signaling in the neural tube has been shown to result in extensive apoptosis of neural epithelial cells (21). Shh has also been shown to regulate proliferation and to inhibit differentiation of central nervous system precursor cells (22). Smo mutants also display neural tube-related defects, including increased apoptosis of cells within the neural
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Fig. 1. Megalin expression during early embryogenesis. Shown are confocal micrographs of a 16-h (A), a 24-h (B), and a 33-h (C–G) zebrafish embryo. In A, megalin was expressed in the central region of the developing eye cup (arrowhead). At all stages, megalin was expressed in the floor plate (A, B, and D, arrowheads). At 24 h, megalin was prominently expressed in the floor plate and more dorsally in cells that line the lumen of the neural tube (B, arrowhead). In a 33-h embryo, megalin was extensively expressed on the luminal surfaces of the ventricles of the midbrain (mb) and forebrain (fb), with intense staining beginning at the midbrain-hindbrain border (C and D, arrowheads). Megalin expression was intense on the interior epithelial surface of the otic vesicle of the developing ear (E). Megalin was also expressed in the paired pronephric ducts (F, arrowheads) of the forming kidney, where it was also associated with the luminal side of the epithelium. A frontal view of the developing oral region (G) shows that megalin was expressed in the ridge of the frontonasal process and maxillary processes (arrowheads).

Fig. 2. Comparative analysis of Shh and megalin expression during early embryogenesis. Shown are confocal micrographs of lateral views of 19-h (20-somite) zebrafish embryos stained with antibodies to N-Shh (A) and megalin (B). Both proteins were coexpressed in the floor plate of the brain (arrowheads). N-Shh was expressed in the notochord (A, arrowhead), whereas megalin expression was not detectable in the notochord (B, arrowhead). Cross-sectional views of regions caudal to the hindbrain (insets) show that megalin was expressed more dorsally in the neural tube, whereas N-Shh was confined to the floor plate.

Fig. 3. Analysis of the integrity of recombinant GST-N-Shh. A shows Coomassie Blue staining of 5 μg of GST-N-Shh and GST. B shows anti-N-Shh immunoblotting of 50 ng of each protein. C shows that GST-N-Shh was capable of stimulating C3H10T1/2 cells to express alkaline phosphatase (ALP), a marker of osteoblast differentiation. C3H10T1/2 cells were treated for 5 days with 111 nM commercial N-Shh, GST-N-Shh, or GST, and alkaline phosphatase levels were measured as described by Williams et al. (30).

and dibutyryl cAMP for 6 days as previously described (24). C3H10T1/2 cells (ATCC CCL226) were obtained from the American Type Culture Collection (Manassas, VA).

Antibodies—Rabbit polyclonal and mouse monoclonal antibodies to megalin (rb6286 and IH2) have been described previously (25). Rabbit anti-megalin IgGs were purified by protein G-Sepharose and megalin-Sepharose chromatography (26). Mouse monoclonal anti-receptor-associated protein (RAP) antibody 7F1 has been described previously (27). Mouse monoclonal anti-N-Shh antibody 5E1 IgG was isolated from the conditioned culture medium of a hybridoma cell line obtained from the Developmental Studies Hybridoma Bank (Johns Hopkins University School of Medicine and University of Iowa). Goat anti-glutathione S-transferase (GST) antibody was obtained from Amersham Biosciences. Fluorescein isothiocyanate- and indocarbocyanine (Cy3)-labeled secondary IgGs were purchased from Jackson Immunoresearch Laboratories, Inc. (West Grove, PA).

Proteins—Megalin was purified from porcine kidney as described previously (28). Human RAP was expressed in bacteria and purified as described by Kounnas et al. (29). Recombinant murine N-Shh (residues 25–198) was obtained from R&D Systems (Minneapolis, MN). A plasmid construct was created to express GST-N-Shh fusion protein in bacteria. Briefly, this involved using reverse transcription-PCR to generate a cDNA encoding amino acids 20–198 of Shh from a cDNA template prepared from day 9.5 postcoitus mouse embryo RNA. The Shh cDNA fragment was inserted into the bacterial expression vector pGEX-2TK (Amersham Biosciences) such that the resulting plasmid encoded a fusion protein composed of GST followed by a thrombin cleavage site (LVPGRS), a five-amino acid phosphorylation target site (RRASV), and the N-Shh polypeptide. The construct was transformed into BL21 bacteria, and the fusion protein was isolated using glutathione-Sepharose affinity chromatography. Recombinant GST was produced from cells transformed with the empty pGEX-2TK vector. Both recombinant protein preparations were adsorbed onto a Detoxigel endotoxin removing gel (Pierce). The biological activity of GST-N-Shh was assayed in C3H10T1/2 cells using the method of Williams et al. (30).

Radiolabeling of GST-N-Shh and GST-RAP—GST-N-Shh and GST-RAP were labeled with [γ-32P]ATP using heart muscle kinase (Sigma) based on the manufacturer’s recommendations and the protocol of Stefansson et al. (31). Briefly, 50 μg of protein was incubated with 125 units of heart muscle kinase in 1× heart muscle kinase buffer (20 mM Pipes (pH 6.5), 1 mM dithiothreitol, 20 mM NaCl, and 12 mM MgCl2) plus 0.1% denatured bovine serum albumin (BSA) and 50 μCi of [γ-32P]ATP (Amersham Biosciences) for 1 h. Labeled fusion protein was purified by size-exclusion chromatography using PD-10 columns. Typical specific activities were 0.5–2 × 107 cpm/nmol.

Whole-mount Embryo Immunolabeling—Zebrafish were maintained, and embryos were collected by standard methods (32). Embryos were fixed for 15 min in 4% paraformaldehyde in phosphate-buffered saline

EXPERIMENTAL PROCEDURES

Cells—Murine sarcoma virus-transformed Brown Norway rat yolk sac cells (BN cells) were provided by Dr. Pierre Verroust (Hospital Tenon, Paris, France). Mouse embryonic teratocarcinoma F9 cells (ATCC CRL1720) were differentiated by treatment with retinoic acid

Based on the manufacturer's recommendations and the protocol of Stefansson et al. (31). Briefly, 50 μg of protein was incubated with 125 units of heart muscle kinase in 1× heart muscle kinase buffer (20 mM Pipes (pH 6.5), 1 mM dithiothreitol, 20 mM NaCl, and 12 mM MgCl2) plus 0.1% denatured bovine serum albumin (BSA) and 50 μCi of [γ-32P]ATP (Amersham Biosciences) for 1 h. Labeled fusion protein was purified by size-exclusion chromatography using PD-10 columns. Typical specific activities were 0.5–2 × 107 cpm/nmol.

Whole-mount Embryo Immunolabeling—Zebrafish were maintained, and embryos were collected by standard methods (32). Embryos were fixed for 15 min in 4% paraformaldehyde in phosphate-buffered saline
FIG. 4. Enzyme-linked immunosorbent assay and competitive radioligand binding assay demonstrate that N-Shh binds to megalin and that RAP inhibits the binding. In A, enzyme-linked immunosorbent assay showed that both GST-N-Shh (○) and commercially available N-Shh (●) bound to megalin. In B, homologous ligand displacement assay (□) was used to demonstrate the interaction between 32P-labeled GST-N-Shh and megalin, and heterologous ligand displacement assay (●) was used to show that RAP inhibited binding of 32P-labeled GST-N-Shh to megalin. The curves shown in B were based on fits of the data calculated using the computer program Ligand.

Solid-phase Binding Assays—Enzyme-linked immunosorbent assay was performed essentially as described previously (33). Briefly, varying concentrations of N-Shh or GST-N-Shh in 150 mM NaCl, 50 mM Tris (pH 7.4), 3% nonfat milk, and 0.05% Tween 20 were incubated for 1 h at 37 °C in microtiter wells coated with megalin (3 μg/ml). Bound N-Shh was detected using monoclonal antibody 5E1, horseradish peroxidase-conjugated sheep anti-mouse IgG (Amersham Biosciences), and the chromogenic substrate 3,3′,5,5′-tetramethylbenzidine (Kirkegaard & Perry, Gaithersburg, MD).

For homologous ligand competition assays, 32P-labeled GST-N-Shh (1 nM) was incubated in microtiter wells coated with megalin (3 μg/ml) in the presence of increasing concentrations of unlabeled competitor (GST-N-Shh or RAP). All other conditions were similar to those described by Williams et al. (34). The algorithm Ligand (35) within SigmaPlot 7.101 was used to analyze the competition data and to determine dissociation (KD) and inhibition (Ki) constants for receptor-ligand interactions.

Kinetic Analysis of N-Shh-Megalin Binding—Kinetic analysis of the interaction of GST-N-Shh with purified megalin was performed using surface plasmon resonance (SPR) measurements made on a BIAcore 3000 instrument. BIAcore sensor chips (type CM5) were activated with a 1:1 mixture of 0.2 M N-ethyl-N′-3-dimethylaminopropylcarbodiimide and 0.05 M N-hydroxysuccinimide in water. Megalin (50 μg/ml, 83 nM in 10 mM sodium acetate at pH 4.8) was immobilized on a CM5 sensor chip using the amine coupling kit (BIAcore) as described by the supplier. Unreacted sites were blocked with 1 M ethanolamine (pH 8.5). The SPR signal from immobilized megalin generated BIAcore response units ranging from 20,000 to 28,000. Control flow cells were activated and blocked in the absence of protein. Binding was evaluated over a range of GST-N-Shh concentrations (25–500 nM) in 150 mM NaCl, 0.005% polysorbate 20, and 100 mM HEPES (pH 7.4) with and without 1 mM CaCl2 at 25 °C. Binding of GST-N-Shh to megalin-immobilized flow cells was corrected for binding to control flow cells. Binding data were fitted to a 1:1 Langmuir binding model using BIAevaluation Version 3.1 software (BIAcore).

To evaluate the effect of pH on the dissociation of megalin-ligand complexes, GST-N-Shh or RAP (each at 3 μM in buffer A (100 mM HEPES (pH 7.4) and 150 mM NaCl)) were passed at 10 μl/min for 2 min over sensor chips containing immobilized megalin. Subsequently, protein-free buffer A or sodium acetate buffer (pH 4.5; sodium ion concen-
Aliquots of BN cell extract were immunoblotted with anti-megalin IgG (H9262) or were incubated with RAP (1 lane 1 GST-N-Shh or GST (20 nM) in the presence of absence of RAP (1 M) for 2 h and immunostained with anti-GST antibody and fluorescein isothiocyanate-labeled anti-goat IgG (green). Nuclei were stained using TOTO-3 (blue). RAP treatment did not affect binding of GST-N-Shh to the cell, but inhibited its internalization. B shows that megalin was the principal RAP-binding protein present in detergent extracts of BN cells. Aliquots of BN cell extract were immunoblotted with anti-megalin IgG (lane 1) or were incubated with RAP (1 M), and the bound RAP was then detected with mouse monoclonal anti-RAP IgG (lane 2). No other RAP-binding proteins were evident even after prolonged exposure of the RAP overlay blot.

**FIG. 6.** N-Shh is endocytosed by BN cells, and uptake is inhibited by the megalin antagonist RAP. In A, BN cells were incubated with GST-N-Shh or GST (20 nM) in the presence of absence of RAP (1 M) for 2 h and immunostained with anti-GST antibody and fluorescein isothiocyanate-labeled anti-goat IgG (green). Nuclei were stained using TOTO-3 (blue). RAP treatment did not affect binding of GST-N-Shh to the cell, but inhibited its internalization. B shows that megalin was the principal RAP-binding protein present in detergent extracts of BN cells. Aliquots of BN cell extract were immunoblotted with anti-megalin IgG (lane 1) or were incubated with RAP (1 M), and the bound RAP was then detected with mouse monoclonal anti-RAP IgG (lane 2). No other RAP-binding proteins were evident even after prolonged exposure of the RAP overlay blot.

**FIG. 7.** Megalin antagonists inhibit uptake of 32P-labeled GST-N-Shh by BN cells and differentiated F9 cells. In A, BN cells were incubated for 2 h with 32P-labeled GST-N-Shh alone or in the presence of RAP (1 M). Cells were rinsed in DPBS, and then treated with serum-free medium containing 0.5 mg/ml trypsin, 0.5 mg/ml insulin, 5 mM selenic acid, and 5 mM selenic acid. After a 1.5-h incubation, the medium was replaced with serum-free medium containing 10% fetal bovine serum, nonessential amino acids, 100 units/ml penicillin, and 100 μg/ml streptomycin (complete medium). Cells were grown for 16 h at 37 °C and 5% CO2, and the medium was replaced with serum-free medium (Eagle’s minimal essential medium containing nonessential amino acids, 100 units/ml penicillin, 100 μg/ml streptomycin, 5 μg/ml insulin, 5 μg/ml transferrin, and 5 μg/ml selenic acid). After a 1.5-h incubation, the medium was replaced with serum-free medium containing 1.5% BSA and either GST-N-Shh (20 nM) or GST (20 nM) with or without competitors and cultured for 2 h. Competitors included RAP (1 M) and GST (1 M).

For immunological detection, GST-N-Shh- and GST-treated cells were rinsed in Dulbecco’s phosphate-buffered saline (DPBS) (pH 7.4), fixed for 20 min in 3.7% paraformaldehyde with 0.2% Triton X-100 in DPBS, and then rinsed in DPBS. Cells were incubated with 2% BSA in DPBS for 1 h, treated with goat anti-GST IgG at 1 μg/ml in 2% BSA in DPBS for 1 h and then with fluorescein isothiocyanate-labeled donkey anti-goat IgG at 3 μg/ml in DPBS for 1 h, and rinsed in DPBS. For nuclear staining, cells were treated with RNase A (100 μg/ml) for 20 min at 37 °C, rinsed in DPBS, and then treated with TOTO-3 (Molecular Probes, Inc. Eugene, OR) at 1 μg/ml in DPBS for 10 min at 37 °C. Cells were rinsed in DPBS, mounted in Vector Shield mounting solution (Vector Laboratories, Burlingame, CA), and then examined by laser scanning confocal microscopy.

**FIG. 8.** Heparin inhibits uptake of 32P-labeled GST-N-Shh by BN cells and differentiated F9 cells. In A, BN cells were incubated for 2 h with 32P-labeled GST-N-Shh alone or in the presence of heparin (1 M). In B, undifferentiated F9 cells (white bars) or F9 cells differentiated with retinoic acid and dibutyryl cAMP (RA/Bt2cAMP; black bars) were incubated for 2 h with 32P-labeled GST-N-Shh alone or in the presence of heparin (1 M).
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RESULTS

Neurodevelopmental Expression of Megalin—Despite indications that megalin is critical to neurodevelopment (19), little was known about the expression of the receptor during early development. Laser scanning confocal microscopic analysis of 16-h zebrafish embryos revealed that megalin was prominent in the floor plate of the neural tube (Fig. 1A, arrow) and on the apical surface of the optic cup (arrowhead). By 24 h, megalin expression was detected in cells of the ventral floor plate (Fig. 1B, arrow) and on the apical surface of cells lining the lumen of the neural tube (arrowhead). At 33 h, ventral floor plate expression persisted, and megalin was also extensively expressed on cells comprising the luminal surfaces of the forebrain and midbrain (Fig. 1D, arrowhead), with strong expression at the midbrain-hindbrain border (Fig. 1C, arrowhead). At the base of the midbrain, intense staining for megalin was seen at the most anterior extent of the floor plate (Fig. 1D, arrow). Outside the central nervous system, megalin was detected on the apical surfaces of cells lining the lumen of the otic vesicle of the developing ear (Fig. 1E, arrowheads). In the area of the developing mouth of 48-h embryos, megalin was distributed medially and laterally in the frontonasal and maxillary processes, respectively (Fig. 1G). These findings demonstrate that early embryonic expression of megalin occurs at specific organizing centers for morphogenesis, including the ventral neural tube, optic and otic vesicles, and orofacial regions.

Many of the observed embryonic sites of megalin expression were the same as those known to express Shh, including the ventral floor plate, eye, otic vesicle, and frontonasal process (2–5, 37). A notable exception was the absence of megalin expression in the notochord (Fig. 2A and B, arrowheads). Also, megalin expression in the neural tube extended more dorsally than Shh (Figs. 2 insets and 1B), detected in areas of the neural tube known to express the receptors for Shh, Ptc-1, and Ptc-2 (38). The results indicate that megalin is expressed in tissues that express Shh or in adjacent tissues regulated by Shh signaling. These observations support the possibility that a functional relationship exists between megalin and Shh during early neurodevelopment.

Megalin Is an N-Shh-binding Receptor —The similarity of megalin- and Shh-null phenotypes and the early embryonic distribution of megalin in relation to sites of Shh production led us to investigate whether megalin and N-Shh are capable of directly binding to one another. Enzyme-linked immunosorbent assay showed that recombinant GST-N-Shh (Fig. 3) and a commercial preparation of N-Shh bound to purified megalin with similar apparent affinities (Fig. 4A). Binding between GST-N-Shh and megalin was also tested using a homologous ligand competition assay. 32P-Labeled GST-N-Shh bound to megalin, and the binding was inhibited in a dose-dependent manner by the addition of unlabeled GST-N-Shh (Fig. 4B). A $K_d$ of 81.3 nm was obtained from fitting the data to a one-site model using the Ligand algorithm. Binding of 32P-labeled GST-N-Shh to megalin was also inhibited by RAP, a well established antagonist of megalin-ligand interaction (39). Interestingly, the RAP competition data could best be fit to a two-site model with $K_v$ values of 3.0 and 2341.9 nm. One interpretation of these findings is that RAP binds to megalin at multiple sites and that one of these binding interactions is a stronger inhibitor of N-Shh binding to megalin. Such an interpretation is consistent with the fact that the megalin family member LRP has multiple RAP-binding sites (34).

Binding of Shh to megalin was also evaluated using SPR. As shown in Fig. 5, GST-N-Shh bound to megalin immobilized on a sensor chip. GST alone displayed no measurable binding to megalin (data not shown). Optimal fitting of SPR data obtained from measuring the binding of various concentrations of GST-N-Shh to immobilized megalin was best achieved using a single class binding site model. As a result, an affinity constant ($K_d$) of 21 nm ($n = 7; \chi^2$ of fit < 10) was determined for GST-N-Shh binding to megalin in the presence of calcium. Recombinant N-Shh cleaved with thrombin to remove the amino-terminal moiety and commercial N-Shh were both found to bind megalin immobilized on a sensor chip with affinities similar to those observed for the fusion protein (data not shown).

Megalin Mediates Endocytosis of N-Shh—The role of megalin in mediating endocytosis of N-Shh was next evaluated. As shown in Fig. 6, confocal analysis of BN cells cultured in the presence of GST-N-Shh showed intracellular GST-N-Shh staining in a punctate pattern consistent with vesicular localization. Cells incubated with GST showed little to no intracellular staining (Fig. 6A). When BN cells were cultured in the presence...
Shh. RAP effectively inhibited internalization of 32P-labeled GST-N-Shh. The uptake of 32P-labeled GST-N-Shh was also evaluated in murine F9 cells. F9 cells express little or no megalin, but can be differentiated with retinoic acid and dibutyryl cAMP, causing induced megalin expression and decreased expression of other LDLR family members (24). As shown in Fig. 7A, differentiated cells exhibited an increased capacity to internalize 32P-labeled GST-N-Shh. RAP effectively inhibited internalization of 32P-labeled GST-N-Shh in differentiated F9 cells, but had little effect on the relatively low level of internalization in undifferentiated cells. These findings further support the interpretation that megalin mediates endocytosis of N-Shh.

N-Shh Endocytosis Involves Proteoglycans—In light of the fact that cell-surface proteoglycans have been implicated as partners with megalin and other LDLR family members in the uptake of numerous ligands (18), we investigated their possible involvement in N-Shh endocytosis. Heparin was an effective inhibitor of the uptake of 32P-labeled GST-N-Shh by both BN cells and differentiated F9 cells (Fig. 8, A and B). This suggests the involvement of cell-surface proteoglycans in the process of N-Shh endocytosis.

N-Shh Is Not Efficiently Targeted to Lysosomes by Megalin—One well characterized consequence of megalin-mediated endocytosis is targeting of ligands to the lysosome for degradation. Inhibition of lysosomal proteinase activity using the drug chloroquine did not inhibit 32P-labeled GST-N-Shh degradation in BN cells (Fig. 9). By contrast, in control experiments, chloroquine efficiently inhibited the degradation of 32P-labeled GST-RAP (Fig. 9), a megalin ligand that is targeted to the lysosomes following megalin-mediated endocytosis. Interestingly, there was a significant level of chloroquine-insensitive N-Shh degradation, suggesting that degradation of N-Shh may occur extracellularly.

Evaluation of Lowered pH upon Dissociation of the N-Shh-Megalin Complex—The effect of low pH on the dissociation of the N-Shh-megalin complex was evaluated by SPR on a BIAcore instrument. Little difference was evident in the dissociation rate constants (k\textsubscript{off}) for the N-Shh-megalin interaction under acidic versus neutral pH conditions: 1.3 × 10\textsuperscript{-3} and 1.28 × 10\textsuperscript{-3} s\textsuperscript{-1}, respectively (Fig. 10A). By contrast, dissociation of the RAP-megalin complex increased ~3-fold from 3.1 × 10\textsuperscript{-3} s\textsuperscript{-1} under neutral pH conditions to 8.36 × 10\textsuperscript{-3} s\textsuperscript{-1} under acidic pH conditions (Fig. 10B). These findings indicate that the N-Shh-megalin interaction is resistant to dissociation by acidic pH as low as 4.5 and suggest that N-Shh may not readily dissociate from megalin under acidic pH within endosomes.

DISCUSSION

Here, we have established that a functional relationship exists between the endocytic receptor megalin and the morphogen N-Shh. Specifically, we found that N-Shh binds to megalin with high affinity and that the interaction is resistant to dissociation by low pH. We have also shown that one consequence of the interaction is endocytosis of N-Shh. Megalin-mediated uptake of N-Shh can be blocked by heparin, suggesting the
involvement of heparan sulfate proteoglycans in the internalization process.

Heparan sulfate proteoglycans have been implicated in N-Shh signaling (40, 41) and in the process of megalin-mediated endocytosis of a number of its ligands (18). In the latter case, evidence suggests that heparan sulfate proteoglycans serve to sequester ligands at or near the cell surface and thereby either facilitate presentation of ligands to megalin or augment the affinity of ligands for megalin (18). Our observation that N-Shh appeared to accumulate pericellularly on BN cells after blocking the ligand-binding activity of megalin suggests the existence of an additional cell-surface or pericellular N-Shh-binding molecule. Considering recent evidence that Ptc is not detected at significant levels on the cell surface (42), this other N-Shh-binding component may very well be heparan sulfate proteoglycans.

The likely significance of the interaction of N-Shh with megalin is that it impacts Shh signaling. Three possibilities are that the interaction leads to 1) direct signal transduction by megalin, 2) modulation of the availability of N-Shh for its receptors, or 3) transcytosis of N-Shh important for long-range N-Shh signaling. Direct signal transduction by megalin is supported by recent evidence that other members of the LDLR family mediate signaling (43). For example, LRP has been shown to interact with the heparin-binding growth factor midkine and to regulate midkine-dependent survival of embryonic neurons (44). LRP has also been shown to interact with platelet-derived growth factor-BB and to function as a co-receptor in the process of platelet-derived growth factor signaling (45, 46). Additionally, the very low density lipoprotein receptor and apoE receptor-2 interact with the neuronal protein reelin and to regulate reelin-dependent survival of embryonic neurons (47).

In addition to megalin mediating long-range signaling via transcytosis of N-Shh, as discussed above, its ability to endocytose N-Shh may also impact N-Shh signaling in the early neural epithelium directly. Whether the mechanism for this involves effects on the bioavailability of N-Shh or on the regulation of Ptc as described above, the end result may be to influence N-Shh dependent survival and differentiation of neural epithelial cells (7, 8, 21). This hypothesis is supported by the megalin-deficient mouse phenotype, which demonstrates that megalin is required for normal viability and development of the neuroepithelium (19).

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