Low Density Lipoprotein Receptor-related Protein-1 (LRP1) Regulates Thrombospondin-2 (TSP2) Enhancement of Notch3 Signaling*§

He Meng†*, Xiaojie Zhang‡*, Soo Jung Lee*, Dudley K. Strickland*, Daniel A. Lawrence§, and Michael M. Wang¶

From the Departments of *Neurology, ‡Cardiology, and §Molecular and Integrative Physiology, University of Michigan, Ann Arbor, Michigan 48109-5622 and the ¶Department of Surgery, School of Medicine, University of Maryland, Baltimore, Maryland 21201

Intracellular trafficking of Notch and Notch ligands modulates signaling, suggesting that choreography of ligand and receptor translocation is essential for optimal Notch activity. Indeed, a major model for Notch signaling posits that Notch trans-endocytosis into the ligand-expressing (signal sending) cell is a key driving force for Notch signal transduction. The extracellular protein thrombospondin-2 (TSP2) enhances Notch signaling and binds to both Jagged1 and Notch3 ectodomains, potentially bridging two essential extracellular components of Notch signaling. We investigated the role of low density lipoprotein receptor-related protein-1 (LRP1), a TSP2 receptor, in the regulation of Notch3 signaling. TSP2 potentiates the activity of Notch as the receptor-associated protein (an inhibitor of low density lipoprotein receptor-related protein function) requires LRP1 expression in the signal-sending cell. TSP2 stimulates Notch3 endocytosis into wild type fibroblasts but not LRP1-deficient fibroblasts. Finally, recombinant Notch3 and Jagged1 interact with the LRP1 85-kDa B-chain, a subunit that lacks known ligand binding function. Our data suggest that LRP1 and TSP2 stimulate Notch activity by driving trans-endocytosis of the Notch ectodomain into the signal-sending cell and demonstrate a novel, non-cell autonomous function of LRP1 in cell-cell signaling.

Notch signaling is an evolutionarily conserved system that mediates direct, short range communication between adjacent cells. This pathway is required during development for cell type specification, which is essential for accurate cell patterning of almost all organs. Increasing evidence suggests that precise regulation of the quantity of Notch signaling is essential for homeostasis and development (dramatically demonstrated by Ref. 1). Thus, factors that modulate the efficacy of Notch signaling may be important in proper function of this important mediator of cell communication.

Unlike signaling systems that utilize soluble ligands, Notch signaling requires juxtaposition of membrane proteins expressed on two adjacent cells, commonly defined as the signal-sending and the signal-receiving cell. Mammalian Notch proteins are constitutively processed by furin, resulting in a noncovalently linked heterodimer (2, 3). Canonical ligands (the DSL (Delta/Serrate/Lag-2) proteins Jagged1–2 and Delta1, -3, and -4) bind to Notch proteins, resulting in sequential processing by two additional proteolytic cleavages (4, 5). Subsequently, the Notch intracellular domain migrates to the nucleus of the cells where it activates gene expression in the Notch signal-receiving cell.

The precise molecular mechanisms by which engagement of DSL ligands result in proteolytic Notch activation are under intense scrutiny. A large amount of work has been devoted to understanding the observation that endocytic function in the DSL-expressing cells is critical for Notch function in genetic studies (6–8). Two basic models have been proposed (9–11). One model proposes that endocytosis may modify DSL ligands, thereby activating them, but putative modifications that are hypothesized to occur after endocytic processing of DSL have not been identified.

An alternative model is that trans-endocytosis of Notch by the DSL ligand-expressing cells is critical for Notch activation. Specifically, a mechanical force provided by the endocytic machinery of the signal-sending cell may facilitate Notch dissociation and subsequent proteolytic cleavage. Indeed, cells expressing Drosophila ligand Delta, trans-endocytose Notch extracellular domain (12–14). Although earlier reports suggested that the uptake of Notch into DSL cells may reflect simple clearance of the protein, recent studies have suggested that trans-endocytosis occurs before regulated proteolysis and correlates with Notch activation (15). Recently, noncanonical Notch-interacting extracellular proteins MAGP were shown to activate signaling through heterodimer dissociation (16).

We recently demonstrated that the matricellular protein TSP2 binds to both Notch3 and Jagged1 and enhances Notch3 signaling (17). TSP2 increases the level of binding of Notch3 to Jagged1, suggesting that TSP2 amplifies Notch3 activity by enhancing the canonical Notch pathway. This study was initiated to identify mechanisms of TSP2 enhancement of Notch3 function. TSP2 acts on cells through multifaceted mechanisms. Multiple membrane receptors for TSP2 have been characterized, including CD36, CD47, HSPG, LRP1, and a variety of integrin subtypes (reviewed in Ref. 18). Each of these receptors...
LRP1 Potentiation of Notch Signaling

binds to a defined region of TSP1 or TSP2 and mediates a variety of cell and context-specific cellular processes.

Among these receptors, LRP1 is of particular interest because it mediates endocytosis of TSP2-matrix metalloproteinase complexes and facilitates their cell-mediated elimination from the extracellular space through endocytosis and targeting to lysosomes (19). Evidence that Notch signaling requires trans-endocytosis of the Notch ectodomain into the signal-sending cell has led us to test the hypothesis that LRP1 mediates TSP2 activation of Notch and facilitates Notch trans-endocytosis.

EXPERIMENTAL PROCEDURES

Cell Culture—MEF, PEA13 (22), 293A, L, Jagged-1, Delta-1, and primary human skin fibroblast cells were all cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum. H460 cells were grown in RPMI 1640 medium with 10% fetal bovine serum. Transfections were performed with Lipofectamine 2000 (Invitrogen) in the presence of serum unless noted. Cocultures involving H460 cells were performed in RPMI 1640-based media. Unless otherwise noted, cocultures were composed of 10,000 ligand-expressing cells freshly trypsinized and then layered onto one well of a 48-well dish that was 50% confluent with Notch3-expressing cells (H460) that had previously been cotransfected with HES-luciferase reporter and the phRG-TK reference reporter the day before. For siRNA studies, serum-starved human skin fibroblasts were transfected with siRNA the day before coculture with H460 cells. All cells were rinsed prior to coculture to remove transfection mixtures. Recombinant proteins TSP2 and RAP were added on the day of the coculture. Cells remained in coculture for 1 day prior to analysis of lysates by a Dual-Luciferase assay. Cell proliferation studies were performed according to a previously published protocol (17). Statistical analysis was performed using Student’s t tests with p < 0.05 as the threshold for significance.

Endocytosis and Trans-endocytosis Assays—A stable HRP-Notch3 293 cell line was first transfected with vector or TSP2 cDNA for 24 h and then incubated with labeled Notch3 protein (R & D Systems; for endocytosis assays) or signal-sending cells (for trans-endocytosis assays). For endocytosis assays, cells were shifted to 37 °C for defined periods prior to trypsin treatment to remove surface-adsorbed protein; endocytosed proteins were quantified by SDS-PAGE and direct imaging of labeled protein. For trans-endocytosis, signal-sending cells (murine) were separated from signal-receiving cells (human) by negative selection using TRA1–85 antibody and magnetic bead removal of human cells (MACS, Miltenyi Biotech). The remaining cells were analyzed for HRP-Notch3 content by immunoblotting. In some trans-endocytosis experiments, cells were detached from the plate and reseeded at a lower density for immunolocalization of HRP-Notch3 within the intracellular compartments; staining could not be performed at high densities used for coculture because of significant detachment from the plate.

Plasmids and siRNA—HES-luciferase is described in Ref. 20. pHRG-TK was purchased from Promega. Mouse TSP2 cDNA was obtained from Dr. Kurt Hankenson. Myc-tagged low-density lipoprotein receptor-related protein B-chain constructs were cloned in pSec-Tag (Invitrogen). siRNAs used to suppress LRP1 in human cells were synthesized by Ambion; the sequences have been described by Bu and co-workers (siRNA2 from Ref. 21).

Materials—RAP was obtained from Molecular Innovations. Notch3-Fc, Jagged, and Fc purified proteins were obtained from R & D Systems. Alexa700-succinimide was purchased from Invitrogen and used to label proteins as described before (17). DAPT (dissolved in DMSO) was obtained from Calbiochem. All other chemicals were obtained at the highest grade possible from Sigma. The TRA1–85 antibody to human CD147 was obtained from the Developmental Studies Hybridoma Bank (University of Iowa).

Transfected Cell Binding Assays—The binding of Notch3 and Jagged1 to the B-chain of LRP1 was assessed by binding labeled Notch3 and Jagged1 recombinant proteins to confluent HeLa cells transiently transfected with the LRP1 B-chain. After 24 h, the cells were transferred to 96-well plates for another 24 h. Another group of transfections was performed with empty vector as a reference for basal binding to HeLa cells. Binding reactions were performed at 4 °C. As another negative control, identical amounts of labeled Fc protein were bound to transfected cells to determine background binding of the Fc fragment, which is present in the recombinant Notch3 and Jagged1 proteins. After binding for 4 h, cells were washed quickly with two rinses of phosphate-buffered saline, and the amount of label remaining on cells was quantified with an Odyssey scanner. Data are presented by subtracting Fc binding values from Notch3 and Jagged1 levels.

Coimmunoprecipitation—293 cells were transiently transfected with plasmid combinations for 24–48 h. Cells were rinsed with phosphate-buffered saline and then lysed with modified RIPA buffer (50 mM Tris-HCl, pH 7.4; 1% Nonidet P-40; 0.25% sodium deoxycholate; 150 mM NaCl; 1 mM EDTA; 1 mM Na3VO4; 1 mM NaF) containing protease inhibitors (Halt protease inhibitor mixture kit, Pierce). Mouse monoclonal antibodies were obtained from Santa Cruz Biotechnology (against hemagglutinin and Myc) and from BD Biosciences (against TSP2); HRP antibodies from goat were obtained from Jackson ImmunoResearch. Lysates were mixed with 1 µg of antibodies for 4 h, followed by solid state capture using 40 µl of protein G-agarose. Immune complexes were washed five times with ice-cold phosphate-buffered saline and eluted by boiling with 30 µl of 2× protein sample buffer. Captured proteins were analyzed by immunoblotting using conventional techniques.

RESULTS

TSP2 Activation of Notch3 Signaling Requires LRP1—We utilized a coculture system to quantitatively measure the strength of Notch3 signaling. H460 lung cancer cells that express Notch3 but not other Notch genes were first transfected with the Notch reporter HES-luciferase with or without TSP2

---

3 The abbreviations used are: MEF, mouse embryonic fibroblast; HRP, horse-radish peroxidase; siRNA, small interfering RNA; RAP, receptor-associated protein.
cDNA. Notch3 was then activated by coculture with fibroblasts expressing Notch ligands. Finally, luciferase activity was measured to determine the strength of Notch3 signaling. In accordance with our prior studies (17), TSP2 transfection magnified Notch3 signaling in H460 cells cocultured with ligand-producing cells (Fig. 1A, 1st six bars).

TSP2 acts through multiple mechanisms mediated by a number of receptors, including LRPI. LRPI is expressed in all fibroblast cell lines used in these experiments; however, LRPI is not expressed in the H460 line (Fig. 1B). To investigate the role of LRPI in the enhancement of Notch3 signaling, we performed Notch3 activation experiments in the presence of RAP, which blocks LRPI. RAP blocked the ability of TSP2 to enhance Notch3 signaling without affecting the basal Notch activation by Jagged-1 or Delta-1 (Fig. 1A).

We confirmed the importance of LRPI in TSP2-dependent regulation of Notch by comparing the effects of coculture with MEF cells or PEA13 cells, which are genetically deficient in LRPI (22). Both MEF and PEA13 cells are capable of acting as signal-sending cells and stimulate an equivalent degree of canonical Notch activity, measured by HES-luciferase activity (Fig. 1C, white bars). TSP2 significantly increased signaling in H460 cells cocultured with MEF cells but did not increase Notch3 signaling when H460 cells were cocultured with PEA13 cells (Fig. 1C, black bars). RAP fully attenuated the ability of TSP2 to enhance Notch3 in cocultures with MEF cells (Fig. 1D).

To test whether LRPI in primary human cells was required for TSP2 stimulation of Notch3, we then tested human skin fibroblasts for their ability to stimulate H460 Notch signaling. Skin fibroblast LRPI was effectively knocked down by transfection of gene-specific siRNA but not control siRNA (Fig. 1E). TSP2 stimulated Notch3 activity in the presence of human skin fibroblasts treated with control siRNA. In contrast, TSP2 failed to stimulate Notch activity in H460 cells cocultured with fibroblasts transfected with LRPI siRNA (Fig. 1F). In summary, three independent lines of experimental evidence implicate LRPI in the signal-sending cell as a critical participant in TSP2 potentiation of Notch3 function.

Previously, we have shown that non-cell autonomous Notch signaling (Notch signaling mediated by coculture of signal-sending cells with Notch3-expressing cells) resulted in decreased cell proliferation of Notch3-positive H460 cells and that TSP2 enhanced Notch-mediated inhibition of cell proliferation (17). To investigate the role of LRPI in TSP2-stimulated
**LRP1 Potentiation of Notch Signaling**

---

**Figure 2.** TSP2-Notch effects on cell proliferation require LRP1. Prior to coculture, H460 cells were transfected with TSP2 cDNA or vector control. On the following day, H460 cells were cocultured with signal sending (ligand-expressing) cells for 48 h, and mitosis was quantified by bromodeoxyuridine incorporation. A, coculture with ligand-expressing Jagged1 cells reduced H460 cell proliferation. The antiproliferative effect of Jagged1 was potentiated by cotransfection with TSP2, which did not affect proliferation in the absence of Jagged1. Inclusion of RAP inhibited the TSP2-mediated inhibition of cell proliferation. Staining of cocultures is shown in supplemental Fig. 1. B, TSP2 reduces proliferation of H460 cells in the presence of MEF cells through Notch signaling. Proliferation of H460 cells was inhibited by coculture with MEF and TSP2 transfection. Retroviral expression of DN-MAML, a dominant negative inhibitor of canonical Notch signaling, failed to respond to TSP2 (Fig. 2B, 2nd pair of bars). The activity of TSP2 also required the presence of LRP1, because expression of TSP2 in H460 cells cocultured with PEA13 cells failed to suppress cell proliferation (Fig. 2C). Our results did not reflect competition of fibroblasts with H460 in coculture, because L and Jagged1 cells and MEF and PEA13 cells did not show significant differences in cell proliferation over the time courses we used (supplemental Fig. 2). In aggregate, our experiments demonstrate that TSP2 potentiation of Notch-dependent inhibition of proliferation of H460 cell requires LRP1.

---

**Low Density Lipoprotein Receptor-related Protein Mediates Trans-endocytosis of Notch Proteins**—Several factors led us to investigate whether LRP1 could participate in the clearance of Notch proteins. First, LRP1 plays an established role in clearance of multiple protein ligands through endocytic trafficking. Second, Notch signaling in multiple species appears to be coupled to endocytosis of the Notch ectodomain by the signal-sending cell. Given that TSP2 potentiation of Notch activity in H460 cells required LRP1 and that LRP1 is only expressed in the signal-sending cell (Fig. 1B), we hypothesized that LRP1 in the signal-sending cell may facilitate the internalization of the Notch ectodomain and provide a “driving force” that improves efficiency of intercellular communication.

We first tested whether LRP1 could mediate TSP2-dependent internalization of Notch3. Purified recombinant Notch3 ectodomain fragments were labeled with Alexa700-succinimide and applied to LRP1-expressing MEF cells. TSP2 significantly enhanced the internalization of Notch3 protein (Fig. 3A). TSP2 enhancement of Notch3 internalization was not observed in PEA13 cells (Fig. 3B). Consistent with these results, inhibition of LRP1 in MEF cells by addition of RAP prevented TSP2-stimulated internalization of Notch3 (Fig. 3C). In sum, these studies show that LRP1 expression stimulates TSP2-dependent Notch3 endocytosis.

We next investigated whether Notch3, expressed at the cell surface, could be endocytosed by LRP1-expressing cells in trans. To unambiguously identify Notch3 expressed by the signal-sending cell, we generated cell lines expressing HRP-tagged Notch3, which could be followed using HRP antibodies; the HRP-Notch3 in these cells functionally responded to Jagged1 and Delta1 (Fig. 4A). Cells lines expressing HRP-Notch3 (human cells) were cocultured with MEF cells (LRP1-expressing mouse cells). After cell coculture, the HRP-Notch3 and MEF cells lines were quantitatively separated using cell depletion mediated by magnetic capture using human-specific monoclonal antibody TRA1–85; the efficacy of this protocol is shown in supplemental Fig. 3. The remaining signal-sending MEF cells were analyzed by Western blotting and by immuno-
cytochemistry to test whether Notch3 could transfer from a signal-receiving cell (human HRP-Notch3 cell line) to a signal-sending cell (MEF).

HRP-tagged Notch3 was transferred to the signal-sending MEF cells under basal conditions. Addition of TSP2 to the culture media resulted in an increase in cellular HRP-Notch3 protein, indicating that trans-endocytosis is stimulated by TSP2 (Fig. 4B). On the other hand, although PEA13 cells internalized HRP-Notch3, this was not stimulated by TSP2 (Fig. 4B). RAP blocked the TSP2-stimulated transfer of HRP-Notch3 into MEF cells (Fig. 4C). Control studies indicated that at the detection level of immunoblotting, HRP was not internalized. Thus, the endocytosis of the HRP-Notch3 fusion was mediated by Notch3 residues (supplemental Fig. 4).

Immunostaining of cells confirmed that HRP-Notch3 protein transferred to ligand/LRP1-expressing cells, appearing in punctate structures within signal-sending cells (Fig. 5A). Examination of cocultures of MEF or PEA13 cells confirmed that TSP2 increased transfer of HRP-Notch3 only into LRP1-expressing cells (Fig. 5B). In aggregate, these studies demonstrate that LRP1 is involved in the TSP2 stimulation of Notch3 trans-endocytosis.
LRP1 Potentiation of Notch Signaling

FIGURE 5. Immunolocalization of Notch3 in signal-sending cells. We analyzed cells by confocal microscopy to identify intracellular transfer of trans-endocytosed Notch3. Cells were processed as in Fig. 4. A, top row, MEF cells that were not cocultured with 293-N3 cells did not react with TRA1–85 (red) or HRP (green) antibodies. Cocultures of MEF and 293-N3 cells showed expression of HRP-Notch3 in both cell types (middle row, red cells are signal-receiving cells). HRP-Notch3 reactivity was clearly within both signal-sending and signal-receiving cells. We confirmed the presence of HRP-Notch3 within signal-sending cells by analyzing cocultures after immunodepletion of 293-N3 cells (bottom row). DIC, differential interference contrast. B, MEF cells that were coincubated with HRP-Notch3-expressing cells transfected with TSP2 showed increased intracellular localization of HRP-Notch3 in signal-sending cells (green only TRA1–85-negative cells). In contrast, TSP2 did not increase trans-endocytosis of HRP-Notch3 into PEA13 cells. Cells were cocultured and then trypsinized to facilitate immunostaining, as cocultured cells at high density easily detached. All cells were stained with 4′,6-diamidino-2-phenylindole. Each experiment was performed at least three times with similar results.

LRP1 Directly Binds to Notch3 and Jagged1—Because LRP1 functionally interacts with Notch3 signal transduction, we tested whether the two proteins bind to each other. To date, Notch3 has been shown to bind canonical ligands (Jagged and Delta), TSP2 (17), and Notch3 itself4 through interactions with epidermal growth factor-like repeats. Sequence alignment of the epidermal growth factor-like repeats from all of these proteins revealed similarity to the epidermal growth factor repeats of Notch3 and enhances its interaction with Jagged1 (17). In new experiments described herein, we test whether the TSP2 receptor LRP1 participates in Notch function and describe three novel findings as follows. 1) LRP1 is required for TSP2 potentiation of Notch3 signaling. 2) LRP1 participates in trans-endocytosis of Notch. 3) Both Notch3 and Jagged1 bind directly to the 85-kDa B-chain of LRP1.

Role for LRP1 in Notch Signaling—LRP1 is a multifunctional receptor and a member of a family of receptors that subserves endocytic and signaling functions. The best described functions of LRP1 include ligand-specific endocytosis and clearance of a large range of secreted proteins (reviewed in Refs. 23, 24), which include but are not limited to TSP1, TSP2, tissue inhibitor of metalloproteinases (TIMP), matrix metalloproteinases, urokinase-type plasminogen activator, tissue plasminogen activator, and amyloid precursor protein. LRP1-mediated clearance requires the nucleation of endocytic components that result in the targeting of complexes to endosomes for recycling or for ultimate disposal by lysosomes.

In addition to endocytic function, LRP1 has rapid signaling properties (reviewed in Ref. 23), such as regulation of c-Jun N-terminal kinase (JNK) (25), disassembly of focal adhesions (26), and activation of cell contraction (27). These are presumed to be cell autonomous functions. Interestingly, the disassembly of focal adhesions is mediated by a three-protein complex involving LRP1, cell surface-expressed calreticulin, and TSP2 (28), demonstrating that the signaling functions of LRP1 involve the collaboration of multiple extracellular proteins.

Our studies implicate LRP1 in a new role in cell signaling; LRP1 modulates Notch signaling by virtue of its expression on the DSL-expressing cell (signal-sending cell), and thus, LRP1 has non-cell autonomous signaling function. Given the broad substrate specificity of LRP1, it may be of interest to examine a more general role for this protein in other signaling systems in which trans-endocytosis plays an important role.

The modification of Notch signal strength by LRP1 significantly expands the potential mechanisms by which Notch is regulated. The large molecular repertoire of LRP1 indicates that multiple extracellular ligands may impair the ability of LRP1 to enhance Notch function by competing for Notch/TSP2 binding. Moreover, engagement of LRP1 signaling results in phosphorylation of the intracellular domain and attenuation of endocytic function (29). As such, the broad range of LRP1 ligands may down-regulate TSP2-driven Notch signaling by

Second, the 85-kDa B-chain avidly bound Notch3 and Jagged1 in coimmunoprecipitation experiments (Fig. 6, C and D). Interestingly, TSP2 coprecipitation with LRP1 was enhanced by Jagged1 expression (Fig. 6D, bottom panel). We conclude that sequences in the B-chain of LRP1 are sufficient for protein-protein interactions with Notch3 and Jagged1.

DISCUSSION

Intracellular regulators of Notch and Notch ligand endocytosis are well known modulators of Notch signaling. In contrast, less is known about modulation of Notch proteins by extracellular proteins. Recent studies demonstrate that TSP2 is a novel extracellular Notch-binding protein that interacts with epidermal growth factor repeats of Notch3 and enhances its interaction with Jagged1 (17). In new experiments described herein, we test whether the TSP2 receptor LRP1 participates in Notch function and describe three novel findings as follows. 1) LRP1 is required for TSP2 potentiation of Notch3 signaling. 2) LRP1 participates in trans-endocytosis of Notch. 3) Both Notch3 and Jagged1 bind directly to the 85-kDa B-chain of LRP1.

4 H. Meng, X. Zhang, S. J. Lee, D. K. Strickland, D. A. Lawrence, and M. M. Wang, submitted for publication.
decreasing LRP1-dependent endocytic rates. Clearly, primary control of LRP1 expression could also influence Notch signal strength as well. Interestingly, LRP1 levels are increased in stroma of breast tumors (30), which are known to be modulated by Notch signaling.

**LRP1 Participates in Notch Endocytosis and Binds Notch and Jagged**—In addition to stimulating Notch activity, the TSP2/LRP1 partnership also activates trans-endocytosis of the Notch ectodomain. Previous work demonstrated that Notch is endocytosed into DSL-expressing cells in Drosophila (12–14). In addition, mammalian Notch1 is endocytosed into DSL-expressing cells (15), and this process is independent of ADAM proteolysis. Although a large array of endocytic proteins that participate in the process have been identified by genetic analysis (reviewed in Ref. 31), regulated extracellular and plasma membrane proteins involved in Notch endocytosis have yet to be identified. Our studies of TSP2/LRP1 regulations of Notch3 endocytosis demonstrate for the first time that extracellular factors (aside from Notch and its ligands) modulate trans-endocytosis and Notch function. This study provides additional support for the model proposed by Nichols et al. (15) that Notch activity is promoted by trans-endocytosis that mechanically stresses the Notch heterodimer, resulting in proteolytic activation of Notch.

Our experiments suggest that multiple molecular mechanisms drive trans-endocytosis of the Notch3 ectodomain. PEA13 cells are still able to functionally activate and endocytose the Notch3 ectodomain, although TSP2 does not enhance this process. RAP inhibition of LRP1 is sufficient to block TSP2-stimulated Notch3 endocytosis and activity, although basal activity of Notch (stimulated by Jagged alone) is not inhibited by RAP. Thus, two modes of endocytosis occur in the same set of cells, with TSP2/LRP1 regulation occurring in the trans-endocytic mode and RAP/LRP1 regulation occurring in the baseline activity of Notch.
LRP1 Potentiation of Notch Signaling

of cells: 1) LRP1 in the signal-sending cell is required for TSP2-mediated Notch signal enhancement; and 2) TSP2-independent signaling may proceed through mechanisms that are independent of LRP1 in the signal-sending cell; the molecules involved in the latter process remain to be elucidated.

What are the mechanisms by which LRP1-mediated trans-endocytosis of Notch is gated by TSP2? At first approximation, the process is analogous to what is seen with many LRP1 ligand complexes. For example, LRP1-mediated endocytosis of MMP2-TSP2 complexes (19) and plasminogen activator inhibitor-urokinase-type plasminogen activator complexes (32) is favored over MMP2 or urokinase-type plasminogen activator monomer endocytosis. We have shown that TSP2 not only enhances endocytosis but also increases the binding of Notch3 and Jagged1 (17). It is conceivable that TSP2 nucleates the formation of multimeric complexes involving Notch and Jagged; such a complex, predicted to have dramatically increased valency for binding to LRP1 (Notch and Jagged bind to LRP1 along with trimeric TSP2), would have significantly increased binding affinity for LRP1. In addition, it is possible that LRP1 and TSP2 binding to Notch3 and Jagged alters conformations in ways that further promote proteolytic processing and signaling of Notch. It is known that these molecules have functional and structural sensitivity to molecular perturbations induced by protein interactions (33), calcium binding (34, 35), and redox state (36–39), which may alter the conformation of the large postulated complex.

The increase in Notch3 signaling stimulated by TSP2 and LRP1 could also result from independent actions on different parts of LRP1. We show that Notch3 and Jagged1 form novel complexes with the 85-kDa A-chain of LRP1. TSP2 and all of the known ligands of LRP1 bind to one or more of the four characterized ligand binding domains of the A-chain. Thus, available data suggest that LRP1 could bind to TSP2 (via the A-chain) and Notch or Jagged (via the B-chain) at the same time, with the possibility that TSP2 simultaneously interacts with Notch3 and/or Jagged1.

RAP has conventionally been used as an inhibitor of LRP1 ligands; its wide success in inhibiting low density lipoprotein receptor-related protein function is likely a result of its ability to bind to multiple domains of the A-chain. All LRP1 ligands described to date bind to the A-chain. However, recent studies have disclosed several RAP-independent effects of LRP1 in peripheral nerve injury and neuropathic pain models (40). Our experiments have disclosed several RAP-independent effects of LRP1 in the signal-receiving cell; the molecules involved in the latter process remain to be elucidated.

In summary, our experiments demonstrate that TSP2 enhancement of Notch activity requires LRP1 activity. The ability of LRP1 to bind TSP2/Notch3/Jagged1 and to facilitate endocytosis of Notch3 suggests that LRP1 drives regulated trans-endocytosis of Notch3, resulting in enhanced signaling. These studies expand the mechanistic repertoire of LRP1 to include a role in non-cell autonomous short range cell signaling. Moreover, these studies suggest a novel link between the dual roles of LRP1 in endocytic function and in signal transduction.

Acknowledgments—We thank Ivan Maillard, Cyrille Alexandre, and Jean-Paul Vincent for reagents and Jim Borjigin and Kurt Hankenson for excellent discussions. TRA1–85 was obtained from the Developmental Studies Hybridoma Bank, University of Iowa.

REFERENCES

1. Schouwey, K., Delmas, V., Larue, L., Zimber-Strobl, U., Strobl, L. J., Radtke, F., and Beermann, F. (2007) *Dev. Dyn.* **236**, 282–289
2. Blummueller, C. M., Qi, H., Zagouros, P., and Artavasnis-Tsakonas, S. (1997) *Cell* **90**, 281–291
3. Logeat, F., Bessia, C., Brou, C., LeBail, O., Jarriault, S., Seidah, N. G., and Israel, A. (1998) *Proc. Natl. Acad. Sci. U.S.A.* **95**, 8108–8112
4. Brou, C., Logeat, F., Gupta, N., Bessia, C., LeBail, O., Doedens, J. R., Cumano, A., Roux, P., Black, R. A., and Israel, A. (2000) *Cell* **5**, 207–216
5. Mumma, J. S., Schroeter, E. H., Saxena, M. T., Griesemer, A., Tian, X., Pan, D. J., Ray, W. J., and Kopan, R. (2000) *Mol. Cell* **5**, 197–206
6. Baron, M., Aslam, H., Flasa, M., Fostier, M., Higgs, I. E., Mazaleyrat, S. L., and Wilkin, M. B. (2002) *Mol. Membr. Biol.* **19**, 27–38
7. Seugnet, L., Simpson, P., and Haenlin, M. (1997) *Dev. Biol.* **192**, 585–598
8. Hagedorn, E. J., Bayraktar, J. L., Kandachar, V. R., Bai, T., Englert, D. M., and Chang, H. C. (2006) *J. Cell Biol.* **173**, 443–452
9. Le Borgne, R., Bardin, A., and Schweigust, F. (2005) *Development* **132**, 1751–1762
10. Wilkin, M. B., and Baron, M. (2005) *Mol. Membr. Biol.* **22**, 279–289
11. Chitnis, A. (2006) *Dev. Dyn.* **235**, 886–894
12. Parks, A. L., Klueg, K. M., Stout, J. R., and Muskavitch, M. A. (2000) *Development* **127**, 1373–1385
13. Moorel, V., Le Borgne, R., and Schweigust, F. (2003) *Dev. Genes Evol.* **213**, 65–72
14. Klueg, K. M., and Muskavitch, M. A. (1999) *J. Cell Sci.* **112**, 3289–3297
15. Nichols, J. T., Miyamoto, A., Olsen, S. L., D’Souza, B., Yao, C., and Weinmaster, G. (2007) *J. Cell Biol.* **176**, 445–458
16. Miyamoto, A., Lau, R., Hein, P. W., Shipley, J. M., and Weinmaster, G. (2006) *J. Biol. Chem.* **281**, 10089–10097
17. Meng, H., Zhang, X., Hankenson, K. D., and Wang, M. M. (2009) *J. Biol. Chem.* **284**, 7866–7874
18. Bornstein, P. (2001) *J. Clin. Invest.* **107**, 929–934
19. Yang, Z., Strickland, D. K., and Bornstein, P. (2001) *J. Biol. Chem.* **276**, 8403–8408
20. Zhang, Y., Jia, L., Lee, S. J., and Wang, M. M. (2007) *Biochem. Biophys. Res. Commun.* **355**, 245–251
21. Li, Y., Lu, W., and Bu, G. (2003) *FEBS Lett.* **555**, 346–350
22. Wiltnow, T. E., and Herz, J. (1994) *J. Cell Sci.* **107**, 719–726
23. Lillis, A. P., Van Dyun, L. B., Murphy-Ullrich, J. E., and Strickland, D. K. (2008) *Physiol. Rev.* **88**, 887–918
24. Herz, J., and Strickland, D. K. (2001) *J. Clin. Invest.* **108**, 779–784
25. Lutz, C., Nimph, J., Jenny, M., Boecklinger, K., Enzinger, C., Utermann, G., Baier-Bitterlich, G., and Baier, G. (2002) *J. Biol. Chem.* **277**, 43143–43151
26. Orr, A. W., Pedraza, C. E., Pallero, M. A., Elzie, C. A., Goicoechea, S., Strickland, D. K., and Murphy-Ullrich, J. E. (2003) *J. Cell Biol.* **161**, 1179–1189
27. Takayama, Y., Takahashi, H., Mizumachi, K., and Takezawa, T. (2003) *J. Biol. Chem.* **278**, 22112–22118
28. Orr, A. W., Elzie, C. A., Kucik, D. F., and Murphy-Ullrich, J. E. (2003) *J. Cell Sci.* **116**, 2917–2927
29. Ranganathan, S., Liu, C. Y., Migliorini, M. M., Von Arnim, C. A., Peltan, I. D., Mikhailenko, I., Hyman, B. T., and Strickland, D. K. (2004) *J. Biol. Chem.* **279**, 40536–40544
30. Christensen, L., Wilborg Simonsen, A. C., Heegaard, C. W., Moestrup, S. K., Andersen, J. A., and Andreassen, P. A. (1996) *Int. J. Cancer* **66**, 441–452
31. Nichols, J. T., Miyamoto, A., and Weinmaster, G. (2007) *Traffic* **8**, 23054 JOURNAL OF BIOLOGICAL CHEMISTRY VOLUME 285 • NUMBER 30 • JULY 23, 2010
32. Stefansson, S., Muhammad, S., Cheng, X. F., Battey, F. D., Strickland, D. K., and Lawrence, D. A. (1998) J. Biol. Chem. 273, 6358–6366
33. Rodrigues, R. G., Guo, N., Zhou, L., Sipes, J. M., Williams, S. B., Templeton, N. S., Gralnick, H. R., and Roberts, D. D. (2001) J. Biol. Chem. 276, 27913–27922
34. Rand, M. D., Grimm, L. M., Artavanis-Tsakonas, S., Patriub, V., Blacklow, S. C., Sklar, J., and Aster, J. C. (2000) Mol. Cell. Biol. 20, 1825–1835
35. Turk, J. L., and Detwiler, T. C. (1986) Arch. Biochem. Biophys. 245, 446–454
36. Speziale, M. V., and Detwiler, T. C. (1991) Arch. Biochem. Biophys. 286, 546–550
37. Sun, X., Skorstengaard, K., and Mosher, D. F. (1992) J. Cell Biol. 118, 693–701
38. Hogg, P. J., Owensby, D. A., Mosher, D. F., Misenheimer, T. M., and Chesterman, C. N. (1993) J. Biol. Chem. 268, 7139–7146
39. Pimanda, J. E., Ganderton, T., Maekawa, A., Yap, C. L., Lawler, J., Kershaw, G., Chesterman, C. N., and Hogg, P. J. (2004) J. Biol. Chem. 279, 21439–21448
40. Gaultier, A., Arandjelovic, S., Li, X., Janes, J., Dragoljovic, N., Zhou, G. P., Dolkas, J., Myers, R. R., Gonias, S. L., and Campana, W. M. (2008) J. Clin. Invest. 118, 161–172