The C-terminal PDZ-Ligand of JAGGED1 Is Essential for Cellular Transformation

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JAGGED1 is a member of the Delta/Serrate/Lag-2 (DSL)1 family of proteins that are cell-bound ligands for Notch receptors. Initiation of Notch signaling occurs through a series of proteolytic events upon the binding of Notch to a DSL protein presented on neighboring cells. Whether DSL proteins themselves are capable of initiating an intrinsic signaling mechanism within the cell they are expressed is not known. Aberrant misexpression of JAGGED1 and DELTA1 has been documented in several human tumors; however, the mechanism by which misexpression of JAGGED1 contributes to oncogenesis has not been elucidated. We report that expression of human JAGGED1 transforms RKE cells in culture, therefore providing a model system to elucidate the function of DSL proteins. JAGGED1-mediated transformation occurs in a dose-dependent manner and requires a PDZ-ligand at the C terminus. Mutation of the PDZ-ligand did not affect the ability of JAGGED1 to initiate Notch signaling in neighboring cells. However, the PDZ-ligand is required for changes in the expression of JAGGED1 target genes and transcriptional activation of luciferase reporter constructs. Our data indicate the existence of a novel PDZ-dependent signaling pathway intrinsic to JAGGED1. We propose a bi-directional signaling model such that DSL proteins may have two distinct functions: to initiate Notch signaling in a neighboring cell and to initiate a PDZ-dependent signaling mechanism in the DSL-expressing cell. Moreover, we conclude that this intrinsic signaling mechanism of JAGGED1 may partly provide a link between aberrant misexpression of JAGGED1 and tumorigenesis.

A developing paradigm in signal transduction is that of the importance of cell-to-cell communication. Many fundamental regulatory decisions are mediated by signal transduction pathways that are initiated by the engagement of a receptor-ligand pair through cell-to-cell contact. The Notch signal transduction pathway, which governs cell fate decisions, is regulated primarily through this sort of mechanism (1–5). JAGGED1 is a member of the Delta/Serrate/Lag-2 (DSL) family of proteins that are thought to be cell-bound ligands that regulate Notch signaling (6–9). DSL genes encode Type I membrane-spanning proteins that have an extracellular domain consisting of multiple highly conserved EGF-like motifs (16 copies in human JAGGED1) and a conserved DSL domain that is rich in cysteine residues (Fig. 2A). The DSL family is classified into either the Delta-like or serratellagged subgroups. The defining motif of serratellagged DSL proteins is the presence of a cysteine-rich region (CR) between the EGF-like repeats and transmembrane domain. The intracellular domains of DSL proteins vary in length and are not conserved in primary amino acid sequence. For example, there is no sequence conservation between the 125 residues in the intracellular domain of JAGGED1 and the 154 residues in the intracellular domain of human DELTA1. Furthermore, the intracellular domains of DSL proteins do not share any significant similarities to other known proteins. However, deletions of the intracellular domains of serratellagged and delta result in similar mutant phenotypes in Drosophila, indicating that there is some function associated with this portion of the molecules (10–12). DSL proteins expressed on the surface of the signal-transmitting cell are thought to function by activating Notch in a neighboring signal-receiving cell (Notch-expressing cell). However, an important question remains to be addressed: Does the signal-transmitting cell itself receive a signal that can be transmitted through the expressed DSL protein? Interestingly, the C terminus of JAGGED1 encodes a putative PDZ (PSD-95/Dlg/Zo-1)-ligand (13). Therefore, a potential role for JAGGED1 in novel PDZ-dependent signaling mechanisms exists. However, biological or biochemical evidence for this type of intrinsic JAGGED1 signaling has not been observed. Although it is not clear if signaling events occur in both the DSL-expressing and Notch-expressing cells upon receptor-ligand binding, bi-directional signaling mechanisms such as the Eph/Ephrin pathway have been documented (14).

There are four mammalian Notch genes (Notch1–4) encoding membrane-spanning receptors that are activated through interaction with DSL proteins across cell boundaries. The binding of DSL and Notch proteins is thought to result in a conformational change that renders Notch susceptible to proteolytic processing mediated by metalloproteases such as TACE converting enzyme (TACE) (15–17). Constitutive presenilin-dependent γ-secretase activity is thought to then mediate an additional proteolytic cleavage that results in the release of the intracellular domain of Notch (NICD) from the plasma membrane.

1 The abbreviations used are: DSL, Delta/Serrate/Lag-2; EGF, epidermal growth factor; CMV, cytomegalovirus; aa, amino acids; GST, glutathione S-transferase; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; PDZ, PSD-95/Dlg/Zo-1.

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(16, 18, 19). Nε translocates to the nucleus and effects gene expression (5, 17, 19, 20). All four Notch receptors have been shown to undergo the constitutive y-secretase proteolysis, indicating that the specificity in signaling is not at this level of processing (21). Where the specificity in DSL-Notch signaling lies is not well understood. Although it is not known which DSL proteins can activate which Notch molecules, the DSL proteins Jagged1, Jagged2, and Delta1 have been reported to bind Notch2 and subsequently induce processing, indicating that multiple DSL proteins can activate a specific Notch molecule (22). Furthermore, soluble forms of the extracellular domain of Jagged1 can physically interact with Notch1, Notch2, and Notch3 in binding assays (23, 24). Taken together, these data indicate that Jagged1 can bind all Notch receptors leading to activation of signaling in a similar manner. One potential mechanism that provides specificity in signaling is at the level of ligand-receptor interactions. Fringe proteins are O-fucose-β1, 3-N-acetylglucosaminyltransferases that modify the extracellular domain of Notch, resulting in an increase in affinity for Delta proteins and a decrease in affinity for Jagged proteins (25–28).

Our laboratory has demonstrated that expression of activated NOTCH proteins (Nε) in RKE cells results in neoplastic transformation. Although there is evidence that JAGGED1 gene expression is altered in several human tumors, such as cervical and colon carcinomas, there is no evidence for a causal role in oncogenesis (29). Here, we report that expression of JAGGED1 results in cellular transformation of RKE cells in a dose-dependent manner. Both the extracellular and intracellular domains are required for this activity since expression of either a soluble form of the extracellular domain or a membrane-tethered intracellular domain fails to transform cells. Furthermore, JAGGED1-mediated transformation requires an intact C terminus that constitutes a PDZ-ligand. Our data indicate that cellular transformation by JAGGED1 expression is likely due to a PDZ-dependent signaling mechanism intrinsic to JAGGED1, providing evidence of a causal role for misexpression of JAGGED1 in oncogenesis and for a bi-directional mode of signaling in the Notch/DSL pathway.

EXPERIMENTAL PROCEDURES

Plasmids—peDNA expression vectors encoding human JAGGED1, DELTA1, and NOTCH1 were kindly provided by S. Artavanis-Tsakonas (29–31). MFG-LacZ and SVSV ectopic retroviral plasmids were kindly provided by R. C. Mulligan and D. R. Littman, respectively (32, 33). Human AF6 expression vector was kindly provided by L. Van Aelst (34). The pcDNA-Nε (Nε) and pcDNA-MATRIX-LIKE-1 (MAML1) vectors were described elsewhere (35).

The following expression plasmids were generated: pcDNA-AF6α203z (deletion of aa 994–1012), pcDNA-J2 (aa 1–1067), pcDNA-J2α300 (deletion of aa 24–1067), pcDNA-J2α300–mυc (J2α300 plus a C-terminal Myc tag), pcDNA-J2α300–mυc (addition of a C-terminal Myc tag) and CMV-Notchα2404 (aa 1–2444). Detailed description of cloning strategies and primer sequences are provided as Supplemental Information.

Jagged1 promoter reporter constructs were used in luciferase assays. For pJPro-α300–98, the cDNA sequence for Jagged1 was used to search the high throughput genomic sequence data base (HTGS) on NCBI. A PAC clone was found to encode the entire genomic locus of Jagged1, including sequences up and downstream. PAC RPC15 was obtained from The Sanger Centre. According to the genomic sequence, a SacI fragment encoded sequences 8 kb upstream of +1 and 2 kb downstream of the +1. This fragment was cloned into pcBlueScript (Stratagene). This clone was then digested with NotI, which digests the fragment immediately 3′ of the 3′ JagPro-xho primer (described below). This fragment was blunt ended using Vent polymerase and dNTPs at 72 °C for 20 min. This product was then digested using XhoI and cloned into pG3LBasic SacI/Smal. A 3.4-kb fragment of the Jagged1 promoter was isolated by PCR from HeLa genomic DNA using primers 5′ JagPro3.4 and 3′ JagPro. Sequences of the primers are 5′-ATTCACCTGGTGAGCTTGAGG-3′ and 5′-AGGGAAGGAGTCTTACCA-3′, respectively.

The PCR product was then cloned into the TA TOPO 2.1 vector (Invitrogen) following the manufacturer’s protocol. pJPro-α203z–α98 was then obtained from this clone using PCR primers 5′ JagPro0.7sacI and 3′ JagPro-xho. The sequences of these primers are 5′-GGCGGAGTCCCAAGATCCCTTCAAGTA-3′ and 5′-GGCGGTCGGAGCTCCGGCCTGTATTAC-3′, respectively. The PCR product was digested with restriction enzymes SacI and XhoI (NEBlod) and cloned into the luciferase vector pGL-basic (Promega). Promoter constructs pJPro-α203z–α98 and pJPro-α300–α98 were as isolated above using the PCR primers 5′ JagPro0.7sacI and 3′ JagPro-UTR (5′-GGCGGAGTCCCAAGTACGGTCTTCT-3′) or 5′ JagProUTR (5′-GGCGGAGTCCCAAGTACGGTCTTCT-3′) and 3′ JagPro-xho, respectively.

Glutathione S-transferase (GST) fusion protein expression constructs were generated by subcloning of the pG3L3 vector (Amerham Biosciences) to coding sequences for the intracellular domain of Jagged1 (GST-C-term 707–1118) or for the deletion mutant that lacks the PDZ-ligand (GST-C-term 557–1118, aa 1094–1218) or for the deletion mutant that lacks the PDZ-ligand (GST-C-term 557–1118, aa 1094–1213). pGEX-Ras was kindly provided by L. Quilliam (36).

Cell Culture and Retroviral Infections—RKE and RKE cells have been previously described (31, 37). All cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mm glutamine (DMEM). All cell culture reagents were purchased from Invitrogen.

In order to establish clonal lines, RKE cells were transduced with pSVSV DNA (10 μg) as indicated in the text using either Lipofectamine (10 μg, Invitrogen) according to manufacturer’s protocols or a modified BES-calcium phosphate method. Cells were seeded in media containing 400 μg/ml G418 (Invitrogen) to select for expression of drug resistance markers.

To analyze transformation efficiency by focus formation, 1 × 10⁶ to 1 × 10⁷ RKE-derived cells expressing Jagged1, 3′, 3′α300, or J1α300 were seeded with 1 × 10⁵ of parental RKE on 100-mm diameter plates. Cultures were maintained for 3 weeks, and DMEMc was replenished every 4–5 days. To enhance visualization of foci, cells were fixed in methanol and stained in 70% isopropl alcohol containing 0.5% methanol for 10 min.

The retroviral vector MFG-LacZ was packaged into retroviral particles by cotransfection of 293T cells with 5 μg of MFG-LacZ and 5 μg SVSV Eco plasmids using Lipofectamine (12 μg). Infections were performed with MFG-LacZ viral supernatant containing 8 μg/ml hexadimethrine bromide (polybrene, Sigma). Cells were washed three times with 1× phosphate-buffered saline prior to seeding non-infected parental RKE cells. Cells were fixed in 0.5% glutaraldehyde (Sigma) and stained with X-gal according to standard protocols to detect β-galactosidase activity in MFG-LacZ-infected cells.

Analysis of Protein Expression—Crude membrane fractions were prepared by hypotonic lysis at 4 °C in hypotonic lysis buffer (25 mm Hepes pH 7.5, 10 mm KC1, 0.5 mm dithiothreitol) supplemented with protease inhibitors (2 mm leupeptin, 5 μg/ml leupeptin, and 2 μg/ml aprotinin (Roche Molecular Biochemicals)). Following dounce homogenization with a B-type pestle (Kontes Glass Company), nuclei were pelleted at 1,600 × g for 10 min at 4 °C. Cell debris were removed from the crude cytoplasmic fraction (supernatant) by centrifugation at 13,000 rpm for 10 min at 4 °C. Membrane fractions were isolated by ultracentrifugation at 100,000 × g for 30 min at 4 °C. Membrane pellets were washed with 1 ml of hypotonic lysis buffer and solubilized in 200 μl of Nonidet P-40 lysis buffer (150 mm NaCl, 50 mm Hepes pH 7.4, 1.5 mm EDTA, 0.5 mm dithiothreitol, 10% glycerol, 0.1% Nonidet P-40, and protease inhibitors). Whole cell lysates were obtained by lysing cells in 1 ml of Nonidet P-40 lysis buffer. Lysates were centrifuged at 13,000 × g for 10 min at 4 °C. Protein concentrations were determined using the bichinoninic acid assay (Pierce).

Equal amounts of each lysate (20–25 μg) were separated in SDS-polyacylamide (8 or 14%) gels, followed by transfer to either nitrocellulose (Schleicher & Schuell) or polyvinylidene difluoride (Millipore) membranes. Western blot analysis was performed by immunoblotting with appropriate antibodies (31, 35) for Jagged1, for Myc, Ref. 38, Na+/K+ ATPase (KETTY) (kindly provided by Dr. Jerry Lingrel)). Proteins were visualized using the appropriate horseradish peroxidase-conjugated secondary antibodies (Jackson Laboratories) followed by enhanced chemiluminescence (ECL, Amersham Biosciences).

RT-PCR Analysis—Total RNA was extracted from confluent cultures using TRIzol reagent according to the manufacturer’s instructions. cDNA was synthesized from total RNA (2 μg) using M-MLV Reverse Transcriptase (Promega) with oligo(dT) primers. PCR reactions were performed in a 50-μl mixture containing 0.5 μM of each gene-specific primer, 1.5 mm MgCl2, 200 μM of each dNTP (Roche Molecular
Expression of JAGGED1 in RKE Cells Results in Cellular Transformation—There is now significant evidence that constitutively active forms of all four Notch receptors can contribute to oncogenesis (31, 40–44). We previously reported that expression of NOTCH	extsuperscript{IC} (N	extsuperscript{IC}) results in neoplastic transformation of E1A-immortalized rat kidney epithelial cells (RKE); in contrast, overexpression of wild-type NOTCH1 failed to transform these cells (31). The current model for Notch signaling proposes that activation of Notch occurs through ligand-induced proteolysis via cell-to-cell contact. We reasoned that wild-type NOTCH1 does not transform RKE cells due to a lack of ligand-induced activation, and therefore, we attempted to establish a coculture focus assay to test for ligand-induced transformation activity in the context of cell-to-cell contact. Although cell-to-cell contact failed to induce the transformation of NOTCH1-expressing cells, we observed that the JAGGED1-expressing cells displayed a transformed phenotype (data not shown and Fig. 1).

To confirm our initial observation that JAGGED1 expression resulted in transformation, several clonal cell lines expressing JAGGED1 were generated (Fig. 1). The JAGGED1 expression vector was transfected into RKE cells, and colonies were obtained by selecting for expression of the linked drug resistance marker. Colonies either displayed a transformed morphology, as indicated by a multilayered growth of cells, or they grew as a flat monolayer, which is consistent with a non-transformed phenotype (data not shown). Cell lines were established from six colonies that appeared morphologically transformed (J1, J3, J12, J4, J7, and J8) and three colonies that appeared to have a flat non-transformed morphology. To determine if the
**Fig. 2.** Deletion of either the extracellular or intracellular domain of JAGGED1 results in the loss of JAGGED1-mediated transformation. A, schematic diagram of JAGGED1: Delta-Serrate-Lag2 (DSL), EGF-like repeats (EGF), cysteine-rich (CR), and transmembrane (TM) domains are indicated. J**

\(^{ex}\)**: a soluble form of the extracellular domain, J**

\(^{tmic}\)**: a membrane-tethered intracellular domain. A Myc epitope (m) was fused to the C-terminal ends of the J**

\(^{ex}\)** and J**

\(^{tmic}\)** deletion mutants. B, focus formation by J**

\(^{ex}\)**, J**

\(^{tmic}\)**, and JAGGED1-expressing (J12) clonal cells (1 \(\times\) 10\(^6\)) was tested in a coculture focus assay with 1 \(\times\) 10\(^6\) parental RKE cells (Ratio = 1:100). C, Western blot analysis of J**

\(^{ex}\)**, J**

\(^{tmic}\)**, and JAGGED1-expressing (J12) clonal cell lines. Proteins were detected in the appropriate subcellular fractions from the following samples: media (0.05% v/v), membrane (mem, 20 µg of protein) and cytoplasmic (cyto, 20 µg of protein) fractions. D, comparison of J**

\(^{ex}\)**, J**

\(^{tmic}\)**-myc, and JAGGED1 protein expression. Whole cell lysates (25 µg of protein) were analyzed for the appropriate proteins. Anti-Jagged1/TS1 antibody was used to detect JAGGED1 in J12 cell lysates (top right) and anti-Myc/9E10 antibody was used to detect J**

\(^{ex}\)** (top left). To compare the avidities of anti-Jagged1 and anti-Myc antibodies for their epitopes, lysate from clonal cell line expressing a Myc-tagged J**

\(^{tmic}\)**-myc, which contains both epitopes, was immunoblotted with both antibodies (bottom panel). Molecular mass markers are indicated to the left (kDa).

JAGGED1-expressing clonal cell lines were transformed, J1 and J12 clonal cell lines were maintained in culture for 3 weeks. J1 and J12 cells did not exhibit contact inhibition and produced a dense multi-layered mat of cells compared with the control plate of parental RKE cells, which formed a uniform monolayer (Fig. 1A). The intensity of methylene blue staining was greater on the J1 plate compared with J12 plate, indicating that J1 cells grew to a greater cell density. Since a dense layer of cells was formed by JAGGED1-expressing cells, it was difficult to assess the differences in transformation efficiency among the clonal cell lines. To determine the degree of transformation efficiency, 1 \(\times\) 10\(^4\) clonal cells were plated in the presence of excess parental RKE cells. Five of the six clonal cell lines that initially displayed a transformed morphology formed foci (Fig. 1B). However, the number and size of the foci were different among the clonal cell lines, indicating that there was a variation in transformation efficiency. J1 and J3 cell lines produced a greater number of foci compared with J12 and J4 cell lines, whereas parental RKE cells and drug resistant clones displaying a non-transformed morphology did not form foci (Fig. 1B and data not shown, respectively).

Crude membranes extracted from the clonal cell lines were analyzed for JAGGED1 expression (Fig. 1C). J1 and J3 cells, which displayed the highest transformation efficiency, expressed the highest level of JAGGED1. In contrast, expression of JAGGED1 was lowest in the J4 clone, which produced the fewest foci, whereas J12 cells displayed an intermediate level of JAGGED1 expression and transformation efficiency, indicating that there is a link between expression and transformation efficiency (Fig. 1C). No JAGGED1 expression was observed in either the J7 clonal cell line that did not form foci or in the drug resistant clones that displayed a non-transformed morphology (Fig. 1 and data not shown). Na\(^+\)/K\(^+\) ATPase expression in the crude membrane fractions is shown as a normalization control for these samples (Fig. 1C). To confirm that the JAGGED1-expressing cell was the transformed cell type in the coculture, JAGGED1-expressing cells were infected with MFG-LacZ retrovirus to mark these cells with \(\beta\)-galactosidase activity prior to coculturing with parental RKE cells. Following focus formation, cells expressing \(\beta\)-galactosidase were visualized by staining with X-gal. The presence of blue foci confirmed that JAGGED1-expressing cells were the transformed cells (Fig. 1D). Furthermore, unstained RKE cells were excluded from the foci and located only in the surrounding monolayer, indicating that only the JAGGED1-expressing cells were transformed.

Deletion of Either the Extracellular or Intracellular Domain of JAGGED1 Results in the Loss of Transforming Activity—To determine the functional domains in JAGGED1 that are necessary and/or sufficient for transformation, we generated deletion mutants of JAGGED1 that separate the extracellular and
intracellular domains (Fig. 2A). While the extracellular domain
is thought to function by binding Notch to initiate signaling, the
intracellular domain has not been associated with any known
function. The extracellular domain of Jagged1 was con-
structed as either a membrane-tethered or soluble protein. The
membrane-bound form of the extracellular domain was trans-
ferred into RKE cells, and isolated drug-resistant cells did not
form foci in transformation assays. However, we were not able
to detect expression of the membrane-tethered protein using an
antibody against the C-terminal Myc tag, but we did detect
protein expression in transiently transfected 293T cells, indi-
cating that the Myc tag must have been cleaved in the clonal
RKE cells (data not shown). Clonal cell lines that expressed
the soluble form of the extracellular domain (Jex) and a membrane-
tethered form of the intracellular domain (Jmic) were gener-
ated and tested for transformation. While focus formation of
J12 cells was readily visible, there were no foci on plates
containing either Jex or Jmic clonal cell lines (Fig. 2B). Western
blot analysis revealed that Jex was found in the media and that
Jmic was targeted to the plasma membrane (Fig. 2C). Fur-
thermore, the expression levels of Jex and Jmic deletion mutants
were greater than the expression of JAGGED1 in the trans-
fected J12 cells (Fig. 2D, top panel). In order to compare the
expression levels of Jex and JAGGED1 using two different
antibodies (anti-Myc and anti-Jagged1 antibodies, respective-
ly), we determined the avidities of these antibodies using a
Myc-tagged Jmic (Jmic-myc), which contains both epitopes.
Western blot analysis of equal amounts of protein from
Jmic-myc cell lysates showed that the signal for Jmic-myc was
slightly more intense with the anti-Jagged1 Ab in comparison
to the anti-Myc Ab indicating that these antibodies have sim-
ilar avidities and can be used for comparing expression levels
(Fig. 2D, bottom panel). Therefore, the expression level of Jex
is much greater than JAGGED1 expression in J12 cells (Fig. 2D,
top panel). In addition, high levels of soluble Jex protein were
found to be stable in the media for at least 3 days (data not
shown). These results indicate that transformation by
JAGGED1 requires an intact protein, supporting that both the
extracellular and intracellular domains might have important
functions.

Transformation by JAGGED1 Requires a C-terminal PDZ-
Ligand—A putative binding site for PDZ-domain proteins was
identified at the C-terminus of JAGGED1 through sequence
comparison with a known PDZ-ligand, EphB2 receptor-protein
tyrosine kinase (Fig. 3A) (13). To determine if these residues
were required for transformation by JAGGED1, we generated a
mutant that has a deletion of the six C-terminal residues that
comprise the PDZ-ligand (J1ΔPDZ). Deletion of the putative PDZ-
ligand resulted in loss of transformation activity, indicated by
the inability of J1ΔPDZ-expressing cells (cl. 4 and cl. 2) to form
foci compared with the transformed J1 and J12 cell lines, which
displayed a matching level of expression, respectively (Fig. 3,
B and C). These results indicate that JAGGED1 may mediate
an intrinsic signaling mechanism involving downstream PDZ
proteins.

The presentation of JAGGED1 proteins to Notch results in
the initiation of Notch signaling in the neighboring cell. This
interaction is thought to be mediated by the extracellular do-
main of JAGGED1; however, it is not known if the intracellular
domain is necessary to cluster and present JAGGED1 proteins.
Whether or not the PDZ-ligand at the C terminus of JAGGED1
is required for ligand presentation remains unknown. In order
to determine if the deletion mutation of the PDZ-ligand did not
effect ligand-induction of Notch proteins, a coculture luciferase
assay was used to measure ligand-induced Notch activity as
described by Wu et al. (45). Briefly, HeLa cells were transiently
cotransfected with a CSL-Luciferase reporter, and expression
plasmids for NOTCH1ΔPEST and MAMLL1. NOTCH1ΔPEST
that lacks the PEST domain (aa 2445–2555) was used in these
assays to generate a more stable form of Notch. We previously
reported that co-expression of MAMLL1 enhances Nγ-induced
transactivation of a CSL reporter gene; therefore, MAMLL1 was
added to this assay to increase detection of ligand-induced Notch
activity (35). 293T cells were transiently transfected with pcDNA vector, EGFR, JAGGED1 (D1), J1ΔPL, or
DELTA1 (D1) and subsequently cocultured with transfected
HeLa cells for 12 h. The J1, D1, and J1ΔPL proteins were able
to induce Notch transactivation by 2.0-fold over pcDNA, indi-
cating that J1ΔPL retained the ability to activate Notch signal-
ing. As a control, we demonstrated that 293T cells expressing
EGFR do not activate Notch signaling (Fig. 3D). Therefore,
deletion of the six C-terminal residues comprising the PDZ-
ligand does not affect the ability of JAGGED1 to initiate Notch
signaling across cell boundaries. However, the PDZ-ligand is
essential for transformation, indicating that there is a poten-
tial for JAGGED1 to initiate Notch signaling in a neighboring
cell and to mediate a PDZ-dependent signaling mechanism
within the JAGGED1-expressing cell.

To determine that the C terminus of JAGGED1 is capable of
binding to PDZ-domain proteins, GST pulldown assays were
performed with GST fusion proteins expressing either the in-
tracellular domain of JAGGED1 with or without the PDZ-
ligand (GST-Jc and GST-JcΔPDZ, respectively). The PDZ-domain
protein AF6 was previously reported to interact with the amino
acids RMELYIV at the C terminus of Jagged1 in a directed yeast
two-hybrid analysis (13). Moreover, AF6 is the mammalian
homolog of Drosophila canoe, which has been genetically linked
to the Notch pathway (46). Therefore, we tested the ability of
the GST-JAGGED1 fusion proteins to bind AF6. While GST-Jc
bound to AF6, GST-JcΔPDZ did not efficiently pulldown AF6,
indicating that the interaction requires the PDZ-ligand (Fig. 3E).
To determine if the PDZ-domain of AF6 was necessary for
this interaction, we generated a mutant AF6 lacking this
domain (AF6ΔPDZ). Although GST-Jc did not pulldown AF6ΔPDZ,
this mutant retained its ability to bind GST-Ras to a similar
extent as AF6, indicating that the structural integrity of AF6
was not compromised in general (Fig. 3F). Therefore, the in-
teraction of JAGGED1 and AF6 occurs in a PDZ-dependent
manner, indicating that JAGGED1 has the potential to signal
through interacting PDZ-domain proteins.

Expression of JAGGED1 Results in Changes in Gene Expres-
sion—To determine the expression levels of Notch signaling
components, RNA was extracted from parental RKE and RKE-
derived clonal cell lines and analyzed by RT-PCR. Primers
were designed to specifically analyze rat mRNA and not mRNA
derived from the ectopic human cDNA in the clonal cell lines. A
basal level of mRNA for Notch1, 2, and 3, Delta 1, Jagged1 and
2, and Radical Fringe was detected in RKE cells (Fig. 4). We
observed that Notch3 and Jagged1 were induced in J1 and
Nγ-expressing cells compared with parental and NOTCH1-
expressing (N) RKE cells, while mRNA levels for Notch1,
Notch2, Jagged2, and Radical Fringe were not affected (Fig.
4A). β-actin mRNA was amplified to serve as a normalization
control for these samples. To show that induction of Notch3 and
Jagged1 gene expression was not due to clonal variation, we
analyzed mRNA isolated from several JAGGED1-expressing
RKE cell lines (J1, J8, J3, and J4) (Fig. 4B). The mRNA levels
of Notch3 and Jagged1 were greater in J1, J8, and J3 clonal
lines, which expressed the highest levels of JAGGED1. In con-
trast, there was only a minor induction of Notch3 and Jagged1
gene expression in the J4 clonal cell line, which expressed the
lowest level of JAGGED1 (Figs. 4B and 1C, respectively). Al-

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though Notch3 and Jagged1 gene expression is up-regulated in N\textsuperscript{cE}-expressing cells, there is no evidence indicating that these genes are direct targets of Notch signaling. Our data indicate that JAGGED1 may be initiating a signal to downstream effectors that cause changes in gene expression.

In order to determine the importance of the PDZ-ligand in regulation of gene transcription, RT-PCR analysis was performed using total RNA extracted from parental RKE and the clonal cell lines J1 and J1\textsuperscript{APL} cl. 4 (Fig. 4C). mRNA levels for both Jagged1 and Delta1 were up-regulated only in the J1 cells and not in J1\textsuperscript{APL} cells. However, the level of Notch3 gene induction was similar in both J1 and J1\textsuperscript{APL} cl. 4 cell lines, indicating that the PDZ-ligand is required for induction of Jagged1 and Delta1, but not required for induction of Notch3. RT-PCR analysis of Radical Fringe is shown for normalization. These data demonstrate the importance of the PDZ-ligand of JAGGED1 for mediating induction of certain JAGGED1 target genes. Moreover, this demonstrates that induction of gene expression by JAGGED1 may involve distinct signaling pathways.

To determine if the induction of Jagged1 mRNA in cells is a consequence of transcriptional activation of the gene, we assayed the ability of JAGGED1 to induce transcription of Jagged1 promoter reporter constructs. There was a 2-fold induction of the reporter constructs containing an 8-kb genomic DNA fragment (pJ1pro\textsuperscript{8000/98}) and a 531-bp genomic DNA fragment (pJ1pro\textsuperscript{321/98}) in cells expressing JAGGED1 compared with the vector control, indicating that the JAGGED1 responsive element was contained between the sequences −629 to −98-bp upstream of the start codon (Fig. 5A). In contrast, there was a lower activation by JAGGED1 of the reporter construct pJ1pro\textsuperscript{321/629} that lacks the sequences between −321 and −98 bp (Fig. 5A). Moreover, JAGGED1 induced the reporter activity by 7-fold using a construct containing the sequences −321 to −98 (pJ1pro\textsuperscript{321/98}), indicating that these sequences contain the JAGGED1 responsive element. To further demonstrate that the PDZ-ligand is required for Jagged1 gene expression, we compared the abilities of JAGGED1 (J1), JAGGED1\textsuperscript{APL} (J1\textsuperscript{APL}), and a C-terminal Myc-tagged JAGGED1 (J1\textsuperscript{MYC}) to activate the Jagged1 promoter reporter constructs, pJ1pro\textsuperscript{629/98} and pJ1pro\textsuperscript{321/629} J1 mediated a 2–4-fold activation of these reporters, whereas J1\textsuperscript{APL} and J1\textsuperscript{MYC} did not activate the Jagged1 promoter (Fig. 5B). Since the PDZ-ligand must be at the C terminus, the addition of a C-terminal Myc tag potentially blocks binding of PDZ-domain proteins to Jagged1, indicating that the PDZ-ligand serves to mediate a downstream signaling pathway through PDZ-domain proteins. These data indicate that there is a vital role for the PDZ-ligand of JAGGED1 to mediate changes in Jagged1 gene expression. Since N\textsuperscript{cE}-expressing cells have an increased level of Jagged1 mRNA, we assayed the ability of N\textsuperscript{cE} to activate the Jagged1 promoter reporter. However, N\textsuperscript{cE} did not activate the Jagged1 promoters pJ1pro\textsuperscript{629/98} and pJ1pro\textsuperscript{321/629} or reporter constructs containing DNA fragments up to 8-kb upstream of the start codon within the Jagged1 promoter (pJ1pro\textsuperscript{321/98}), indicating that the Jagged1 gene is not likely
to be a direct target of Notch signaling (Figs. 4A and 5C and data not shown). Interestingly, there is no induction of Jagged1 mRNA upon a 12-hour hormone induction of RKE cells expressing Notchic-ER. Therefore, the Jagged1 promoter is responsive to a PDZ-dependent signaling mediated by JAGGED1 and not to activated Notch proteins, indicating that JAGGED1 has an intrinsic signaling mechanism that likely is Notch-independent.

**DISCUSSION**

**Cellular Transformation by Human JAGGED1**—Although aberrant misexpression of DSL proteins has been documented in several tumor types, a causal role in oncogenesis has not been demonstrated (29, 47–49). Here, we report that expression of JAGGED1 transforms RKE cells in culture. These cells readily escape contact inhibition, but fail to form colonies in soft agar or tumors in nude mice (data not shown), indicating that these cells are not malignantly transformed. This is in contrast to expression of activated alleles of NOTCH proteins (N	extsuperscript{ic}) in RKE cells, which results in complete malignant transformation. We present evidence that cellular transformation by JAGGED1 expression is due, at least in part, to a PDZ-dependent signaling mechanism intrinsic to JAGGED1 and may be independent of Notch activation.

The current model of Notch signaling proposes that DSL proteins serve to activate Notch. Binding of DSL ligands to Notch across cell boundaries induces proteolytic processing that results in the release of N	extsuperscript{ic} from the plasma membrane. N	extsuperscript{ic} subsequently translocates to the nucleus and regulates a specific set of genes. RKE cells express a basal level of mRNA for Notch1, 2, and 3 (Fig. 4). Since both Jagged1 and Notch1 proteins are expressed in JAGGED1-expressing cells, it is plausible that Notch signaling may be activated in an autocrine-like manner where Notch1 is activated by interaction with JAGGED1 within the same cell. However, we have been unable to demonstrate any differences in Notch processing, localization, or signaling between parental and JAGGED1-expressing RKE cells. One possible explanation for these results is that the presence of Radical Fringe in RKE cells may render Notch insensitive to JAGGED1-induction (Fig. 4). There is substantial evidence that Fringe proteins have N-acetylglucosaminyltransferase activity that decreases the affinity of Notch for serrate/Jagged proteins and increases the affinity of Notch for Delta proteins (25–28,50). Although it remains a possibility that the level of Notch activation is sufficiently low so that changes at protein level are undetectable in RKE cells, we do

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* C. Ronchini and A. J. Capobianco, unpublished results.
not think this is the case. Moreover, we present evidence that JAGGED1 is able to mediate cellular transformation through a PDZ-dependent signaling mechanism.

A PDZ-Ligand at the C-terminal End of JAGGED1 May Be Involved in an Intrinsic Signaling Pathway Independent of Notch Activation—An emerging paradigm is the existence of bi-directional signaling, in which cell-to-cell contact stimulates cellular responses from both the signal-receiving cell and the signal-transmitting cell. Receptor-ligand interactions result in receptor clustering and recruitment of cytoplasmic proteins such as kinases and adaptor proteins that ultimately transmit the signal (51). Although there is much more known about the downstream events that occur upon activation of Notch within the Notch-expressing cell, there is little known about the effects of the receptor-ligand interaction within the DSL-expressing cell. Here, we present a model indicating that Jagged1 can mediate a signal through a PDZ-dependent mechanism. We have demonstrated that the six C-terminal residues of Jagged1 that comprise a PDZ-ligand are required for Jagged1-mediated transformation, as shown by a dramatic reduction in focus formation by J1APL-expressing cell lines compared with expression matched JAGGED1-expressing cell lines (Fig. 3B). However, both J1 and J1APL proteins are capable of activating Notch signaling to a similar extent, indicating that deletion of the PDZ-ligand does not interfere with Notch activation across cell boundaries (Fig. 3D). RT-PCR analysis of gene expression in J1 and J1APL cell lines demonstrated that the PDZ-ligand is required for induction of Jagged1 and Delta1, since only the J1 cell line and not the J1APL cell line had increased levels of Jagged1 and Delta1 mRNA (Fig. 4C). Furthermore, J1APL did not activate the Jagged1 promoter construct, indicating that the PDZ-ligand is required for downstream signaling events that lead to changes in gene expression (Fig. 5, B and C). Therefore, we provide evidence for the existence of a novel signaling mechanism intrinsic to JAGGED1 that involves a PDZ-dependent pathway.

In our initial analysis to determine which domain of JAGGED1 is necessary and sufficient for transformation, we created deletion mutations to separate the two major domains. The extracellular domain is thought to function by binding Notch to initiate signaling, and therefore we generated a soluble form of the extracellular domain (Jexo). Overexpression of Jexo did not transform RKE cells even though Jexo molecules were expressed at a higher level compared with JAGGED1 in the J12 cell line (Fig. 2). Soluble forms of Jagged1 have been reported to physically interact with Notch1, 2, and 3 receptors, indicating that Jexo molecules should be capable of binding to endogenous Notch proteins in RKE cells (24). Furthermore, soluble forms of Jagged1 have been reported to have biological function (52–55). A soluble form of the extracellular domain of JAGGED1 is not sufficient to transform RKE cells, even though a large amount of Jexo remains in the media, indicating that the intracellular domain is required for some signaling event. However, expression of a membrane-tethered intracellular domain of JAGGED1 (Jmex) failed to transform cells even though it encodes the PDZ-ligand. Why then does the intracellular piece not transform cells? One simple explanation is that the extracellular domain is required to localize or cluster the intracellular domain in order to initiate downstream signaling, this model is consistent with other receptor-mediated signals.

In order to determine if JAGGED1 could interact with PDZ-domain proteins, GST pulldown assays were performed. More specifically, we assessed if the PDZ-domain protein AF6 could bind the intracellular domain of JAGGED1, since AF6 was previously reported to interact with six residues (RMEYIV) found at the C terminus of Jagged1 using a directed yeast two-hybrid analysis (13). Furthermore, AF6 is the mammalian homolog of Drosophila canoe which has been genetically linked to the Notch pathway (46). Our data demonstrates that the interaction between JAGGED1 and AF6 occurs in a PDZ-dependent manner, since deletion of the PDZ-ligand of JAGGED1 or of the PDZ-domain of AF6 abolished this interaction (Fig. 3, E and F). Although AF6 may link JAGGED1 to downstream signaling proteins such as members of the RAS superfamily, the role of AF6 in JAGGED1-mediated transformation remains to be elucidated (56).

A Bi-directional Signaling Mechanism through DSL Proteins—Jagged2 has been reported to facilitate cell cycle progression through sustained activation of Notch signaling, which results in a modest increase in CDK2 kinase activity (57). Although Jagged2-expressing fibroblasts no longer exhibit contact inhibition upon confluency, these cells do not form foci or grow in soft agar. We previously reported that there are
increased levels of CDK2 kinase activity upon induction of Notch activity (58). However, we are unable to detect a difference in CDK2 kinase activity between RKE and J1 cell lines, indicating that Jagged1 may be signaling independently of Notch activation (data not shown). Although the functional differences between Jagged1 and Jagged2 proteins remain unclear, the presence of the PDZ-dock likely provides a functional difference between these DSL proteins (Fig. 6A). Jagged1 proteins have a highly evolutionarily conserved sequence (RMYIV) that comprises a PDZ-dock; however, there is no resemblance of the PDZ-dock consensus sequence at the C-terminal end of Jagged2 (RYAGKE). Therefore, the DSL protein Jagged1 has the potential to mediate a PDZ-dependent signaling mechanism through downstream PDZ-domain proteins. Furthermore, we have compared the different classes of DSL proteins and found the consensus sequences for putative proteases. Furthermore, we have compared the different classes of DSL proteins and found the consensus sequences for putative proteases.

What are the factors that mediate the specificity in DSL/Notch signaling? It is proposed that temporal and spatial expression of DSL proteins provide some level of specificity in Notch signaling during development and growth. Fringe proteins provide another level of specificity between the two classes of DSL proteins. Fringe modifies Notch such that there is a decreased affinity for Jagged proteins and a higher affinity for Delta proteins (Fig. 6) (25–28). Therefore, upon expression of Fringe, Delta proteins would still be able to initiate Notch signaling, while Jagged proteins could not. Here, we have provided evidence that a PDZ-dock of Jagged1 is required to mediate transformation and changes in gene expression, indicating that DSL proteins have an intrinsic signaling mechanism that can be activated in the cells in which they are expressed. We propose a bi-directional signaling model such that DSL proteins may have two distinct functions: (1) to initiate Notch signaling in a neighboring cell and (2) to initiate an intrinsic PDZ-dock-dependent signaling mechanism (Fig. 6B). For example, Jagged1 could signal in both directions in the absence of Fringe, whereas Jagged2 would always be bi-directional. If the case of Jagged2, signaling would only be in the Notch direction and this signal could be attenuated by Fringe. In contrast, Delta3 is insensitive to Fringe and would always allow signaling in the Notch direction. This model provides exquisite flexibility for signal specificity and accounts for the multiple distinct DSL proteins.

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