Mouse Jagged1 Physically Interacts with Notch2 and Other Notch Receptors

ASSESSMENT BY QUANTITATIVE METHODS*

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Kiyoshi Shimizu‡§, Shigeru Chiba‡§, Keiki Kumano‡§, Noriko Hosoya‡§, Tokiharu Takahashi‡§, Yoshinobu Kanda‡§, Yoshio Hamada‡, Yoshio Yazaki‡§**, and Hisamaru Hirai‡§***

From the Departments of ¥Hematology and Oncology, ¥Cell Therapy and Transplantation Medicine, ¥Transfusion and Immunohematology, and **Cardiology, Graduate School of Medicine, University of Tokyo, Tokyo, 113-8655 Japan and the ¥National Institute of Basic Biology, Okazaki, Aichi, Japan 444-8585

The Delta/Serrate/LAG-2 (DSL) domain containing proteins are considered to be ligands for Notch receptors. However, the physical interaction between DSL proteins and Notch receptors is poorly understood. In this study, we cloned a cDNA for mouse Jagged1 (mJagged1). To identify the receptor interacting with mJagged1 and to gain insight into its binding characteristics, we established two experimental systems using fusion proteins comprising various extracellular parts of mJagged1, a "cell" binding assay and a "solid-phase" binding assay. mJagged1 physically bound to mouse Notch2 (mNotch2) on the cell surface and to a purified extracellular portion of mNotch2, respectively, in a Ca2+-dependent manner. Scatchard analysis of mJagged1 binding to BaF3 cells and to the soluble Notch2 protein demonstrated dissociation constants of 0.4 and 0.7 nm, respectively, and that the number of mJagged1-binding sites on BaF3 is 5,548 per cell. Furthermore, deletion mutant analyses showed that the DSL domain of mJagged1 is a minimal binding unit and is indispensable for binding to mNotch2. The epidermal growth factor-like repeats of mJagged1 modulate the affinity of the interaction, with the first and second repeats playing a major role. Finally, solid-phase binding assay showed that Jagged1 binds to Notch1 and Notch3 in addition to Notch2, suggesting that mJagged1 is a ligand for multiple Notch receptors.

The gene Notch was originally identified in Drosophila melanogaster as playing an important role in appropriate cell specification during embryogenesis (1–4). Although only one Notch gene has been identified in Drosophila (5), multiple Notch homologs have been described in higher vertebrates, including Notch1 through Notch4 in rodents and humans (6–12). The basic structure of the Drosophila and mammalian Notch proteins comprises 29–36 epidermal growth factor (EGF)-like repeats and 3 copies of a Lin-12/Notch/Glp motif in the extracellular region, and cdc10/Ankyrin repeats and a PEST-containing domain in the intracellular region.

Genes encoding Notch ligands and their homologs isolated to date include Delta (13) and Serrate (14) in Drosophila; LAG-2 and APX-1 in Caenorhabditis elegans (15–17); Delta1 (mDelta1) and Jagged2 (mJagged2) in mice (18, 19); and Jagged1 and Jagged2 in rats (20, 21) and humans (22, 23). All share two important extracellular features: the DSL domain (17) and tandem EGF-like repeats. Jagged1 and Jagged2 share an additional homology with Serrate in the cysteine-rich domain.

On the basis of the finding that Notch family proteins can autonomously transduce a signal in various organisms if most of the extracellular region (and transmembrane region) is truncated, they are considered to represent activated forms of Notch proteins (24–28). Studies with activated forms of Notch1 provide evidence that this protein can regulate differentiation in various types of cells, including C2C12 myoblasts and 32D myeloid progenitors (29–31). Activated Notch1 also controls CD4/CD8 cell fate (32) as well as αβγδ T cell lineage (33) decisions during normal T lymphocyte development in mice. An attractive model which ties the activated Notch and Notch ligands was recently proposed wherein the intracellular domain of Notch is processed by stimulation with a Notch ligand and translocates into the nucleus. This suggests that ligand stimulation of Notch switches on the intracellular machinery in a manner similar to that of activated Notch (34–36). Stimulation with a Notch ligand actually inhibits differentiation of various mammalian cells. Jagged1 prevents granulocyte colony-stimulating factor-induced granulocytic differentiation of 32D cells (23) and horse serum-induced myocytic differentiation of C2C12 cells (20, 21) and humans (22, 23). It has also been reported that mDelta1 and mJagged2 can inhibit myocytic differentiation of wild-type C2C12 cells (22, 23).

To better understand the biology of the Notch system in mammals, further investigation of the interaction between DSL ligands and Notch receptors is required. The only information obtained to date has come from Drosophila cell-aggregation assay (38, 39). A better quantitative approach to the assessment of ligand–receptor association is required.

In the present study, we isolated a cDNA encoding the mouse homologue of Jagged1 (mJagged1) and obtained various forms of this protein containing an extracellular region. Two methods were developed to characterize molecules interacting with mJagged1 and their binding features, a "cell" binding assay using intact cells and a "solid-phase" binding assay using pu...
rified proteins immobilized to a plastic tray. These systems provided important information on the interaction between mJagged1 and the Notch receptors.

**EXPERIMENTAL PROCEDURES**

**Oligonucleotides and Probes**—A probe to screen a cDNA library by low-stringency hybridization was obtained by polymerase chain reaction using degenerate oligonucleotide primers and mouse embryo cDNA as a template. The primers were synthesized based on the peptide sequences FCPRPRD (amino acid (aa) 199–205) and PWQCCLCE (aa 279–285), corresponding to the DSL domain and second EGF-like repeat of mDelta1 (19), respectively. Amplified fragments of around 260 bp were subcloned into a TA-cloning vector. Sequencing of these the inserts revealed the amplification of three kinds of cDNA. The inserts were mixed and used as a probe. Northern blot analysis of Notch mRNA was done using the 585- bp 5′ EcoRV-HindIII fragment and 532-bp SacI fragment, corresponding to similar intracellular domains of mouse Notch1 (mNotch1) (6) and mNotch2, respectively. Mouse Notch2 sequence data is from GenBank under accession number D32210. The 660-bp fragment at the 5′ end of a newly obtained mJagged1 cDNA was used as a probe to detect Jagged1 mRNA in mouse tissues. Each probe was 32P-labeled with the Megaprime DNA labeling system (Amer- sham Pharmacia Biotech) according to the manufacturer’s instructions.

**cDNA Clones**—A mouse cDNA library in λ gt11 made from an 11-day postcoitum embryo (CLON- TECH) was plated out at 5 × 10^6 plaque forming units on Luria broth/MgCl_2 agar according to the manufacturer’s instructions. Following incubation for 6 h, plaques were transferred to a nylon membrane (Hybond-N, Amersham Pharmacia Biotech), denatured, neutralized, and hybridized at 42 °C in a solution containing 5 × SSC, 1% SDS, 5 × Denhardt’s solution, and 30% formamide. Following hybridization, filters were washed three times with 2 × SSC, 1% SDS for 15 min each. Positive clones were isolated through a second and third cycle of hybridization under the same conditions. The cDNA inserts in the isolated clones, DSL20-1 and DSL47-1, which harbored a novel sequence, were bridged by a second cycle of hybridization under the same conditions. The cDNA inserts in the isolated clones and a 5′ end clone. The extracellular portion of mNotch1 cDNAs were truncated at the codon GAA corresponding to glutamic acid (606th aa for soluble Notch1-Fc (sN1-Fc), 610th aa for the Flag(His)6-tagged proteins, an anti-Flag monoclonal antibody (bhN6) (gift from Dr. Spyros Artavanis-Tsakonas) (42) was used at a dilution of 1:20. For Western blot analysis and cell-binding and solid-phase binding assays for the Fc-fused proteins, a horseradish peroxidase (HRP)-conjugated anti-human Fc antibody (Dako) was used at a dilution of 1:5000. A rabbit polyclonal antibody was raised against the intracellular domain of mNotch2 fused in-frame to glutathione S-transferase and used at a dilution of 1:1000 for immunoprecipitation. For the Northern blot analysis of Notch2, an anti-Notch2 monoclonal antibody (bhN6) (gift from Dr. S. Shirahata) were maintained in 2% SDS and 0.1% SDS at 65 °C for 30 min and the blot was visualized with a Fuji BioImage Analyzer BAS2000 (Fuji Film).

**Preparation of Soluble Fusion Proteins**—Stable CHO(r) cells expressing each soluble Notch receptor or soluble Jagged1 at the highest level were plated out at 5 × 10^6 cells in 200 μl of binding buffer (phosphate-buffered saline containing 2% FBS, 100 μg/ml CaCl_2, and 0.05% NaN_3). After blocking with 5 μl of rabbit serum, the cells were incubated in cell-binding buffer containing the Fc- or Flag(His)_6-tagged soluble mJagged1 proteins at room temperature for 1 h. After washing three times with the binding buffer, the cells were incubated with a PE-conjugated anti-mouse IgG antibody or an enzyme-linked immunosorbent assay using an antibody against the hIgG Fc. The cell counting was done using a FACS Calibur (Becton Dickinson Immunocytometry Systems). A colorimetric method was adopted for the cell binding assay to determine the affinity of mJagged1 to the surface of BaF3. In this method, a binding procedure similar to those above was performed using 0.1% bovine serum albumin instead of 2% FBS in the blocking buffer and a HRP-conjugated anti-hIgG instead of a PE-conjugated antibody as second antibody. In this method, the amount of bound FLAG-Fc was estimated by measuring cell-bound HRP activity using an HRP development reagent (Sumilon). To determine the absolute molar value of incubated and bound FLAG-Fc, the optical density (OD) value given by the enzyme-linked immunosorbent assay for a concentration-determined hIgG (Xymed) was utilized. The intensity of color was then measured using a microplate reader (MR700; Dynatech Laboratories).

**Northern Blot Analysis**—Poly(A)^+ RNA was isolated using the PolyATract mRNA isolation system (Promega) according to the manufacturer’s instructions. Two micrograms of mRNA from different adult mouse tissues was separated in a 1.2% agarose-formamide gel, transferred to a nylon membrane (Hybond-N, Amersham Pharmacia Biotech), and hybridized with a probe labeled with a random priming method and used as a probe. Hybridization was carried out for 24 h at 42 °C in 50% formamide, 5 × SSPE, 5 × Denhardt’s solution, 1% SDS, and 0.1 mg/ml salmon sperm DNA. The membrane was subjected to stringent washing twice with 0.2 × SSC and 0.1% SDS at 65 °C for 30 min and the blot was visualized with a Fuji BioImage Analyzer BAS2000 (Fuji Film).
Immunoprecipitation and Western Blot Analysis—A total of 1 $\times$ 10^7 32D or BaF3 cells were subjected to cell binding assay as described above with 1.7 nM FE-J1-Fc. The cells were then solubilized in a TNP buffer containing 20 mM Tris-Cl (pH 7.4), 150 mM NaCl, 1.0% Nonidet P-40, 5 μg/ml aprotinin, and 100 μg/ml CaCl2 for 30 min at 4 °C. The lysates were precipitated with Protein G beads which were washed four times with TNP buffer and boiled in the SDS sample buffer under reducing conditions. The samples were subjected to 7.0% SDS-polyacrylamide gel electrophoresis and electrophoretically transferred to a nylon membrane (Immobilon, Millipore). The membranes were blocked with 5% nonfat dry milk in TBST (20 mM Tris-HCl (pH 7.4), 500 mM NaCl, 0.1% Tween 20) for 1 h and incubated overnight at room temperature with an anti-Notch2 monoclonal antibody, bhN6. The membrane was washed three times with TBST and incubated with an alkaline phosphatase-conjugated anti-rat IgG antibody (Promega) for 1 h. Following three washes with TBST, the membrane was visualized using BCNP and nitro blue tetrazolium (Promega).

Results

Isolation and Sequencing of mJagged1 cDNA—In an attempt to isolate a novel mouse Notch ligand, we screened a cDNA library constructed from an 11-day postcoitum mouse embryo using a low stringency hybridization method. The probe used was a mixture of three DSL sequences, which were obtained by polymerase chain reaction using degenerated primers and mouse embryo cDNA as a template ("Experimental Procedures"). The sequences of these fragments corresponded to the DSL region of mouse Delta1, Jagged1, and Jagged2, the latter two of which have to date only been identified in rats and humans. The nucleotide sequence of a number of cDNA clones isolated revealed that two, DSL20-1 and DSL47-1, represented partial cDNA clones encoding a mouse homologue of Jagged1 and which covered the entire coding region when combined. DSL20-1 contained the 5′ end of mJagged1 with 268-bp non-coding and 3.2-kb coding regions. DSL47-1 contained 3.6-kb coding and 160-bp 3′-end noncoding regions (Fig. 1A). The full-length mJagged1 clone contained an open reading frame of 7527 bp.
mJagged1 mRNA. The blot was initially hybridized with a probe for mJagged1 and then stripped and rehybridized with a probe for glyceraldehyde-3-phosphate dehydrogenase (G3PDH).

3,654 bp, which predicted a protein consisting of 1,218 amino acids. Amino acid sequence alignment of mouse, rat, and human Jagged1 shows mJagged1 protein having overall amino acid identities with rat and human Jagged1 (mJagged1 and hJagged1) of 97 and 96%, respectively (Fig. 1B). Amino acid conservation in the DSL and EGF-like repeat regions was high at 98 and 97%, respectively.

mJagged1 Gene Expression in Various Mouse Tissues—Examination of mJagged1 expression by Northern blot analysis with mRNAs from various adult mouse tissues and a 14.5-day postcoitum embryo showed that mJagged1 was expressed in various tissues. Highest expression was in the embryo (Fig. 2). Expression in adult tissues was highest in brain, heart, muscle, and thymus. An mRNA transcript of 6.5 kb was detected as a major band; tissues showing this transcript also showed two minor bands of about 4.0 and 3.6 kb. These short transcripts may be alternatively spliced forms of mJagged1 mRNA.

Construction of a Cell Binding Assay System: Specific Binding of Extracellular Region of mJagged1 Protein to Hematopoietic Cell Lines—Two kinds of soluble protein comprising the full-length extracellular region of Jagged1, one with hIgG Fc and the other with Flag(His)$_6$ tagged to the C terminus, designated FE-J1-Fc and FE-J1-Flag(His)$_6$, respectively, were stably produced by cDNA-transfected CHO(r) cells. Coomassie Brilliant Blue staining of Protein G-purified FE-J1-Fc and Ni-purified FE-J1-Flag(His)$_6$ revealed that purity was over 95 and 90%, respectively (Fig. 3A). FE-J1-Fc was found at positions of about 210 kDa under reducing and over 400 kDa under nonreducing conditions, whereas FE-J1-Flag(His)$_6$ migrated to similar positions of about 140–180 kDa under both conditions (Fig. 3A). This indicates that, as expected, FE-J1-Fc is dimerized at the Fc portion.

We first investigated the binding of FE-J1-Fc to various cell lines by FACS analysis. FE-J1-Fc bound to all of the cell lines screened, including BaF3 (early B lineage cells), 32D (myeloid lineage cells), CTLL2 (T lineage cells), NF/SF60 (myeloid leukemia cells), NIH3T3 (embryonic fibroblasts), and C2C12 (myoblastic cells) (data not shown), with binding to 32D and BaF3 greater than to the other cells (Fig. 3B; other data not shown). FE-J1-Flag(His)$_6$ also bound to these hematopoietic cells (Fig. 3B). With regard to the characteristics of the Notch/Notch ligand interaction, it is known that both Delta and Serrate interact with Notch in a Ca$^{2+}$-dependent manner in the Drosofila cell system (39). To determine whether this feature was also conserved in a mammalian system, EGTA, a Ca$^{2+}$-chelating reagent, was added to the binding mixture. Binding of FE-J1-Fc and FE-J1-Flag(His)$_6$ to either the 32D or BaF3 cells was clearly abolished by the addition of EGTA (Fig. 3B).

We further found that the amount of BaF3-bound FE-J1-Fc increased in accordance with the incubation concentration of FE-J1-Fc, reaching a plateau at 1 nM (Fig. 3C). Competition assay in the same experimental system but with an excess of purified FE-J1-Flag(His)$_6$ added to the cell-binding mixture showed complete abrogation of FE-J1-Fc binding to 32D and BaF3 at an approximately 500-fold molar excess of FE-J1-Flag(His)$_6$ (Fig. 3D). These results indicate that the binding observed represents specific interaction of the extracellular domain of mJagged1 with a Notch receptor on the surface of the hematopoietic cells.

Determination of the dissociation constant ($K_d$) of mJagged1 binding to its receptor on the BaF3 cell surface was done using a colorimetric rather than FACS method. Specific colorimetric activity was calculated as 0.092 OD/fmol of hIgG or FE-J1-Fc. Re-evaluation of a saturation binding assay and associated Scatchard plot (Fig. 3E) gave a $K_d$ value of about 0.4 nM. The number of binding sites was estimated at 5,548/BaF3 cell (Fig. 3E).

Involvement of Jagged1 DSL Domain and EGF-like Repeats in Binding to Hematopoietic Cells—The roles of the DSL domain and EGF-like repeats in the binding capacity of Jagged1 were examined with three deletion mutants of Jagged1. We first confirmed that FE-J1-Fc derived from COS1 possessed binding activity equivalent to that derived from CHO(r) cells (data not shown). When COS1-derived purified FE-J1-Fc and three deletion mutants (Fig. 4, A and B) were allowed to bind to 32D at identical molar concentrations, binding activity was greatest for FE-J1-Fc, slightly lower for EGF-1,2-J1-Fc and profoundly lower for DSL-J1-Fc (Fig. 4C). In all cases, binding activity was dependent on Ca$^{2+}$ (data not shown). $\Delta$DSL-J1-Fc did not bind at all (Fig. 4C). Essentially identical results were obtained when the same experiment was performed using proteins tagged with Flag(His)$_6$ instead of hIgG Fc (data not shown). These observations indicate that the DSL domain of Jagged1 confers the minimum binding capability to these hematopoietic cells and that the EGF-like repeats of Jagged1, in particular the first and second, substantially stabilize the interaction.

Interaction of Jagged1 with Notch2 Receptor on the Surface of 32D and BaF3 Cells—We next attempted to identify the Notch receptor interacting with the soluble Jagged1. Candidate receptors were selected through Notch receptor expression in hematopoietic cells by Northern blot analysis. Notch2 mRNA was strongly expressed in both 32D and BaF3. In contrast, full-length (9.5 kb) Notch1 mRNA was not expressed in these cells, although the shorter mRNA species with approximate sizes of 6.0 and 7.2 kb were detected in 32D (Fig. 5A). Furthermore, mRNA for Notch3 or Notch4 was also not detected. Notch2 was therefore selected as the major candidate for receptor interaction with Jagged1. Using an anti-Notch2 monoclonal antibody recognizing a cytoplasmic domain of Notch2, Western blot analysis for the protein G-bound fraction of the lysates of 32D and BaF3 was performed after FE-J1-Fc binding. The results showed that the cytoplasmic domain-containing Notch2 fragments were immunoprecipitated with FE-J1-Fc but not with control hIgG (Fig. 5B). One protein species with an approximate molecular mass of 115 kDa was similar in size to that found by other investigators as a fragment of the Notch2 protein (42, 45). In contrast, full-length 300-kDa Notch2 was detected in precipitates with an anti-Notch2 polyclonal antibody but not in the Protein G precipitate. These results indicated that the soluble Jagged1 protein binds to Notch2 on the cell surface.
Fig. 3. Specific binding of soluble mJagged1 to live cells. A, generation and purification of soluble mJagged1 proteins comprising the full-length extracellular region, FE-J1-Fc and FE-J1-Flag(His)6. FE-J1-Fc and FE-J1-Flag(His)6 derived from CHO(r) cells were purified with Protein G or Ni-bound beads, respectively. Integrity and purity of the soluble Jagged1 proteins were verified by Coomassie Brilliant Blue (CBB) staining and Western blot analysis. Antibodies used for Western blot analysis were an HRP-conjugated anti-hIgG Fc antibody and an anti-Flag antibody. The samples were electrophoresed in the presence or absence of a reducing reagent (dithiothreitol; DTT). Arrows show the soluble Jagged1 proteins of interest. B, Ca2+ dependence of soluble Jagged1 binding to the hematopoietic cell lines 32D and BaF3. 32D and BaF3 were incubated with FE-J1-Fc or FE-J1-Flag(His)6 in the absence (green) or presence (red) of 2 mM EGTA. As a control, hIgG was incubated with the cells in the absence of EGTA (black). C, binding of increasing concentrations of FE-J1-Fc to BaF3. The extent of fluorescence brightness which gives the highest frequency (vertical axis) was plotted against each concentration of FE-J1-Fc (horizontal axis). D, displacement of FE-J1-Fc binding by a 500-fold molar excess of FE-J1-Flag(His)6. E, Scatchard analysis of FE-J1-Fc binding to BaF3 by colorimetric cell binding assays as described in the text. A saturation binding curve (inset, mean value of duplicates and standard deviation in each protein) and Scatchard plot are shown. Calculated Kd and the number of FE-J1-Fc-binding sites are also described.
Establishment of a Solid-phase Binding Assay using Purified Recombinant Soluble Proteins—To further understand the interaction between Jagged1 and Notch, we established a binding assay system (solid-phase binding assay). CHO-derived purified soluble Notch2 proteins were used, namely sN2-Flag(His)6 and sN2-Fc, consisting of the N terminus through the 15th EGF-like repeat. Purity estimated by Coomassie Brilliant Blue staining using a densitometer was greater than 90 and 95%, respectively (Fig. 6A). As shown in Fig. 6B, direct and specific binding of FE-J1-Fc to the immobilized sN2-Flag(His)6 was clearly demonstrated. As in the cell binding assay, this interaction was again dependent on the presence of Ca\(^{2+}\) (Fig. 6C).

Next, to evaluate the affinity of FE-J1-Fc binding to sN2-Flag(His)6, a solid-phase binding assay was performed by adding increasing concentrations of FE-J1-Fc. Results again showed that binding was concentration-dependent and saturable (Fig. 6D). Conversion of the data into a Scatchard plot gave an estimated $K_d$ value of about 0.7 nM (Fig. 6C), within a magnitude of that calculated in the cell binding assays. Furthermore, examination of the interaction under the opposite conditions, in which FE-J1-Flag(His)6 was immobilized and sN2-Fc was used as a probe, showed that sN2-Fc interacted with immobilized FE-J1-Flag(His)6 but not with the control (Fig. 6D). This interaction was again abolished by the addition of EGTA (Fig. 6D).

Analysis of the functional domain by addition of the deletion mutants of soluble Jagged1 at the same molar concentration (3.3 nM) to the sN2-Flag(His)6-immobilized plate showed that both FE-J1-Fc and EGF-1,2-J1-Fc strongly interacted with sN2-Flag(His)6, although the amount of EGF-1,2-J1-Fc binding was a little less than that of FE-J1-Fc (Fig. 6E). DSL-J1-Fc bound weakly to sN2-Flag(His)6 while ΔDSL-J1-Fc did not bind at all (Fig. 6E). These findings for mutant mJagged1 proteins binding to purified soluble Notch2 were closely similar to those for binding to the 32D cell surface.

Interaction of Jagged1 with Notch1 and Notch3—The solid-phase binding assay using purified and immobilized FE-J1-Flag(His)6 allowed the evaluation of its association with Notch proteins other than mNotch2, namely mNotch1 and mNotch3. Coomassie Brilliant Blue staining of CHO-derived and purified sN1-Fc and sN3-Fc (purity greater than 95%) is shown in Fig. 7A. FE-J1-Flag(His)6 interacted with sN1-Fc and sN3-Fc as well as sN2-Fc in a dose-dependent manner, although with different binding affinities (Fig. 7B). Among these three soluble Notch proteins, sN3-Fc had highest affinity, followed by sN2-Fc and sN1-Fc in that order. As with Notch2, Ca\(^{2+}\) dependence was maintained (Fig. 7C).

DISCUSSION

In this study, we cloned a cDNA for mouse Jagged1 (mJagged1) and used it to establish two experimental systems for the assessment of the Notch-ligand interaction. Results using these systems have shown that mJagged1 binds to multiple Notch receptors in a saturable and Ca\(^{2+}\)-dependent manner, and that the DSL domain and EGF-like repeats of mJagged1 are critical for binding to Notch2. They have also revealed the affinity of mJagged1 binding to BaF3 cells as well as soluble Notch2 and the number of mJagged1-binding sites on BaF3.

A cDNA obtained from a mouse embryo library showed 97 and 96% homology with rat and human Jagged1 cDNAs, respectively, at the deduced overall amino acid level (Fig. 1), indicating that this isolated cDNA represents the mouse homolog.
though it was precipitated by an anti-Notch2 polyclonal antibody. This is consistent with the recent finding that plasma membrane Notch protein comprises two polypeptide chains as a result of proteolytic cleavage of the full-length Notch protein (45, 46). A 115-kDa protein, found by other investigators as a fragment of the Notch2 protein (42, 45), and multiple shorter Notch2 species of about 70–80 kDa were coprecipitated with Jagged1. We suggest that the 70–80-kDa proteins are the products of the 115-kDa species degraded by metalloproteinases during the immunoprecipitation procedure. EDTA, an inhibitor of metalloproteinases, inhibited the appearance of these fragments (data not shown) but was not added to the ligand-Notch interaction due to the expectation that it would cancel the binding.

Binding assays with added EGTA showed that the interaction of Jagged1 with the cell surface, as well as with purified Notch1, Notch2, and Notch3, depends on the presence of Ca$^{2+}$. This characteristic has also been described in the Drosophila Notch system (39), in which it was reported that a Ca$^{2+}$-binding site exists in each of the 11th and 12th EGF-like repeats, which are necessary and sufficient for the interaction with both Delta and Serrate (39). This 11th and 12th EGF-like repeat sequence which included Ca$^{2+}$-binding sites is strongly conserved among all the mammalian Notch receptors (39, 47).

Scatchard analysis in the cell-binding and solid-phase binding assays gave $K_d$ values of approximately 0.4 and 0.7 nM, respectively, indicating that the affinity of the full-length extracellular domain of mJagged1 for the cell surface of BaF3 is slightly higher than that for the purified partial extracellular portion of Notch2. As the difference is surprisingly small, however, we suggest that the N-terminal region through to the 15th EGF-like repeat sequence which included Ca$^{2+}$-binding sites is strongly conserved among all the mammalian Notch receptors (39, 47).

With respect to the roles of each domain in Jagged1, deletion mutant analyses of Jagged1 clearly demonstrated in both binding assays that the DSL domain and the EGF-like repeats are critical for binding to Notch2 (Figs. 4C and 6E). The DSL domain is indispensable and the minimal unit for binding to Notch. Our finding that the DSL domain is a receptor-binding site is consistent with previous speculation that an oligonucleotide corresponding to the DSL domain could be biologically active (23, 48, 49). The DSL domain is essential to the biological activity of LAG-2 and APX-1 in C. elegans (48, 49), and it has been speculated that the lack of the DSL domain in LAG-2 or APX-1 impairs binding activity of DSL proteins for Notch. Our results support this idea. Regarding the function of the EGF-like repeats in the DSL ligands, the 4th EGF-like repeat in Drosophila Delta is speculated to be involved in maintaining stable affinity for Notch (50). Results from both the present assays show that the EGF-like repeats, particularly the 1st and 2nd, function in the formation of the high affinity complex with Notch2 (Figs. 4C and 6E).

The solid-phase binding assay showed that Jagged1 bound to Notch3, Notch2, and Notch1 with diverse affinities in this order (Fig. 7). However, it cannot be concluded that this apparent difference invariably reflects the characteristics of interaction between mJagged1 and each Notch polypeptide, due to the possibility that binding affinities between mJagged1 and Notch receptors are modified by Fringe proteins, putatively secreted glycosyltransferases which may confer glycosyl chains to Notch. In this regard, it is known that Fringe modulates the biological activity of Serrate and Delta in Drosophila (51–53). Thus far, stress has been put on Notch1 as a natural ligand for Jagged1 (20, 23). However, our observations suggest that
Jagged1 is potentially a natural ligand for multiple Notch receptors. This suggestion agrees with the results from in situ hybridization analyses (20, 54).

In this paper, we describe a useful experimental approach which provides direct evidence that one of the DSL proteins, mJagged1, directly associates with multiple mouse Notch pro-
teins. We believe these findings add to the further understanding of Notch signaling.

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