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Physical Interaction of Delta1, Jagged1, and Jagged2 with Notch1 and Notch3 Receptors

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The Delta/Serrate/LAG-2 (DSL) domain-containing proteins, Delta1, Jagged1, and Jagged2, are considered to be ligands for Notch receptors. However, the physical interaction between the three DSL proteins and respective Notch receptors remained largely unknown. In this study, we investigated this issue through the targeting of Notch1 and Notch3 in two experimental systems using fusion proteins comprising their extracellular portions. Cell-binding assays showed that soluble forms of Notch1 and Notch3 proteins physically bound to the three DSL proteins on the cell surface. In solid-phase binding assays using immobilized soluble Notch1 and Notch3 proteins, it was revealed that each DSL protein directly bound to the soluble Notch proteins with different affinities. All interactions between the DSL proteins and soluble Notch proteins were dependent on Ca^{2+}. Taken together, these results suggest that Delta1, Jagged1, and Jagged2 are ligands for Notch1 and Notch3 receptors.

The Notch gene, which was originally identified in *Drosophila melanogaster,* plays an important role in appropriate cell fate specification during embyogenesis (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.

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Two proteins, Delta and Serrate, characterized by a common structure of a Delta-Serrate-Lag2 (DSL) domain, have been identified as Notch ligands in *Drosophila* (13, 14). A cell-aggregation assay demonstrated that the two proteins bind to *Drosophila* Notch in a Ca^{2+}-dependent manner and that the 11th and 12th EGF-like repeats of Notch are necessary and sufficient for the interaction with both Delta and Serrate (15).

Vertebrate counterparts of the two *Drosophila* genes, *Delta* and *Serrate,* have been identified; *Delta* (*like-*) *1* in mice and chickens (16, 17), *Delta-like-3* (*Dll3*) in mice (18), *Delta2* in *Xenopus* (19), and the potential orthologs of *Serrate, Jagged1* and *Jagged2* in rats, mice and humans (20–25). We have shown that the soluble Jagged1 protein binds to Notch2 on the cell surface and to a purified extracellular portion of Notch2 and that the DSL domain and EGF-like repeats of mJagged1 are critical for binding to Notch2 (25). In addition, the soluble form of Jagged1 binds to Notch1 and Notch3 in addition to Notch2, which is shown in a solid-phase binding assay (25). We have further demonstrated that Delta1, Jagged1, and Jagged2 have transducing activity in the Notch2 signaling in addition to their binding activity to Notch2 (26). However, no substantial evidence has been reported demonstrating that the DSL proteins other than Jagged1 physically interact with Notch1 and Notch3 receptors. Further investigation of interactions between them has been awaited for better understanding of biology of the Notch system in mammals.

MATERIALS AND METHODS

Plasmid constructions. To generate soluble Notch1 and Notch3, their cDNAs were truncated at the codon GAA corresponding to glutamic acid (606th amino acid (a.a.) for soluble Notch1-Flag$(His)_6$ (sN1-Flag$(His)_6$) and 586th a.a. for sN3-Flag$(His)_6$). A Flag$(His)_6$ sequence was fused to the 3′ end of the truncated cDNAs, respectively. These cDNAs were constructed in an expression vector pTraserCMV (Clontech).

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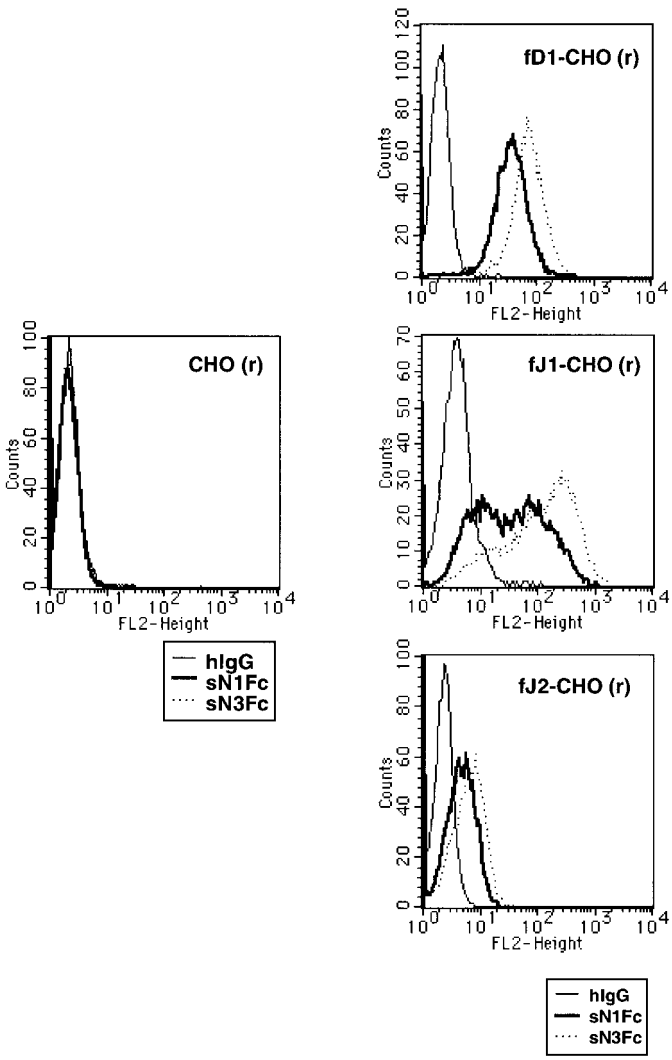

FIG. 1. Binding of soluble Notch receptors to three DSL proteins on the cell surface. Fc-fused soluble Notch proteins (sN1-Fc and sN3-Fc) were allowed to bind to the CHO (r) cells expressing the respective full-length DSL protein (fD1-CHO (r), fJ1-CHO (r) and fJ2-CHO (r)) at the same molar concentration (6.7 nM). The interaction between them was examined as described in the cell-binding assay section under Materials and Methods.

Antibodies. For Western blot analyses using Flag(His)$_6$-tagged proteins, an anti-Flag monoclonal antibody (M2, Eastman Kodak) was used at a dilution of 1:15000. An alkaline phosphatase-conjugated anti-mouse secondary antibody (Promega) was used at a dilution of 1:5000. For cell-binding assay using Fc-fused proteins, a PE-conjugated anti-human Fc antibody (Chimicon) was used at a dilution of 1:200. For solid-phase binding assays using Fc-fused proteins, a horseradish peroxidase (HRP)-conjugated anti-human Fc antibody (DAKO) was used at a dilution of 1:5000.

Cell lines and cell culture. CHO ras clone-I (CHO(r)) (27) were maintained in alpha-minimal essential medium containing 10% FBS. Stable CHO(r) cell lines expressing respective full-length DSL protein (fD1-CHO(r), fJ1-CHO(r) and fJ2-CHO(r)) were prepared as described previously (26).

Preparation of soluble fusion proteins. Soluble DSL proteins (sD1-Fc, sJ1-Fc and sJ2-Fc) and soluble Notch proteins (sN1-Flag(His)$_6$ and sN3-Flag(His)$_6$) were prepared as described previously (25). The same protocol was used for sN1-Flag(His)$_6$ and sN3-Flag(His)$_6$.

Cell-binding assay. Binding of each soluble Notch1 and Notch3 protein to the CHO(r) cells expressing their respective full-length DSL protein was performed as previously described (25). Briefly, a total of 3×10^5 CHO(r) cells was incubated in suspension in cell-binding buffer (PBS containing 2% fetal bovine serum (FBS), 100 μg/ml $CaCl_2$, 0.05% NaN_3) containing soluble Notch proteins (6.7 nM) at 37°C after blocking with 5 μl of rabbit serum. After 15 min incubation, the cells were washed three times with the cell-binding buffer and further incubated with a PE-conjugated anti-hIgG antibody. The cells were then analyzed using a FACS Caliber (Becton Dickinson Immunocytometry Systems).

Solid-phase binding assay. Solid-phase binding assay was performed as described previously (25). Briefly, 200 ng of purified Flag(His)$_6$-tagged soluble Notch proteins was immobilized on the wells of a 96-well plate (Dynatech). After blocking the wells with PBS containing 2.5% gelatin, DMEM medium containing purified sD1-Fc, sJ1-Fc or sJ2-Fc was added. After 2 h incubation, the wells were stained with an HRP-conjugated anti-human IgG antibody. The color was developed with an HRP-development reagent (Sumilon). The amount of binding was measured using a micro-plate reader.

RESULTS

Binding of Soluble Notch Receptors to Three DSL Proteins on the Cell Surface

We recently established a system to assess binding of a DSL protein to live cells using its soluble form comprising the extracellular portion (cell-binding assay) (25). We used a similar assay to evaluate binding of the tagged extracellular portion of Notch proteins (sN1-Fc and sN3-Fc) to three kinds of DSL proteins, Delta1, Jagged1, and Jagged2 on the cell surface. We found that both sN1-Fc and sN3-Fc proteins bound to the

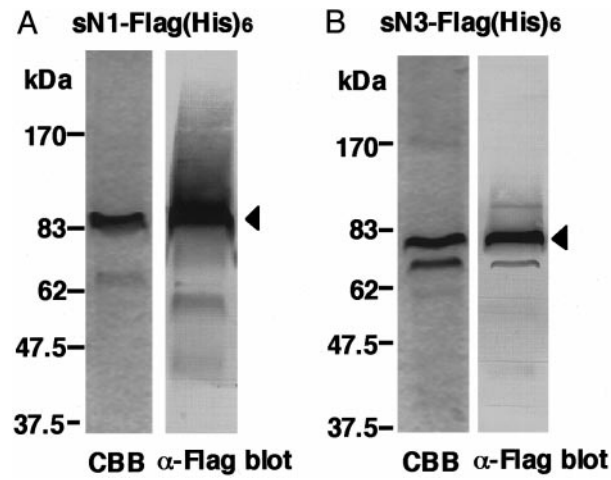

FIG. 2. Preparation of soluble Notch proteins comprising a partial extracellular region tagged with Flag(His)$_6$. Two kinds of Flag(His)$_6$-tagged soluble Notch proteins, sN1-Flag(His)$_6$ and sN3-Flag(His)$_6$, produced in CHO (r) cells were purified with Ni-bound beads, respectively. Integrity and purity were verified by Coomassie Brilliant Blue (CBB) staining and Western blots for sN1-Flag(His)$_6$ (A) and sN3-Flag(His)$_6$ (B).

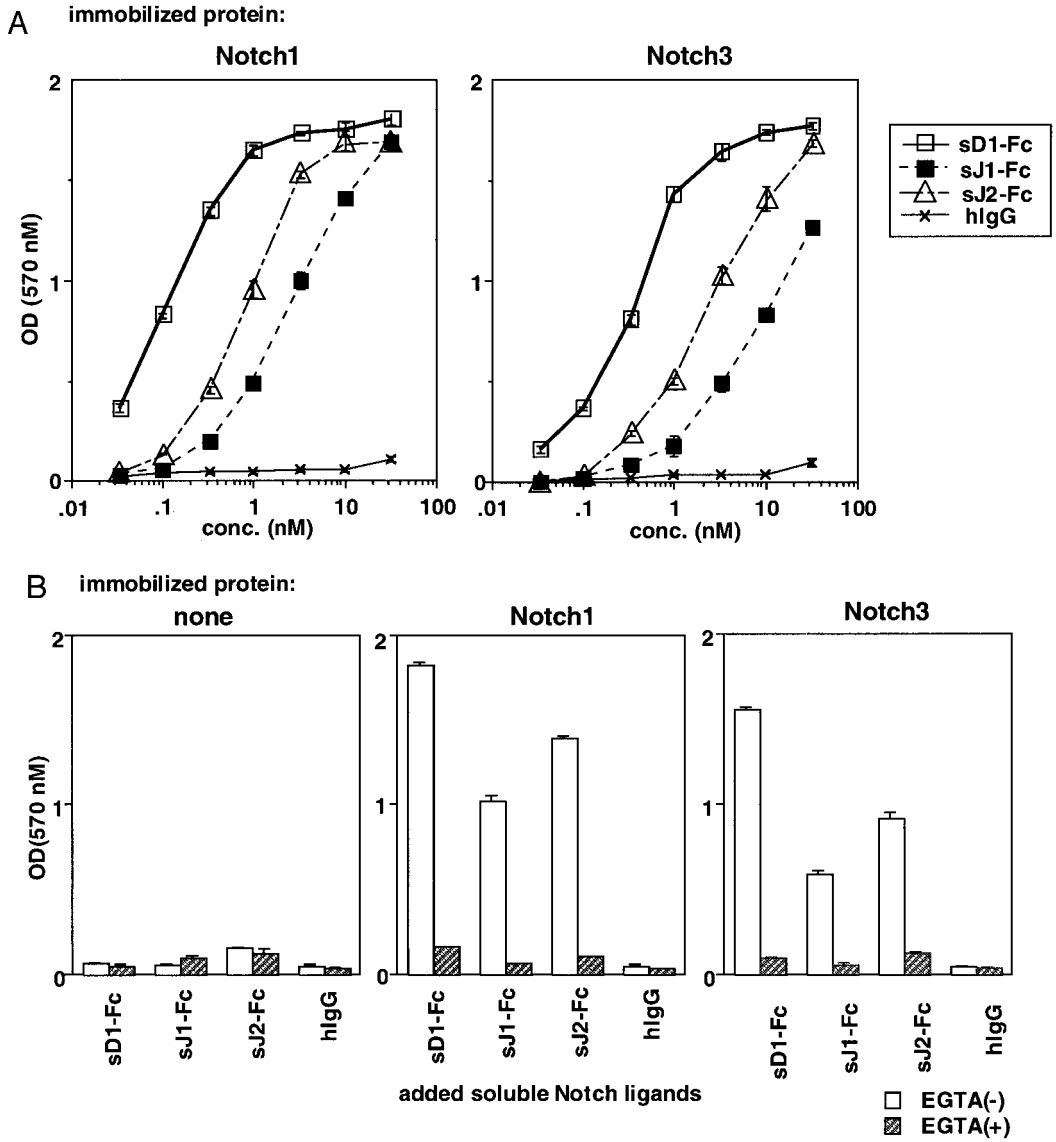

FIG. 3. Binding of soluble DSL proteins to soluble Notch1 and Notch3. (A) Saturation binding. Various concentrations of soluble DSL proteins were allowed to bind to sN1-Flag(His)$_6$ and sN3-Flag(His)$_6$ immobilized to the plastic surface, respectively. OD: Optical Density. (B) Dependence on Ca^{2+} for binding of sD1-Fc, sJ1-Fc, and sJ2-Fc to sN1-Flag(His)$_6$ and sN3-Flag(His)$_6$. sD1-Fc, sJ1-Fc, sJ2-Fc, and control hIgG at the same molar concentration (6.7 nM) were allowed to bind to immobilized sN1-Flag(His)$_6$ and sN3-Flag(His)$_6$ in the presence (hatched box) or the absence (open box) of 2 mM EGTA. Mean value of duplicates and standard deviation are shown for each protein.

CHO(r) cells expressing full-length DSL proteins (DSL-CHO(r); fD1-CHO(r), fJ1-CHO(r) and fJ2-CHO(r)) (Fig. 1). In contrast, neither sN1-Fc nor sN3-Fc bound at all to parental CHO(r) used as a control (Fig. 1). For all the DSL-CHO(r), sN3-Fc showed affinities slightly higher than sN1-Fc (Fig. 1). Binding of the soluble Notch proteins to DSL-CHO(r) was Ca^{2+} dependent (data not shown).

Binding of Soluble DSL Proteins to Soluble Notch1 and Notch3

To further characterize the interaction between the DSL proteins and Notch receptors, we examined it in a solid-phase binding assay using purified and immobilized sN1-Flag(His)$_6$ and sN3-Flag(His)$_6$. The partial extracellular region of Notch1 and Notch3 which comprised the N-terminus through the 15th EGF-like repeat, were tagged with a Flag(His)$_6$ sequence at the C-terminus to give sN1-Flag(His)$_6$ and sN3-Flag(His)$_6$. These proteins were produced in a stable expression system using Chinese Hamster Ovary ras (CHO(r)) cells. It was confirmed by Coomassie brilliant-blue staining that the purity of the two soluble Notch-Flag(His)$_6$ proteins was over 90% (Fig. 2) and that sN1-Flag(His)$_6$ and sN3-Flag(His)$_6$ were migrated into positions of about 125 kDa and 110 kDa, respectively

(Fig. 2). The positions of sN1-Flag(His)$_6$ and sN3-Flag(His)$_6$ migrated were larger than the molecular weights predicted from their cDNA sequences, indicating that both soluble Notch proteins should be highly modified.

Fc-fused soluble DSL proteins, Delta1 (sD1), Jagged1 (sJ1), and Jagged2 (sJ2), were added into microtiter wells, in which Flag(His)$_6$-tagged soluble Notch1 and Notch3 proteins were immobilized, and binding of the DSL proteins to the soluble Notch-Flag(His)$_6$ proteins was evaluated. Results showed that each of the DSL-Fc proteins bound to both immobilized sN1-Flag(His)$_6$ and sN3-Flag(His)$_6$ in a concentration-dependent manner (Figs. 3A and 3B). It was also revealed that each of the DSL-Fc binds to sN1-Flag(His)$_6$ and sN3-Flag(His)$_6$ with different affinities. Among the three DSL-Fc proteins, sD1-Fc had the highest binding activity for both soluble Notch proteins, followed by sJ2-Fc and sJ1-Fc (Fig. 3B). As shown previously in the case of sJ1-Fc (25), the addition of EGTA abolished the binding of both sD1-Fc and sJ2-Fc to any of the Notch receptors, indicating that these interactions are dependent on Ca^{2+} (Fig. 3C).

DISCUSSION

In the present study, we assessed binding activities of three DSL proteins, Delta1, Jagged1, and Jagged2, for soluble Notch1 and Notch3. It was shown that membrane-bound and soluble forms of three DSL proteins bound to soluble Notch1 and Notch3 proteins (Figs. 1 and 3). Interaction between the DSL proteins and Notch1 and Notch3 *in vivo* has been previously suggested by *in situ* hybridization analyses, in which a partially overlapping expression pattern is shown between the DSL proteins and the Notch receptors (20, 28–30). Integrating this information into our result, any one of the three DSL proteins could be a natural ligand for Notch1 and Notch3 *in vivo.* Furthermore, given that they also function as ligands for Notch2 (26), they may be ligands for all Notch receptors. Our observation that the three DSL proteins bound to Notch1 corresponded to the facts that they have signal-transducing activity for Notch1 (20, 22, 31). It remains to be elucidated whether they indeed function as ligands for Notch3.

The addition of EGTA revealed that all interactions between the DSL proteins and soluble Notch proteins were dependent on Ca^{2+} (Fig. 3). This characteristic has also been described in the *Drosophila* Notch system (15). Ca^{2+} dependency would therefore be a feature common in *Drosophila* through mammals. Since it has been found that the 11th and 12th EGF-like repeats of *Drosophila* Notch containing a Ca^{2+} binding site was a minimal binding unit for Delta and Serrate (15), the three mammalian DSL proteins would also bind to the corresponding regions of Notch1 and Notch3. In fact, in the solid-phase binding assay, the N-terminus through 15th EGF-like repeat of Notch1 and Notch3, which includes this region, interacted with all the soluble DSL proteins (Fig. 3). The experiments using soluble Notch1 and Notch3 proteins also revealed that the binding of the DSL proteins to Notch1 and Notch3 is direct (Fig. 3).

Furthermore, the solid-phase binding assay showed that the DSL proteins bound to Notch1 and Notch3 with diverse affinities. However, this experimental data may not directly indicate that the affinity of each DSL protein binding to Notch1 or Notch3 is invariable, due to the possibility that binding affinities among them are modified by Fringe proteins, putatively secreted glycosyltransferases which may confer glycosyl chains to Notch (32–34). In fact, we recently observed that binding of Notch2 to DSL proteins was significantly modified by fringe proteins (unpublished data).

In summary, three DSL proteins, Delta1, Jagged1, and Jagged2, have binding activity for Notch1 and Notch3. This suggests that the DSL proteins could be ligands for Notch1 and Notch3 receptors.

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