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Integrity of intracellular domain of Notch ligand is indispensable for cleavage required for release of the Notch2 intracellular domain

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The biological activity of the soluble form of the Notch ligand (sNL) and requirement of the intracellular domain (ICD) of the Notch ligand have been debated. Here we show that soluble Delta1 (sD1) activates Notch2 (N2), but much more weakly than full-length Delta1 (fD1). Furthermore, tracing the N2 molecule after sD1 stimulation revealed that sD1 has a defect in the cleavage releasing ICD of N2 (intracellular cleavage), although it triggers cleavage in the extracellular domain of N2. This represents the molecular basis of the lower activity of sD1 and suggests the presence of an unknown mechanism regulating activation of the intracellular cleavage. The fact that Delta1 lacking its ICD (D1Δ^{ICD}) exhibits the phenotype similar to that exhibited by sD1 indicates that the ICD of D1 (D1^{ICD}) is involved in such an as yet unknown mechanism. Furthermore, the findings that D1Δ^{ICD} acts in a dominant-negative fashion against fD1 and that the signal-transducing activity of sD1 is enhanced by antibody-mediated cross-linking suggest that the multimerization of Delta1 mediated by D1^{ICD} may be required for activation of the N2 intracellular cleavage.
Keywords: extracellular cleavage/intracellular cleavage/multimerization/Notch/Notch ligand

Introduction

The Notch family of genes encodes transmembrane receptors that are involved in the cell fate decision in vertebrates and invertebrates (Weinmaster, 1997; Greenwald, 1998; Artavanis-Tsakonas, 1999). In mammals, multiple Notch homologs have been identified, including Notch1 to Notch4 (Ellisen *et al*., 1991; Weinmaster *et al*., 1991, 1992; Kopan and Weitraud, 1993; Lardelli *et al*., 1994; Uyttendaele *et al*., 1996). The extracellular region comprises 29–36 epidermal growth factor (EGF)-like repeats and three copies of a Lin-12/Notch/Glp motif. The intracellular region contains cdc10/Ankyrin repeats and a PEST-containing domain. The Notch receptors are initially synthesized as ~300 kDa proteins, which are then proteolytically processed in the Golgi apparatus into an extracellular subunit (N^{EC}) containing multiple EGF repeats and lin-12/Notch repeats (Blaumueller *et al*., 1997; Logeat *et al*., 1998), and a single-pass transmembrane subunit (N^{TM}) containing a short extracellular tail and an intracellular domain (ICD; N^{ICD}). These subunits are reassembled in the *trans*-Golgi network and are presented as a heterodimeric, mature receptor at the cell surface (Blaumueller *et al*., 1997). The lin-12/Notch repeats and Ca^{2+} ion are involved in maintaining the heterodimeric complex of N^{EC} and N^{TM} (Rand *et al*., 2000).

Binding of a Notch ligand (NL) to N^{EC} triggers cleavage of Notch, releasing N^{ICD} from the cell membrane, which is then translocated into the nucleus to activate transcription of target genes in cooperation with RBP-Jκ (Kopan *et al*., 1996; Chan and Jan, 1998; Jarriault *et al*., 1998; Schroeter *et al*., 1998; Struhl and Adachi, 1998; Shimizu *et al*., 2000). This cleavage is mediated by a presenilin-containing complex and occurs within the transmembrane domain of Notch (intracellular cleavage) (De Strooper *et al*., 1999; Struhl and Greenwald, 1999; Ye *et al*., 1999). It has recently been proposed that prior to this cleavage, an additional cleavage at the extracellular domain of N^{TM} occurs in a ligand-dependent manner (Brou *et al*., 2000; Mumm *et al*., 2000) and that the extracellular cleavage autonomously promotes intracellular cleavage (Mumm *et al*., 2000). However, these proposals were based on experiments using Notch1 (N1) proteins with most of the extracellular domain truncated, or experiments using a partial peptide of N1. Therefore, the relationships between ligand stimulation and cleavage of the extracellular region of the native Notch protein, and between ligand-induced extracellular cleavage and subsequent intracellular cleavage, have not been fully addressed.

Delta and Serrate (Jagged), comprising a Delta/Serrate/Lag-2 motif, tandem EGF repeats and a short ICD, are known to be ligands for the Notch receptor. As a natural protein *in vivo*, *Drosophila* Delta exists in both the transmembrane and soluble forms (Klueg *et al*., 1998). It has recently been proposed that the soluble form of Delta is generated by Kuzbanian, a metalloprotease of the ADAM family (Qi *et al*., 1999). Results of examinations of the biological activity of the soluble Notch ligands have been controversial. Whereas all experiments using cell-culture systems have shown that they behave as agonists (Li *et al*., 1998; Qi *et al*., 1999; Han *et al*., 2000; Karanu *et al*., 2000; Morrison *et al*., 2000), *in vivo* experiments have demonstrated that soluble Delta and Serrate act as antagonists (Hukriede *et al*., 1997; Sun and Artavanis-Tsakonas, 1997). It remains to be elucidated why such contradictory conclusions are drawn. To explain the discrepancy, the difference in the activity between the soluble and full-length forms should be clarified.

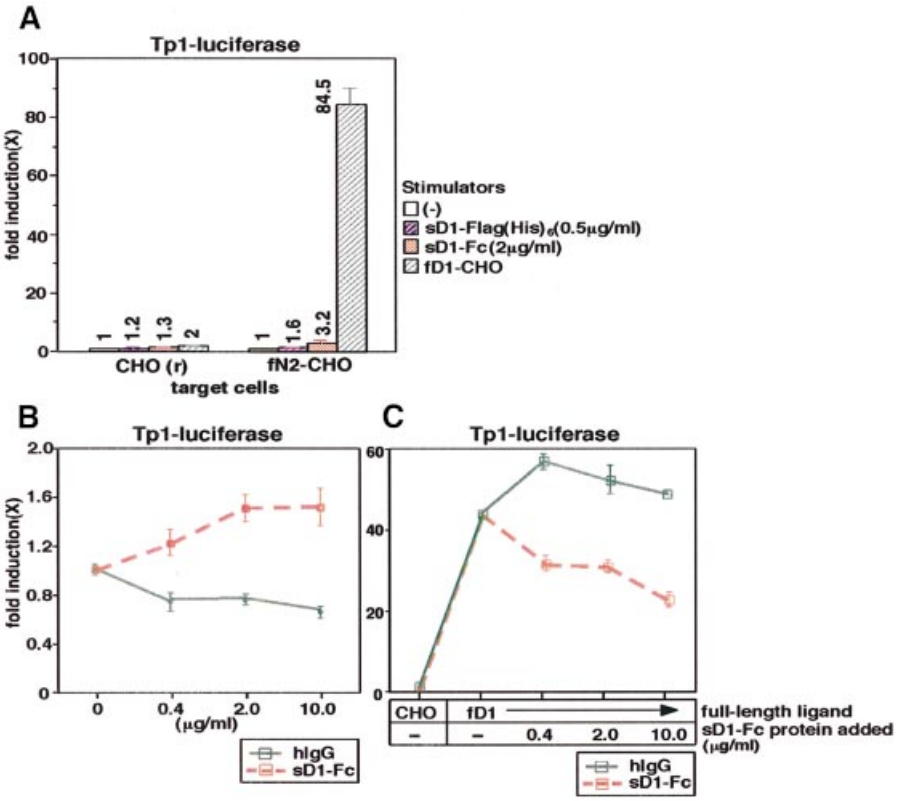

Fig. 1. Lower signal-transducing activity of soluble Delta1 protein. (**A**) Comparison of signal-transducing activity of sD1–Fc, sD1-Flag(His)$_6$ and fD1. A transient reporter assay with a TP1-luciferase reporter plasmid, pGa981-6, was performed using fN2-CHO cells. Following transfection of pGa981-6 into fN2-CHO, sD1–Fc, sD1-Flag(His)$_6$ or fD1-CHO was added to the transfected cells. Fold induction of the luciferase activity for each sample (mean of triplet measurements with standard deviation) was calculated against the control. The values are also shown in the graph. (**B**) N2-mediated transcriptional activation by sD1–Fc at increasing concentrations. Various concentrations of sD1–Fc were added to fN2-CHO cells transfected with pGa981-6. (**C**) The inhibitory effect of sD1–Fc on fD1-induced N2 signaling. fD1-CHO cells and sD1–Fc proteins at various concentrations were added simultaneously to the pGa981-6-transfected fN2-CHO cells. The same concentration of hIgG was added as a control.

In the present study, we show that the signal-transducing activity of the soluble form of Delta(-like-)1 (sD1) for Notch2 (N2) is obviously lower than that of full-length Delta1 (fD1), and that in coexistence with fD1 it inhibits the fD1-triggered N2 signal. This implies that sD1 is a partial agonist, while fD1 is a full agonist. Furthermore, we demonstrate the molecular basis of the impaired signal-transducing activity of sD1; it triggers cleavage of the extracellular domain of $N2^{TM}$, but promotes the cleavage step that releases $N2^{ICD}$ only very little. This indicates that, although the extracellular domain of NL alone is sufficient for extracellular cleavage, intracellular cleavage requires some other domain of NL, and that extracellular cleavage does not autonomously promote intracellular cleavage, suggesting the existence of an unknown mechanism that regulates the activation of the intracellular cleavage. Experiments using Delta1 without the ICD ($D1\Delta^{ICD}$) demonstrate that NL^{ICD} is important for the intracellular cleavage. Furthermore, the findings that $D1\Delta^{ICD}$ acts as a dominant-negative molecule against fD1 when they coexist and that the signal-transducing activity of sD1–Fc (sD1 fused to hIgG Fc portion) is enhanced by the addition of anti-Fc antibody suggest that oligomerization of NL is involved in Notch signaling.

Results

Lower signal-transducing activity of sD1

To define a biological activity of a soluble form of Notch ligand (sNL), we assessed the signal-transducing activity of mouse sD1 encompassing the entire extracellular region by comparing it with that of the full-length form in a transient reporter assay with CHO(r) cells overexpressing mouse full-length N2 (fN2-CHO), which is a highly sensitive assessment system for N2 signaling. Results showed that both Fc-fused and Flag(His)$_6$-tagged sD1 proteins [sD1–Fc and sD1-Flag(His)$_6$] activated the transcription of a reporter gene driven by the RBP-Jκ-responsive promoter, TP-1 (Figure 1A and B), but the transcriptional activity was obviously lower than that of fD1 [represented by the stimulation with CHO(r) expressing fD1 (fD1-CHO)] (Figure 1A). On the other hand, in coexistence with fD1, sD1 inhibited the fD1-mediated N2 activation compared with control hIgG (Figure 1C). These data indicate that sNL is a partial agonist, while full-length NL (fNL) is a full agonist.

We further evaluated the difference in the signal-transducing activity between the two molecules from another viewpoint, i.e. the nuclear accumulation of $N2^{ICD}$.

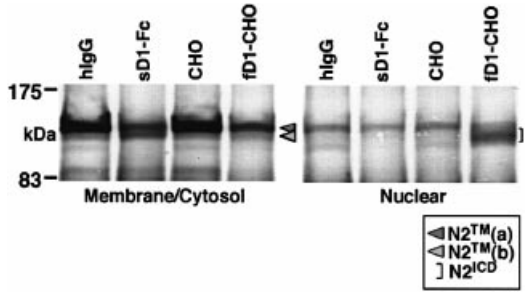

Fig. 2. N2 molecule traced after sD1–Fc and fD1 stimulations. BaF3 was stimulated for 1.5 h under the conditions indicated in the figure and then separated into membrane/cytosol-rich and nucleus-rich fractions. In each fraction, N2 fragments containing an ICD were analyzed by western blot analysis using the bhN6 antibody after immunoprecipitation with an anti-N2 polyclonal antibody.

It is generally accepted that the nuclear accumulation of N^{ICD} generated by cleavage within the transmembrane domain of the Notch receptor after fNL stimulation is associated with activation of the transcription of downstream genes in Notch signaling. To evaluate the cleavage and nuclear accumulation in a serial manner, we used BaF3 cells capable of displaying these two events following stimulation with fD1. As previously reported, stimulation with fD1 decreased the amount of $N2^{TM}$ in the membrane/cytosol fraction [designated $N2^{TM}$(a); Figure 2] and, instead, N2-derived fragments representing $N2^{ICD}$ were accumulated in the nuclear-rich fraction (Shimizu *et al*., 2000). In contrast, the stimulation with sD1–Fc did not result in detectable $N2^{ICD}$ in the nuclear-rich fraction, although it also reduced the amount of $N2^{TM}$(a) in the membrane/cytosol fraction. Instead, a new band representing a protein smaller than $N2^{TM}$(a) emerged in the membrane/cytosol fraction [designated $N2^{TM}$(b); Figure 2]. The fact that hardly any $N2^{ICD}$ was generated after stimulation with sD1–Fc was compatible with the results of reporter assays (Figure 1).

sD1 has a defect in the cleavage required for release of $N2^{ICD}$, although it can trigger the extracellular cleavage of N2

To understand better the lower signal-transducing activity of sD1, we then characterized $N2^{TM}$(b) generated by sD1 stimulation, which was scarcely seen after fD1 stimulation (Figure 2). The decrease in the amount of $N2^{TM}$(a) and the appearance of $N2^{TM}$(b) in the membrane/cytosol fraction after stimulation with sD1–Fc (Figure 2) indicated that $N2^{TM}$(b) represented a molecule derived from $N2^{TM}$(a). A further fractionation of the membrane/cytosol fraction demonstrated that $N2^{TM}$(b) and $N2^{TM}$(a) were present in the membrane but not in the cytosol fraction (Figure 3A), suggesting that $N2^{TM}$(b) was a membrane-associated molecule lacking either the N- or the C-terminal tail of $N2^{TM}$(a). To determine which side of $N2^{TM}$(a) was cleaved to generate the $N2^{TM}$(b) fragment, we performed the two experiments. In the first, we used fN2-CHO(r), [CHO(r) with exogenous fN2 tagged with a Flag sequence at the C-terminus] to investigate whether the Flag tag remained in $N2^{TM}$(b) generated after sD1–Fc stimulation. The result was that the anti-Flag antibody detected $N2^{TM}$(b) (Figure 3B), indicating that $N2^{TM}$(b) lacks the N-terminus

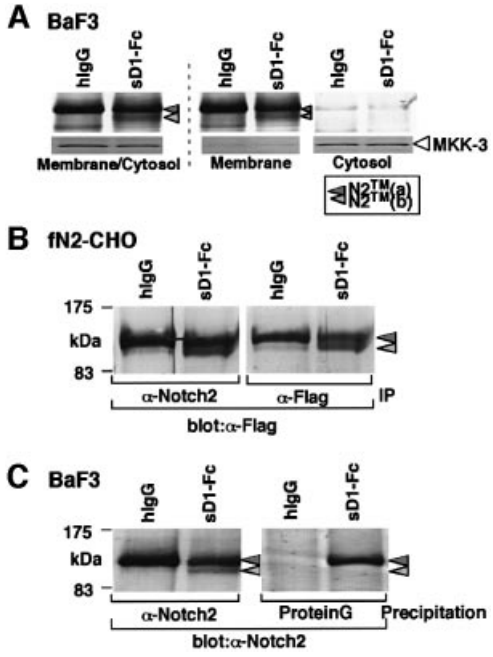

Fig. 3. Characterization of the $N2^{TM}$(b) fragment induced by sD1–Fc-stimulation. (**A**) To determine whether $N2^{TM}$(b) is a transmembrane protein, membrane/cytosol-rich fraction prepared from BaF3 after the sD1–Fc stimulation was then separated into membrane and cytosol fractions. N2 proteins in each fraction was subjected to western blot after immunoprecipitation with an anti-N2 polyclonal antibody. As a control for correct fractionation of membrane and cytosol franctions, an antibody against MKK3, MAP kinase, was used for each fraction in western blot analysis. (**B**) Generation of $N2^{TM}$(b) fragment containing a Flag$(His)_6$ tag at the C-terminus. fN2-CHO [CHO(r) with exogenous N2 with a Flag$(His)_6$ tag at the C-terminus] was incubated in the presence of either sD1–Fc or hIgG at 6.7 nM. After 1.5 h, the stimulated cells were collected and solubilized in a TNE buffer. The cell lysates were precipitated with an anti-Flag monoclonal (M2) or an anti-N2 polyclonal antibody. The precipitates were analyzed by western blot with the M2 antibody. IP, immunoprecipitation. (**C**) Co-precipitation analysis. BaF3 was incubated in RPMI medium containing sD1–Fc or hIgG at 6.7 nM for 1.5 h, then subjected to a cross-linking reaction to form the binding complex of sD1–Fc and N2. Following the reaction, the BaF3 lysates were divided into two aliquots. One was precipitated with an anti-N2 polyclonal antibody to identify N2 protein fragments. To precipitate sD1–Fc-containing complex, protein G beads were added directly to the other. These precipitates were analyzed by western blot with the bhN6 antibody.

but not the C-terminus of $N2^{TM}$(a). In the second experiment, we assessed whether $N2^{TM}$(b) was coprecipitated with sD1–Fc. In a previous report, we described that $N2^{TM}$(a) is precipitated with sD1–Fc (Shimizu *et al*., 2000). If the cleavage after sD1 stimulation occurs within the short extracellular domain of $N2^{TM}$(a), sD1–Fc-bound $N2^{EC}$ probably loses the association with $N2^{TM}$(b), and thus $N2^{TM}$(b) is not coprecipitated with sD1–Fc. As expected, sD1–Fc coprecipitated only $N2^{TM}$(a) and not $N2^{TM}$(b) (Figure 3C). This result also suggests that $N2^{TM}$(b) was generated from $N2^{TM}$(a) by the cleavage in the juxtamembrane portion of the extracellular region (see Figure 7).

We then investigated whether the same cleavage occurred during the process of fD1-mediated N2 signaling, to verify that $N2^{TM}$(b) generated by sD1 was not an artifact. The amount of sD1–Fc binding to BaF3 was significantly reduced when the binding assay was performed after the co-culture of BaF3 with fD1-CHO, as

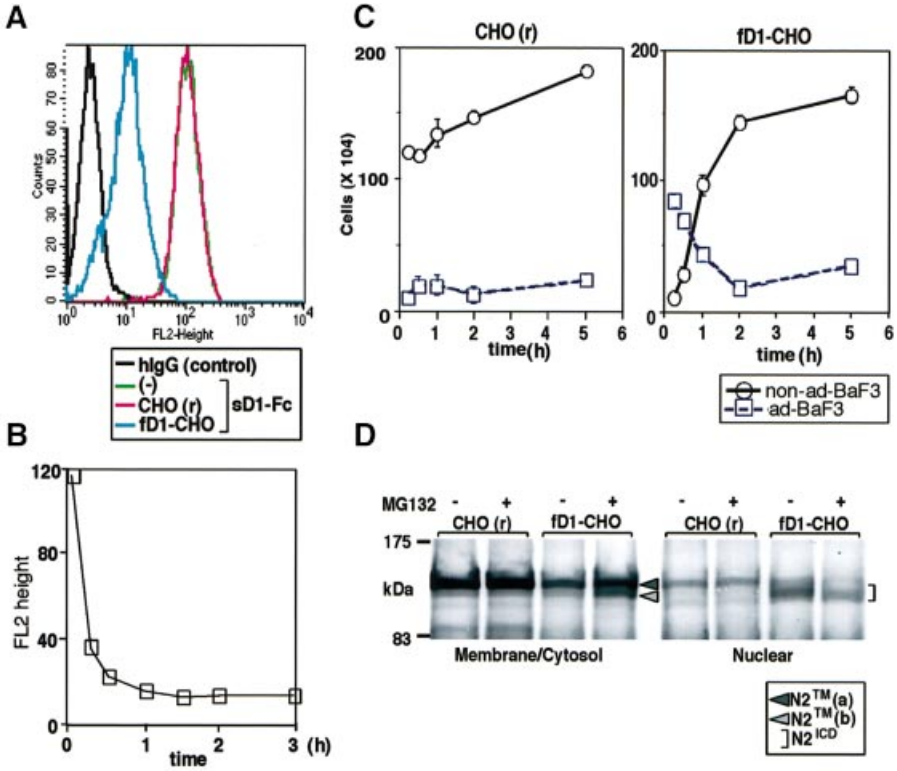

Fig. 4. Involvement of extracellular cleavage in fD1-mediated N2 activation. (**A**) Reduction in the amount of sD1–Fc binding to BaF3 after co-culture with fD1-CHO. BaF3 cells were collected at 1.5 h after co-culture with either CHO(r) or fD1-CHO. Cell-binding assay using sD1–Fc at 6.7 nM was performed for BaF3 cells recovered from the co-culture. (**B**) Time-course analysis of binding of sD1–Fc to BaF3 after co-culture with fD1-CHO. BaF3 cells co-cultured for the times indicated in the figure were subjected to cell-binding assays. The extent of fluorescence brightness giving the highest frequency (*y*-axis) was plotted against time (*x*-axis). (**C**) Time-dependent detachment of BaF3 from fD1-CHO. The time-course of the number of detached BaF3 cells was recorded in a cell–cell association assay. ad-BaF3, BaF3 that adhered to CHO cells; non-ad-BaF3, BaF3 that did not adhere to CHO cells. (**D**) Relationship between extracellular cleavage and nuclear transport of $N2^{ICD}$. MG-132, an inhibitor of cleavage for release of N^{ICD}, was added to a co-culture system of BaF3 and fD1-CHO at a final concentration of 25 μM. After 1.5 h of co-culture, the BaF3 cells were collected and separated into membrane/cytosol-rich and nucleus-rich fractions. In each fraction, N2 fragments containing an ICD were analyzed by western blot using the bhN6 antibody after immunoprecipitation.

compared with the co-culture with control CHO(r) (Figure 4A). A time-course analysis showed that the reduction in sD1–Fc binding started within 15 min and reached a plateau 1.5 h from the initiation of the co-culture (Figure 4B). During the co-culture, we observed that BaF3 cells, which previously adhered to fD1-CHO within 10 min, were detached from it in a time-dependent fashion (Figure 4C). One possible explanation for these phenomena is that the fD1-induced N2 extracellular cleavage results in the dissociation of $N2^{EC}$ together with the bound fD1 molecule from $N2^{TM}$, which then results in the reduction in fD1-bindable N2 receptors on BaF3 cell surface (see Figure 7).

To obtain more direct evidence of the extracellular cleavage of $N2^{TM}$(a) by fD1 and to determine the relationship between this extracellular cleavage and the cleavage following it, we added MG-132, a known inhibitor of the intracellular cleavage that results in the release of N^{ICD} (De Strooper *et al*., 1999; Mumm *et al*., 2000), into the co-culture system of BaF3 and fD1-CHO. The addition of MG-132 in fact reduced the amount of fD1-induced $N2^{ICD}$ in the nucleus-rich fraction (Figure 4D), implying that it prevented fD1-induced intracellular cleavage. In addition, $N2^{TM}$(b) was detected in the membrane/cytosol fraction when MG-132 was added (Figure 4D). This indicated that extracellular cleavage also occurred during the process of fD1-mediated N2 signaling, as in the case of sD1, and that stimulation with fD1 induced cleavage of $N2^{TM}$(a) in the extracellular region, prior to cleavage in the transmembrane region. The above findings lead to the conclusions that the extracellular cleavage does not autonomously trigger the N2 intracellular cleavage and that sD1 has a defect in the cleavage required for release of $N2^{ICD}$, although it can trigger extracellular cleavage of N2 (see Figure 7).

Requirement of NL^{ICD} for full activation of N2

To determine which region of Delta1 is involved in progression of the intracellular cleavage, we generated a CHO(r) cell line expressing $D1\Delta^{ICD}$ ($D1\Delta^{ICD}$-CHO) and investigated its signal-transducing activity. Using cell-binding assays with sN1, we first confirmed that sN1 bound to fD1-CHO and $D1\Delta^{ICD}$-CHO in an indistinguishable manner (Figure 5A), indicating that fD1 and $D1\Delta^{ICD}$ were approximately equally expressed on the cell surface. We also observed that the amount of sD1–Fc binding to BaF3 after co-culture with $D1\Delta^{ICD}$-CHO was reduced to a degree similar to that after the co-culture with fD1-CHO

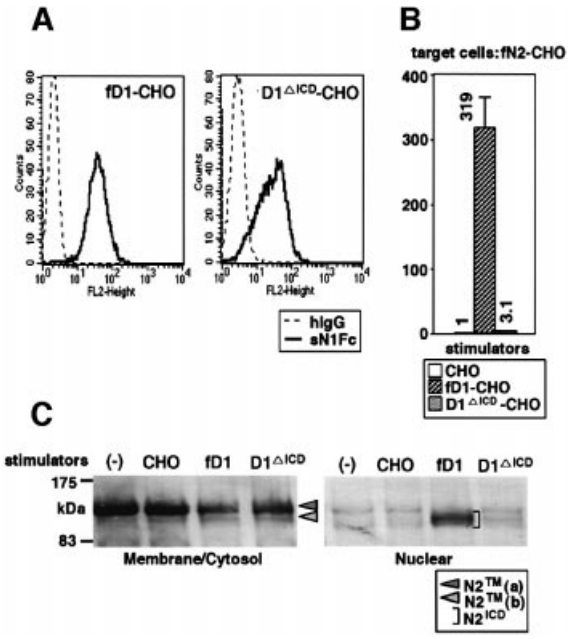

Fig. 5. Requirement of the intracellular domain of Delta1 for full activation of N2. (**A**) Generation of D1Δ^{ICD}-CHO [CHO(r) cells expressing the truncated Delta1 lacking its intracellular domain]. To investigate the expression of fD1 and D1Δ^{ICD}, a cell-binding assay using sN1-Fc (6.7 nM) was performed against the fD1-CHO and D1Δ^{ICD}-CHO cells. (**B**) Comparison of signal-transducing activity of fD1 and D1Δ^{ICD}. To examine activity of the two molecules, a transient reporter assay with a TP1-luciferase reporter plasmid was performed using fN2-CHO cells. Fold-induction of luciferase activity for fD1-CHO and D1Δ^{ICD}-CHO (mean of triplicate measurements with standard deviation) was calculated against luciferase activity when parental CHO(r) was used as stimulator. (**C**) N2 fragments after fD1 and D1Δ^{ICD} stimulations. BaF3 was stimulated for 1.5 h under the conditions indicated in the figure and then separated into two fractions, membrane/cytosol-rich and nucleus-rich. In each fraction, N2 fragments containing an intracellular domain were analyzed by western blot using the bhN6 antibody after immunoprecipitation.

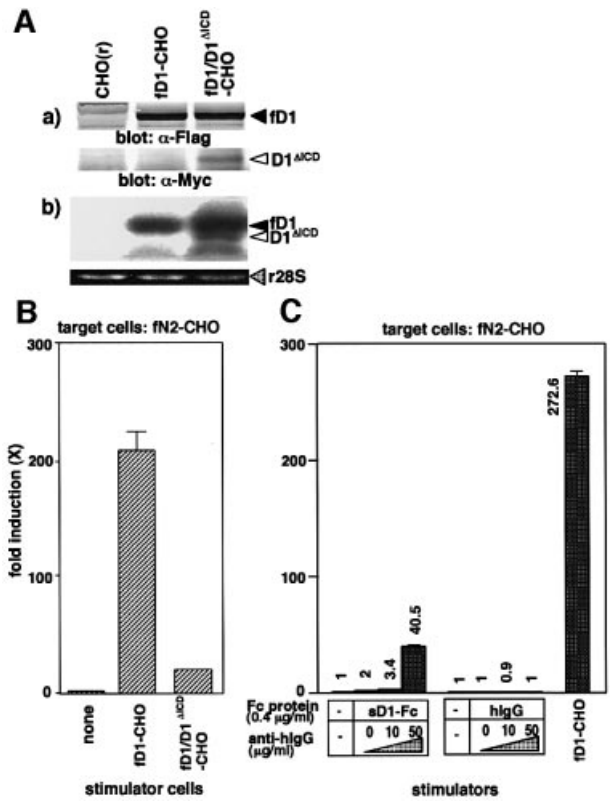

Fig. 6. Involvement of multimerization of Delta1 in the N2 activation. (**A**) Generation of fD1-CHO cells expressing Myc-tagged D1Δ^{ICD} (fD1/D1Δ^{ICD}-CHO). (a) Expression of Myc-tagged D1Δ^{ICD} and Flag-tagged fD1 proteins in fD1/D1Δ^{ICD}-CHO cells were examined by western blot analysis with an anti-Flag or an anti-Myc antibody. (b) To compare the expression levels of mRNA of fD1 with D1Δ^{ICD} in the fD1/D1Δ^{ICD}-CHO cells, total RNA (10 μg) extracted from the cells was subjected to northern blot using the 5′-end fragment of mouse *Delta1* cDNA as a probe. The lower panel shows ethidium bromide-stained 28S ribosomal RNA (r28S) in each lane. (**B**) Enhancement of the signal-transducing activity of sD1–Fc by addition of an anti-Fc antibody. A transient reporter assay was performed using pGa981-6 plasmid-transfected fN2-CHO cells in the presence of sD1–Fc and the anti-Fc antibody at various concentrations. hIgG was added as a control for sD1–Fc. The relative induction of luciferase activity in each sample (mean of triplicate measurements with standard deviation) was calculated against luciferase activity in the presence of hIgG alone. (**C**) A dominant-negative effect of D1Δ^{ICD} on fD1-triggered N2 activation. fD1/D1Δ^{ICD}-CHO [CHO(r) cells co-expressing fD1 and D1Δ^{ICD}] was generated and its signal-transducing activity was examined by a transient reporter assay with pGa981-6 plasmid-transfected fN2-CHO cells.

(data not shown), and that once D1Δ^{ICD}-CHO-adhered BaF3 cells were detached from it exactly like BaF3 cells co-cultured with fD1-CHO (data not shown). In contrast, the reporter assays using these cell lines showed that the signal-transducing activity of D1Δ^{ICD} was obviously lower than that of fD1 (Figure 5B). Correspondingly, N2^{ICD} was hardly detected in the nucleus-rich fraction after stimulation with D1Δ^{ICD}, unlike after stimulation with fD1, while D1Δ^{ICD} reduced the amount of N2^{TM}(a) in the membrane/cytosol fraction (Figure 5C). These observations indicate that D1Δ^{ICD} can bind to N2 and induce its extracellular cleavage, but cannot facilitate the ensuing intracellular cleavage, being similar to the phenotype exhibited by sD1, although emergence of N2^{TM}(b) was less clear when stimulated with D1Δ^{ICD} than that with sD1. Therefore, it was concluded that the ICD of D1 (D1^{ICD}) is essential for D1-induced N2 intracellular cleavage and full activation of N2, and that the lower signal-transducing activity of sD1 is a consequence of the lack of the ICD rather than the lack of the membrane anchorage.

Importance of multimerization of NL for full activation of N2

To see an effect of D1Δ^{ICD} on fD1-triggered N2 activation in the coexistence of the two molecules, we generated the fD1-CHO cell line expressing D1Δ^{ICD} (fD1/D1Δ^{ICD}-CHO) (Figure 6A) and investigated its signal-transducing activity. The result showed that the intensity of the N2 signal transduction by fD1/D1Δ^{ICD}-CHO was about one-tenth of that by fD1-CHO, indicating that the activity of fD1 was reduced to about one-tenth in the presence of D1Δ^{ICD} (Figure 6B). This suggests that D1Δ^{ICD} acts in a dominant-negative fashion against fD1, in agreement with previous report indicating that the Delta proteins lacking the ICD act as dominant-negative proteins in *Drosophila* and vertebrates (Chitnis *et al.*, 1995; Sun and Artavanis-Tsakonas, 1996; Jen *et al.*, 1997). Since the expression level of D1Δ^{ICD} was less than that of fD1 in the fD1/ D1Δ^{ICD}-CHO cells [Figure 6A, (b)], the strong

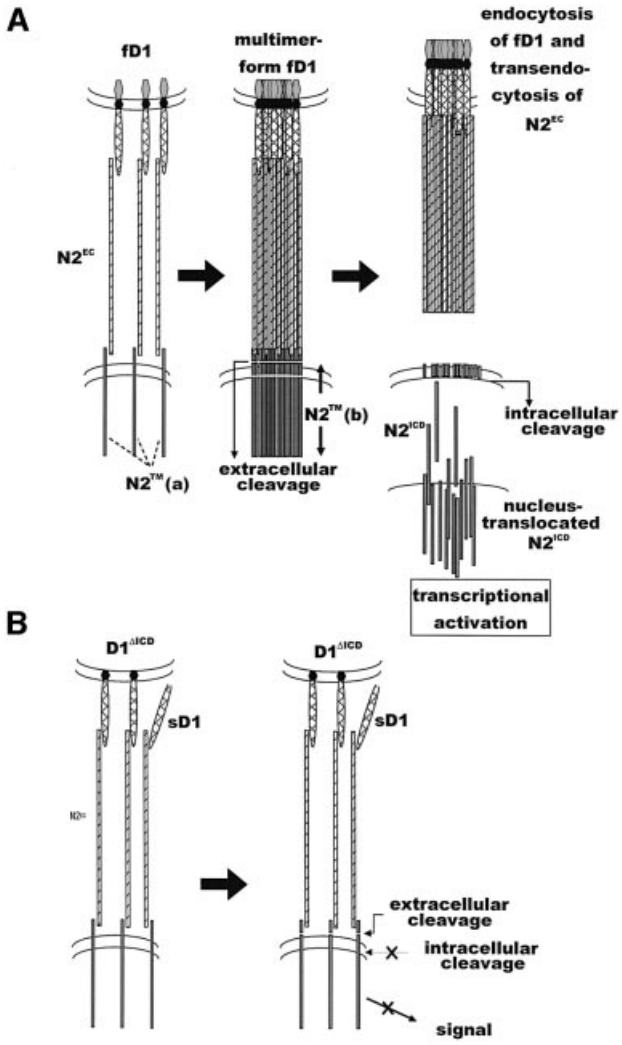

Fig. 7. Scheme of the ligand-induced cleavage of Notch receptor. (**A**) Upon binding to the Notch receptor, fNL forms appropriate homomultimers, leading to the intracellular cleavage of Notch in addition to the extracellular cleavage. Cleaved N^{ICD} translocates into the nucleus. Endocytosis of NL and N^{EC} occurs at some time point during the series of process. (**B**) $NL\Delta^{ICD}$ or sNL can bind to the Notch receptor and cleave Notch at the extracellular site, but do not form appropriate multimers or excute the intracellular cleavage of Notch, resulting in the failure of the Notch activation.

dominant-negative activity of $D1\Delta^{ICD}$ was likely to occur via interaction with fD1 rather than simple binding competition, raising the possibility that NL molecules interact with each other. Therefore, we investigated whether the signal-transducing activity of sD1–Fc was enhanced by addition of an anti-Fc polyclonal antibody, which can cross-link the sD1–Fc molecules. Results showed that addition of the anti-Fc antibody significantly increased the signal-transducing activity of sD1–Fc, but not of control IgG, in a dose-dependent manner (Figure 6C). We also note that the same phenomenon also occurred using soluble Jagged1 protein fused to Fc (data not shown). These results suggest that multimerization of the Notch ligand plays an important role in full activation of N2 (see Figure 7).

Discussion

In this study, we compared the signal-transducing activity of sD1 and fD1 and used the resultant information to analyze the activation process of N2 after ligand stimulation. We found that sD1 functions as a partial agonist and that the mechanism of such a function could stem from the incomplete activity of sD1 in the intracellular cleavage required to release $N2^{ICD}$. Furthermore, experiments using sD1 and $D1\Delta^{ICD}$ demonstrated that the extracellular cleavage at the extracellular domain of N2 does not autonomously induce intracellular cleavage, which takes place in the transmembrane domain, and that $D1^{ICD}$ is involved in some unidentified mechanisms that exist between the two cleavage processes. Given that the signal-transducing activity of sD1–Fc was enhanced by the addition of an anti-Fc antibody and that $D1\Delta^{ICD}$ acted in a dominant-negative fashion against fD1 for N2 signaling, we suggest that multimerization of NL would be important for intracellular cleavage (Figure 7).

A soluble form of *Drosophila* Delta does exist *in vivo* (Klueg *et al*., 1998), possibly generated by Kuzbanian (Qi *et al*., 1999). Therefore, it is important to understand the exact physiological function of sNL, although the precise C-terminal sequence of naturally occurring soluble Delta is unknown. However, conclusions regarding the biological activity of sNL to date have been discordant, as manoeuvered mammalian sD1 and soluble Jagged1 have been characterized as having an agonistic activity in *in vitro* experiments (Li *et al*., 1998; Qi *et al*., 1999; Han *et al*., 2000; Karanu *et al*., 2000; Morrison *et al*., 2000) and an antagonistic activity has been proposed for soluble Delta and Serrate in *Drosophila in vivo* assessments (Hukriede *et al*., 1997; Sun and Artavanis-Tsakonas, 1997). Examination of this issue was enabled by transcriptional activation assay using CHO(r) cells overexpressing mouse fN2 (fN2-CHO) (Shimizu *et al*., 2000), which is a very sensitive system. We have demonstrated that sD1 comprising entire extracellular domain induces N2 activation, but the activity is markedly lower than that induced by fD1 (Figure 1A). This indicates that sD1 is a partial agonist, while fD1 is a full activator for Notch signaling. The fact that the coexistence of sD1 led to inhibition of fD1-induced N2 activation (Figure 1C) further supports the nature of sD1 as a partial agonist. We also note that the signal-transducing activity of soluble Jagged1-Fc is lower than that of full-length Jagged1 (data not shown). Hence, a partial activity in the soluble form may be common to all kinds of NL, including naturally existing sNL, since they do not harbor NL^{ICD} that is essential for full activation of Notch signaling (Figure 5); this scenario explains the contradiction concerning the biological activity of sNL. Regarding the physiological function of sNL *in vivo*, we speculate that it is associated with the strict control of the Notch signaling, which is known to be critical for the exact cell fate decision, because the abnormal phenotype is seen in patients with haploinsufficiency of Jagged1 (Li *et al*., 1997; Oda *et al*., 1997).

In an analysis tracing the N2 fragments, we ascertained the molecular basis of the incomplete function of sD1. Consistent with the low transcriptional activity of sD1 (Figure 1A), the stimulation with sD1–Fc did not result in

nuclear accumulation of $N2^{ICD}$ (Figure 2), whose amount is considered to determine the level of subsequent RBP-Jκ-mediated transcriptional activation (Jarriault *et al*., 1995; Schroeter *et al*., 1998). Furthermore, the experiments using sD1–Fc incidentally unveiled the mechanism of the cleavage of $N2^{TM}$ in the extracellular region (Figures 2 and 3), which was recently demonstrated by different approaches (Brou *et al*., 2000; Mumm *et al*., 2000). This cleavage was also evident after the binding of fD1 (Figure 4), but only when the intracellular cleavage that takes place in the transmembrane portion of $N2^{TM}$ was blocked by an inhibitor (Figure 4D). Taken together, these data imply that sD1 induces the extracellular cleavage of N2, but fails to sufficiently promote the intracellular cleavage that releases $N2^{ICD}$, while fD1 efficiently triggers both cleavages. This indicates that the extracellular domain of Notch ligand alone is sufficient for extracellular cleavage of N2, and that extracellular cleavage is not necessarily followed by progression to intracellular cleavage, suggesting the existence of an as yet unknown mechanism regulating activation of the intracellular cleavage.

However, this notion was controversial, being in contrast to the recent report on extracellular cleavage of N1 in which the intracellular cleavage and subsequent signal transduction are described as autonomous events after extracellular cleavage (Mumm *et al*., 2000), which was drawn from experiments using the truncated N1 protein lacking N^{EC}. Regarding this discrepancy, we raise the possibility that the intracellular cleavage in the truncated Notch protein lacking N^{EC} progresses through a mechanism different from the intracellular cleavage induced by a ligand in the natural Notch protein. Indeed, the difference in intracellular cleavage between the two molecules was reported; the intracellular cleavage in the natural Notch protein occurred within 15 min of ligand binding (Shimizu *et al*., 2000), whereas that in the truncated Notch protein required >60 min (Schroeter *et al*., 1998) after protein synthesis. In addition, we also found that the amino acid sequence surrounding the extracellular cleavage site identified using truncated N1 (Brou *et al*., 2000; Mumm *et al*., 2000) is not conserved in N2. Consistent with this, addition of 1,10-*o*-phenanthroline, a reagent identified as an inhibitor of the extracellular cleavage in truncated Notch1 (Mumm *et al*., 2000), did not prevent sD1–Fc-induced extracellular cleavage of N2 (data not shown). Therefore, the extracellular cleavage site and the activation mechanism required for intracellular cleavage in the mutant Notch protein lacking N^{EC} may be somewhat diverse from those in the natural Notch proteins. Alternatively, the discrepancy may result from an unidentified difference between N1 and N2.

One clue to the mechanism regulating activation of the intracellular cleavage was found in the data from experiments using the truncated Dl protein lacking its ICD, $D1\Delta^{ICD}$ (Figure 5). The characteristics of $D1\Delta^{ICD}$ are similar to those of sD1 rather than those of fD1, i.e. a low level of transcriptional activity from the TP-1 promoter, sufficiency in cleaving the extracellular domain of $N2^{TM}$, and insufficiency in cleaving $N2^{TM}$ within the transmembrane portion and in releasing $N2^{ICD}$, although there was a slight difference in the emergence of $N2^{TM}$(b) (Figure 5B and C). These indicate that ICD of Delta1 is indispensable for the cleavage of $N2^{TM}$ within its transmembrane portion, which is essential for full activation of N2 (Figure 7).

Another clue to the mechanism lies in the data from experiments using sD1–Fc plus an anti-Fc antibody. Addition of the anti-Fc antibody resulted in enhancement of the signal-transducing activity of sD1–Fc (Figure 6C), suggesting that multimerization of the ligand is associated with the full activation of N2 (Figure 7). Dimerization of NL is inefficient, since the signal-transducing activities of sD1–Fc (dimer) and sD1-Flag(His_6) (monomer) were the same (Figure 1). It is possible that an assembly of a large number of NL molecules is required for fN activation, since a monoclonal anti-Fc antibody did not work, unlike a polyclonal antibody (data not shown). Putting together the inevitable role of $D1^{ICD}$ for D1-induced N2 intracellular cleavage and subsequent N2 activation, as we discussed above, NL^{ICD} may be the region that is used for the multimerization of fNL. As for the strong dominant-negative effect of $D1\Delta^{ICD}$ against fD1 (Figure 6), we raise the possibility that $D1\Delta^{ICD}$ can interact with fD1 using its transmembrane and/or extracellular domains to participate in the fD1 assembly, which interfere with the appropriate fD1 multimerization suitable for the intracellular cleavage of N2.

As is well known with regard to cytokines and their receptor systems (reviewed in Heldin, 1995), the multimerization of NL may be associated with the assembly of the Notch receptor, which may result in its conformational change and allow the transmembrane domain of Notch to be subjected to the action of the presenilin-containing protease complex and subsequent cleavage. Upon fD1 binding, N2 extracellular cleavage must be preceeded by fD1 multimerization and N2 assembly, although these steps are not necessary for N2 extracellular cleavage itself (Figure 7).

It was reported recently that transendocytosis by the ligand-expressing cells of the ligand-bound Notch extracellular domain together with the ligand appears to be necessary for efficient Notch processing and signal transduction (Parks *et al*., 2000). Integrating this into our model of Notch receptor activation, the endocytosis process may be positioned after the ligand multimerization or Notch assembly. However, we are not certain whether the transendocytosis is always necessary for the Notch signaling, since cell-free ligands can activate Notch signaling, particularly when the antibody-mediated ligand cross-linking or ligand-coating technique is used (Figure 6; Morrison *et al*., 2000; Varnum-Finney *et al*., 2000). Given the well-established notion in the G-coupled receptor and the cytokine receptor systems that endocytosis is associated with receptor/ligand degradation rather than directly associated with signal activation, the possibility may remain that transendocytosis in the Notch signaling pathway participates in degradation of Notch and NL.

Comparing the effect of $D1\Delta^{ICD}$ with that of sD1, emergence of $N2^{TM}$(b) was less clear when stimulated with the former (Figures 2 and 3) than that with the latter (Figure 5C), despite our assumption that $N2^{TM}$(b) was indeed generated after $D1\Delta^{ICD}$ stimulation, since the amount of $N2^{TM}$(a) was obviously reduced. As an explanation of this phenomenon, we speculate that some

unknown degradation process for $N2^{TM}$(b) is accelerated by stimulation with $D1\Delta^{ICD}$ but not with sD1.

In the present study, we demonstrated the biological activities of sNL and the existence of a novel mechanism involved in the activation of the Notch receptor by its ligand. Although many investigators are using sNL to determine the involvement of the Notch signal in the regulation of cell differentiation in various experimental approaches, we have to be careful of the interpretation of results, since the demonstrated phenotype could be caused by the inhibition, rather than activation, of the Notch signal. We believe that the findings described here will facilitate understanding of the complexities of Notch signaling in higher vertebrates.

Materials and methods

Plasmid construction

To generate $D1\Delta^{ICD}$, mouse *Delta1* cDNA (a gift from A.Gossler; Bettenhausen *et al*., 1995) was truncated at the codon CGG corresponding to arginine (amino acid 570). The resulting $D1^{DICD}$ was constructed in an expression vector pTraserCMV (Clontech) after addition of a Flag or a Myc tag.

Soluble fusion proteins

sD1 proteins [sD1–Fc and sD1-Flag$(His)_6$] and sN1 protein (sN1-Fc) were prepared as described previously (Shimizu *et al*., 1999, 2000).

Cell culture

BaF3 cells were maintained in RPMI medium supplemented with 10% fetal bovine serum (FBS) and 0.5 ng/ml recombinant mouse interleukin-3 (a gift from Kirin Brewery, Japan). CHO(r) (a gift from S.Shirahata, Kyushu University), fN2-CHO (Shimizu *et al*., 2000), fD1-CHO (Shimizu *et al*., 2000), $D1\Delta^{ICD}$-CHO and fD1/$D1\Delta^{ICD}$-CHO cells were maintained in alpha-minimal essential medium containing 10% FBS.

Antibody

An anti-human IgG goat polyclonal antibody used for multimerization of sD1–Fc was purchased from DAKO Japan Co., Ltd.

Cell-binding assay

Binding of sD1–Fc to the pro-B cell line BaF3 was performed as described previously (Shimizu *et al*., 1999), with the minor modification that the binding reaction was terminated at 5 min.

Co-precipitation using sD1–Fc

Co-precipitation using sD1–Fc has been described elsewhere (Shimizu *et al*., 2000). Disuccinimidyl glutarate (Pierce) was used to cross-link sD1–Fc and the bound Notch receptor.

Cell–cell association assay

Cell–cell association assay was performed as described previously (Shimizu *et al*., 2000). Briefly, CHO(r) and fD1-CHO cells were inoculated at 1×10^6 into a 6 cm plate. After overnight culture, 1×10^6 BaF3 cells were spread over the monolayer of cells. Following co-culture at 37°C for the time indicated in Figure 4C, BaF3 cells that did not adhere to the cell layer were collected by swirling the plate very gently and washing the wells gently once with RPMI medium. The population obtained through these procedures was defined as non-adhered BaF3. Next, phosphate-buffered saline containing 2 mM EGTA was added to the wells and the BaF3 cells adhering to the cell layer were allowed to dissociate by tapping the plate. These BaF3 cells, together with additional cells collected by washing with RPMI medium, were defined as adhered BaF3. The cells in each fraction were then counted.

Transient transcription assay

A total of 4×10^4 fN2-CHO was inoculated into a 24-well plate and transfected with a TP1-luciferase reporter plasmid (Minoguchi *et al*., 1997), pGa981-6, by a liposome-based method (SuperFect, Qiagen). Following transfection, sD1–Fc and sD1-Flag$(His)_6$ were added at the respective concentrations shown in Figure 1A and cultured for 30–40 h. When fD1 or $D1\Delta^{ICD}$ were used as a stimulator, these cells were added at 5×10^4 to the pGa981-6-transfected fN2-CHO and co-cultured for 30–40 h. The mixture of cells was then used for luciferase assay.

Subcellular fractionation of BaF3

After 1.5 h co-culture with fD1-CHO or $D1\Delta^{ICD}$-CHO, BaF3 was collected and membrane/cytosol-rich and nucleus-rich fractions were prepared as described elsewhere (Shimizu *et al*., 2000). The membrane/cytosol-rich fraction was further centrifuged at 105 000 *g* for 30 min at 4°C to separate the membrane (pellet) and the cytosol (supernatant) fractions. These fractions were used for immunoprecipitation with an anti-N2 polyclonal antibody.

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