The adhesion force of Notch with Delta and the rate of Notch signaling

Francois Ahimou, Lee-Peng Mok, Boris Bardot, and Cedric Wesley

Department of Microbiology and Molecular Genetics, The University of Vermont, Burlington, VT 05405

Notch signaling is repeatedly used during animal development to specify cell fates. Using atomic force microscopy on live cells, chemical inhibitors, and conventional analyses, we show that the rate of Notch signaling is linked to the adhesion force between cells expressing Notch receptors and Delta ligand. Both the Notch extracellular and intracellular domains are required for the high adhesion force with Delta. This high adhesion force is lost within minutes, primarily due to the action of Presenilin on Notch. Reduced turnover or Delta pulling accelerate this loss. These data suggest that strong adhesion between Notch and Delta might serve as a booster for initiating Notch signaling at a high rate.

Introduction

Notch (N) and Delta (Dl) are cell surface proteins that are required for differentiation of almost all tissues in animals from worms to humans. Their actions specify two cell types from a population of equipotent cells (in a process called lateral inhibition) or establish boundaries between two different cell populations (for reviews see Artavanis-Tsakonas et al., 1999; Mumm and Kopan, 2000).

When Dl on one cell binds N on the other, N gets proteolytically cleaved, first by the Kuzbanian or TACE metalloproteases (S2 cleavage) and subsequently by the Presenilin (Psn)/γ-secretase complex (S3 cleavage). The Notch intracellular domain (N^{intra}) is released from the plasma membrane, translocated to the nucleus, and in association with the transcription factor Suppressor of Hairless (SuH) activates transcription of target genes such as the *Enhancer of split Complex* (E(spl)C) genes (see Fig. 1 A). One group of cells accumulates a high level of N activity to become one cell type, whereas the other group accumulates very little or none to become the other cell type (Artavanis-Tsakonas et al., 1999; Brou et al., 2000; Mumm and Kopan, 2000; Lieber et al., 2002). From here onwards, N activity dependent on SuH and N^{intra} will be referred to as the SuH/N^{intra} signaling. Dl also gets cleaved in a manner similar to N; its consequence is not clear but is thought to down-regulate N signaling (Qi et al., 1999; Mishra-Gorur et al., 2002; Bland et al., 2003; Ikeuchi and Sisodia, 2003; LaVoie and Selkoe, 2003).

Lateral inhibition is a rapid process. Production of the precursor cells for the embryonic epidermis and the central nervous system, or the R8 photoreceptor cell in the developing compound eye, takes less than 20 min (Campos-Ortega and Hartenstein, 1985; Hoppe and Greenspan, 1990; Baker and Yu, 1998). Time might be a very important element, as the process often takes place in the context where morphogenetic events are rapidly progressing. Even in vertebrates, Notch receptors are involved in time-driven processes such as somitogenesis (Pourquie, 2003). Thus, a delay or acceleration in the rate of SuH/N^{intra} signaling might adversely affect proper differentiation of tissues. Although we know quite a bit about the mechanisms regulating the level of SuH/N^{intra} signaling, we know very little about the mechanisms regulating its rate.

Cell–cell adhesion has been a conspicuous but enigmatic part of N and Dl functions. When the function of N or Dl is reduced, loss of cell/tissue adhesion is often observed in addition to loss of cell types (de Celis and Garcia-Bellido, 1994; Goode et al., 1996; Renaud and Simpson, 2001). Furthermore, when cultured *Drosophila* Schneider (S2) cells expressing N are mixed with S2 cells expressing Dl, huge cell aggregates form (Fehon et al., 1990), suggesting that N and Dl themselves could act as adhesion molecules in addition to their signaling activities. However, their adhesive functions would require membrane anchoring, whereas their signaling functions would require proteolytic cleavage that would oppose adhesive functions. At least partly due to this paradox, the significance of the adhesive capabilities of N and Dl to development has remained obscure. Here, we show that the high adhesion strength between the N-expressing cells and the Dl-expressing cells promotes initiation of SuH/N^{intra} signaling at a high rate. These data provide

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Correspondence to Cedric Wesley: cwesley@uvm.edu

Abbreviations used in this paper: AFM, atomic force microscopy; Dl, Delta; EPC, epidermal precursor cell; E(spl)C, Enhancer of split Complex; N, Notch; NPC, neuronal precursor cell; Psn, Presenilin; RNAi, RNA interference; SuH, suppressor of Hairless.

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a basis for resolution of the paradox and suggest that the very physical adhesive capabilities of N and Dl regulate their very chemical signaling activities.

We used atomic force microscopy (AFM) and the S2 cell model system to study N/Dl adhesion and SuH/N^{intra} signaling. AFM is an excellent tool for studying cell surface ultrastructure and molecular interactions under physiological conditions (Schabert et al., 1995; Benoit et al., 2000; Ahimou et al., 2003). It measures the force applied to make contact between two surfaces (the contact force) and the force applied to detach them after contact (the detachment force). One of the surfaces is mounted on a probe called the cantilever that is lowered onto, or retracted from, a receptacle containing the other surface. Using a laser beam, a photodiode detects the deflections or bending of the cantilever caused by attraction, repulsion, or adhesion, and a computer processes them to produce a "force–distance graph" from which the maximum contact and detachment forces can be measured. The cantilever can also be made to scan a surface to construct its "image" based on attraction, repulsion, and physical features affecting the cantilever movements. We mounted one type of live cells on the "tip-less" cantilevers, placed the other type of live cells in a Falcon plate well, and measured the maximum contact or adhesion forces, under 1× PBS or 1× PBS + Ca^{2+}. We used the silicon nitride cantilevers with pyramidal tips to scan the surfaces of live cells.

S2 cells do not express the endogenous N or Dl (Fehon et al., 1990). They can be made to express N, Dl, or their variant/mutant forms using exogenous inducible or constitutive promoters. All known aspects of SuH/N^{intra} signaling are reproduced in S2 cells expressing N or Dl (Fehon et al., 1990; Lieber et al., 1992; Klueg and Muskavitch, 1999; Parks et al., 2000; Wesley and Saez, 2000; Mishra-Gorur et al., 2002; Wesley and Mok, 2003). Using S2 cells made to express various proteins, we studied the adhesion force between Notch receptors and Dl and its effect on SuH/N^{intra} signaling, with minimal disruptive procedures, maximum experimental controls, minimal variation in physiological or developmental states of cells, minimal interference from other pathways, and maximum control over the expression of desired molecules.

Results

Definitions and the basic features of molecules and S2 cells used in experiments

N will refer to the wild-type Notch receptor; additional characters will identify the mutant or modified Notch receptors. The word "Notch" will refer to all Notch receptors. The tip-less cantilevers will be identified by the type of cell they carry. The structures of the various Notch receptors used and their relative in vivo activities are shown in Fig. 1 B. All S2 cell lines used were stable, with >90% of the cells expressing the expected proteins, except N^{Ax59d} and NΔI cell lines that had only 40% expressing high levels. The frequency of cells showing significant detachment force with Dl corresponded with the frequency of cells expressing the Notch receptor able to bind Dl. Measurements were collected from at least 10 Notch-expressing cells in each experiment. With NΔ1-18 that lacks the Dl binding site, significant detachment force was never detected with over 150 cells, although ∼95% of the cells express this Notch receptor. The size of cells was not significantly different between the S2 cell lines; they were all ∼10 μm. The total and cell surface expression of all Notch receptors were equivalent to or higher than the levels of N (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200407100/DC1).

Detachment forces between S2-Dl cells and S2 cells expressing Notch receptors

A force–distance graph generated between an S2-Dl cantilever and an S2-N cell is shown in Fig. 2 A. The contact force between an S2-Dl cantilever and an S2-N cell was generally ∼19 ± 8 nanoNewtons (nN), and the detachment force ∼14 nN (see also Fig. 2 B, set 1; Fig. 2 C, top graph). These values were very similar in over 25 defined experiments. The contact forces were more variable than the detachment forces, but there was no correlation between the two within each cell line (unpublished data). For measurement of detachment force, we consider only the highest peak, as it is most likely to represent detachment from Dl. The distance of "pull" between S2-N and S2-Dl cells was generally ∼750 nanometers (nm) and could include minor detachment, nonspecific events, stretching of N and/or Dl molecules, or stretching of S2-N and/or S2-Dl cells.

When the same S2-Dl cantilever was used first on an S2-N cell, then on an S2-NΔ1-18 cell (lacking the Dl binding region), and back on an S2-N cell, the contact and detachment forces were similar on the S2-N cells but low or negligible on the S2-NΔ1-18 cell (Fig. 2 C; also Fig. 2 B, compare set 1 with set 10). This experiment, repeated many times, indicated the following: (1) the significant forces measured were specific to the first 18 EGF-like repeats of N, which includes the Dl-binding region (Rebay et al., 1991); (2) if the S2-Dl cell had detached from the cantilever after the first contact with an S2-N cell, the lectin-coated cantilever would have attached to the S2-NΔ1-18 cell and would have required ∼27 nN to detach this cell from the plate (unpublished data; the presence of the S2-Dl cell was also confirmed microscopically); (3) if the S2-N cell had detached from the bottom of the Falcon plate and held on to the S2-Dl cell on the cantilever, the force–distance graph with the second S2-N cell would have resembled the one obtained with the S2-NΔ1-18 cell, as force is not required to detach an S2-N cantilever from an S2-N cell (Fig. 2 B, set 11); (4) if the S2-Dl cantilever had contacted the bottom of the receptacle, the force required to detach them would have been ∼31 nN (Fig. S2 A, available at http://www.jcb.org/cgi/content/full/jcb.200407100/DC1); and (5) if the S2-N cells had contacted the cantilever at a place not holding the S2-Dl cell, ∼32 nN would have been required for detachment of the S2-N cell from the plate (Fig. S2 A). N requires calcium for binding Dl (Rand et al., 2000), and in the absence of calcium the detachment force was only ∼4 nN (Fig. 2 B, set 1). These observations indicated that the detachment forces we measured represented the forces required to detach an S2-Dl cell from an S2-N cell.

The contact force required for the S2-Dl cantilever to contact S2-N cells was 19 ± 8 nN, 3 ± 2 for S2-NΔ1-18 cells,

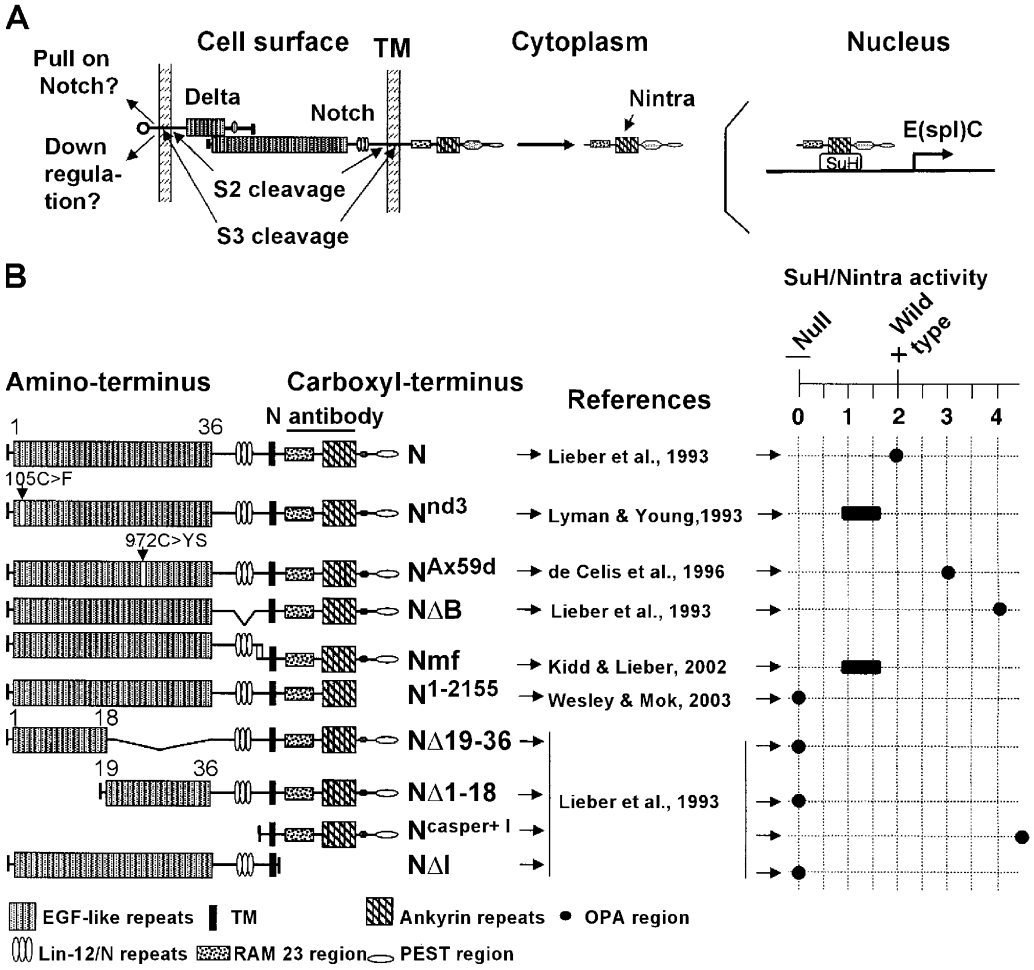

Figure 1. **Notch signaling and the various Notch receptors used in this paper.** (A) The SuH/N^{intra} signaling pathway. (B) The structures of Notch receptors used in this paper; relative activities shown on an arbitrary scale. TM, transmembrane domain.

3 ± 1.3 for S2-$N^{1\text{-}2155}$ cells, 2.5 ± 1.2 for S2-N^{nd3} cells, 4.7 ± 1 for S2-Nmf cells, and 2 ± 1 for S2-Dl cells. This suggests that Notch receptors and Dl do not experience much attraction force between them. In particular, N and Dl have to be "pushed" hard toward each other for binding. However, once they bound a force of ~14 nN was required to detach them. This is ~50–250 times the force estimated for separation of streptavidin from biotin or an antibody from its antigen (Moy et al., 1994; Hinterdorfer et al., 1996; Allen et al., 1997).

The other detachment forces we measured are also shown in Fig. 2 B and they indicated the following: (1) both small mutations or large deletions in the extracellular domain not including the Dl-binding site (in N^{nd3}, N^{Ax59d}, and NΔ19-36) affected the binding strength with Dl (Fig. 2 B, compare set 1 with 3, 5, and 6); (2) the predominantly heterodimeric Nmf, resembling the mammalian Notch receptors, bound Dl with a significantly lower strength than the colinear N predominant in flies (Fig. 2 B, compare sets 1 and 4); (3) the absence of the carboxyl-terminal half of the intracellular domain (in $N^{1\text{-}2155}$) or of the entire intracellular domain (in NΔI) dramatically reduced the binding strength with Dl (Fig. 2 B, compare set 1 with 2 and 7); (4) the detachment forces were significantly higher than zero with all Notch molecules containing the Dl-binding site, but not with Notch receptors lacking the Dl-binding site (Fig. 2 B, compare sets 1–7 with 9–10); (5) the detachment force was related to the type of Notch receptors and not to their relative total or cell surface levels (compare Fig. 2 B with Fig. S1). Furthermore, S2 cells expressing N through the heat-shock promoter or the actin promoter (representing almost an order of magnitude difference in the levels of N) showed very similar detachment forces with either S2 cells expressing Dl through the heat shock or the actin promoter (unpublished data); (6) the detachment force was independent of the clustering abilities of Notch receptors at contact points with Dl: N, $N^{1\text{-}2155}$, Nmf, and NΔI cluster and N^{nd3} does not (data for N and $N^{1\text{-}2155}$ is shown in Fig. S3; data for others not shown); $N^{1\text{-}2155}$ and NΔI showed the lowest detachment force, N the highest, and Nmf and N^{nd3} intermediate levels; (7) Dl showed weak binding to itself (Fig. 2 B, compare sets 1 and 8), consistent with the report of Dl homotypic interactions (Fehon et al., 1990); (8) N molecules expressed on different cells do not bind each other (Fig. 2 B, compare sets 1 and 11); (9) calcium significantly enhanced the binding strength of Dl with all Notch receptors, except with $N^{1\text{-}2155}$, NΔI, and itself (Fig. 2 B, compare hatched bars with the

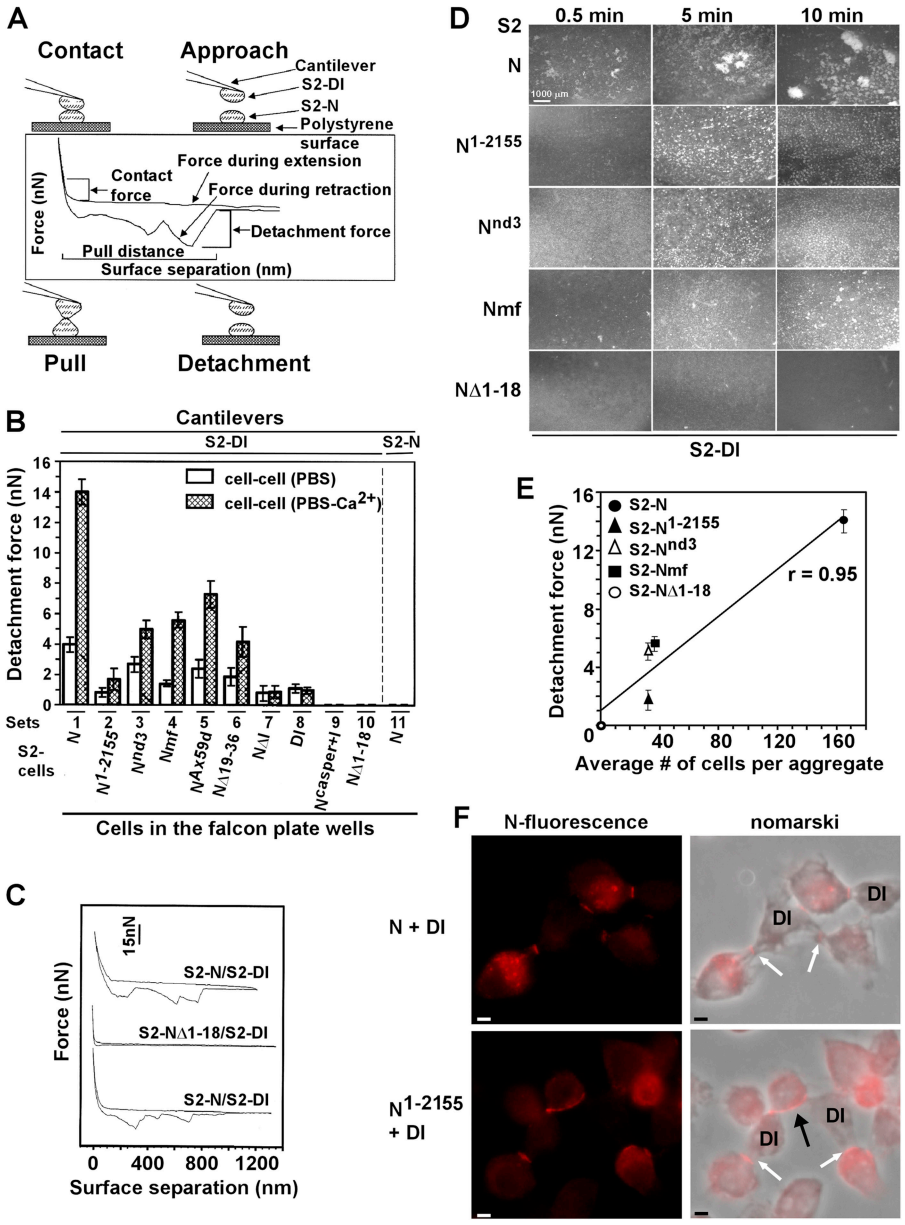

Figure 2. **The detachment force between Notch receptors and Delta.** (A) A force–distance graph generated between an S2-Dl cantilever and an S2-N cell in 1× PBS+Ca $^{2+}$. (B) Detachment forces between S2-Dl or S2-N cantilevers and different cells. (C) Force–distance graphs generated when the same S2-Dl cantilever was used successively on an S2-N cell, an S2-NΔ1-18 cell, and an S2-N cell, in 1× PBS+Ca $^{2+}$. (D) Cell aggregates of S2-Dl or S2 cells with S2 cells expressing Notch receptors. (E) Correlation (r) between the detachment force and the average number of cells in aggregates (at 10 min). Cell number = aggregation size/S2-NΔ1-18+S2-Dl aggregate size (~1 cell); SD: N = 20.44, N^{1-2155} = 8.4, N^{nd3} = 13.9, Nmf = 16.7). (F) Aggregated cells after forceful separation. White arrows = Notch cluster regions coextensive with Dl contact regions; black arrow = an N^{1-2155} cluster region partially coextensive with Dl contact region. Left, Texas red images with an N antibody (C458.2H); right, Nomarski images of the same cells. Bars, 2 μm (in F).

clear bars in sets 1–8); (10) the low detachment force with N^{1-2155} was not due to it being plucked out of the membrane by the S2-Dl cantilevers, as the "serial binding" experiments (as in Fig. 2 C) showed that the same S2-Dl cantilever adhered equally well to the first and the second S2-N^{1-2155} cells (Fig. S2 C); (11) the differences in detachment forces between the cell lines were unlikely to be due to differential adhesion of the cells to the Falcon plates, as the forces required for detaching each cell type from the Falcon well plates were all at least twice the values observed with S2-Dl cantilever (Fig. S2 A); and (12) the differences in detachment forces between the Notch receptors were due to the differences in their interaction with Dl, as they all showed similar maximum detachment forces with the silicon nitride cantilevers (Fig. S2 B). The differences in interactions with Dl are expected to be due to the differences in the number of Notch receptors binding Dl, the strength of each one of these bindings, and other Notch-specific aspects affecting these two factors (protein modifications, multimerization, ratio of functioning to defective molecules, rate of signaling or turnover, etc.).

Cell aggregates form when S2-N cells and S2-Dl cells are mixed and gently rotated. The size of cell aggregates, at a particular rotation speed, is a measure of the strength of adhesion between S2-Dl and S2-N cells. Cell aggregation sizes have been used previously to assess the binding of modified or mutant Notch receptors to Dl (Rebay et al., 1991; Lieber et al., 1992). If the differences in the detachment forces measured by AFM reflected real difference in adhesion, the different Notch receptors and Dl were expected to form cell aggregates of sizes in accordance with their detachment force. Experiments showed that they do so: S2-N cells produced the largest cell aggregates, most rapidly after initiation of aggregation; S2-N^{1-2155}, S2- N^{nd3}, and S2- Nmf produced smaller cell aggregates at a lower rate, even though they initiated aggregation at about

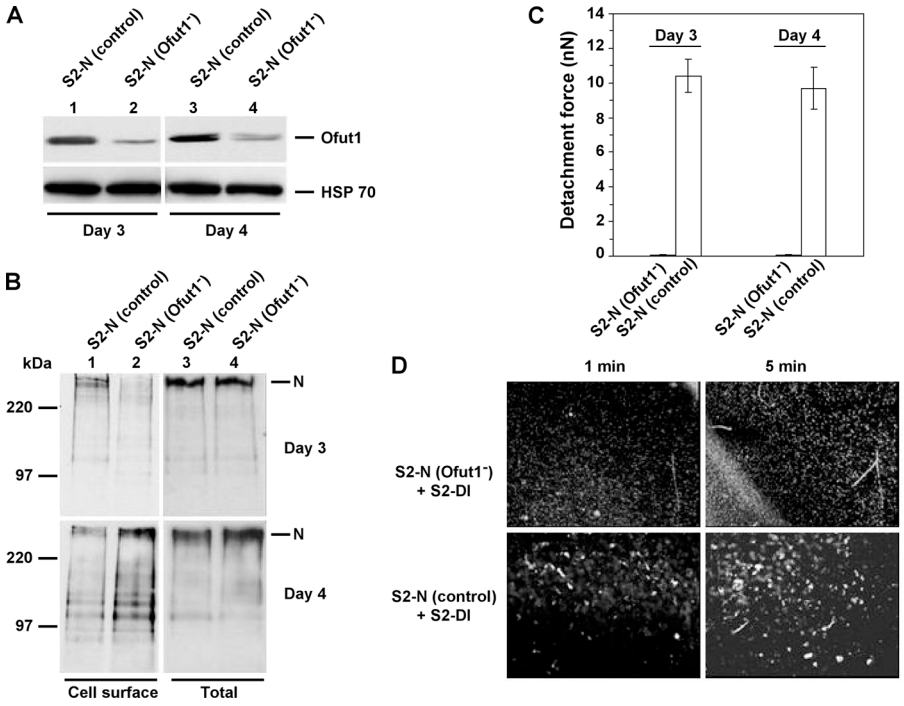

Figure 3. **Ofut1 RNAi abolishes the detachment force between N and Dl.** (A) Western blots showing the levels of Ofut1 in S2-N cells treated with *Ofut1* dsRNA [S2-N(Ofut1⁻)] or not [S2-N(control)]. (B) N levels on S2-N cell surfaces after treatment with *Ofut1* dsRNA. Cell surface = streptavidin bead precipitates in 40 μl; Total = 40 μl total extracts used. Same extracts were used for A and B. (C) Detachment forces between S2-Dl cantilevers and S2-N(Ofut1⁻) or S2-N(control) cells, in 1× PBS+Ca^{2+}. (D) Aggregates of S2-Dl and S2-N(Ofut1⁻) or S2-N(control) cells.

the same time (Fig. 2 D). By measuring the dimensions of cell aggregates in defined areas within each population, we estimated the average number of cells in the aggregates. A plot of the estimates showed a strong correlation between the detachment force and the size of cell aggregates (Fig. 2 E). The wild-type N was about four times more adhesive, and held four times the number of cells in groups than the mutated or $N^{1\text{-}2155}$ receptors.

As mentioned earlier, N molecules cluster at contact points between S2-N and S2-Dl cells (Fehon et al., 1990). The position and size of the N cluster region is an excellent indicator of the place and extent of contact with an S2-Dl cell at the time of fixation for immunostaining (unpublished data). When S2-N and S2-Dl cell aggregates were forcefully separated, the cells often tore apart near the contact regions rather than yield at the contact points between cells, as indicated by the N cluster region remaining coextensive with the length of contact between cells despite the cells being extremely stretched at the contact regions (Fig. 2 F, top row). On the other hand, S2-$N^{1\text{-}2155}$ and S2-Dl cells often yielded at the contact points between cells rather than tear each other apart, as indicated by the $N^{1\text{-}2155}$ cluster region often remaining partially coextensive with the length of contact between cells (Fig. 2 F, bottom row, black arrow). Not yielding or yielding at the contact region could be related to the S2-N cells adhering to S2-Dl cells more strongly than the S2-$N^{1\text{-}2155}$ cells (Fig. 2 B, compare sets 1 and 2).

Ofut1 RNAi in S2-N cells abolished the detachment force with S2-Dl cells

O-fucosyl transferase *Ofut1* is known to modify N, and its RNA interference (RNAi) knock-out in S2 cells has been shown to significantly reduce N binding to Dl (Okajima and Irvine, 2002; Okajima et al., 2003). To confirm that we are indeed measuring Dl binding ability, we knocked down *Ofut1* expression in S2-N cells by RNAi. Ofut1 protein levels were significantly reduced on the third and fourth day after ds*Ofut1* RNA treatment, when compared with S2-N control cells (Fig. 3 A). Cell surface biotinylation and streptavidin immunoprecipitation showed that although the levels of N at the cell surface were lower than control levels on d 3, it was higher on d 4 (Fig. 3 B). AFM measurements on the same population of cells showed that detachment forces with S2-Dl cantilever was almost zero on both days (Fig. 3 C). To confirm by an independent method that S2-N (Ofut1⁻) cells have lost their ability to bind S2-Dl cells, we performed cell aggregation assays using the same batch of d 4 cells used in AFM and expression assays. Results showed that although the control S2-N cells formed aggregates as usual, S2-N (Ofut1⁻) cells did not (Fig. 3 D). Thus, all our experiments indicated that the detachment forces we measured with the different Notch receptors were due to the differences in their Dl binding strengths.

The surfaces of S2 cell–expressing Notch receptors or Delta

To get some idea regarding the physical basis for the adhesion strength between N- and Dl-expressing cells, we scanned the surfaces of S2-N, S2-Dl, S2-$N^{1\text{-}2155}$, S2-N^{nd3}, S2-N^{Ax59d}, S2-NΔ1-18, and S2-N (Ofut1⁻) cells, and many others using the pyramidal silicon nitride cantilever. Although the surface of heat-shocked untransfected S2 cells appeared plain and devoid of features, the surfaces of S2 cells expressing the different heat shock–induced cell surface molecules showed characteristic topologies (Fig. 4 A). For the following reasons, we consider these topologies to be related to the expression of cell surface proteins induced in the cells: (1) they are not an unrelated consequence of heat shock because heat-shocked S2 cells did not show these topologies; (2) the characteristic topology of each

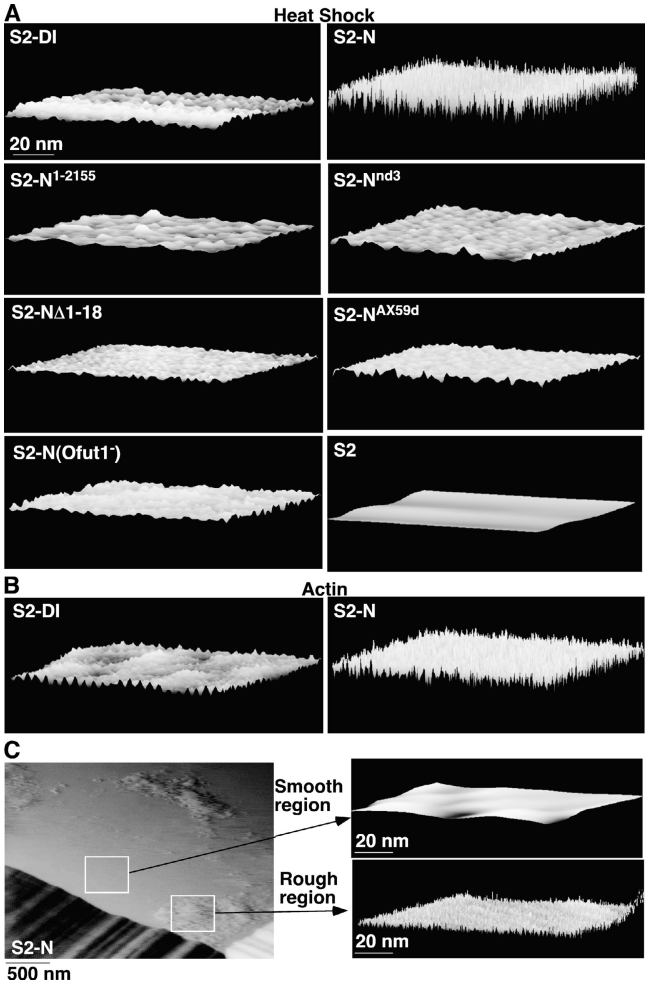

Figure 4. **Surface scans of cells expressing the different Notch receptors or Dl.** (A) High-resolution AFM height images (100 × 100 nm; z range, 7 nm) of the surfaces of live heat-shocked S2 cell or S2 cells expressing the different proteins, scanned in 1× PBS+Ca^{2+}. (B) Similar images of the surfaces of live S2 cells expressing Dl or N through the actin promoter. (C) Deflection images (z range, 16 nm) of the surface of an in situ "heat-shocked" S2-N cell. Higher resolution height images (100 × 100 nm; z range, 10 nm) on the right.

cell line was highly reproducible and other cell surface receptors like Dfrizzled 2 showed a different topology (unpublished data); (3) the topologies of the surfaces of S2 cells expressing N and Dl from the actin promoter were very similar to the topologies observed with S2-N and S2-Dl, respectively (Fig. 4 B); (4) when uninduced S2-N cells were placed in the AFM and the solution was heated in situ, the characteristic fibrous topology appeared in patches and spread to cover the whole surface (Fig. 4 C); (5) these characteristic topologies slowly reverted to the plain surface seen with S2 cells 2 d after the heat shock induction subsided (unpublished data); and (6) if the topologies are due to other proteins or molecules, it would raise the unlikely possibility that these were different for each Notch receptor, or responded differently to each Notch receptor.

Each ~15-kD lysozyme molecule has been measured at 8–10 nm (diameter) × 2–2.5 nm (height) by AFM, and the crystallographic size estimate is 4.5 × 3 × 3 nm (Raab et al., 1999). N is ~350 kD and Dl ~110 kD. Thus, each "fiber" or "bump" we observe is probably composed of only a few molecules. The bump or fiber topology does not seem to affect the access of Notch receptors to Dl, as S2-Dl cells initiate aggregation with S2-N and S2-N^{nd3} cells at the same time. Also, Dl coimmunoprecipitations of cell surface–biotinylated proteins in the presence of cross-linkers (to recover Notch proteins independent of binding strength) also showed that the access of N^{nd3} receptors to Dl was similar to that of N (unpublished data). However, the topology could be a factor determining the appropriate conformation or configuration of Notch receptors, both of which could affect the Dl binding strength.

Detachment force between S2-N and S2-Dl cells initially increased and then decreased to zero, all in 10 min

To determine the kinetics of the detachment forces between Dl and the Notch receptors, we rested the S2-Dl cantilevers on S2-N, S2-N^{nd3}, S2-Nmf, S2-N^{1-2155}, and S2-NΔ1-18 cells for various lengths of time and measured detachment forces. The same batch of S2-Dl cells and the same AFM settings were used on all Notch receptors. Any uncontrolled factors (like drift of the cantilever over time, etc.) were expected to be common to all Notch receptors. Results, shown in Fig. 5 A, indicate the following: (1) the detachment force increased in the first few minutes with all Notch receptors that are able to bind Dl and then decreased for all Notch receptors; (2) the decrease in detachment forces was most rapid with S2-N cells (from 14–18 nN to 0 nN in 10 min), followed by S2-N^{nd3} and S2-Nmf cells (from 5–11 nN to 0 nN in 20 min); (3) the detachment force with S2-N^{1-2155} cells decreased at the slowest rate and did not reach zero even after 60 min (unpublished data); and (4) the adhesion force with S2-NΔ1-18 cell was zero at all times. Thus, there was a positive correlation between the initial detachment force and the rate of decrease in detachment force.

To determine the basis for the increase in detachment force in the first few minutes, we relied on Western blotting analyses of cell aggregations. S2-N and S2-Dl cells initiate formation of cell aggregates in less than a minute after mixing. The size of these cell aggregates increases in the first 10 min and then gradually decreases; few and small aggregates remain after 3 h, with neither the aggregated cells or the free cells showing any change in their morphology (unpublished data). Although individual N and Dl cells lose adhesion within 10 min (AFM data), cells in a population remain in aggregation over longer periods, possibly because of contacts with multiple cells and cell replacement (Lieber et al., 1992).

We discovered that the amount of N increased up until 5 min after Dl binding and then decreased (Fig. 5 B; compare the S2-Dl–treated samples with the S2-treated samples). The increase in N levels in Dl-treated samples from 0–5 min was 2.3× ± 0.21 ($P < 0.05$; $n = 5$). Similar changes over time were observed with S2-N^{nd3}, S2-Nmf, and S2- N^{1-2155} cells, but not with S2-NΔ1-18 cells; Dl levels on the same blots did not change significantly in the same periods (unpublished data). It appears that the initial increase in adhesion is possibly due to the increase in Notch amounts in response to Dl binding. This increase is likely to be post-transcriptional, as the endogenous *Notch* gene in S2 cells is disrupted (Fehon et al., 1990).

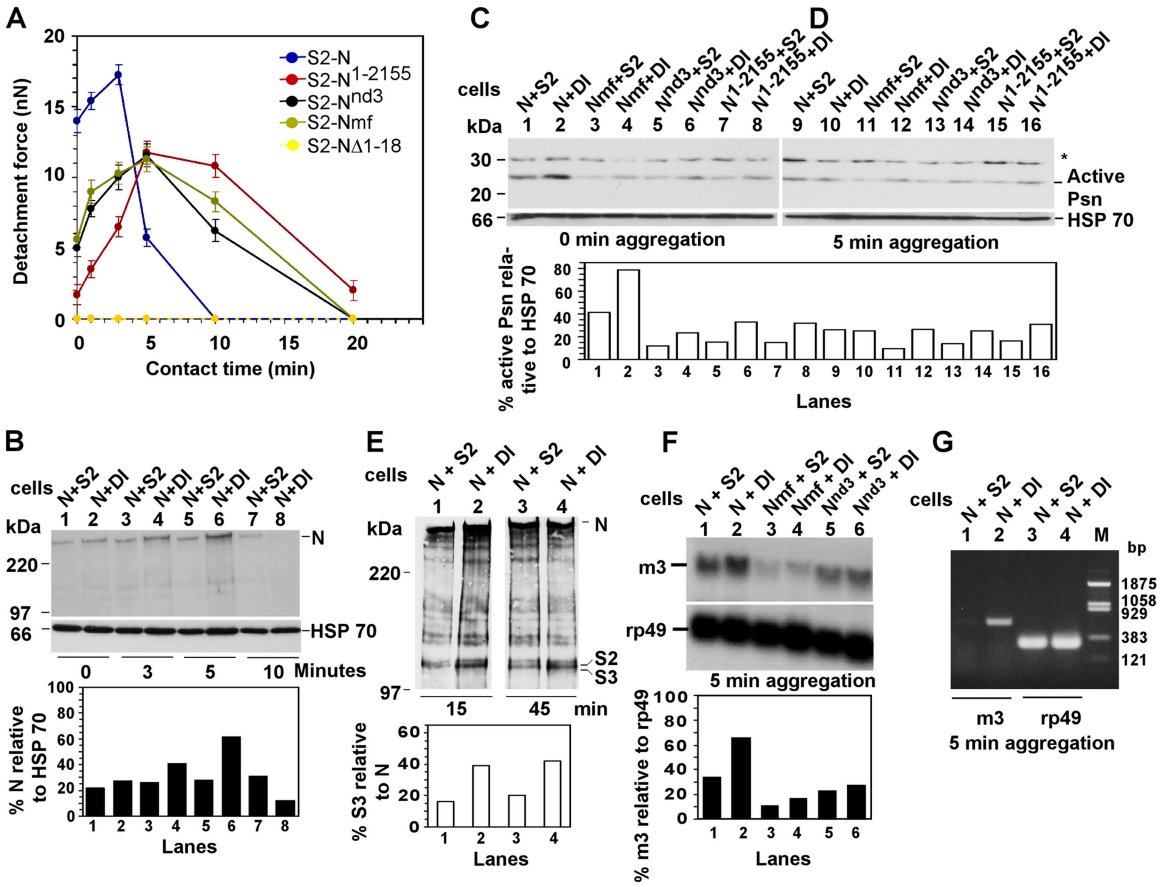

Figure 5. **Temporal change in detachment force and SuH/N^{intra} signaling.** (A) Detachment forces between the S2-Dl cantilevers and the S2 cells expressing Notch receptors, in 1× PBS+Ca^{2+}. (B) Western blot showing the levels of N after treatment with S2-Dl cells. (C and D) Western blots showing the levels of active Psn in the different cell mixtures. Asterisk corresponds to a cross-reacting band. (E) Western blots showing the increase in levels of S2- and S3-cleaved N fragments in S2-Dl–treated S2-N cells. (F) Northern blots showing the levels of *E(spl)C m3* RNA in the different cell mixtures. (G) Semi-quantitative PCR showing relative levels of *E(spl)C m3* RNA. rp49 RNA amplification shows levels of total RNA in the samples.

To determine the basis for the decrease in the detachment force, we checked the levels of active Psn on Western blots. We found that the amount of active Psn increased immediately after mixing S2 cells expressing Notch receptors with S2-Dl cells (Fig. 5 C). The response was strong with S2-N cells (2.1× ± 0.13, $P < 0.05$; $n = 3$), and this strong response subsided after 5 min (Fig. 5 D). We also examined the accumulations of S2 and S3 fragments of N in S2-N/S-Dl cell aggregates. They were not robust after 5 min (apparent only when multiple blots were examined), but very much so after 15 min of aggregation (Fig. 5 E). However, if Psn was activated and the S3-cleaved fragment (N^{intra}) had been produced in 5 min, the expression of N-signaling target gene, *E(spl)C*, was expected to increase in that time. An *E(spl)C* gene, *E(spl)C m3*, responds to N signaling in S2 cells (Mishra-Gorur et al., 2002; Wesley and Mok, 2003). The expression of *E(spl)C m3* RNA in S2-N and S2-Dl cell aggregations was clearly increased (2.1× ± 0.07, $P < 0.05$; $n = 3$); increase in S2-Dl aggregations with S2-N^{nd3} cells or S2-Nmf cells was barely detectable and not statistically different (Fig. 5 F). We confirmed the rapid response by an independent method, semi-quantitative PCR (Fig. 5 G). Thus, the decrease in the detachment force between S2-N cells and S2-Dl cells was likely to be due to the production of N^{intra} by Psn.

Treatment of S2 cells expressing Notch receptors with a Psn inhibitor blocked the decrease in detachment force

To determine if the decrease in detachment force was indeed due to Psn cleavage, we treated the S2 cells expressing various Notch receptors with DFK-167 Psn inhibitor for 30 min, washed the cells, and then measured the detachment force with the S2-Dl cantilever. DFK-167 is dissolved in DMSO. So, we first measured the temporal change in detachment force between the S2-Dl cantilever and the S2 cells expressing the various Notch receptors that were pretreated with the same concentration of DMSO used with the Psn inhibitor. The level of adhesion force at all time points was lower and the rate of decrease to zero was retarded (particularly with S2-N cells), possibly due to the effect of DMSO on the plasma membranes (compare Fig. 6 A with Fig. 5 A). When the S2 cells expressing various Notch receptors were treated with DMSO + Psn inhibitor, the decrease of the detachment force to zero was blocked with N, N$^{1-2155}$, N^{nd3}, and Nmf receptors (Fig. 6 B). The detachment forces between the S2-Dl cantilevers and S2-Dl cells or S2 cells treated with the Psn inhibitor did not increase (unpublished data). Also, when Psn inhibitor–treated S2-Dl cells were used on the cantilever and tested against untreated S2-N cells, the loss in detachment force was

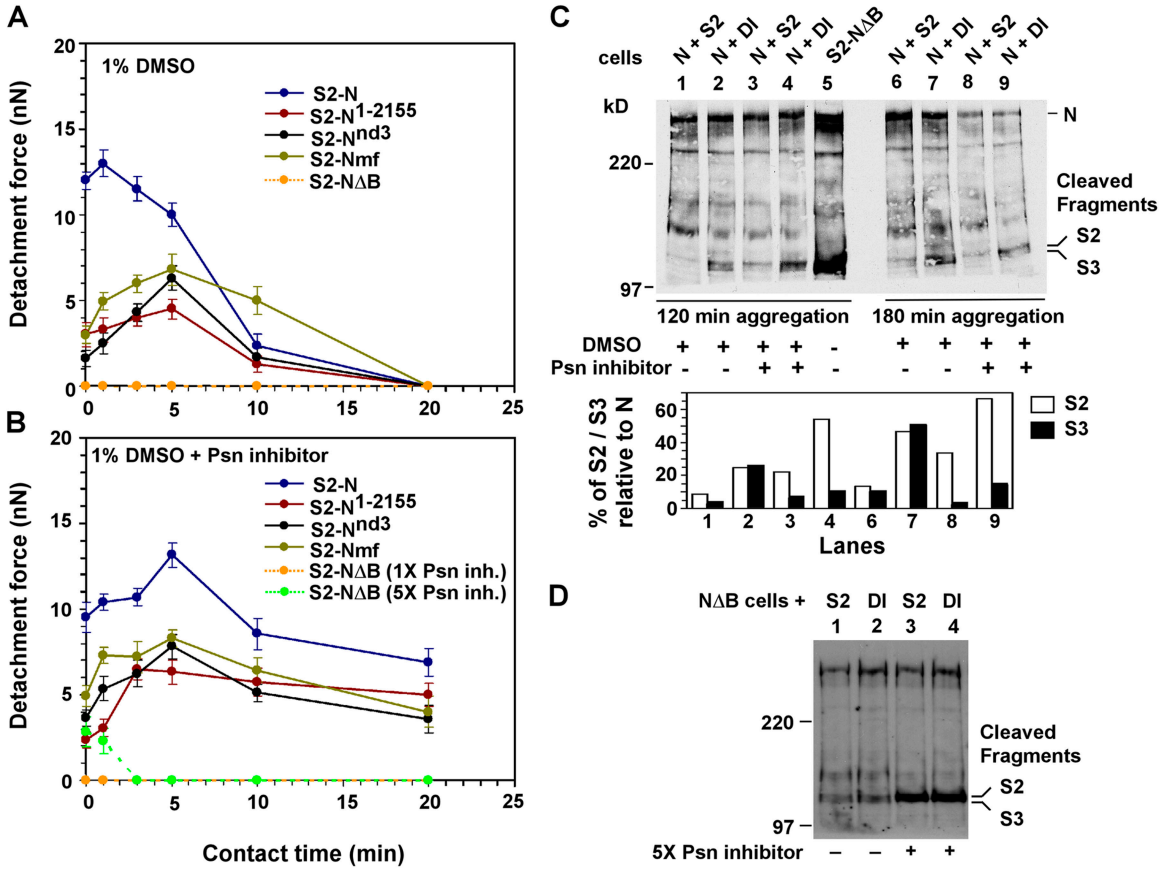

Figure 6. **Presenilin inhibitor blocks the loss of detachment force between Notch receptors and Dl.** (A) Detachment forces between the S2-Dl cantilevers and 1% DMSO-pretreated S2 cells expressing Notch receptors, in 1× PBS+Ca^{2+}. (B) Detachment force between the S2-Dl cantilevers and 1% DMSO + Psn inhibitor–pretreated S2 cells expressing Notch receptors, in 1× PBS+Ca^{2+}. (C) A Western blot showing the amounts of Notch in the presence or absence of the Psn inhibitor. (D) A Western blot showing the amounts of NΔB molecules in the absence or presence of 5× Psn inhibitor.

not blocked (see later, Fig. 8 E). These observations indicated that the block in the decrease in detachment force was due to the Psn inhibitor affecting N rather than some other general cell adhesion molecules. Western blotting showed that the level of the S2-cleaved N fragment in S2-N cells increased after treatments with the Psn inhibitor, even in the absence of Dl; in the presence of Dl, the levels of the S2 fragment further increased (2.6 ± 0.24 at 120 min, $P < 0.05$, $n = 4$) in association with reduced levels of the S3 fragment (Fig. 6 C). Although the effect of the Psn inhibitor was apparent even at 10 min of aggregation, data for longer periods are shown to indicate the robustness of the effect.

NΔB is a constitutively active Notch receptor (Lieber et al., 1993) that produces high levels of S2 and S3 fragments (Fig. 6 C, lane 5) and *E(spl)C* RNA (unpublished data), even in the absence of Dl. Possibly because of its very brief residence time at the membrane, we did not detect significant detachment force between NΔB and the S2-Dl cantilever (Fig. 6 A). If the time-dependent decrease in the detachment forces was due to Psn activity on Notch receptors, we expected to see an increase in the detachment force when S2-NΔB cells are treated with the Psn inhibitor. We did not see it in the presence of 1× Psn inhibitor (unpublished data), but we did at 5× concentration (Fig. 6 B, yellow and green lines). The gain in detachment force is likely to be due to the high level of the S2 fragment in 5× Psn inhibitor samples (Fig. 6 D; the loss in S3 fragment is not very apparent due to the high level of the S2 fragment). Thus, we conclude that the loss in detachment force is related to Psn activity on Notch and Notch S3 fragment production.

Protein turnover block in S2-N cells promoted the decrease in detachment force

S2 and S3 fragments are produced at low levels in S2-N cells, and N levels in these cells turn over even in the absence of S2-Dl cells (unpublished data), suggesting a connection between the turnover rate and N signaling. Therefore, we used chloroquine to block turnover in S2 cells expressing the various Notch receptors and measured the detachment force between them and the S2-Dl cantilevers. We found that the decrease in detachment force was accelerated (Fig. 7 A). Western blot analysis showed that chloroquine treatment of S2-N cells resulted in accumulation of the S3 cleaved fragment even in the absence of Dl (Fig. 7 B; 7.8 ± 0.96, $P < 0.05$; $n = 3$). We also examined the level of *E(spl)C m3* expression and found that it was increased in chloroquine-treated samples (unpublished data). Thus, it appeared that blocking N turnover advanced the rate of S3 cleavage and SuH/N^{intra} signaling, thereby accelerating the rate of decrease in the detachment force after Dl binding.

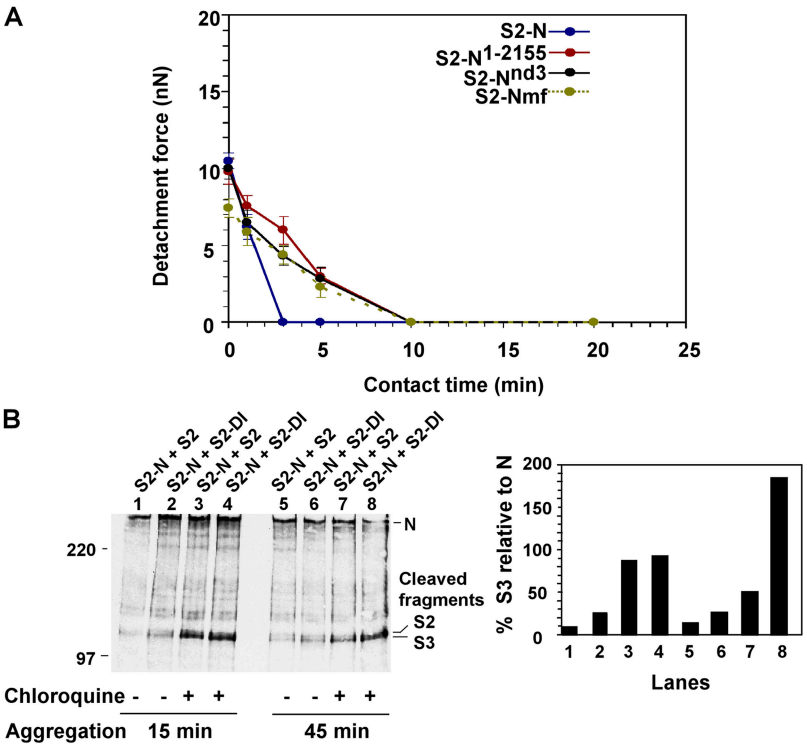

Figure 7. **Chloroquine accelerates the loss of detachment force between Notch receptors and Dl.** (A) Detachment forces between the S2-Dl cantilevers and chloroquine-pretreated S2 cells expressing Notch receptors, in 1× PBS+Ca^{2+}. (B) A Western blot showing the levels of N molecules, in chloroquine-pretreated or untreated S2-N cells, in the presence of S2 or S2-Dl cells.

Dl cell pulling could promote N signaling

Some recent data have suggested that Dl pulling on N would promote SuH/N^{intra} signaling (Parks et al., 2000; Struhl and Adachi, 2000; Pavlopoulos et al., 2001). We tested this proposal and also examined if the strength of adhesion between Notch receptors and Dl had a role in it.

The speed of cantilever retraction is directly proportional to the force of retraction at a particular piezo displacement. Therefore, we studied the effect of different speeds of retraction on the detachment force after 0 and 3 min of contact between the S2-Dl cantilevers and S2-N cells, S2-N^{1-2155} cells, or S2-N^{nd3} cells. We chose one speed higher than the normal speed (1.6 μm/s, chosen for comparison with other AFM studies) and four lower speeds, yielding the following series: 0.1, 0.2, 0.4, 0.8, 1.6, and 3.2 μm/s. We chose 3 min because SuH/N^{intra} signaling is minimal at this point and adhesion the highest, so we would be able to detect even a subtle impact of pulling. Results of our experiments showed that the detachment force between S2-Dl cantilevers and S2-N cells under 1× PBS+Ca^{2+} was high at 0.1 μm/s retraction, decreased until 0.8 μm/s, and increased again at 1.6 and 3.2 μm/s. The differences were all significant, except between 1.6 and 3.2 μm/s (Fig. 8 A). As our data show that the loss of detachment force between N and Dl is due to Psn cleavage (Fig. 5 and Fig. 6), we repeated the experiments with S2-N cells pretreated with 1× PBS+Ca^{2+} + 1% DMSO, or 1× PBS+Ca^{2+} + 1% DMSO + Psn inhibitor. The results with 1× PBS+Ca^{2+} + 1% DMSO pretreatment was similar to the result with 1× PBS+Ca^{2+}, except that the overall levels of detachment force were lower, as expected from data in Fig. 6 A (unpublished data). The results of experiments under 1× PBS+Ca^{2+} + 1% DMSO + Psn inhibitor showed that the detachment forces between S2-Dl cantilevers and S2-N cells were not significantly different between different speeds of retraction (Fig. 8 B). The same series of experiments conducted with S2-Dl cantilevers and S2-N^{1-2155} cells or S2-N^{nd3} cells showed that Dl pulling had minimal effect, if at all, on the detachment force with N^{1-2155} or N^{nd3} receptors (Fig. 8, C and D; data for S2-N^{nd3} cells are not depicted).

If Dl is able to pull N and promote S3 cleavage, and thereby SuH/N^{intra} signaling, it seemed logical to expect that Psn cleavage of N would happen first, followed by Psn cleavage of Dl. It would be difficult for Dl to pull if it were cleaved first. If this surmise were correct, the treatment of S2-N cells with Psn inhibitor, and not of the S2-Dl cells, was expected to block the decrease in detachment force with time. To examine this, we pretreated the S2-Dl cells with the Psn inhibitor before mounting them onto the cantilevers. We used these cantilevers, S2-Dl (t) cantilevers, and the regular nontreated S2-Dl (nt) cantilevers against Psn inhibitor–pretreated S2-N (t) cells and nontreated S2-N (nt) cells. The adhesion force between the untreated S2-Dl (nt) cantilever and nontreated S2-N (nt) cells decreased between 0 and 5 min of contact, as expected (Fig. 8 E, set 1). The detachment force between nontreated S2-Dl (nt) cantilever and treated S2-N (t) cells increased between 0 and 5 min of contact (Fig. 8 E, set 2), but the detachment force between the treated S2-Dl (t) cantilever and the nontreated S2-N (nt) cell was low, even at the 0-min contact, and it decreased further after 5 min of contact (Fig. 8 E, set 3). This confirmed our prediction and suggested that blocking Dl cleavage possibly promoted SuH/N^{intra} signaling. Northern blot analysis on the same samples showed that SuH/N^{intra} signaling (based on *E(spl)C m3* expression in response to Dl binding) was lower in combinations including Psn inhibitor–

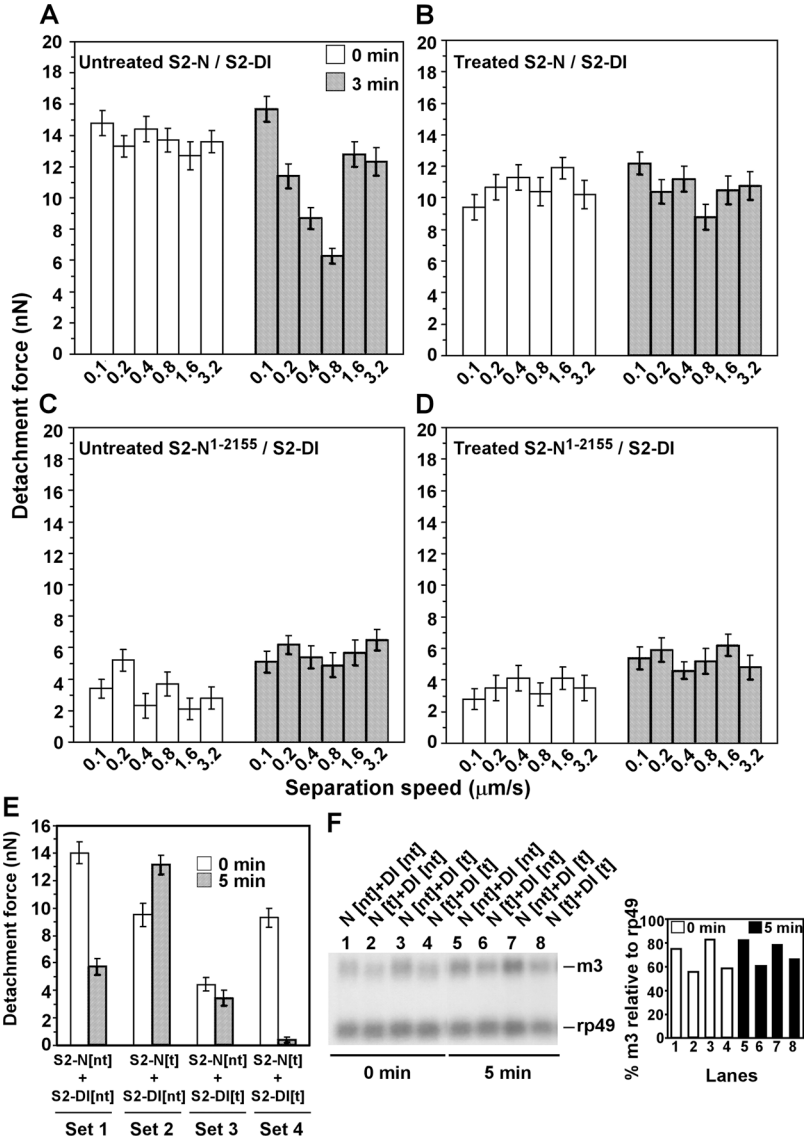

Figure 8. **Dl pulling promotes the loss of detachment force between N and Dl.** Detachment forces at various speeds of retraction of S2-Dl cantilevers from S2-N (A and B) or S2-$N^{1\text{-}2155}$ (C and D) cells that were either not pretreated (A and C) or pretreated with 1% DMSO + Psn inhibitor (B and D), in 1× PBS+Ca^{2+}. (E) Detachment forces between cantilevers carrying untreated (nt) or 1% DMSO + Psn inhibitor–pretreated (t) S2-Dl cells and untreated (nt) or 1% DMSO + Psn–inhibitor pretreated (t) S2-N cells, in 1× PBS+Ca^{2+}. (F) Northern blots showing the levels of *E(spl)C m3* RNA in the different cell mixtures, at different times of aggregation.

treated S2-N cells compared with combinations including Psn inhibitor–treated S2-Dl cells (Fig. 8 F; 1.3 to 1.6× in three replications; the response was weaker presumably due to the presence of DMSO). The detachment force between treated S2-Dl (t) cantilever and treated S2-N (t) cells at 0 min was high, but decreased dramatically after 5 min of contact (Fig. 8 E, set 4). We do not know the reason for this precipitous drop in detachment force when both cells are treated, but we obtained similar results in three replications of the experiments. We interpret these data to mean that Dl pulling promotes Psn S3 processing of N, best at the speed of 0.8 μm/s (we did not use this speed for all other experiments because the speed of response prevented us from doing experiments properly). Dl might be unable to exert the same level of pull on NΔCterm-like receptor $N^{1\text{-}2155}$ or the mutant receptor N^{nd3} as a consequence of lower adhesion strength (see Fig. 2 B and Fig. 5 A). On the other hand, these latter receptors might be deficient in responding to Dl pulling.

Discussion

We will discuss the relevance of our findings to cell differentiation and development using the following instance of lateral inhibition as a model for N and Dl actions. During embryonic development, lateral inhibition initiates when N on one cell binds Dl on the other within clusters of 5–20 proneural cells that form within a monolayer of cells in the periphery of the embryo. The majority of cells in the proneural clusters accumulate a high level of N signaling, suppress expression of achaete–scute complex genes, become the epidermal precursor cells (EPCs), remain in the periphery of the embryo, and differentiate the epidermis. One or a few cells in the proneural clusters accumulate a high level of a truncated N receptor NΔCterm (a poor activator of N signaling) and a low level of N signaling, increase the expression of the achaete–scute complex genes, become the neuronal precursor cells (NPCs), move inside of the embryo, and differentiate the central nervous system (see Artavanis-Tsakonas et al., 1999, and Wesley and Mok, 2003).

N in the above description is comparable to N in our experiments; NΔCterm to $N^{1\text{-}2155}$ (Wesley and Saez, 2000).

In our experiments, N showed the strongest adhesion with Dl and produced the highest rate of SuH/N^{intra} signaling (Figs. 2, 5, and 6). In these experiments, the rate of SuH/N^{intra} signaling is indicated by the slope of the line showing time- and Psn-dependent loss of adhesion from peak adhesion to zero or the lowest point. Our data suggest that the high adhesion strength between N and Dl might serve to initiate SuH/N^{intra} signaling at a high rate and level. It is also possible that for a brief period, a minute or two, the strong adhesion between N and Dl is used to sort nascent cell types (e.g., the EPCs and the NPCs). Embryos homozygous for the hypomorphic N^{nd3} allele (Lyman and Young, 1993) show symptoms of poor cell adhesion, such as gaps between cells (unpublished data). As N^{nd3} receptors bind Dl at a significantly lower strength than N (Fig. 2 and Fig. 5), it is possible that development of proper adhesion between cells initially requires stronger N and Dl adhesion, and therefore a higher rate or level of SuH/N^{intra} signaling, than for cell type specification. This might be the basis for the much-reported loss of tissue adhesion associated with reduced activities of N or Dl (de Celis and Garcia-Bellido, 1994; Goode et al., 1996; Renaud and Simpson, 2001). Once the high rate or level of SuH/N^{intra} signaling required for both EPC production and development of proper adhesion is attained, Dl or the extracellular domain of N might be dispensable. This would explain the production of the EPCs that adhere well when N^{intra} is overexpressed (Lieber et al., 1993; Struhl et al., 1993). Thus, the high rate or level of signaling, which would eliminate the adhesion capabilities of N and Dl as a consequence of N processing, might trigger alternate mechanisms for development of strong adhesion between the EPCs.

Lateral inhibition is accomplished in less than 20 min (Campos-Ortega and Hartenstein, 1985; Hoppe and Greenspan, 1990; Baker and Yu, 1998), suggesting that the rate of SuH/N^{intra} signaling might be as important as its level. Our data show that the SuH/N^{intra} signaling circuit can be completed almost instantly. In 10 min, this signaling either reaches such a rapid rate that binding to Dl cannot be detected or is shut down at that contact point. It appears that this rate can be further accelerated as our pulling data suggest. We seem to have hit upon the optimum pulling for SuH/N^{intra} signaling in S2 cells (0.8 μm/s; see Fig. 8, A and B). It is possible that Dl pulling also accelerates the rate of SuH/N^{intra} signaling in vivo, as lateral inhibition often takes place in association with movements of some kind (for example, the NPC segregation in the embryos or the movement of the morphogenetic furrow in the developing eye). Also, E(spl)C proteins are detected in the surrounding cells only after the NPC (neuroblast) delamination has begun (Jennings et al., 1994). As the EPCs are enriched for N and the NPCs for NΔCterm (Wesley and Saez, 2000), it is possible that the NPCs exert a unidirectional pull on the EPCs (as N responds to pulling but not NΔCterm), thereby increasing the rate of SuH/N^{intra} signaling in the EPCs. With assistance from computer modeling, it might be possible in the future to examine the involvement of force in SuH/N^{intra} signaling in vivo, and whether this force is comparable to the optimal force in S2 cells.

Shaye and Greenwald (2002) have shown that down-regulation of LIN12/Notch signaling in *Caenorhabditis elegans* is an important part of lateral inhibition. Our data show that a block in down-regulation by chloroquine treatment enhanced the rate of SuH/N^{intra} signaling (Fig. 7). Thus, the rate and level of SuH/N^{intra} signaling could be regulated by a down-regulation mechanism similar to the RAS-activated endocytosis mediated mechanism involved in *C. elegans* (Shaye and Greenwald, 2002). Alternatively, but not mutually exclusively, S2 and S3 cleavage enzymes could be constitutively active at the cell surface, ready to mount a rapid response to Dl binding. It is possible that N has only two fates after synthesis: turnover or signaling. Dl binding might simply increase the residence time of N at the cell surface and tilt the balance in favor of signaling.

The surface topologies we observe provide important clues regarding the structure and function of N and Dl. For example, if the fibers and bumps are taken to represent cohesive units, it appears that in the absence of any binding partners the cell surface N is a monomer or an oligomer, whereas the cell surface Dl is a multimer. The structure of N and its adhesion strength with Dl appear to be dependent on the complete N intracellular domain (that binds many proteins) and are very sensitive to point mutations, truncations, and glycosylation; N^{nd3}, that appears to be aberrantly glycosylated (unpublished data), and $N^{1\text{-}2155}$, that lacks the carboxyl terminus, both show lower detachment force with Dl. With our approach, it would be possible to pursue these clues and determine how the various factors/proteins that modify the extracellular domain or bind the intracellular domain of N affect the presentation of N at the cell surface or its adhesion strength with Dl. The images of N and Dl cell surfaces bear some superficial resemblance to the surfaces of a Velcro. There might be something more to it than mere resemblance if we consider the observations that N and Dl do not show much attraction, they have to be forced together for binding, and they tear cells apart rather than let go when forcefully separated. If conventional approaches for studying physical structure and biochemical parameters fail, it might be worthwhile to consider the possibility that N and Dl interact through a Velcro-like mechanism.

N and Dl need to be forced to bind. However, once they bind the rapid response might make it difficult to put the brakes on SuH/N^{intra} signaling. Thus, it is possible that the intracellular and extracellular mechanisms regulating N and Dl binding might be more important regulators of SuH/N^{intra} signaling than the mechanisms regulating events downstream of N and Dl binding. The approach we have taken opens the door for fine dissection of mechanisms regulating N and Dl binding. Further elaboration of this approach might make it possible to also extract the underlying biophysical and biochemical parameters for computer modeling to better understand developmental processes or diseases involving Notch receptors and Dl. It might be possible to extend the current two-cell model of SuH/N^{intra} signaling or lateral inhibition to models based on interactions among all cells in a population. As our approach incorporates the element of time into the process, newer models could be made to more closely reflect the well-known dynamism of the functions of Notch receptors and Dl during animal development.

Materials and methods

Cell procedures

All stable cell lines except the following have been described previously (Lieber et al., 1992; Wesley, 1999; Wesley and Saez, 2000). cDNAs of *Nmf* (Kidd and Lieber, 2002), N^{nd3} (hsN cDNA with the amino-terminal KpnI fragment from the N^{nd3} allele), and N^{Ax59d} (hsN cDNA with the NheI–BglII fragment from the Ax^{59} allele) were used to establish others using pCaSpeR-hs or our pUAct vector. All cells were heat shocked for 30 min at 37°C, and were used after 150 min or indicated times. For aggregation assays, 5×10^6 cells of each cell type were mixed in round-bottom Falcon tubes or siliconized Falcon well plates and rotated at ~120 RPM.

AFM procedures

A BioScope (Digital Instruments) on an inverted microscope (IX70; Olympus) was used. Tip-less silicon nitride cantilevers (Veeco) were functionalized according to Benoit et al. (2000). Cells were washed and resuspended in 1× PBS+Ca^{2+} at a density yielding ~50 cells/mm^2 in a glass dish. The tip-less cantilever was lowered at a force of 1–2 nN to attach a cell to it. The other type of cells were in a monolayer in Falcon 3046 plates, under 1× PBS or 1× PBS+Ca^{2+}. The detachment force was measured at a velocity of 1.6 μm/s, unless otherwise mentioned, and the indentation was <10% of cell diameter (Costa and Yin, 1999). The Set Point was zero, and horizontal line of the force curve close to zero, to control contact force over long periods. The silicon nitride cantilever spring constants were between 0.12 and 0.58 N/m, the scan rate was 2–3 Hz, and the indentation force was below 1 nN for force measurement.

For imaging of live-cell surfaces (under 1× PBS+Ca^{2+}), oxide-sharpened pyramidal-tipped silicon nitride cantilevers with a spring constant of ~0.06 N/m (Advanced Surface Microscopy, Inc.) were used in contact mode. Cells were trapped in a polycarbonate membrane (Millipore) with 8-μm pore size (Ahimou et al., 2002), cut to 1 × 1 cm strips, turned upside-down, attached to a glass slide with adhesive tape, and scanned under 1× PBS+Ca^{2+}. For pretreatment, cells were incubated for 30 min in 1× PBS+Ca^{2+} + 1% DMSO, in 1× PBS+Ca^{2+} + 1% DMSO + 300 μM DFK-167 (1× Psn inhibitor, Hu et al., 2002; Enzyme Systems Products), or in 1× PBS+Ca^{2+} + 100 μM chloroquine and washed before use. AFM data were analyzed using Nanoscope III (Digital Instruments) and Force Curve Convert (Dr. Yves Dufrene) programs. For in situ induction of N, cells were placed under the laser beam for >5 h in the presence of a table lamp (N is induced at 27°C). New cantilevers were used each time. All measurements are based on at least three experiments (10 cell measurements in each) using different batches of cells.

Molecular procedures

Western and Northern blotting were performed as described in Wesley (1999); RT-PCR in Mishra-Gorur et al. (2002); RNAi in Okajima and Irvine (2002) and Clemens et al. (2000); immunofluorescence in Fehon et al. (1990); and cell surface biotinylation in Kidd and Lieber (2002; with only a 5-min incubation on ice). Dl (C594.9B) and N (C458.2H for immunofluorescence) antibodies were from Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA); αNI (for Westerns) from Toby Lieber (The Rockefeller University, New York, NY); αPsn from Mark Fortini (National Cancer Institute, Frederick, MD); and αOfut1 from Ken Irvine (Rutgers University, Piscataway, NJ). Aggregation time is the time when the cells were placed on ice before processing. The blots were probed with heat-shock protein 70 (HSP70) or ribosomal protein 49 RNA (rp49) probes for assessing total proteins or RNA in the lanes. All experiments were repeated at least three times. As pooling of data obscured the response or misrepresented the data (due to cell batch to batch variance), quantification is shown only for the blots in figures. Images were processed in Photoshop, Canvas, or Word programs, with any adjustment applied to whole images.

Online supplemental material

Total and cell surface expression levels of the various Notch receptors are shown in Fig. S1. Additional force measurements indicating the specificity of the detachment force between N and Dl are shown in Fig. S2. Clustering of N and $N^{1\text{-}2155}$ receptors in response to Dl binding is shown in Fig. S3. Online supplemental material available at http://www.jcb.org/cgi/content/full/jcb.200407100/DC1.

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