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Immobilization of Notch ligand, Delta-1, is required for induction of Notch signaling

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SUMMARY

Cell-cell interactions mediated by Notch and its ligands are known to effect many cell fate decisions in both invertebrates and vertebrates. However, the mechanisms involved in ligand induced Notch activation are unknown. Recently it was shown that, in at least some cases, endocytosis of the extracellular domain of Notch and ligand by the signaling cell is required for signal induction in the receptive cell. These results imply that soluble ligands (ligand extracellular domains) although capable of binding Notch would be unlikely to activate it. To test the potential activity of soluble Notch ligands, we generated monomeric and dimeric forms of the Notch ligand Delta-1 by fusing the extracellular domain to either a series of myc epitopes (Delta-1$^{ext\text{-}myc}$) or to the Fc portion of human IgG-1 (Delta-1$^{ext\text{-}IgG}$), respectively. Notch activation, assayed by inhibition of differentiation in C2 myoblasts and by HES1 transactivation in U20S cells, occurred when either Delta-1$^{ext\text{-}myc}$ or Delta-1$^{ext\text{-}IgG}$ were first immobilized on the plastic surface. However, Notch was not activated by either monomeric or dimeric ligand in solution (non-immobilized). Furthermore, both non-immobilized Delta-1$^{ext\text{-}myc}$ and Delta-1$^{ext\text{-}IgG}$ blocked the effect of immobilized Delta. These results indicate that Delta-1 extracellular domain must be immobilized to induce Notch activation in C2 or U20S cells and that non-immobilized Delta-1 extracellular domain is inhibitory to Notch function. These results imply that ligand stabilization may be essential for Notch activation.

Key words: Notch, Delta, Myoblast development, Ligand immobilization

INTRODUCTION

Cell-cell interactions play significant roles in cell fate determination in both invertebrate and vertebrate developmental systems. Many cell-cell interactions are mediated by the cell surface receptors encoded by the *Notch* superfamily (Artavanis-Tsakonas et al., 1999). Well-characterized cell fate decisions mediated by *Notch* occur during central and peripheral nervous system development, wing, eye, bristle, and ovary development in *D. melanogaster*, germinal vesicle and nervous system development in *C. elegans*, and eye development in *Xenopus* (Dorsky et al., 1997; Greenwald and Rubin, 1992; Heitzler and Simpson, 1991). At present, four *Notch* homologs, *Notch-1*, *-2*, *-3* and *-4*, have been identified in vertebrates.

Notch receptors are transmembrane proteins that consist of several EGF repeats in the extracellular domain followed by Notch/lin-12 repeats (LNR). Removal of the EGF repeats while retaining the LNR region results in a non-functional receptor. However, removal of both the EGF repeats and the LNR region while retaining the transmembrane and intracellular domains results in a constitutively active receptor, suggesting that the LNR region must be removed for receptor activation. It has recently been shown that following ligand binding, the Notch extracellular domain (NotchECD) is proteolytically cleaved adjacent to the transmembrane domain by the Disintegrin-Metalloprotease TACE (Brou et al., 2000; Mumm et al., 2000), resulting in the removal of the LNR repeats. Following cleavage and removal of the extracellular domain, the Notch intracellular domain (NotchICD) is then cleaved by a presenilin dependent process. NotchICD is then translocated to the nucleus, where it interacts with nuclear proteins collectively known as CSL proteins (CBF1/RBP-Jκ, Suppressor of Hairless, Lag) to affect expression of downstream factors including Hairy Enhancer of Split (HES) (Schroeter et al., 1998; Struhl and Adachi, 1998).

Notch ligands have been thought to activate signaling through direct cell-cell contact where an interaction with ligand on the signaling cell induces successive proteolytic cleavages of Notch in the receptive cell. Notch ligands are also transmembrane proteins and, in vertebrates, include the *Serrate* homologs *Jagged-1* and *-2* and the *Delta* homologs *Delta-1*, *-2* *-3* and *-4*. The extracellular domain of Notch ligands consists of a highly homologous DSL (Delta, Serrate, and Lag-2) domain (Tax et al., 1994) at the amino terminus followed by several EGF repeats. It has recently been shown that in at least

some instances, endocytosis of both Notch ECD and Notch ligand by the signaling cell is required to induce signaling in the responding cell (Parks et al., 2000). It was hypothesized that endocytosis mediates removal of the Notch extracellular domain allowing subsequent activation of the receptor. Although the structural basis for this requirement is not known, it may indicate a need for ligand stabilization sufficient to induce conformational changes in the Notch receptor, rendering it susceptible to further proteolytic cleavage events. Regardless of the mechanism, these results suggest that truncated ligands consisting of only the ligand extracellular domain would be non-functional due to either their instability or inability to remove the receptor. Studies using truncated ligands consisting of the extracellular domain, while deleting the transmembrane and intracellular domains, have produced various effects on Notch function. In some systems these ligands inhibit Notch activation, while in other systems they induce Notch activation.

We previously reported that cell bound, full-length Jagged induced a 2- to 3-fold increase in the formation of a precursor cell HPP-mix during in vitro expansion of murine hematopoietic progenitor cells (Varnum-Finney et al., 1998). In the same study, Jagged-1 extracellular domain (Jagged^{ext}) affected differentiation in a similar manner. In that case, Jagged^{ext} was immobilized on a solid surface with substrate-bound antibody. These results suggested that both the cell bound full length Jagged molecule and the immobilized Jagged^{ext} induced Notch activation.

For the present studies, we investigated the effect of monomeric (Delta-1$^{ext\text{-}myc}$) and dimeric (Delta-1$^{ext\text{-}IgG}$) forms of extracellular Delta-1 on C2 myoblast differentiation and on HES1 transactivation in U20S cells. Notch activation in C2 cells has been reported to inhibit myotube formation, both when these cells express a constitutively active form of Notch intracellular domain (Kopan et al., 1994), and upon co-culture with fibroblasts expressing Notch ligands Jagged-1 (Lindsell et al., 1995) or Jagged-2 (Luo et al., 1997). Our results show that ligand immobilization is required for Notch signal induction in C2 myoblasts and in U20S cells, indicating that ligand tethering may be an essential function for Notch activation. Our studies further show that non-immobilized ligand has a dominant negative effect, presumably by sequestering the Notch receptor and preventing Notch interactions with immobilized ligand.

MATERIALS AND METHODS

Generation of *Delta-1$^{ext\text{-}myc}$*, *Delta-1$^{ext\text{-}IgG}$* and *Control^{IgG}*

Delta-1$^{ext\text{-}myc}$ was generated by methods similar to those used to prepare *Jagged$^{ext\text{-}myc}$* (Varnum-Finney et al., 1998). Briefly, sequences encoding amino acid 537 to the carboxy terminus including the transmembrane and cytoplasmic domain were deleted from full-length human *Delta-1* (Gray et al., 1999) GenBank accession number AF003522 and subcloned into pcDNA3, to add a *Cla*I site to the 3′ end. A *Hin*dIII, *Cla*I fragment was subcloned into PCS2 (generous gift from David Turner and Hal Weintraub) that contained sequences encoding six consecutive myc epitopes. An *Eco*RI fragment containing *Delta-1* extracellular domain and the myc epitopes was subcloned into pcDNA 3.1/amp (Invitrogen, Carlsbad, CA) adding sequences encoding six histidines and a stop codon to the carboxy terminus. To generate *Delta-1$^{ext\text{-}IgG}$* a *Stu*I, *Cla*I fragment of *Delta-1$^{ext\text{-}myc}$* missing the sequences encoding the myc epitopes and poly-histidines was subcloned into a pBluescript vector containing sequences encoding the Fc portion of human IgG1 (generous gift from Immunex). A *Kpn*I, *Xba*I fragment was subcloned into pcDNA3.1/amp (Invitrogen) for expression. *Control^{IgG}* was generated using 500 bp PCR fragment encoding 125 amino acids of *Jagged-1*. The PCR fragment was generated from the following primer pair: upstream 5′CAATCGGCGGAGTATATTAGA3′ and downstream 5′ACTGAAAGGACGCACGATGC3′ using *Jagged$^{ext\text{-}myc}$* as a template (Varnum-Finney et al., 1998). *Control^{IgG}* sequences encoded the Jagged-1 signal peptide but did not include sequences encoding the DSL domain or the EGF repeats of *Jagged*. The upstream primer encoded a *Kpn*I site and the downstream primer, a *Cla*I site. The purified PCR product was cut with *Kpn*I and *Cla*I and inserted into pBluescript vector containing sequences encoding the Fc portion of human IgG1 cut with *Kpn*I and *Cla*I. A *Kpn*I, *Xba*I fragment was subcloned into pcDNA3.1/amp (Invitrogen) for expression.

Expression and purification of Delta extracellular domain

Cells from a myeloma cell line, NSO, were electroporated with constructs to generate stable cell lines for protein production. G418 resistant clones were screened for secretion of the fusion proteins using a quantitative ELISA assay. In brief, wells were coated with the myc-tag antibody (9E10) for testing Delta-1$^{ext\text{-}myc}$ and an anti-human-IgG antibody (Sigma, St Louis, MO) for Delta-1$^{ext\text{-}IgG}$ and Control^{IgG}. Wells were then washed and blocked with a 0.5% solution of gelatin and further incubated with the respective supernatant to be tested. A biotinylated anti-myc antibody followed by Streptavidin-HRP was used for detection of Delta-1$^{ext\text{-}myc}$ and an HRP-conjugated Fc specific anti-human-IgG antibody (Sigma) for detection of Delta-1$^{ext\text{-}IgG}$ and Control^{IgG}. Clones with the highest expression were expanded into roller bottles (Dulbecco's modified Eagle's medium (DMEM) with 1.0% Nutridoma NS (Boehringer Mannheim, Indianapolis, IN)) for mass production of proteins. Two liters of conditioned medium were generated for each batch of ligand.

To purify Delta-1$^{ext\text{-}myc}$ from conditioned medium generated from *Delta-1$^{ext\text{-}myc}$* transfected NSO cells, we used methods previously reported for purifying Jagged$^{ext\text{-}myc}$ (Varnum-Finney et al., 1998). For a control for studies with Delta-1$^{ext\text{-}myc}$, an equal volume of conditioned medium generated by untransfected NSO cells was similarly prepared. In brief, conditioned medium from either control untransfected cells or cells expressing Delta-1$^{ext\text{-}myc}$ was concentrated, dialyzed against PBS (10 mM Na_2HPO_4, 100 mM NaCl, pH 7.2) and subsequently bound to a Nickel column (NiNTA Agarose, Qiagen, Chatsworth, CA) using the His-Bind buffer Kit (Novagen, Madison, WI). Bound protein was washed extensively with wash buffer (His-Bind buffer supplemented with 1.0% Tween-20 and 20 mM β-mercaptoethanol) to remove non-specific binding proteins. Protein was then eluted with increasing concentrations of imidazole. Fractions containing Delta-1$^{ext\text{-}myc}$ were identified with western blots and subsequently pooled, dialyzed with PBS and concentrated about eightfold. The same fractions from the control elutions were also pooled, concentrated to the same amount, and dialyzed. For experiments, the same volume of control solution was used as the volume of Delta$^{ext\text{-}myc}$ solution that was added. To assess purity, proteins were separated using 8% SDS-PAGE and Coomassie stained. Westerns were performed as previously described for Jagged$^{ext\text{-}myc}$ (Varnum-Finney et al., 1998).

To purify Delta-1$^{ext\text{-}IgG}$ and Control^{IgG,} conditioned medium was slowly pumped (1 ml/minute) over a Protein G column (HiTrap Protein G, Amersham Pharmacia Biotech, Piscataway, NJ) to allow maximal binding of the Fc fusion proteins. The column was washed with 20 mM phosphate, pH 7.0. Bound protein was eluted with 100 mM glycine, pH 2.7 and neutralized with 1 M Tris, pH 9.0. Fractions containing Delta-1$^{ext\text{-}IgG}$ or Control^{IgG} were identified using a western blot, pooled and dialyzed with PBS. To assess purity, proteins were separated using 8% SDS-PAGE in the presence or absence of β-mercaptoethanol and Coomassie stained. For westerns, separated proteins were transferred to nitrocellulose (Schleicher and Schull, Keene NM) and immunoblotted with the respective monoclonal antibodies. For detection of Delta-1$^{ext\text{-}IgG}$ or Control^{IgG}, a biotinylated

anti-human IgG antibody was used followed by HRP streptavidin (Amersham, Arlington Heights, IL) and enhanced chemiluminescence reagents (NEN, Boston MA).

HES1 activation assay

Using the Superfect Transfection Reagent (QIAGEN), U20S cells in 60 mm plates were transfected with 75 ng of TK-*Renilla* luciferase control reporter construct (pRL-TK, Promega), 1.5 μg/ml HES-1-luc that contains the –194 to +160 promoter fragment of the HES-1 gene cloned upstream of the luciferase gene in the pGL2-basic vector (Jarriault et al., 1995), and 1.5 μg of pFLAG-CMV-2 plasmid encoding Mam1 (L. Wu, J. Aster and J. D. Griffin, unpublished) and incubated for 24 hours. Cells were then replated into 4 wells in 24-well plates, 2 previously coated with immobilized human-IgG and 2 with immobilized Delta, or 2 with growth medium containing non-immobilized human IgG and 2 with non-immobilized Delta. To immobilize ligands, plates were first coated with 20 μg/ml goat polyclonal anti-human IgG for 30 minutes, washed, blocked and then coated with 10 μg/ml human-IgG (Sigma) or Delta-1$^{ext\text{-}IgG}$ for 2 hours and washed. Cell extracts were then prepared and luciferase activity was measured using the Dual Luciferase Reporter Assay System (Promega). Luciferase activity, corrected for *Renilla* luciferase activity, was expressed as fold activation relative to cells cultured on human-IgG.

C2 differentiation assay

C2 cells were maintained in growth medium (DMEM and 20% fetal bovine serum (FBS)). For the differentiation assay, cells were trypsinized and replated in growth medium at a low density (about 2,000 cells/cm^2) in chamber slides (LabTek, Santa Clara, CA) with or without immobilized ligand. After 24 hours, the growth medium was removed and replaced with differentiation medium (DMEM, 2% FBS and 10 μg/ml insulin). Fusion could be seen after 2-3 days. Cultures were fixed with 2% paraformaldehyde dissolved in PBS for 5 minutes, incubated with a 0.1% solution of Triton X-100 and stained with a monoclonal antibody for myosin heavy chain (MF 20; Bader et al., 1982). Antibody staining was detected using a fluorescein tagged anti-mouse IgG 2b antibody (Pharmingen, San Diego, CA). Nucleii were stained with Dapi to enable cell counts. Five random fields were counted for each chamber of the slide using a microscope equipped with fluorescent optics. The percentage of Dapi stained nucleii that also expressed myosin heavy chain were quantitated.

To immobilize ligand on the plastic surface of the chamber slides, slides were first incubated with a solution of myc tag antibody (9E10) (10 μg/ml) or anti-human IgG antibody (20 μg/ml) in PBS for 30 minutes at 37°C. Slides coated with antibody were blocked with growth medium for at least 30 minutes. These coating conditions sufficiently bound ligand, but did not affect C2 differentiation. Slides were incubated with a solution of ligand at the indicated concentrations in growth medium for 2 hours at 37°C.

Detection of Delta-1$^{ext\ -myc}$ or Delta-1$^{ext\ -IgG}$ binding to C2 cells

C2 cells were detached from the culture flask using 0.5 mM EDTA. Residual EDTA was removed and cells were suspended in PBS containing 2% FBS (PBS/FBS). All reagents for flow analysis were diluted in PBS/FBS. 50 μl of cells was incubated with a 50 μl solution containing the appropriate concentration of ligand for 20 minutes at 4ºC. Unbound ligand was removed and cells previously incubated with Delta-1$^{ext\text{-}myc}$ or Delta-1$^{ext\text{-}IgG}$ were then incubated with 9E10 followed with FITC-conjugated goat anti-mouse IgG1 (Cappel, Durham, NC) or FITC-conjugated goat anti-human IgG1Fc (Cappel) for 20 minutes at 4ºC, respectively. To measure inhibition of binding, cells were first incubated with Delta-1$^{ext\text{-}myc}$ or Delta-1$^{ext\text{-}IgG}$ for 20 minutes at 4ºC and then with Delta-1$^{ext\text{-}IgG}$ or Delta-1$^{ext\text{-}myc}$ for an additional 20 minutes, respectively. Unbound ligand was removed and FITC-conjugated goat anti-human IgG1Fc or 9E10 followed by FITC-conjugated goat anti-mouse IgG1 was used to detect binding, respectively. Binding for all studies was detected using a Vantage flow cytometer (Becton Dickinson Co. Mountain View, CA).

RESULTS

Binding of Delta-1 extracellular domain to C2 cells

Delta-1 extracellular domain (amino acids 1-356), including the DSL domain and all eight EGF repeats was fused at the carboxy terminus to six myc epitopes followed by poly-histidines to generate a monomeric ligand (Delta-1$^{ext\text{-}myc}$, Fig. 1A). In PAGE this protein had a molecular mass of about 100 kDa in non-reducing conditions and in reducing conditions a higher molecular mass of about 110 kDa (Fig. 1C). This observed increase in molecular mass under reducing conditions has been previously reported by other investigators (Qi et al., 1999) and indicates that Delta-1$^{ext\text{-}myc}$ is not dimerized. In addition, Delta-1 extracellular domain was fused to the Fc portion of human IgG1 to generate a dimeric ligand (Delta-1$^{ext\text{-}IgG}$, Fig. 1B). In PAGE, this protein had a molecular mass of about 200 kDa under non-reducing conditions compared to 120 kDa under reducing conditions (Fig. 1D), indicative of a dimer. Flow cytometry analysis showed that both forms of Delta bound to C2 cells (Fig. 2A and B). In addition, each form inhibited the binding of the other form (Fig. 2C and D) indicating that binding was specific. Ligand binding was Ca^{2+} dependent, and chelation of Ca^{2+} with EDTA abolished binding (data not shown), consistent with previous studies showing that depletion of Ca^{2+} leads to separation and release of the extracellular domain of Notch (Rand et al., 2000).

Immobilized Delta-1 extracellular domain inhibits C2 differentiation and induces HES1 transactivation in U20S cells

To assay the effect of Delta on C2 differentiation, C2 cells were plated at low density in growth medium in plastic chamber slides, and 24 hours later, growth medium was replaced with

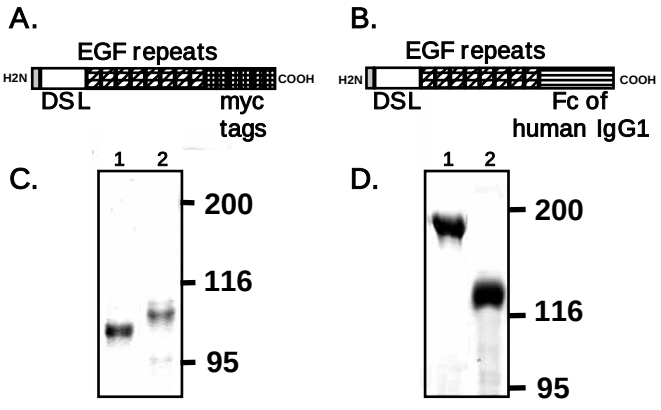

Fig. 1. A schematic drawing of Delta-1$^{ext\text{-}myc}$, Delta-1$^{ext\text{-}IgG}$ and PAGE of these purified proteins. (A) The protein products of *Delta-1$^{ext\text{-}myc}$*, and (B) *Delta-1$^{ext\text{-}IgG}$* are depicted. Indicated are the DSL domain, and the EGF repeats. (C) Purified preparations of Delta-1$^{ext\text{-}myc}$ and (D) Delta-1$^{ext\text{-}IgG}$ that were separated with 8% SDS-PAGE and Coomasie stained. Preparations were incubated without β-mercaptoethanol (non-reducing conditions, lane 1) or with β-mercaptoethanol (reducing conditions, lane 2) to indicate secondary structure.

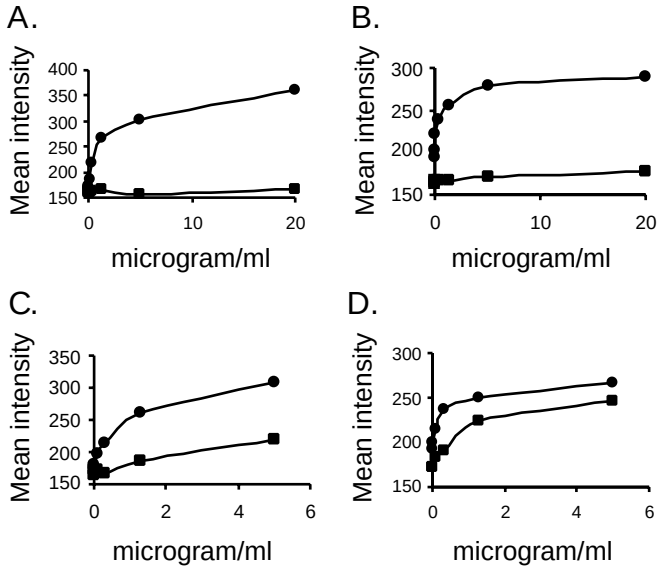

Fig. 2. Specific binding of purified proteins Delta-1$^{ext\text{-}myc}$ and Delta-1$^{ext\text{-}IgG}$ to C2 cells. (A) Mean intensity of fluorescence was determined from fluorescence histograms generated after incubating C2 cells with increasing concentrations of Delta-1$^{ext\text{-}myc}$ (●) or equal volume of the similarly prepared control solution generated from conditioned medium from untransfected NSO cells (■). (B) Mean intensity of fluorescence was determined from fluorescence histograms generated after incubating C2 cells with increasing concentrations of Delta-1$^{ext\text{-}IgG}$ (●) or the Control^{IgG} (■). (C) Mean intensity of fluorescence was determined after incubating in increasing concentrations of Delta-1$^{ext\text{-}myc}$ in the presence of 20 μg/ml Delta-1$^{ext\text{-}IgG}$ (■) or 20 μg/ml Control^{IgG} (●). (D) Mean intensity of fluorescence was determined after incubating in increasing concentrations of Delta-1$^{ext\text{-}IgG}$ in the presence of 20 μg/ml Delta-1$^{ext\text{-}myc}$ (■) or the same volume of control solution (●). In both A and C, an antibody to the myc-epitopes (9E10) was used to detect binding. In both B and D, an antibody to the human IgG-1 epitopes was used to detect binding.

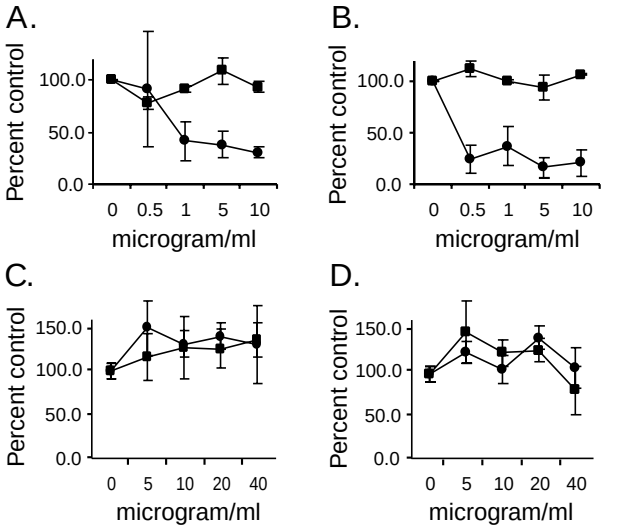

Fig. 3. Effect of immobilized (A and B) and non-immobilized (C and D) purified Delta-1$^{ext\text{-}myc}$ (A and C) and Delta-1$^{ext\text{-}IgG}$ (B and D) on C2 differentiation. (A) C2 cells were incubated in growth medium in chamber slides where the plastic was coated first with specific antibody and then with increasing concentrations of Delta-1$^{ext\text{-}myc}$ (●) or an equal volume of control solution (■). (B) C2 cells were incubated with Delta-1$^{ext\text{-}IgG}$ (●) or Control^{IgG} (■) immobilized as in A. In both cases, after 24 hours, growth medium was replaced with differentiation medium. (C) C2 cells were incubated in growth medium with increasing concentrations of non-immobilized Delta-1$^{ext\text{-}myc}$ (●) or an equal volume of control solution (■). (D) Cells were incubated with increasing concentrations of non-immobilized Delta-1$^{ext\text{-}IgG}$ (●) or Control^{IgG} (■). In both cases, after 24 hours, growth medium was replaced with differentiation medium and fresh increasing concentrations of non-immobilized Delta-1$^{ext\text{-}myc}$ or Delta-1$^{ext\text{-}IgG}$. In all cases, after 3 days in differentiation medium, the cells were stained with an antibody to the differentiation marker, myosin heavy chain, and the percentage of cells that displayed the marker was quantitated. The percentage of control differentiation (culture that had no additional molecules) was then determined. Each point represents the mean from two separate experiments ± a range.

differentiation medium containing a lower serum concentration and insulin. After 2-3 days in differentiation medium, 30-60% of the cells had begun to fuse and express the muscle differentiation marker, myosin heavy chain. To test whether immobilized ligand affects C2 differentiation, Delta-1$^{ext\text{-}myc}$ was bound to plastic coated with an anti-myc monoclonal antibody 9E10, and Delta-1$^{ext\text{-}IgG}$ was bound to plastic coated with a goat polyclonal antibody to the Fc portion of human IgG1. When C2 cells were cultured with either Delta-1$^{ext\text{-}myc}$ or Delta-1$^{ext\text{-}IgG}$ coated on plastic, cell number was not affected (data not shown). However, the extent of differentiation was markedly reduced in the presence of immobilized ligand, with 75% fewer differentiated cells in cultures incubated with the highest concentrations of Delta-1$^{ext\text{-}myc}$ compared to control cultures, and 75-80% fewer differentiated cells in cultures incubated with Delta-1$^{ext\text{-}IgG}$ compared to control cultures (Fig. 3A and B).

Notch signaling was further assayed by measuring HES1 promoter activation (Jarriault et al., 1995) after incubation with immobilized Delta-1$^{ext\text{-}IgG}$. U20S cells overexpressing the human Mam1 gene (L. Wu, J. Aster and J. D. Griffin, unpublished) were transfected with the HES1 reporter gene and incubated for 24 hours. Cells were then incubated with immobilized Delta-1$^{ext\text{-}IgG}$ or human-IgG for 20 hours and lysed to assay luciferase activity (Fig. 4A). Immobilized Delta-1$^{ext\text{-}IgG}$ produced an approximately 6-fold increase in HES1 promoter activity, compared to immobilized human-IgG. For comparison, incubation with NIH3T3 cells overexpressing Jagged2 typically produced an 8- to 10-fold increase in HES1 promoter activity in U20S cells, compared to cells cultured with control NIH3T3 cells (not shown).

Non-immobilized Delta-1 extracellular domain does not affect C2 differentiation nor does it induce HES1 transactivation in U20S cells

Although both Delta-1$^{ext\text{-}myc}$ and Delta-1$^{ext\text{-}IgG}$ specifically bound to Notch on C2 cells, neither protein in solution perturbed C2 differentiation (Fig. 3C and D). The extent of C2 myotube formation obtained in the presence of both non-immobilized monomeric and dimeric Delta-1 extracellular domain (5 to 40 μg/ml) was comparable to that obtained with each respective control (Fig. 3C and D), and cell number/well remained unaffected (data not shown). This was true whether non-immobilized Delta-1 extracellular domain was added with growth medium on Day 0 and again with differentiation medium on Day 1 (Fig. 3C and D), or just with differentiation

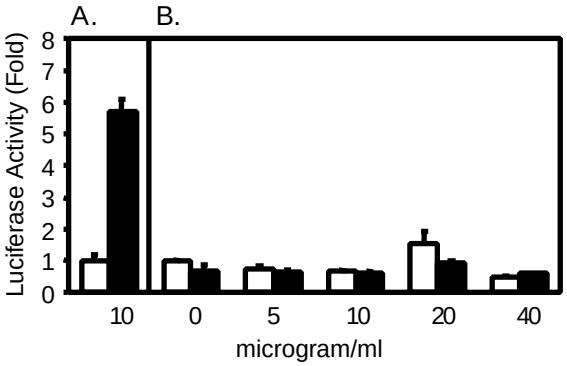

Fig. 4. Effect of immobilized (A) and non-immobilized (B) purified Delta-1$^{ext\text{-}IgG}$ on HES1 transactivation. U20S cells were transfected with a TK-*Renilla* luciferase control reporter construct, a HES1 luciferase construct containing HES1 promoter sequences upstream of the luciferase gene and pFLAG-CMV-2 plasmid encoding Mam1. Twenty-four hours post infection, cells were split and replated into treated wells. (A) Wells were coated with immobilized human-IgG (white bar) or immobilized Delta-1$^{ext\text{-}IgG}$ (black bar). (B) Wells contained growth medium with increasing concentrations of non-immobilized human-IgG (white bar) or non-immobilized Delta-1$^{ext\text{-}IgG}$ (black bar). Luciferase activity, corrected for *Renilla* luciferase activity, was expressed as fold activation relative to cells cultured on human-IgG.

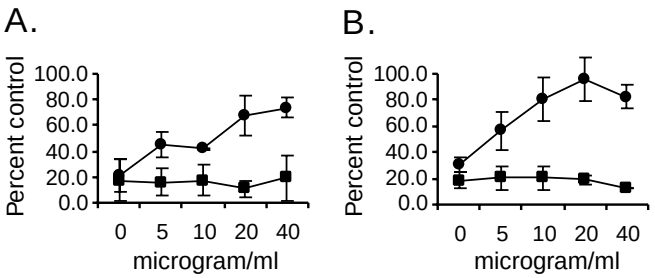

Fig. 5. Inhibitory effect of non-immobilized purified Delta-1$^{ext\text{-}myc}$ and Delta-1$^{ext\text{-}IgG}$ on C2 differentiation in cultures incubating with immobilized (A) Delta-1$^{ext\text{-}IgG}$ and (B) Delta-1$^{ext\text{-}myc}$, respectively. (A) C2 cells were incubated in growth medium in chamber slides where the plastic was coated first with specific antibody and then with 20 μg/ml Delta-1$^{ext\text{-}IgG}$ and after 24 hours, growth medium was replaced with differentiation medium containing increasing concentrations of Delta-1$^{ext\text{-}myc}$ (●) or equal volume of the control solution (■). (B) Cells were incubated with 10 μg/ml of Delta-1$^{ext\text{-}myc}$ immobilized as in A and after 24 hours, growth medium was replaced with differentiation medium containing increasing concentrations of Delta-1$^{ext\text{-}IgG}$ (●) or Control^{IgG} (■). After 3 days, the cells were stained with an antibody to the differentiation marker, myosin heavy chain, and the percentage of cells that displayed the marker was quantitated. The percentage of control differentiation (culture that had incubated with just the specific antibody coated on the plastic and no additional molecules) was then determined. Each point represents the mean from two separate experiments ± a range.

medium (data not shown). The use of Delta-1$^{ext\text{-}IgG}$ at concentrations up to 100 μg/ml had no effect on either differentiation or growth (data not shown).

To assay whether non-immobilized extracellular ligand could induce HES1 activation, U20S cells overexpressing the human Mam1 gene were transfected with a HES1 reporter and then incubated with non-immobilized Delta-1$^{ext\text{-}IgG}$ or human-IgG. Neither non-immobilized Delta-1$^{ext\text{-}IgG}$ nor human-IgG induced HES1 promoter activity (Fig. 4B).

Non-immobilized Delta extracellular domain inhibits Notch function invoked by immobilized Delta

To further assess the potential effects of non-immobilized Delta-1 extracellular domain, increasing concentrations of Delta-1 in solution were added to cells incubating with Delta-1 fixed to the plastic surface with its respective antibody. To avoid using ligand in solution that was recognized by antibody on the substrate, non-immobilized Delta-1$^{ext\text{-}myc}$ was added to C2 cultures stimulated with immobilized Delta-1$^{ext\text{-}IgG}$, and non-immobilized Delta-1$^{ext\text{-}IgG}$ was added to cultures stimulated with immobilized Delta-1$^{ext\text{-}myc}$ (Fig. 5A and B). At concentrations as low as 5 μg/ml, both Delta-1$^{ext\text{-}IgG}$ and Delta-1$^{ext\text{-}myc}$ partially inhibited the Notch effect induced with immobilized Delta-1$^{ext\text{-}myc}$ or Delta-1$^{ext\text{-}IgG}$, respectively, and at higher concentrations, both nearly completely blocked function (Fig. 5A and B). Again, there was no affect of any condition on cell number.

DISCUSSION

In this study, we report that monomeric and dimeric forms of Delta-1 extracellular domain activate Notch only when they are immobilized on the plastic surface. However, non-immobilized monomeric and dimeric forms fail to induce Notch signaling. In fact, non-immobilized Delta-1 blocks the effects of immobilized Delta-1.

Other studies have reported inhibition of Notch function by non-immobilized Delta and Serrate extracellular domains in *Drosophila* eye and wing development (Hukriede et al., 1997; Sun and Artavanis-Tsakonas, 1997). In these settings, ligands that bind but fail to activate Notch would be expected to competitively inhibit the effect of endogenous ligand or, in our case, immobilized ligand. There are several possible explanations for why non-immobilized ligand can bind to receptor but not activate Notch. A recent study showed that trans-endocytosis of Notch extracellular domain by the signaling cell is required for Notch activation in the receptive cell (Parks et al., 2000). These results indicate that the signaling cell is providing an additional required function for activation of receptor than simply a ligand to bind with receptor.

In our studies, ligand extracellular domain activated Notch only if it was first immobilized on the plastic, although it was neither inserted in a membrane nor was there trans-endocytosis. Taken together, these studies imply a required mechanical function that is met by immobilization either in a membrane or on a plastic surface. Although the structural basis for this requirement is not known, it may be that stabilized ligand is capable of altering the conformation of Notch, enabling access of enzymes or other accessory molecules required for cleavage events necessary for activation.

A number of studies from other laboratories have found that non-immobilized ligand extracellular domains induce Notch signaling. In *C. elegans*, exogenous expression of a truncated Notch ligand consisting only of the extracellular domain or the DSL domain was able to rescue a ligand null mutant (Fitzgerald and Greenwald, 1995). In vitro studies have found that monomeric and dimeric forms of Delta extracellular domain could induce Notch activation in cortical neurons, oligodendrocytes and hematopoietic cells (Han et al., 2000;

Qi et al., 1999; Wang et al., 1998). Qi et al. (1999) also demonstrated that proteolysis occurring within the embryo generated monomeric Delta extracellular domain and that generation of the soluble form was associated with normal Notch function (Qi et al., 1999). Other studies have shown that high concentrations of peptides corresponding to regions of the DSL domain induced Notch activation in an in vitro culture system (Li et al., 1998). However, the possibility exists that in the in vitro cases ligand became immobilized by attachment to the unblocked tissue culture wells or to secreted extracellular matrix. Further, it is possible that in the in vivo cases, ligand was bound to extracellular matrix or to molecules on adjacent cells.

It is also possible that ligand forms in solution are capable of inducing Notch signaling in some cell types or under certain conditions. For example, if either the Notch receptor or enzymes and accessory molecules required for proteolysis are present in greater concentrations, a requirement for ligand induced conformational changes of Notch to induce cleavage may be reduced. Hence, C2 myoblasts or U20S cells may express a lower concentration of these molecules and may therefore require a higher density of ligand or longer ligand induced conformational changes before cleavage occurs.

Alternatively, it may be that in our studies only immobilized ligand is adequately concentrated to sufficiently cluster receptor to induce proteolysis and activation. Our studies indicate that non-immobilized dimeric ligand is insufficient for receptor clustering to induce activation, but do not exclude the possibility that multimers in solution might activate Notch. Therefore, non-immobilized ligand forms in other systems may cause activation of the Notch receptor because they form ligand clusters or aggregates in vitro. Consistent with this, previous studies have shown that a dimeric form of Delta1 was also unable to activate Notch in Neural Crest cells. However, when the appropriate amount of a clustering antibody was added, Notch was activated (Morrison et al., 2000). Hence, when clusters bind to multiple receptors on the receiving cell, the aggregated ligand may be sufficiently stabilized to induce the required conformational changes with the Notch extracellular domain to cause activation.

In this report we demonstrate that to induce Notch activation in C2 myoblasts or U20S cells, the extracellular domain of Delta-1 must be immobilized, and that non-immobilized ligand inhibits Notch activation. These results indicate that events leading to activation of Notch may require that ligand be stabilized either in the membrane of the signaling cell or on the plastic surface of the culture flask. They further suggest that, depending on the target cells, the nature and method of presentation of Notch ligand is critical for inducing agonistic or inhibitory effects on Notch signaling.

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