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Notch-induced Proteolysis and Nuclear Localization of the Delta Ligand*

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The Delta protein is a single-pass transmembrane ligand for the Notch family of receptors. Delta binding to Notch invokes regulated intramembrane proteolysis and nuclear translocation of the Notch intracellular domain. Delta is proteolytically processed at two sites, Ala^{581} and Ala^{593} in the juxtamembrane and transmembrane domains, respectively (Mishra-Gorur, K., Rand, M. D., Perez-Villamil, B., and Artavanis-Tsakonas, S. (2002) *J. Cell Biol.* 159, 313–324). Controversy over the role of Delta processing in propagating Notch signals has stemmed from conflicting reports on the activity or inactivity of soluble extracellular domain products of Delta. We have examined Delta proteolysis in greater detail and report that Delta undergoes three proteolytic cleavages in the region of the juxtamembrane and transmembrane domains. Only one of these cleavages, analogous to cleavage at Ala^{581}, is dependent on the Kuzbanian ADAM metalloprotease. The two additional cleavages correspond to the previously described cleavage at Ala^{593} and a novel unidentified site within or close to the transmembrane domain. Delta processing is up-regulated in co-cultures with Notch-expressing cells and is similarly induced by *p*-aminophenylmercuric acetate, a well documented activator of metalloproteases. Furthermore, expression of a truncated intracellular isoform of Delta shows prominent nuclear localization. Altogether, these data demonstrate a role for Notch in inducing Delta proteolysis and implicate a nuclear function for Delta, consistent with a model of bi-directional signaling through Notch-Delta interactions.

The Notch receptor pathway is a highly conserved signal transduction mechanism that is critical for cell fate decisions during tissue patterning and morphogenesis in a number of organ systems in metazoans (1, 2). Signals through the Notch receptor are initiated by interaction with one of two known single-pass transmembrane ligands, Delta or Serrate (mammalian Jagged) (3–5), presented on an adjacent cell. Delta is cleaved to release its extracellular domain by a mechanism involving Kuzbanian (Kuz),[1] an ADAM metalloprotease (6) required for appropriate Notch signaling in several contexts (7–10). Mishra-Gorur *et al.* (11) recently elucidated that Delta is cleaved at Ala^{581} and Ala^{593} to give soluble but inactive extracellular products, Dlec581 and Dlec593. Nonetheless, controversy continues about the activity or inactivity of extracellular forms of Notch ligands (6, 11–13), and thus the functional role of Delta proteolysis in propagating Notch signals is unclear.

There is growing evidence for a functional role of the intracellular domain of Notch ligands. In *Drosophila* and mammalian systems, truncated forms of Delta and Serrate lacking the intracellular sequences are ineffective activators of Notch, indicating the intracellular domain of these ligands is critical for appropriate Notch signals (14–16). Even more intriguing, Jagged1 has an intrinsic signaling activity that relies upon a C-terminal PDZ-ligand domain (17). Thus, the role of proteolytic processing in modulating the activity of Delta, Serrate and Jagged has become an increasingly important question.

We now show that *Drosophila* Delta undergoes three proteolytic cleavages in the region of the juxtamembrane and transmembrane domains. Only one of these cleavages, corresponding to Ala^{581}, is dependent on Kuz. The two additional cleavages correspond to the previously described cleavage at Ala^{593} and a novel unidentified site within or close to the transmembrane domain. Processing of Delta is up-regulated when exposed to Notch expressing cells and is similarly induced by APMA, a well documented activator of metalloproteases. Finally, we show that a truncated intracellular isoform of Delta shows prominent nuclear localization. Altogether, these data demonstrate Notch-induced Delta proteolysis and implicate a nuclear function for Delta and therefore support a model of bi-directional signaling in the Notch pathway.

EXPERIMENTAL PROCEDURES

Cloning of Delta Expression Constructs—The cDNA for *Drosophila* Delta (gift from Spyros Artavanis-Tsakonas, Harvard Medical School) was cloned into the pIZV5His vector (Invitrogen) inframe with the C-terminal V5 epitope tag and polyhistidine sequence to create the pIZDlV5His plasmid. Delta intracellular domain expression constructs were generated by first creating a hemagglutinin (HA) epitope with a start codon by annealing the oligonucleotides: 5′phos-AGCTTATGTACCCCTACGACGTGCCCGACTACGCCGAGCT and 5′phos-CGGCGTAGTCGGGCACGTCGTAGGGGTACATA and cloning into the *Hin*dIII/*Sac*I site of the pIZV5His vector to generate pIZ5′HAV5His. The Delta intracellular domain (amino acids 620–833, which includes the natural stop codon) was PCR amplified with primers 5′-TGGAATTCTAAGCGCAAGCGTAAGCGTGC and 5′-CCGCTCGAGTTACATATGCGGAGTGCCGCAG and cloned into the *Eco*RI/*Xho*I site in pIZ5′HAV5His to create an N-terminal HA epitope inframe with the Delta intracellular domain (pIZDlicHA).

Cell Culture—*Drosophila* S2 and Kc167 cells were routinely cultured at 25–27 °C in Sang's M3 medium (JRH Biosciences, Lenexa, KS) with 10% fetal bovine serum (FBS) and *b*acto*p*eptone (2.5 g/liter) and *ye*astolate (1 g/liter) (1× BPYE) supplement (Difco). *Spodoptera frugiperda* (SF9) cells were cultured in Graces medium (JRH Biosciences) with 10% FBS and 0.5× BPYE. The stable S2 cell lines, Dl-S2 and N-S2, which express *Drosophila* Delta or *Drosophila* Notch under the metallothionien promoter are described previously (18). Protein expression in

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[1] The abbreviations used are: Kuz, Kuzbanian; ADAM, a disintigrin and metalloprotease; APMA, *p*-aminophenylmercuric acetate; N-S2, stable *Drosophila* Notch S2 cells; Dl-S2, stable *Drosophila* Delta S2 cells; Dlfl, full-length Delta; Dlec, Delta extracellular domain; Dlcd, Delta C-terminal domain; HA, hemagglutinin; FBS, fetal bovine serum.

these cells was routinely induced with 0.7 mM $CuSO_4$ addition to the medium for 16 h prior to assay.

Assays for Cleavage of Delta—Both stable Dl-S2 and transiently transfected S2, Kc167, and SF9 cells with pIZDlV5 were analyzed. After transient transfection using Cellfectin (Invitrogen) and 16–20 h of recovery, assays were carried out in serum-free M3 medium/BPYE to better resolve the Dlec product in the medium. APMA (5 mM in H_20 or Me_2SO (Sigma)) was added to the medium at various concentrations. Cells were incubated 4 h, and the medium and cells were harvested separately. Cells were lysed with 50 mM Tris, 1% IGEPAL CA-630 (Sigma), 150 mM NaCl containing the protease inhibitors EDTA (5 mM), phenylmethylsulfonyl fluoride (2 mM), aprotinin, leupeptin, and pepstatin (5 μg/ml each). Samples were run on SDS-PAGE and Western blotted by standard procedures with the anti-Delta 9B antibody (6) (gift from Spyros Artavanis-Tsakonas, Harvard Medical School) and anti-V5 antibody (Invitrogen).

Inhibitory RNA—Double-stranded RNA (RNAi) treatment of S2 or Kc167 cells was done essentially as described (19) (dixonlab.biochem.med.umich.edu/protocols/RNAiExperiment.html). A PCR-amplified segment created using T7 primers with the Kuz sequences (5′-ATGTCATCAAAATGTGCTTTCAAC and 5′-GTGACTGTTGTTGCTGAGGATTGT) was used as a template for RNA synthesis. RNAi was added to cells at concentrations up to 6 μg/well of a 12-well culture plate and incubated 3 days prior to expression of Delta by transient transfection of pIZDlV5. Cell lysates and media were prepared and analyzed by Western blotting as described above.

Notch-induced Cleavage of Delta—pIZDlV5-transfected Kc167 or S2 cells were incubated with S2 or N-S2 cells in M3/BPYE without FBS for various time points prior to harvest and preparation of cell lysates for Western blot analysis.

Immunohistochemistry—DlV5 and DlicHA expressing S2 and Kc167 cells were grown on poly-L-lysine-coated glass coverslips. Cells were fixed with paraformaldehyde and stained in phosphate-buffered saline with 1% normal goat serum and 0.1% Triton X-100. The anti-V5 (1/500 dilution, Invitrogen) and anti-HA (1/1000 dilution, Babco, Berkeley, CA) antibodies were used and detected with fluorescein isothiocyanate-conjugated anti-mouse secondary antibody (1/250 Jackson Immunologicals, West Grove, PA). Nuclei were stained with 4′,6-diamidino-2-phenylindole. Images were captured using Leitz Orthoplan2 fluorescent microscope and Spot Insight QE digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI). Images were processed in Adobe Photoshop (Adobe, San Jose, CA).

RESULTS

APMA-induced Cleavage of Delta—Two isoforms of Dlec are generated by endogenous proteolytic activity in *Drosophila* S2 cells raising the possibility that more than one enzyme acts upon the Delta protein (11). We have therefore explored the potential role of additional sheddase activity in Delta processing using the organomercurial compound APMA, which is an effective activator of cysteine-switch metalloproteases used in numerous experimental systems (20, 21). Incubation of Delta-expressing cells in increasing concentrations of APMA results in a reduction of full-length Delta (Dlfl) and a corresponding increase in the amount of Dlec released in the medium (Fig. 1*A*). Interestingly, two Dlec isoforms are seen that migrate identically with the previously described Dlec581 and Dlec593 isoforms (not shown) (11). We have assigned these products DlecP1 and DlecP2, respectively (see Fig. 1*A*). This APMA-induced proteolysis clearly indicates that there are latent pools of endogenous protease(s) in S2 cells, most likely metalloproteases, that are able to act on Delta.

To clarify the nature of these proteolytic cleavages in Delta, we examined the fate of the C-terminal portion of the protein using a V5-epitope-tagged construct, DlV5. In addition, we examined Delta processing in three distinct insect cell lines: *Drosophila* S2 and Kc167 lines and the Lepidopteran SF9 cell line. Treatment of DlV5-expressing S2 cells with increasing concentrations of APMA showed a progression of C-terminal cleavage products with three distinct bands that migrate at ~30, 28, and 26 kDa (Fig. 1*B*). We have designated these bands DlcdP1, DlcdP2, and DlcdP3, respectively (see Fig. 1, *B* and *C*). In the absence of APMA, the predominant band seen in S2 cells

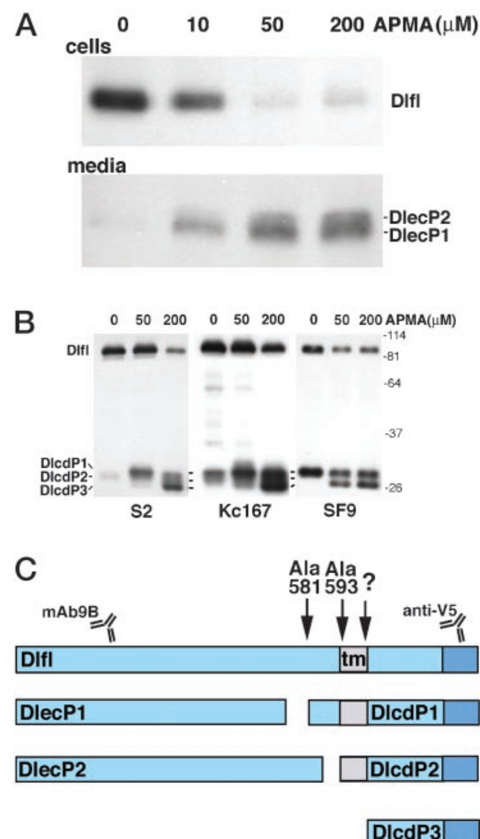

FIG. 1. **Delta proteolysis with APMA.** *A*, Dl-S2 cells were incubated in medium containing the indicated amount of APMA. Full-length (Dlfl) and extracellular (Dlec) Delta were detected in cell lysates and medium, respectively, by Western blotting with monoclonal antibody 9B directed to the extracellular domain of *Drosophila* Delta. An increase in two Dlec products, designated DlecP1 and DlecP2, is seen in the media with a corresponding decrease in Dlfl in the cells with increasing APMA. *B*, DlV5-transfected S2, Kc167, and SF9 cells were incubated with the indicated concentration of APMA and cell lysates analyzed by Western blotting with anti-V5 antibody. Three C-terminal domain products (DlcdP1/P2/P3) are seen in the cell lysates of S2 and Kc167 cells with increasing APMA. The DlcdP2 product is not generated in SF9 cells. *C*, a schematic representation of the cleavage sites in *Drosophila* Delta is shown based on Mishra *et al.* (11) and the results in Fig. 1*B*.

is the DlcdP2 band indicating that it is the major product of steady state levels of endogenous protease activity in these cells. With 50 μM APMA DlcdP1 is the predominant band detected, whereas at 200 μM APMA the predominant product is the DlcdP3 band (Fig. 1*B*, *first panel*). By apparent molecular weight, the DlcdP1 and DlcdP2 bands are predicted to correlate with the cleavages that generate DlecP1 (DlEC581) and DlecP2 (DlEC593), respectively (see Fig. 1*C*). It follows that a third uncharacterized cleavage must occur with APMA induction that gives rise to the DlcdP3 product. Furthermore, given that the DlecP2/DlcdP2 products likely result from cleavage at Ala593 (*i.e.* DlecP2 co-migrates with Dlec593 (not shown)), which is positioned at the N-terminal portion of the transmembrane domain, the DlcdP3 band must arise from a cleavage at an intramembrane or intracellular site (see Fig. 1*C*).

Delta processing also occurs in Kc167 and SF9 cells (Fig. 1*B*). In contrast to the S2 cells, the DlcdP1 band is seen as the major product in the untreated Kc167 and SF9 cells. Furthermore, a significantly greater amount of the DlcdP1 band, in proportion to the full-length Delta, is seen in SF9 cells suggesting that the enzyme activity required for this product is present at a much higher level than in S2 or Kc167 cells. It is of note that the DlcdP2 is absent in SF9 cells (Fig. 1*B*, *third panel*). Altogether,

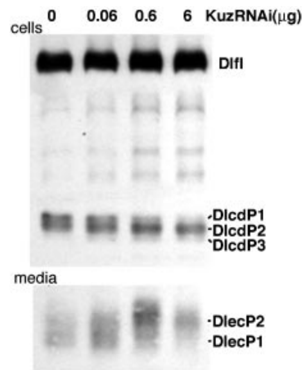

FIG. 2. **Kuzbanian RNAi inhibition of Delta Cleavage.** Kc167 cells treated with Kuz RNAi at the concentrations indicated were transfected to express DlV5. Delta processing was determined by Western blot analysis of cell extracts with anti-V5 antibody (*top panel*) to detect C-terminal domain products and of medium with monoclonal 9B (*bottom panel*) to detect extracellular domain products.

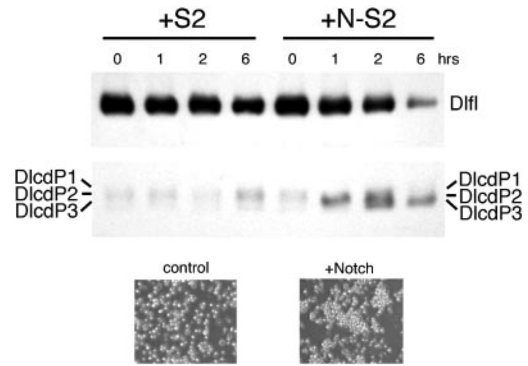

FIG. 3. **Notch-dependent cleavage of Delta.** DlV5-expressing S2 cells were co-cultured with S2 cells (*+S2*) or Notch S2 cells (*+N-S2*) for the times indicated. Cell extracts were analyzed by Western blotting with anti-V5 antibody to detect C-terminal domain products. *Bottom panels* are bright field images of the corresponding cultures at 1 h of co-culture.

the data confirm that Delta is a substrate for three distinct proteolytic processing events and that the activity of the enzymes responsible for these cleavages varies among different cell lines, both at a resting state and upon APMA induction.

Kuzbanian-dependent Delta Processing—To begin identifying the enzymes responsible for these cleavages, we examined which of the three Dlcd products arises from Kuz-dependent cleavage using RNA interference (RNAi) to remove Kuz activity. Reduction of Kuz in Kc167 (Fig. 2) or S2 (not shown) cells resulted in the disappearance of the DlcdP1 band, while the DlcdP2 and DlcdP3 bands remain unaffected. The Dlec in the medium shows a shift to predominantly the DlecP2 with increasing KuzRNAi, confirming that the DlecP1 and DlcdP1 are the corresponding products of the same cleavage.

Notch-induced Cleavage of Delta—APMA induction of Delta cleavage indicates that Delta processing is susceptible to activation of latent proteases and raises the possibility that Delta processing may be modulated in the context of cell signaling events. We therefore asked whether Delta processing could be induced through interaction with its receptor, Notch. Incubation of DlV5-expressing S2 (Fig. 3) or Kc167 (not shown) cells with N-S2 cells caused a transient accumulation of the DlcdP2 band at 1–2 h and resolved to the DlcdP3 product at 6 h (Fig. 3, *+N-S2 lanes*). A corresponding decrease in the full-length Dl was observed. Obvious aggregates were formed in the DlV5/Notch cell mixtures (Fig. 3, *lower right panel*), indicative of a Delta-Notch binding event described previously (18). In contrast, when DlV5 cells were incubated with control S2 cells only a slight increase in the DLcdP2 band was seen at 6 h (Fig. 3, control lanes), and no significant cell aggregates were observed (Fig. 3, *lower left panel*). These data confirm that Notch expressing cells promote processing of Delta.

Localization of Full-length and Intracellular Delta—The cleavages within or close to the membrane that give rise to DlcdP3 predict a cytoplasmic product that is untethered from the membrane (see Fig. 1*C*). We therefore asked what the fate of the intracellular domain of Delta is by examining its subcellular localization. Expression of the entire Delta intracellular domain in either S2 (Fig. 4) or Kc167 (not shown) cells showed prominent nuclear localization. In contrast, full-length Delta was localized to the plasma membrane and intracellular compartments (Fig. 4). These data indicate that the products that result from Delta processing can potentially translocate to the nucleus.

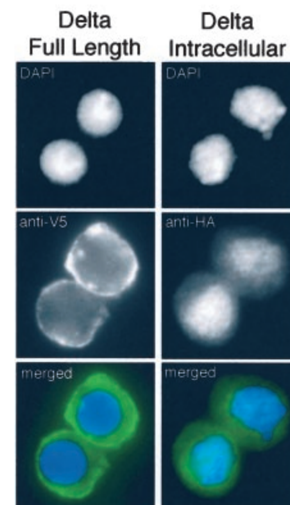

FIG. 4. **Localization of full-length and intracellular Delta.** S2 cells were transfected with DlV5 (full-length) or DlicHA (intracellular). The localization of the Delta proteins relative to the nucleus (indicated by 4′,6-diamidino-2-phenylindole (*DAPI*) staining) was revealed by immunostaining with anti-V5 or anti-HA, respectively.

DISCUSSION

The question of how signals conveyed by the Notch receptor direct distinct cellular fates in development remains complex; however, several advances have been made at the molecular level. Perhaps most provocative is the discovery that regulated proteolysis by ADAM proteases and Presenilin γ-secretases are essential activation steps to generate an intracellular Notch product with the ability to localize to and act in the nucleus to modulate gene transcription (reviewed in Refs. 22 and 23). It is now clear that Delta undergoes a similar pattern of proteolytic processing. We demonstrate that Kuzbanian is required for processing to give the DlecP1/DlcdP1 products and that the DlcdP2 and DlcdP3 products point to additional intramembranous/intracellular cleavages. Consistent with our observations, a very recent report by Ikeuchi and Sisodia (24) shows presenilin-dependent processing of human Delta1 and Jagged2. Altogether, the data suggest a receptor-like function for the Delta protein: 1) processing is induced by binding to another protein (*e.g.* Notch), 2) DlcdP3 predicts an untethered cytoplasmic product, and 3) the cytoplasmic domain of Delta localizes to the nucleus. Our data support a model whereby Notch-Delta interaction between neighboring cells results in bi-directional signaling.

The pattern of Delta processing is in accordance with a growing body of evidence showing that juxtamembrane and intramembrane proteolysis is a widely conserved mechanism for modulating activity in a variety of signaling pathways (25,

26). The ErbB-4 receptor and the amyloid precursor protein are remarkably similar to Notch in that they undergo sequential ADAM and presenilin-dependent cleavages and nuclear translocation of their intracellular domains for subsequent modulation of gene transcription (27–30). Interestingly, the Neuregulin1 ligand for ErbB receptors is also a substrate for ADAM protease activity and undergoes intramembrane proteolysis resulting in nuclear translocation of its intracellular domain and changes in gene transcription (31, 32). Together with Notch/Delta, these data suggest the existence of a common mechanism of proteolytic regulation for bi-directional cell signaling in diverse signaling pathways.

A bi-directional Notch/Delta signaling model is consistent with the prevailing feedback-regulation model whereby transcription of Notch and Delta genes in neighboring cells is modulated to elaborate the asymmetries that propagate cell fate decisions (33–36). While the function of DlcdP3 awaits further characterization, several molecular and genetic studies implicate a role for the intracellular domain of Notch ligands. Truncated forms of Delta and Serrate, which lack the intracellular domain, act as non-autonomous dominant-negative inhibitors of Notch signaling *in vivo* (14, 15). Likewise, a truncated form of the human Delta1 ligand shows significantly diminished activity toward activating Notch in a cell-based luciferase reporter assay (16). Furthermore, a recent study by Ascano *et al.* (17) describes a novel intrinsic signaling activity of the mammalian Jagged1 ligand that relies upon interaction of the Jagged1 intracellular domain with PDZ proteins. Importantly, these authors also demonstrate that ectopic Jagged1 expression up-regulates Notch3 and Jagged1 mRNA levels in cultured cells, providing further evidence for transcriptional regulation in elaboration of Notch signals. Our data clearly show that the cytoplasmic domain of *Drosophila* Delta has the intrinsic property of localizing in the nucleus. We have thus far been unable to detect a Notch-induced DlcdP3 product in the nucleus by conventional immunostaining methods (not shown). However, data from Ikeuchi and Sisodia (24) indicate a Delta-Gal4VP16 chimera acts in the nucleus in a luciferase reporter based assay. Altogether, these data highlight the potential signal-receiving capacity of Notch ligands. However, questions of how proteolysis might contribute to this activity remain the subject of further investigation.

Alternatively, a model proposed recently for the role of Kuz cleavage of Delta (11) argues that cleavage and inactivation of Delta on the Notch/Delta-expressing cell is required to make that cell preferentially a signal receiving cell. In this regard, the additional cleavages of Delta reported here may similarly contribute to Delta inactivation. However, it is important to note that the cleavages generating DlcdP2 and DlcdP3 occur independent of Kuz activity and would therefore represent alternative pathways to down-regulate Delta ligand activity. Furthermore, the fact that the DlcdP2 and DlcdP3 cleavages, but not the Kuz-dependent DlcdP1 cleavage, are promoted by interaction with Notch suggests these novel cleavages are potentially linked directly to a Notch signaling mechanism.

There is growing evidence that several classes of enzymes can mediate proteolysis at and within the plasma membrane (for review, see Refs. 26 and 37). In addition to the ADAM metalloproteases, aspartyl and serine proteases are known to act on transmembrane protein substrates (26, 37). Our observations indicate that all three cleavage events in Delta are up-regulated in the presence of APMA. Consistent with its properties as a metalloprotease activator, APMA induces the Kuz-dependent cleavage to generate the DlecP1/DlcdP1 products. The enzyme activities responsible for the DlcdP2 and DlcdP3 products are yet to be identified. However, it is of note that DlcdP2 is generated in S2 and Kc167 cells but not in SF9 cells, indicating that the enzyme required for this cleavage is absent in SF9 cells. The fact that SF9 cells lack endogenous γ–secretase activity (20) implicates presenilin as a candidate for cleavage that generates DlcdP2. The role of presenilins and other proteases in Delta processing warrants further investigation.

In conclusion, our results demonstrate Notch-induced processing of the Delta ligand and nuclear localization of the Delta intracellular domain. These events, which are analogous to the proteolytic activating steps in Notch and a number of other cell surface signaling molecules, point to a receptor function for Delta and thus support a model of bi-directional signaling in the Notch-Delta pathway.

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