Analysis of Notch Lacking the Carboxyl Terminus Identified in Drosophila Embryos

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Abstract. The cell surface receptor Notch is required during development of Drosophila melanogaster for differentiation of numerous tissues. Notch is often required for specification of precursor cells by lateral inhibition and subsequently for differentiation of tissues from these precursor cells. We report here that certain embryonic cells and tissues that develop after lateral inhibition, like the connectives and commissures of the central nervous system, are enriched for a form of Notch not recognized by antibodies made against the intracellular region carboxy-terminal of the CDC10/Ankyrin repeats. Western blotting and immunoprecipitation analyses show that Notch molecules lacking this region are produced during embryogenesis and form protein complexes with the ligand Delta. Experiments with cultured cells indicate that Delta promotes accumulation of a Notch intracellular fragment lacking the carboxyl terminus. Furthermore, Notch lacking the carboxyl terminus functions as a receptor for Delta. These results suggest that Notch activities during development include generation and activity of a truncated receptor we designate N ΔC termin.

Key words: Notch • Delta • neurogenesis • daughterless • differentiation

Introduction

Notch (N) is required throughout development of Drosophila melanogaster for differentiation of tissues as diverse as the nervous systems, cuticle, internal organs, and muscles (for a review of Notch signaling, see Artavanis-Tsakonas et al., 1999; see also Zecchini et al., 1999; Wesley, 1999; Brennan et al., 1999a,b). N is a cell surface receptor which generates intracellular signals when a ligand binds its extracellular domain (Artavanis-Tsakonas et al., 1999). During embryogenesis, N is required to produce neuronal and epidermal precursor cells in a process termed lateral inhibition (Cabrera, 1990; Skeath and Carroll, 1992).

During lateral inhibition, the ligand Delta (Dl) binds the extracellular domain of N, leading to transmission of signals to the nucleus by the intracellular protein, Suppressor of Hairless (Su(H)). Cells that respond to these signals by turning on the expression of Enhancer of split Complex genes (E(spl)C), and turning off the expression of the pro-neural Achaete scute Complex genes, become the epidermal precursor cells; cells that do not turn on the expression of E(spl)C but continue to express Achaete scute Complex genes, become the neuronal precursor cells (see Artavanis-Tsakonas et al., 1999). N function continues to be required during differentiation of neurons from the neuronal precursor cells (Giniger et al., 1993; Giniger, 1998) and epidermis from the epidermal precursor cells (Hopp and Greenspan, 1990; Couso and Martinez-Arias, 1994; Wesley, 1999). Requirement of N function at successive stages is also observed during differentiation of tissues like the adult compound eyes and sensory bristles (Cagan and Ready, 1989; Guo et al., 1996; Wang et al., 1997). This implies that N is required continuously during differentiation of a cell lineage to maintain the cell fates specified during lateral inhibition and/or generate additional differentiation signals at post-lateral inhibition stages.

Su(H) activity is affected by some proteins that also bind the N intracellular domain. Deltex contributes to the Su(H)-mediated N signaling pathway (Matsumoto et al., 1995), while Numb, Dicersa, and Hairless antagonize this pathway (Axlrod et al., 1996; Frise et al., 1996; Guo et al., 1996; Spana and Doe, 1996; Wang et al., 1997). On the other hand, Disabled, which functions with N during differentiation of neurons from neuronal precursor cells (i.e., after lateral inhibition), is not known to affect Su(H) activity (Giniger et al., 1993; Giniger, 1998). Su(H) interacts with the RAM 23 region and the CDC10/Ankyrin repeats.
peptides region in the N intracellular domain (Fortini and Artavanis-Tsakonas, 1994; Tamura et al., 1995; see Fig. 1). Deltex interacts with the CDC10/A nkyrin repeats region (Diederich et al., 1994; Matsuno et al., 1995), Numb with the RAM 23 and PEST regions (Guo et al., 1995), D ishevelled with the unique region carboxy-terminal of the CDC10/A nkyrin repeats (Axelrod et al., 1996), and Dishevelled with the R AM 23 region (Gninger, 1998). The binding domain of Hairless has not been mapped (Wang et al., 1997; see Fig. 1). These different activities and affinities suggest that regulation of activities of different proteins that bind the intracellular domain might be an important component of N functions at successive stages of differentiation.

In this study, we describe results showing that a truncated form of N lacking the sequence carboxy-terminal of the CDC10/A nkyrin repeats is produced during embryogenesis. This truncated receptor, which would lack the Dishevelled and one of the two Numb-binding sites, may function as a receptor for DI. Its differential accumulation in interacting cells may play a role in choice of cell fates during lateral inhibition and regulation of activities of different proteins that bind the N intracellular domain.

Materials and Methods

Immunostaining of N Protein in Embryos

αNPCR antibody was generated against the intracellular segment of N, amino acids 2,115–2,536, between the CDC10/A nkyrin repeats and the OPA repeats (Lieber et al., 1993; K i dd et al., 1998; numbering of the 2,703-amino-acid-long N protein is according to K i dd et al., 1986). The αN203 antibody was generated in rats against a glutathione-S-transferase fusion peptide including N EGF-like repeats 1–3 (amino acids 59–177) following standard procedures (Harlow and Lane, 1988). αN203 immunoprecipitates and detects only N forms from embryos and S2-Notch cells. It gives N immunostaining patterns in embryos, imaginal discs, and larval brains that is indistinguishable from other published N staining patterns. All the N antibodies used in this study are N-specific antibodies; they do not give signals in N-embryos or N molecules recognized by each are recognized by at least two other independently generated N antibodies (K i dd et al., 1986, 1998; Lieber et al., 1993). These polyclonal antibodies also recognize N only when their respective epitope regions are included and even a small terminal segment of the epitope region is sufficient for recognition by the respective antibody.

Immunostaining procedure described in Lieber et al. (1993) was followed and signals detected with HRP and N anti-β-galactosidase antibody made in rabbit and alkaline phosphatase reactions were used to sort out FM 7 lac-Z or TM 6 lac-Z chromosome carrying embryos laid by N 264-47, FM 7 lac-Z or DI1/TM 6 lac-Z flies. Embryos shown in Fig. 2, a–i and j–q were collected in separate batches and samples within each batch were processed identically.

Immunoprecipitations

For immunoprecipitation of N molecules from embryos, ~50–100-μl vol of dechorionated embryos, of appropriate ages (laid by circadian cycle entrained flies to minimize age variance in embryos), were crushed using a Wheaton Dounce GrINDER, in the presence of 0.75 mM Tris-HCl, pH 7.5, 1.2 μl of cold 2-M Tris-HCl, pH 7.5, 0.1 μl of 1% Triton X-100 and 0.5% deoxycholate. The rest of the procedure was identical to that described for immunoprecipitation of Notch proteins from embryos except that the wash buffer included 10 mM Tris, pH 7.5. 100 μl of the monoclonal αDI was used per immunoprecipitation. The amounts of proteins in different extracts were standardized using absorbance values at 280 nM and the B i o R ad D C protein assay kit. See also Wesley (1999) for description of these immunoprecipitation procedures.

Western Blot Analyses

Embyros. Populations of flies were transferred to the appropriate temperature, eggs collected for 2 or 3 h (or 6 h at 18°C), and reared for the indicated period of time at the indicated temperatures (with appropriate corrections for differences in developmental rate).

Cultured Cells. Cells were heat-shocked for 30 min at 37°C, allowed to synthesize proteins for 1 or 2 h at room temperature, and washed 2× in Shields and Sang’s M3 media plus antibiotics.

N and DI Cell Aggregates. N 25 S-N and N 1997; see Fl Fig. 1 a). These different activities and affinities were collected in separate batches and samples within each batch were processed identically.

Cloning of N 1991). These were cotransfected into S2 cells with hsGal4 UAS-N and UAS-N 1997; see Fl Fig. 1 a). These different activities and affinities were collected in separate batches and samples within each batch were processed identically.

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Results

An Antibody Made against the Carboxyl Terminus of N Does Not Stain Certain Embryonic Tissues Expressing N

Immunostaining experiments were done with αN203, which recognizes the amino terminus of N, and with αNPCR, which recognizes the carboxyl terminus of N (see Fig. 1 and Materials and Methods for information about these antibodies). Stage 8-9 Canton S embryos immunostained with αN203 showed relatively intense punctate staining in the region involved in lateral inhibition, whereas the embryos stained with αNPCR showed a homogenous staining of the same region (Fig. 2, a–e). The intense punctate signals in embryos treated with αN203 are derived from segregating neuroblasts; cell morphology identify them as neuroblasts and the pattern of αN203 staining rapidly changed during this stage of embryogenesis (Fig. 2, compare b with c). Furthermore, αN203 staining corresponded with the expression pattern of the proneural achaete gene accompanying neuroblast segregation (Campos-Ortega and Hartenstein, 1985; Cabrera, 1990; Skeath and Carroll, 1992; data not shown). Differences in the staining patterns of αNPCR and αN203 were more striking at later stages of embryogenesis: αNPCR antibody did not stain the commissures and connectives of the central nervous system (CNS; Fig. 2 f), while αN203 showed strong staining of the same tissues as previous studies of N distribution have shown (Fig. 2 g; Kidd et al., 1989; Johansen et al., 1989; Fe-hon et al., 1991; Gijger et al., 1993; Kooh et al., 1993; Giniger, 1998). The previous studies cited used antibodies made against the first two EGF-like repeats, the last six EGF-like repeats, extensive regions of the EGF-like repeats, or intracellular regions including both the CDC10/A nkyrin repeats and the unique region in the carboxyl terminus. None of these studies used an antibody that is specific to the unique region carboxy-terminal of the CDC10/A nkyrin repeats, like αNPCR. At some other stages, the two antibodies gave similar patterns (Fig. 2, h and i).

Lack of staining of commissures and connectives of the CNS by αNPCR was not because this antibody fails to recognize N in the embryos: (a) omission of αNPCR from the immunostaining procedure resulted in complete loss of signals in the embryos (Fig. 2, compare k with j); (b) αNPCR failed to generate any signals in the neurogenic N264-47/Y embryos which have lost expression of N (see Kidd et al., 1989), but generated strong signals in the neurogenic Dl/Dlx embryos which continue to express N (Fig. 2, l and m); (c) αNPCR generates a patchy staining pattern in N251 embryos raised at 30°C (Fig. 2, n and o); patchy loss of N in N251 embryos is expected since only ~70% of these embryos fail to complete embryogenesis at the restrictive temperature of 30°C, see Shellenbarger and Mohler, 1978); and (d) non-recognition of N in connectives and commissures of the CNS was a property of αNPCR as these tissues were stained with αN203 (Fig. 2 g) and the nervous system-specific anti-HRP antibody (Fig. 2, compare q with p stained with αNPCR). If both αN203 and αNPCR antibodies recognized the same N molecules at all

![Figure 1](http://rupress.org/jcb/article-pdf/149/3/683/1290565/9909059.pdf)
stages of development, similar staining patterns would be expected at all stages. Instead, only \( \alpha \)N203 showed higher levels of N in the connectives and commissures of the developing CNS (Fig. 2, b, c, e, and g, compare with \( \alpha \)NPCR staining in a, d, and f). The pattern of N expression in E(spl)C embryos deficient in lateral inhibition signaling was the same as in Dl embryos (detected by \( \alpha \)N203 and \( \alpha \)NPCR antibodies): expression of N is higher than in Canton S embryos and limited to the neurogenic region (data not shown). These results indicated that a subset of differentiating tissues that express N, produced after lateral inhibition signaling, are enriched for a form of N that either does not contain the region known to be present carboxy-terminal of the CDC10/Ankyrin repeats, or has masked the antibody epitopes in that region.

Embryos Produce Notch Molecules Lacking Sequence Carboxy-terminal of the CDC10/Ankyrin Repeats

SDS-PAGE analysis of N immunoprecipitated from Canton S embryonic extracts showed that \( \alpha \)N203 and \( \alpha \)NI recover a triplet of N proteins in the \( \sim \)350-kD range (Fig. 3 a, lanes 1 and 2; \( \alpha \)NI is made against the intracellular region between the transmembrane domain and the end of CDC10/Ankyrin repeats, Lieber et al., 1993; see Fig. 1). The three forms are referred to as NFull, N350.2, and N\( \Delta \)Cterm in increasing order of electrophoretic mobilities (see later for the basis for these names). Similar forms of N have been reported previously, detected using an antibody made against the last six EGF-like repeats (Johansen et al., 1989). However, \( \alpha \)NPCR, made against the intracellular region carboxy-terminal of the CDC10/Ankyrin repeats, immunoprecipitated only NFull and N350.2 (Fig. 3 a, lane 3) indicating that N\( \Delta \)Cterm is not recognized by this antibody.

As immunoprecipitations were done with a buffer approximating physiological conditions, it is possible that physiological N\( \Delta \)Cterm masked \( \alpha \)NPCR epitopes and this prevented immunoprecipitation by \( \alpha \)NPCR. To evaluate this possibility, N was immunoprecipitated from Canton S embryos with \( \alpha \)NI (which recovers all three forms), two equal aliquots of the immunoprecipitates were separated by SDS-PAGE, and the resultant Western blots probed with \( \alpha \)NI and \( \alpha \)NPCR. N\( \Delta \)Cterm was detected by \( \alpha \)NI (as
expected) but not by αNPCR (Fig. 3 b) indicating that non-recovery of NΔTerm with αNPCR is due to absence, rather than masking, of αNPCR epitopes.

The absence of αNPCR epitopes and the faster SDS-PAGE migration (compared with NFull containing the αNPCR epitopes) suggested that NΔTerm lacked the carboxyl terminus sequence. To determine whether N molecules truncated to remove the carboxyl terminus αNPCR epitope region migrate alongside NΔTerm in SDS-PAGE, and to get a rough estimate of how much of the carboxyl terminus region is lost in NΔTerm, the following cell lines were generated: S2-N1–2155 cells producing N molecules truncated after amino acid 2,155, immediately after the CDC10/A nkyrin repeats, and S2-N1–2262 cells producing N molecules truncated after amino acid 2262. Extracts from these cells were separated in SDS-PAGE alongside extracts from embryos, from S2 cells expressing N, and from S2 cells expressing N60g11. N60g11 is N protein produced from the mutant N60g11 allele. N60g11 contains a frame shift mutation that results in deletion of the intracellular region carboxy-terminal of amino acid 2,123 (580 amino acids are deleted and 19 random amino acids added before termination; Lyman and Young, 1993). Western blotting analysis showed that NΔTerm migrates alongside N1–2155 and
The migration of all N molecules in SDS-PAGE reflected the size of truncation in the carboxyl terminus (see diagram in Fig. 3 d). A difference in mobility due to a difference of ∼107 amino acids (in ∼2,300 amino acids) is clearly apparent in SDS-PAGE. N ΔCterm is not recognized by αNPCR because it is truncated for ∼500 amino acids in the carboxyl terminus and therefore lacks the αNPCR epitope region. The nature of differences between N350.2 and NFull and between N350.2 and N ΔCterm are presently unknown. The slowest migrating ∼350-kD form is named N Full because it contains the complete sequence; the fastest migrating ∼350-kD form is called N ΔCterm because it lacks the carboxyl terminus (half of the intracellular domain); and the form migrating between NFull and N ΔCterm is named N350.2 because it is the second of three forms in the ∼350-kD range.

N Full, N350.2, and N ΔCterm are colinear N molecules as they are recognized by an amino terminus antibody (αN203) and at least one of the intracellular antibodies (αNI and αNPCR) in SDS-PAGE-based Western blot analysis. Therefore, these colinear forms may be substrates of Kuzbanian or Furin-like Convertase enzymes for production of heterodimeric cell surface molecules as proposed by Pan and Rubin (1997), Blaumueller et al. (1997), and Logeat et al. (1998). Our data related to activities of NFull and N ΔCterm do not distinguish between the colinear and the proposed heterodimeric forms of the receptors. Therefore, NFull and N ΔCterm would refer to the colinear receptors on Western blots but to both the colinear and the proposed heterodimeric receptors with regard to activities. N, without any numbers, acronyms, or abbreviated names, will be used to refer to the N protein in general (inclusive of all forms). The proposed or inferred structures of the various forms of N referred to in this study and the caveats, if any, associated with inference of their structures or usage of names are shown in Fig. 1 b.

**N ΔCterm Is Associated with Delta during Embryogenesis**

Antidiotopes were performed from different stages of embryos to determine whether N ΔCterm is associated with Dl during embryogenesis. Embryos laid by circadian cycle entrained adult flies were used to minimize age variance and maximize chances for detection of any developmental stage-specific recovery of different forms of N. Proteins interacting at the cell surfaces were...
cross-linked, and the complexes immunoprecipitated by anti-Dl antibody were analyzed with antibodies made against different regions of N. The cross-linking/immunoprecipitation procedure employed recovered only complexes of proteins known to interact at cell surfaces during Drosophila embryogenesis (Wesley, 1999).

The monoclonal anti-Dl antibody used here (mAb 202, Fehon et al., 1990) does not recover detectable levels of NFull in the absence of cross-linkers (Fig. 4 a, lanes 1 and 2; see also Wesely, 1999). It does not recover even the intracellular and extracellular fragments of the proposed heterodimeric NFull (data not shown). This may be due to a disruption of N-Dl complexes when cells are lysed for immunoprecipitation (Fehon et al., 1990; Wesley, 1999) or due to inefficient recovery of NFull by this anti-Dl antibody. Also, none of the anti-N antibodies produced in our laboratory (six have been tested), nor anti-Dl antibodies tested, detect or recover significant levels of the intracellular domain of the proposed heterodimeric NFull receptor, either from cultured cells expressing N or wild-type embryos (Kidd et al., 1998; Wesley, 1999). Significant levels of a 250–300-kD N extracellular fragment is detected in Western blots or immunoprecipitations with antibodies made against the extracellular domain (Wesley, C.S., personal observation). The ~180-kD extracellular domain fragment described in Blaumueller et al. (1997) is not detected by these extracellular N antibodies. We do not know the reason for this. Failure to recover the proposed intracellular domain of the heterodimeric NFull receptor may be due to the fact that most NFull molecules in these cells or embryos are nonfunctional (see Struhl and Adachi, 1998; Schroeter et al., 1998). In the cross-linking/immunoprecipitation procedure employed here, the intracellular and extracellular fragments composing the heterodimeric cell surface receptor are expected to be cross-linked along with the ligand.

N immunoprecipitated by anti-Dl antibody from 0- to 3-h embryonic extracts was recognized by αNPCR, αNI, and αNT (the last antibody was made against the first two EGF-like repeats, Kidd et al., 1989; see Fig. 1 for their epitope regions), indicating that N in these complexes contains all domains of N (Fig. 4 a, lanes 5, 7, 9). N immunoprecipitated from 3–6-h embryonic extracts was recognized by αNI and αNT, but not by αNPCR (Fig. 4 a, lanes 6, 10, 8) suggesting that this form of N is not recognized by αNPCR. Immunoprecipitation in the absence of cross-linkers, or without the anti-Dl antibody, failed to recover any N containing complexes (Fig. 4 a, lanes 1–4), indicating that the complexes recovered in these experiments contained both N and Dl. Recognition of N by αNPCR in one extract and not in the other (when both were extracted at the same time, with the same procedure) ruled out any experimental variation influencing antibody recognition and indicated that N molecules in the two complexes are indeed different.

Western blot analysis of a 3-h interval sampling of proteins showed that while NFull was the predominant form in 0-3 h embryos, it was expressed at very low levels in the 3–6-h-old embryos (Fig. 4 b, lanes 1 and 2; N350.2 and N ΔCterm are present at similar levels in 3–6-h extracts and migrate close to each other in 4.25% SDS-PAGE gels). This suggested that the form of N associated with Dl in 0–3-h embryos is NFull and the form of N associated with Dl in 3–6-h embryos is N ΔCterm. The form of N associated with Dl in 0–3-h embryos is unlikely to be N350.2 (which is also recognized by αNPCR) because it is present at equivalent levels in both 0–3- and 3–6-h embryos (see Fig. 4 b) and would have been recovered from both embryos if it associated with Dl. The low level of NFull in 3–6-h embryos and the association of Dl with N ΔCterm in embryos of the same age are consistent with the observations that a form of N not recognized by αNPCR is enriched in 3–6-h embryos (Fig. 2, a–e) and that both N and Dl are required for neurogenesis after lateral inhibition (Giniger et al., 1993; Giniger, 1998). The majority of embryos in the 3–6-h sample will be between 4 to 5 h of develop-
An Intracellular Fragment of Notch Lacking the Carboxyterminus Accumulates when S2 Cells Expressing NFull Are Treated with S2-DI Cells

To determine whether NΔCterm or the intracellular domain of this cell surface receptor is produced when DI binds NFull, in vitro experiments were performed with S2-DI and S2-N cells. N and DI produced in S2 cells bind each other (Fehon et al., 1990; Rebay et al., 1999). Most of N produced in S2-N cells is NFull (see later and Wesley, 1999). S2 cells (untransfected) and S2-DI cells do not express N and the Notch gene in S2 cells is rearranged (Wesley, C.S., unpublished data; Fehon et al., 1990; Y e et al., 1999).

S2-N cells were treated with S2-DI cells or S2 cells, and protein extracts analyzed by Western blotting with αNI and αNPCR antibodies. S2-N cells treated with S2-DI cells for 2 h accumulated higher levels of a ~120-kD fragment (designated NIntra) and a ~55-kD fragment (designated NΔCtermMIntra) that are recognized by αNI (Fig. 5 a, lanes 1 and 2; see later for the basis for these names). The same blot probed with αNPCR recognized NIntra but not NΔCtermMIntra (Fig. 5 a, lanes 3 and 4). NIntra, recognized by both αNI and αNPCR (see Fig. 5 a, lanes 2 and 4), is the full-length N intracellular domain. It migrates along-
tively designated it NΔCtermTM1(r7) (see Fig. 1 b). As the cell surface N molecules are proposed to be a heterodimers of the extracellular domain and the intracellular domain (Blaumueller et al., 1997; Pan and Rubin, 1997; Logeat et al., 1998), NΔCtermTM1(r7) could very well be the intracellular domain of heterodimeric NΔCterm receptor. In all experiments with S2 cells, the N extracellular domain (Nextra) detected by our antibodies (i.e., the ~250–300-kD fragment) did not enrich in response to Dl although its level relative to NFull increased (data not shown).

NΔCterm Promotes Expression of daughterless in Response to Dl

The staining pattern shown in Fig. 2 indicates that NΔCterm is involved in development of commissures and connectives of the CNS. This raised the possibility that NΔCterm might function as a receptor for Dl. We examined this possibility in cultured cells. S2-N cells express NFull, whereas S2-N60g11, S2-N1–2155, and S2-N1–2262 express NΔCterm-like receptors (see Fig. 3 c). All N molecules have the complete extracellular domain and form aggregates with S2-Dl cells indicating that they bind Dl (data not shown; see R. Rebay et al., 1991). We treated S2-N, S2-N60g11, S2-N1–2155, and S2-N1–2262 with S2-Dl cells and assayed RNA extracted from these cells for expression of numerous genes known to interact genetically with Notch and Delta. The RNA s of Achaete Scute Complex, Enhancer of Split Complex, and wingless were not detected in our experiments. RNA of many other genes were not responsive to Dl treatment. However, the expression of the daughterless (da) gene was responsive to NΔCterm-like receptors. Expression of both NFull and NΔCterm-like receptors in S2 cells suppressed da expression (Fig. 6 a, lanes 1–3, 8, and 10). This indicated that the presence or absence of sequence carboxy-terminal of the CDC10/Ankyrin repeats per se does not affect da expression. Treatment of NΔCterm-like receptors with Dl promoted accumulation of da RNA, while treatment of NFull did not (Fig. 6 a, lanes 2–5 and 8–11). A more comparison of NFull and NΔCterm-like receptor, N60g11, treated with Dl is shown in Fig. 6 a,
Nintra1790 (Fig. 6 c). N molecules express of
N60g11 and N1–2155 declines rapidly. The results show that
acids). The cells were treated for only 1 h as the expression
with S2-Dl cells. These two N molecules are indistinguish-
the difference between the carboxyl termini of N1–2155
and N 1–2262. This indicates that N
termintra does not contain the transmembrane domain.
E(spl)Cterm-like receptors, Western blot analysis was per-
and N1893–2155 should overproduce E(spl)C R.N.A. Probing
of the same blots with m5 and m8 genes of E(spl)C shows
that these genes are overexpressed in both (Fig. 6 e). A s
expected, m5 and m8 genes of E(spl)C are also overex-
present in N60g11 embryos (Fig. 6 d).

The differential response of da and the E(spl)C might be
due to expression of N60g11 and N1893–2155 in both neu-
ronal and epidermal precursor cells, and expression of
N intra1790 only in epidermal precursor cells (see Fig. 2).
O nly the neuronal precursor cells increase da expression
during embryogenesis (Vassan et al., 1994). However,
the differential expression could be also due to
Cterm promoting da expression and not the full-length N (as in S2
cells). A citation of E(spl)C by N Cterm may have come
about through the proneural genes rather than through
lateral inhibition signaling (see Discussion). Thus, it is pos-
sible that genes like da are responsive to signals
from N Cterm, not from N Full, and genes like m5 and m8 of
E(spl)C are responsive to signals from both receptors.

nd3 Embryos Overproduce N Cterm and
Related Molecules

nd3 is a temperature-sensitive, homozygous viable allele of
N (Shellenbarger and Mohler, 1975) with an amino acid
replacing point mutation in the EGF-like repeat 2 (L yman
and Yong, 1993). In a screen of Notch mutants, we dis-
covered that nd3 embryos reared at 25°C accumulate
higher levels of a form of N that is recognized by α NT
and α NI but not by α NPRC, which migrates close to the
full-length form (Fig. 7 a). 4% SDS-PAGE gels were used here
as N that are recognized by α NPRC (N Full and N350.2),
migrate together in these gels and the levels of N Cterm
can be unambiguously determined. Embryos heterozygous
or hemizygous for the null allele, N264–47, the homozygous
viable allele, split, and several A bruptalus alleles of Notch
showed no alteration in levels of N Cterm (data not
shown). The overexpressed form in nd3 embryos (25°C) is
N Cterm because: (a) there is no other N molecule in D.
melanogaster that migrates close to the full-length form
and is recognized by α NT and α NI, but not by α NPRC
(Wesley, C .S., unpublished data); (b) it is recognized by
α NT made against the first two EGF-like repeats indicating
that the amino terminus is intact in this form (Fig. 7 a); and
(c) α NPRC failed to immunoprecipitate a form of N
migrating alongside N Cterm from nd3 embryos (25°C)
(expected if the faster mobility is due to a truncation in the
amino terminus rather than in the carboxyl terminus, data
not shown).

A higher percentage SDS-PAGE analysis of extracts
prepared from 25°C reared embryos revealed that ~55-
and ~40-kD N intracellular fragments, having the same
SDS-PAGE migration properties as N Cterm TMintra and
N Cterm intra from cultured cells, are also overexpressed in
nd3 (25°C) embryos (Fig. 7 b). Overexpression of these
lates production of these truncated molecules. Of no less significance, nd^{3} allele provided us with a means to identify the putative in vivo NΔCterm^{TM}_{intra} and NΔCterm^{intra} molecules from among the many minor N molecules generally detected in a N Western blot.

**Discussion**

Our analysis of N molecules in embryos and S2 cells show the following: (a) whereas the cells undergoing lateral inhibition in the developing embryo are enriched for N molecules recognized by both the amino and carboxyl terminus antibodies, the cells and tissues produced subsequent to lateral inhibition are enriched for N molecules not recognized by the carboxyl terminus antibody (Fig. 2). (b) Correspondingly, D1 forms complexes with the full-length N during lateral inhibition period, and with the N molecule lacking the carboxyl terminus in the period after lateral inhibition (Fig. 4). (c) N molecules lacking the carboxyl terminus (NΔCterm, NΔCterm^{TM}_{intra}, and NΔCterm^{intra}) are produced during embryogenesis (Figs. 3 and 5–7). (d) S2 cells expressing N receptors containing the carboxyl terminus (NFull) treated with S2-D1 cells accumulate an intracellular N molecule lacking the carboxyl terminus, NΔCterm^{TM}_{intra} (Fig. 5). (e) NΔCterm is the most likely substrate for production of NΔCterm^{TM}_{intra} (Figs. 5 and 7). (f) NΔCterm functions as a receptor for D1, with the NΔCterm^{intra} (comprised mostly of the CDC10/Ankyrin repeats) as its activated signaling molecule, and the da gene is responsive to its signals (Fig. 6).

Based on the results summarized above, we propose the following hypothetical model for N functions during embryogenesis. Lateral inhibition starts with NFull receptor containing the full signaling potential. The back and forth lateral inhibition signaling between interacting cells leads to carboxyl terminus processing of the full-length N molecules present inside the cells (i.e., those not involved in D1 binding) and production of the NΔCterm receptors. Cells expressing higher levels of NΔCterm become the neuronal precursor cells and cells expressing higher levels of NFull become the epidermal precursor cells. NFull disappears in neuronal precursor cells and NΔCterm, a secondary receptor with restricted signaling potential, functions during differentiation of the nervous system. Epidermal precursor cells expressing only NFull, or appreciable levels of both NFull and NΔCterm, continue the same process during differentiation of the epidermis. A advance from signaling by NFull to signaling by NΔCterm would mean that those cells have attained a degree of irreversibility in their differentiation process. For example, once NΔCterm becomes the sole N receptor in the neuronal precursor cells, these cells can only proceed along the neuronal differentiation path. N would continuously function in this manner to both specify and restrict cell fates during differentiation of a cell lineage.

NΔCterm would lack the Dishevelled-binding region, one of the Numb-binding regions, the OPA sequence, and the PEST sequence (see Fig. 1 a). Therefore, it is likely that loss of one or more of these features is involved in restricting the differentiation possibilities for a cell. Dishevelled and Numb are known to antagonize Su(H) activities (Axelrod et al., 1996; Frise et al., 1996; Guo et al., 1996; Spana and Doe, 1996; Wang et al., 1997). Proteolytic re-
moval of their binding sites is likely to eliminate antagonisms to Su(H) activities and promote activities of facilitators like Delta. This might contribute to the lateral inhibition process and selection of precursor cells for neuronal fates. On the other hand, production of NΔCterm^intra lacking the Su(H)-binding sites from NΔCterm receptor might promote neuronal fates by promoting activities of Hairless or Numb or A chaete (through Daughterless; Schweisguth and Posakony, 1994; Schweisguth, 1995; Frise et al., 1996; Guo et al., 1996; Spana and Doe, 1996). It is also possible that Disabled, which functions with N during differentiation of the CNS after lateral inhibition, can signal from NΔCterm and not NFfull. Thus, production and functions of NFfull and NΔCterm might provide directionality to N functions at successive stages of differentiation. All these properties of NFfull, NΔCterm, and the proteins interacting or not interacting with these two receptors, may be involved during differentiation of the adult sensory organ (bristle) wherein Su(H) activity is required for determination of some fates and not others (Schweisguth and Posakony, 1994; Schweisguth, 1995; Wang et al., 1997).

We have no evidence, one way or the other, about involvement of Su(H) in transducing signals from NΔCterm. Regulation of expression of E(spl)C genes by NΔCterm seems to indicate that the canonical Su(H)-mediated lateral inhibition pathway is involved. However, E(spl)C genes expression could be regulated by an alternate pathway. NΔCterm regulates da, not NFfull. Daughterless protein, is an activator of proneural proteins (Dambly-Chaudiere et al., 1988; Murre et al., 1989; Cabrera and Alonso, 1991; van Doren et al., 1991) and proneural genes also activate expression of E(spl)C (Kramatschek and Campos-Ortega, 1994; Singson et al., 1994). Some differences in the activities of the intracellular domains of NFfull and NΔCterm seem very likely. One, the R A M 23 region in the intracellular domain of N (see Fig. 1a) is important for Su(H) activities related to NFfull, N^intra and lateral inhibition (Fortini and Artavanis-Tsakonas, 1994; Tamura et al., 1995; Matsuno et al., 1997). It appears that NΔCterm^intra lacks most of this region, if not all. Two, the sequence carboxy-terminal of the CDC10A^nykyn repeats is required for transcriptional activation upon binding DNA (Kidd et al., 1998). Since NΔCterm lacks this sequence, it might activate genes indirectly through inactivation of a constitutive repressor or stabilization of RNA. NFfull containing the carboxy terminus would activate genes directly from DNA. Thus, it is possible that NFfull and NΔCterm might signal through different pathways with some shared outcomes at certain stages of development, like expression of E(spl)C genes. Su(H) might be functioning with both pathways, albeit in different ways.

Production of N receptors with restricted signaling potential may be important for another reason. NFfull binds different ligands and regulates different genes in response to them (see Artavanis-Tsakonas et al., 1999; Wesley, 1999). Removal of the carboxy terminus after initiation of NFfull signaling by one ligand might set the cell on a differentiation path specific to that ligand. For example, removal of the carboxy terminus in neuronal precursor cells after Delta-specific lateral inhibition signaling might make NΔCterm in these cells either unresponsive to Wingless functioning in the epidermis differentiation pathway, or responsive to Wingless in the manner specific to neuronal differentiation pathway. Treatment of full-length N with Wingless results in accumulation of a N molecule lacking the DI-binding region (Wesley, 1999). This secondary N receptor may be produced during epidermogenesis to eliminate the antagonism to Wingless functions presented by the DI-binding site. Non-response or pathway-specific response to a second ligand may be necessary for development given the broad overlap in distributions of different N ligands. Thus, expression of a particular secondary N receptor might indicate both the differentiation path taken by a cell and the degree to which this cell has differentiated from cells in the parent population.

The molecular phenotypes of nd^3 allele suggest that EGF-like repeat 2 might be an important component in the regulation of NΔCterm production during embryogenesis. It seems possible that the EGF-like repeat array of N might include two classes of repeats, one containing repeats that bind ligands outside the cells and the other containing repeats that target Notch for different kinds of processing inside the cell. Such a function for EGF-like repeats might explain why N^intra do not produce NΔCterm^intra. These molecules might lack the appropriate EGF-like repeats to target them to the right place for carboxyl terminus processing. An interesting extension of this possibility is that there are different targeting EGF-like repeats responsive to different ligands.

The regulation of da expression by NΔCterm may be significant for embryogenesis. da genetically interacts with Notch (Brand and Campos-Ortega, 1988, 1990), it is required for development of the nervous system from neuroblasts but not for lateral inhibition (Caudy et al., 1988a,b; Vaessin et al., 1994), and the Daughterless protein promotes DNA-binding activities of the proneural A chaete-Scute Complex proteins (Dambly-Chaudiere et al., 1988; Murre et al., 1989; Cabrera and Alonso, 1991; van Doren et al., 1991). Both NΔCterm and Daughterless protein (Vaessin et al., 1994) accumulate in segregating neuroblasts raising the possibility that NΔCterm is involved in this upregulation of da expression. Accordingly, nd^3 embryos which overproduce NΔCterm also overproduce da RNA in the neuroblasts (data not shown).

In the embryo, da is expressed at low levels in almost all cells (Murre et al., 1989; CSW, personal observation) but is upregulated in certain cells including the segregating neuroblasts (Vaessin et al., 1994). In our experiments, S2 cells expressing NFfull and NΔCterm receptors had lower levels of da RNA than S2 cells without N. In response to DI, only S2-NΔCterm cells increased expression of da RNA, but only to the level observed in cells without N (Fig. 6a). Therefore, it appears possible that with the expression of different forms of N, developing cells acquire an ability to differentially regulate the otherwise constitutive da expression. Such differential regulation might be important for suppressing the activities of A chaete-Scute Complex proteins in the developing epidermis where NFfull is expected to function, but not in the developing nervous system where NΔCterm is expected to function. Since both N receptors have the ability to activate E(spl)C, the timing and sequence of expression of NFfull and NΔCterm may also be important for development.
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