Ligand-induced cleavage and regulation of nuclear entry of Notch in Drosophila melanogaster embryos

Simon Kidd,1 Toby Lieber,1 and Michael W. Young2

Laboratory of Genetics, The Rockefeller University, New York, New York 10021-6399 USA

Notch, a transmembrane protein found in a wide range of organisms, is a component of a pathway that mediates cell-fate decisions that involve intercellular communication. In this paper, we show that in Drosophila melanogaster, Notch (N) is processed in a ligand-dependent fashion to generate phosphorylated, soluble intracellular derivatives. Suppressor of Hairless [Su(H)] is predominantly associated with soluble intracellular N. It has been demonstrated by others that N has access to the nucleus, and we show that when tethered directly to DNA, the cytoplasmic domain of N can activate transcription. Conversely, a viral activator fused to Su(H) can substitute for at least some N functions during embryogenesis. We suggest that one function of soluble forms of N is to bind to Su(H), and in the nucleus, to act directly as a transcriptional transactivator of the latter protein. Although N has functional nuclear localization signals, the N/Su(H) complex accumulates in the cytoplasm and on membranes suggesting that its nuclear entry is regulated. Localization studies in cultured cells and embryos suggest that Su(H) plays a role in this regulation, with the relative levels of Delta, N and Su(H) determining whether a N/Su(H) complex enters the nucleus.

[Key Words: Notch; processing; Delta; Suppressor of Hairless; nuclear entry; transcriptional transactivator]

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Notch (N) is a 2703-amino-acid transmembrane protein that is found in a wide range of organisms from Caenorhabditis elegans to humans. The N gene was originally identified in Drosophila melanogaster in which N is a component of a pathway involved in cell-fate decisions that involve intercellular communication. During Drosophila embryogenesis, this process, which is known as lateral inhibition, operates in all three germ layers and ensures that only one cell of several equipotential cells, termed an equivalence group, will adopt the primary cell fate. Because this pathway was first characterized in the neuroectoderm, in which the absence of any member of the pathway results in overproliferation of neuroblasts, the genes involved in lateral inhibition are known as neurogenic genes. N, and other members of the neurogenic gene family are also conserved during evolution (for review, see Artavanis-Tsakonas et al. 1995; Greenwald 1998).

Genetic mosaic analysis has demonstrated that N is cell autonomous, suggesting that it is a receptor (Hoppe 1986; Heitzler and Simpson 1991). Delta (Dl), also a transmembrane protein, has been identified as the ligand for N in its role in lateral inhibition (for review, see Artavanis-Tsakonas et al. 1995; Nye and Kopan 1995). Activation of N results in transcription of genes of the Enhancer of split (E(spl)) complex. This transcriptional activation is mediated by Suppressor of Hairless (Su(H)), a DNA binding protein that has been demonstrated to bind to the cytoplasmic domain of N (Fortini and Artavanis-Tsakonas 1994; Jennings et al. 1994; Jarriault et al. 1995; Lecourtois and Schweisguth 1995; Tamura et al. 1995; Kato et al. 1997; Schroeter et al. 1998).

Previous work has shown that expression of N proteins deleted for the extracellular domain results in gain of function phenotypes indicative of ligand-independent activation (for review, see Artavanis-Tsakonas et al. 1995; Greenwald 1998). The cytoplasmic domain of N contains functional nuclear localization signals (Stifani et al. 1992; Lieber et al. 1993), and it has been proposed that on binding DI, the transmembrane N protein is cleaved releasing the cytoplasmic domain that translocates to the nucleus, where it is tethered to DNA via Su(H) and behaves as a transcriptional transactivator (Lieber et al. 1993; Struhl et al. 1993; Jarriault et al. 1995; Kopan et al. 1996). In tissue culture, Schroeter et al. (1998) have mapped a site in the transmembrane domain of mouse Notch-1 at which it undergoes ligand-dependent proteolytic cleavage. Mutating this site reduces the activity of Notch-1 in cell culture. In addition, it has been found that in vivo the cytoplasmic domain of Drosophila N has access to the nucleus, although the biochemical nature of this access was not determined (Lecourtois and Schweisguth 1998; Struhl and Adachi 1998).

1These authors contributed equally to this work.
2Corresponding author.
E-MAIL young@rockvax.rockefeller.edu; FAX 212-327-8695.
In this paper, we present data indicating that in wild-type Drosophila, N is processed in a ligand-dependent manner to generate a cytoplasmic domain that, on the basis of size and solubility, does not span the membrane. This domain is phosphorylated and Su(H) preferentially associates with this form. The N/Su(H) complex is found associated with membranes and predominantly in the cytoplasm, indicating that there is a mechanism for regulating its subcellular location. Our data suggest that Su(H) can inhibit nuclear entry of the soluble N proteins, and that nuclear entry occurs in a fashion dependent on the relative abundance of soluble N and Su(H). In prior genetic studies of N nuclear activity, it was suggested that nuclear N participates in the transcriptional regulation of downstream target genes (Lecourtois and Schweisguth 1998; Struhl and Adachi 1998). We show that in the nucleus, N behaves as a transcriptional trans-activator, and that a heterologous activator fused to Su(H) can substitute for activated N function in embryos.

Results

Su(H) interacts with phosphorylated Notch proteins

N and Su(H) proteins have been shown to physically interact (Fortini and Artavanis-Tsakonas 1994; Jarriault et al. 1995; Tamura et al. 1995; Kato et al. 1997; Schroeter et al. 1998). To characterize the nature of the associated N and Su(H) proteins in vivo, immunoprecipitations with antibodies against either N or Su(H) were performed on detergent extracts of Drosophila embryos. Following electrophoresis, N proteins in these immunoprecipitates were detected by Western blot. When anti-N and anti-Su(H) immunoprecipitations are probed with anti-N antibodies, only a small subset of the proteins immunoprecipitated by anti-N antibodies are found in the corresponding Su(H) immunoprecipitate [cf. Fig. 2A, below, lanes 1 and 2, anti-Su(H) immunoprecipitation, with Fig. 2C, lanes 1 and 2, anti-N immunoprecipitation]. This suggests that the interactions between N and Su(H) detected in this assay occurred in vivo and not during the course of the immunoprecipitation, as the latter might be expected to result in similar array of N proteins in both immunoprecipitates.

Anti-Su(H) immunoprecipitates contain two major size classes of N proteins, both of which are recognized by antibodies raised against 2 different regions of the intracellular domain of N (see Fig. 1 for antibodies used in this work); one the size of full length N and another, substantially enriched, which migrates as smear of proteins of ~114 kD (Fig. 2A, lane 1). The existence of a smear of bands at 114 kD suggests that these proteins are N. As no alternatively spliced N transcripts have been observed, and no appropriately positioned methionine exists at which internal translation could initiate to give rise to a protein with the size and antigenic determinants of Npp114 (Wharton et al. 1985; Kidd et al. 1986; Kopan et al. 1996), it is most likely that these smaller N proteins are the result of proteolytic cleavage.

The proteins used for the previous experiment were from an overnight collection of embryos. We wondered if production of the various N proteins associated with Su(H) is developmentally regulated. Figure 2B shows that whereas Npp114 is present throughout embryogenesis with Np100B as its major component, the amount of Np100A appears to increase with age, and Np100C is only found late in embryogenesis. In addition, embryos that are young (0–4 hr) contain significantly more processed N protein that comigrates with hypophosphorylated Np100A (indicated by an asterisk) relative to Npp114. Late in embryogenesis, proteins that comigrate with both hypophosphorylated Np100B and Np100C are found.
Figure 2. Su(H) is associated with phosphorylated N proteins. (A,B) Western blot analysis of anti-Su(H) immunoprecipitates from Drosophila embryos probed with anti-N (NPCR) antibody. For A, an overnight collection of embryos was used and in B, staged embryos (age indicated in hours at the top of lanes; H denotes hatching) were used for detergent extracts. Prior to electrophoresis, some of the samples were treated with alkaline phosphatase with or without inhibitor. (+) Presence of phosphatase and/or inhibitor. (A, Lane 4 and B, lane 9) In vitro-translated N^intra1768, the size of which is not affected by phosphatase treatment; (A, lane 5) Antibody but no extract; (A, lane 6) Embryo extract recovered on protein A-Sepharose beads that were not conjugated to antibody. The locations of N (N), N^intra1768, N^pp114, N^p100A, N^p100B, and N^p100C proteins are shown along the side of each blot, and also by dots on the blot. The x in A indicates a cross-reacting protein nonspecifically bound to the protein A-Sepharose beads; the asterisk (*) in B indicates a hypophosphorylated N protein that comigrates with N^p100B, (Lane 3–6) 500 µg of protein was used for each immunoprecipitation; the remaining lanes in B contain 2.5 mg (lanes 1, 2) and 1.75 mg (lanes 7, 8). (C) Western blot analysis of anti-N (NII) immunoprecipitates from Drosophila embryos probed with anti-N (NPCR) antibody. (Lanes 1, 2) Immunoprecipitates from yw embryos; (lanes 3, 4) Immunoprecipitates of embryos resulting from a cross of Su(H) hs^N^intra1790/CyO males to Su(H)^hs^FRT40A/Cyo^virgins; (lanes 5, 6) Immunoprecipitates of embryos resulting from a cross of Su(H) hs^N^intra1790/Cyo^virgins to hs^flp12/yw; Su(H)^FRT40A/ovo^FRT40A^virgins. All N^intra expressing embryos are both zygotically and maternally Su(H) null. For all genotypes, 3–5 hr-old embryos were subjected to a 30 min heat shock at 37°C and allowed to recover for 15–30 min prior to collection. (+) Phosphatase treatment. The locations of N (N) and N^intra are indicated next to the blot.

The above experiment indicates that phosphorylated, processed N proteins interact with Su(H). To address whether phosphorylation is a consequence of Su(H) binding, we asked whether N^intra1790 (Fig. 1) is phosphorylated in embryos that lack Su(H). As can be seen in Figure 2C, lanes 5 and 6, N^intra1790 expressed in embryos that are both maternally and zygotically Su(H)^− is phosphorylated, indicating that phosphorylation of processed N proteins is not dependent on Su(H) binding. Interestingly, even after phosphatase treatment, N^intra1790 immunoprecipitated from Su(H)^− embryos migrates slower than N^intra1790 immunoprecipitated from embryos that contain Su(H) (cf. lanes 4 and 6). This might be because N^intra1790 bound to Su(H) has undergone additional proteolytic processing, or N^intra1790 not bound to Su(H) has been subject to additional post-translational modification. However, in the Su(H)^− extracts there is also a smear extending upward from endogenous N. This suggests that in Su(H)^− embryos, N^intra1790, as well as being phosphorylated, has undergone additional post-translational modification.

We have also analyzed Su(H) coimmunoprecipitated with anti-N antibodies (data not shown). The Su(H) associated with N comigrates with the Su(H) immunoprecipitated by anti-Su(H) antibodies. In both immunoprecipitates, there is no change in mobility on phosphatase treatment, suggesting that Su(H) is not extensively phosphorylated.

Cleavage of N is ligand dependent

If N signaling is dependent on ligand-induced cleavage of N, then N^pp114 might result from the binding of a N ligand. The two experiments shown in Figure 3 demonstrate that the presence of ligand and the ability of N to bind ligand is required for the presence of N^pp114. All the known N ligands bind to the extracellular EGF-like repeats, deletion of which results in a nonfunctional protein (Rebay et al. 1991, 1993; Lieber et al. 1993). Extracts from embryos expressing a form of N that spans the membrane but lacks the EGF-like repeats and is tagged at the Carboxyl terminus with the DNA-binding domain of LexA [N^EGF1-36-LexA (Fig. 1)] were immunoprecipitated with anti-N and anti-Su(H) antibodies, treated with phosphatase, and the Western blot probed with anti-LexA antibody. As can be seen in Figure 3A, lane 4, the anti-Su(H) immunoprecipitates from N^EGF1-36-LexA embryos do not contain N^p100-LexA. Expression of N^EGF1-36-LexA will not rescue the neurogenic phenotype of a zygotically N^− embryo (data not shown). As a control for this experiment, immunoprecipitations from embryos expressing LexA-tagged N (N^L-loxP) were carried out. In these experiments, a LexA-tagged N^p100 protein is seen (Fig. 3A, lane 3, N^p100-LexA, indicated by an asterisk, presumably the hypophosphorylated form of N^pp114-LexA). Expression of N^L-loxP will rescue the neurogenic phenotype of zygotically N^− embryos (data not shown).
We then examined the effect of lowering the level of ligand by using temperature-sensitive mutants of the Notch ligand Delta. $\text{Dl}^{60}/\text{TM6}$ (a strong temperature-sensitive DI allele) and $\text{Dl}^{RF}/\text{TM6}$ (a weak temperature-sensitive DI allele) males were mated to $\text{Dl}^{y}/\text{TM6}$ (an amorphic DI allele) females. All the embryos resulting from the above crosses were incubated at either room temperature (lanes 1,3,5) or at the nonpermissive temperature, 29°C (lanes 2,4,6). N proteins coimmunoprecipitated from detergent extracts by anti-Su(H) antibody were detected with the NPCR anti-N antibody. Beneath each lane the ratio of processed N to N ($\text{N}_{\text{pp}114}/\text{N}$) is shown. The strong temperature-sensitive DI allele (lanes 3,4) has a considerably greater effect on the level of processed $\text{N}$ than the weaker allele (lanes 5,6)

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duction of a hypophosphorylated N protein the size of N\textsuperscript{p100B} fused to LexA that is immunoprecipitated with anti-N antibody. Lane 7 lacks embryo extract; lane 8 contains N\textsubscript{p100B} in vitro translation products. After immunoprecipitation, lanes marked with a + were treated with alkaline phosphatase prior to electrophoresis. N proteins were detected with the anti-N antibody NPCR. (Labels) Locations of N (N) and N\textsubscript{p100B} (N\textsubscript{p100B}).

Intracellular location of the Su(H) bound N proteins

The three dephosphorylated components of N\textsuperscript{pp114} are small enough to be soluble. To see if this was the case, immunoprecipitations were carried out on subcellular fractions of Drosophila embryos. Equal fractions of each subcellular fraction were immunoprecipitated to allow the relative abundance of the proteins in each fraction to be determined. When the fractionation is carried out under hypotonic conditions (10 mM KCl), the majority of N proteins, full-length as well as N\textsuperscript{pp114}, immunoprecipitated by both N and Su(H) antibodies are in the membrane fraction (Fig. 5A, lanes 1, 2, 7, 8). Some N\textsuperscript{pp114} is found in the soluble fraction (Fig. 5A, lanes 3, 4, 9, 10) and little or no N is detectable in the nuclear fraction (Fig. 5A, lanes 5, 6, 11, 12). In addition to N\textsuperscript{pp114}, the anti-N immunoprecipitate of the soluble fraction is also enriched for two proteins of ~99 kDa and 86 kDa, which are superimposed over an 86-114 kDa smear (Fig. 5A, lane 3 and B, lane 1). Phosphatase treatment reduces the smear to the hypophosphorylated components of N\textsuperscript{pp114} and a protein of 86 kDa (termed N\textsuperscript{p86}) (Fig. 5A, lane 4 and B, lane 2) suggesting that N\textsuperscript{pp99} is a phosphorylated form of N\textsuperscript{p86}. Despite being soluble, N\textsuperscript{pp99} was not found associated with Su(H) (Fig. 5A, cf. lanes 3 and 4 with lanes 9 and 10).

In contrast, when subcellular fractionations are carried out under physiological salt conditions (100 mM KCl) the majority of N\textsuperscript{pp114} associated with Su(H) (cf. lanes 1 and 3). In this experiment, there was an ~40%–50% increase in the amount of N\textsuperscript{pp114} bound to Su(H) (cf. the ratios of N\textsuperscript{pp114} to N\textsuperscript{N} in lane 5 with lane 7, and in lane 6 with lane 8). A histogram comparing the relative amounts of processed vs. full-length N associated with Su(H) in the absence or presence of ectopic Dl. The average of the results of four immunoprecipitations are plotted. The fold increase in the amount of processed N compared with full-length N in the presence of UAS Dl for each of the four experiments was as follows: 1.7, 1.5, 2.0, and 1.8.

Su(H) is capable of retaining the cytoplasmic domain of N in the cytoplasm

The biochemical data presented above suggest that some processed N complexed with Su(H) protein is still associated with membranes even though, on the basis of size, it probably lacks a transmembrane domain. In addition, there are larger amounts of N, principally N\textsuperscript{pp114}, in the soluble fraction. This is surprising, as we and others have...
shown previously that the intracellular domain of N has functional nuclear localization signals and can localize to nuclei (Stifani et al. 1992; Lieber et al. 1993).

Given our fractionation studies of N^{pp114}, subcellular localizations of N^{Intra} were further examined. Whereas in S2 cells N^{Intra,768} (Fig. 1) is totally nuclear (Fig. 6A), in embryos, a substantial fraction of N^{Intra,768} is retained in the cytoplasm. This is illustrated in Figure 6B by use of an anti-Flag-antibody to recognize Flag-tagged N^{Intra,768}. Using an anti-N antibody, we found that it is in cells in which N^{Intra,768} is expressed at higher levels that it is found in nuclei. In cells in which N^{Intra,768} is expressed at lower levels, it is primarily cytoplasmic (Fig. 6C). This suggests that there is something in embryos that is retaining N^{Intra,768} in the cytoplasm, and that this retention mechanism can be saturated by high levels of N^{Intra,768}. It has been shown that the cdc10 repeats of N are a phosphorylated form of N^{pp99}. (C) Some N^{pp114} is associated, but not stably, with membranes. Postnuclear supernatants were incubated with increasing amounts of KCl prior to fractionation by centrifugation into membrane bound (lanes marked M) or soluble proteins (lanes marked S). Fractionated proteins were then immunoprecipitated with anti-Su(H) antibody and detected with the anti-N antibody. The autoradiograph has been overexposed to show the presence of processed N in the soluble fraction. Because the amount of N in the membrane fraction is so high, lanes 1 and 2 are from a shorter exposure. (B) Evidence for additional processing of the cytoplasmic domain of N. Soluble proteins extracted in 0.4 M KCl were immunoprecipitated with anti-N1 antibody and detected with anti-NPCR antibody. As well as immunoprecipitating the same N proteins as those immunoprecipitated by anti-Su(H) antibodies, anti-N antibodies immunoprecipitate two novel proteins of ~99 kD (N^{pp99}) and 86 kD (N^{pp86}) (lane 1), treatment with alkaline phosphatase (lane 2) reduces the amount of N^{pp99} and increases the amount of N^{pp86}, suggesting that N^{pp99} is a phosphorylated form of N^{pp86}. (D) Some N^{pp114} is associated, but not stably, with membranes. Postnuclear supernatants were incubated with increasing amounts of KCl prior to fractionation by centrifugation into membrane bound (lanes marked M) or soluble proteins (lanes marked S). Fractionated proteins were then immunoprecipitated with anti-Su(H) antibody and detected with the anti-N antibody. A longer exposure of the relevant region of the resulting autoradiograph is shown at bottom. Some N^{pp114} can be seen to be associated with membranes when extracted at 0.1 M KCl but is removed at higher salt concentrations. The protein that comigrates with N^{pp100B} and appears to be stably associated with membranes is marked by an asterisk.

The cytoplasmic domain of N behaves as an activator when bound to DNA

The data we have presented above indicate that N is processed and associates with Su(H), and the entry of this complex into the nucleus appears to be dependent on the relative levels of processed N and Su(H). During the course of yeast two-hybrid experiments, it was found that the cytoplasmic domain of N was a strong activator. Figure 8A shows a comparison in yeast of the activating ability of the N cytoplasmic domain with that of the well-characterized transcriptional activator GAL4. It can be seen that the cytoplasmic domain of N has almost as much activator activity (85%) as GAL4. Smaller derivatives of the N cytoplasmic domain activate to a lesser degree. Thus, in a heterologous system, the cytoplasmic domain of N strongly activates transcription from a homologous promoter. We tested this in two ways. First, we fused the DNA-binding domain of the bacterial repressor LexA to the cytoplasmic domain of N. In S2 cells, this N^{LexA} fusion protein (N^{Intra-LexA}; Fig. 1), but not N^{Intra,790}, activates transcription from a LexA reporter (data not shown). In Fig-
Figure 6. \(N^{\text{intra}}\) is retained in the cytoplasm in embryos. Confocal images showing the localization of \(N^{\text{intra}}\) in embryos and Drosophila S2 cells as detected by immunofluorescence. With the exception of the left panel in C, \(N^{\text{intra}}\) protein is represented in green and the nuclei in red. This panel is a pseudocolored representation to illustrate the relative amounts of \(N^{\text{intra}1768}\). The correspondence of the colors with the intensity of the signal is indicated by the pseudocolor bar, with the more intense signals being depicted by colors higher up the bar. (A) Heat shock-induced \(N^{\text{intra}1768}\) appears to be totally nuclear in S2 cells. UAS \(N^{\text{intra}1768}\) was cotransfected along with HS GAL4 and detected with a rabbit anti-N (N1) antibody. The nucleus was detected with SYTOX Green. (B, C) In embryos (hGAL4; UAS \(N^{\text{intra}1768}\)) there is retention of \(N^{\text{intra}1768}\) in the cytoplasm. (B, C, right) The merged images of the N signal in green and the nuclei in red. (C, left) \(N^{\text{intra}1768}\) is primarily nuclear in those cells in which it is most highly expressed. Mouse anti-NPCR antibody was used to detect \(N^{\text{intra}1768}\). (B) Mouse anti-Flag antibody was used to detect Flag-tagged \(N^{\text{intra}1768}\). The nuclei were detected by propidium iodide. (D) \(N^{\text{intra}1768}\) is still retained in the cytoplasm in embryos that are maternally and zygotically N null (N264-47 FRT/ovoD FRT; hGAL4/hslp X FM7/Y; \(N^{\text{intra}1768}\). (D, left) Mouse NPCR antibody was used to detect \(N^{\text{intra}1768}\). (D, right) a merged image of N (green) and nuclei (red). (E) \(N^{\text{intra}1790}\) is predominantly nuclear in embryos with reduced levels of Su(H). Anti- myc antibody was used to detect myc-tagged N\(^{\text{intra}1790}\) in embryos that are maternally Su(H)~ (hsflp/yw;Su(H)268 FRT/ovo D FRT;hGAL4 X Su(H)/CyO;UASN\(^{\text{intra}1790}\)). In embryos that are maternally Su(H)~ (hGAL4 X Su(H)/CyO;UASN\(^{\text{intra}1790}\)) there is retention of \(N^{\text{intra}1790}\) in the cytoplasm (data not shown).

Discussion

Previous work has led to the model that on ligand binding, N is cleaved, and the cytoplasmic domain enters the nucleus where, in concert with Su(H), it activates transcription of genes such as m8, a member of the E(spl) complex (Lieber et al. 1993; Jarriault et al. 1995; Kopan et al. 1996; Lecortois and Schweisguth 1998; Schroeter et al. 1998; Struhl and Adachi 1998). In this study, it has been shown that (1) soluble cytoplasmic N proteins are produced in vivo in response to the N ligand, D1 (2) Su(H) is recovered in association with these soluble forms of N, and (3) intracellular forms of N appear to function as transcriptional activators in embryos when physically associated with Su(H).

We have used antibodies against Su(H) and N to examine the structure of the N proteins associated with Su(H). During most of Drosophila embryogenesis, two size classes of N proteins are coimmunoprecipitated by antibodies against Su(H). These include full-length N proteins and, to a greater extent, phosphoproteins of ~114 kD, N\(^{p114}\). Unlike mammalian systems in which N exists predominately as a heterodimer, during Drosophila embryogenesis, the bulk of N exists as the full-length form (Results; Kidd et al. 1989; Blauemueller et al. 1997). When dephosphorylated, N\(^{p114}\) resolves into three proteins, N\(^{p100A}\), N\(^{p100B}\), and N\(^{p100C}\) of ~100 kD. Through most of embryogenesis, the most abundant of these proteins is N\(^{p100B}\), N\(^{p100C}\) being found only late in development. The size difference between the two proteins might be because N\(^{p100C}\) has been cleaved further into...
the intracellular domain than Np100B, or the two proteins may both have the same amino termini, but Np100C might have been additionally cleaved at the carboxyl terminus. It is also possible that there is a precursor product relationship between the two. In any case, the occurrence of Np100C only late in embryogenesis suggests that production of these forms of N is under developmental control.

Throughout most of embryogenesis, the majority of processed N proteins that are associated with Su(H) show some level of phosphorylation. Full-length N has been shown previously to be phosphorylated on serines (Kidd et al. 1989). We do not know how the latter relates to the phosphorylation described here, although the presence of hypophosphorylated forms of N bound to Su(H) suggests that the two events are unrelated. How this phosphorylation is effected and how it influences N function is not known. There are two lines of evidence that suggest that phosphorylation is not an immediate consequence of ligand binding and cleavage. First, most if not all of NIntra1768 are expressed in nuclei and retain NIntra1768 in the cytoplasm at higher magnification in G. As cytoplasmic localization of NIntra1768 is never seen in cells lacking ectopic Su(H) (D), the upper cell in G must have received more Su(H) relative to NIntra1768 than the lower one, resulting in NIntra1768 being retained in the cytoplasm. NIntra1768 was detected with a rabbit anti-N (NI) antibody and the nuclei with SYTOX Green. (F) When low levels of NIntra1768 are expressed along with Su(H), NIntra1768 is retained in the cytoplasm. A pseudocolored confocal image showing the relative levels of N at right and Su(H) at left is portrayed. The intensity of staining is depicted by the pseudocolor bar with the colors representing the more intensely stained regions being higher up the bar. In the lower cell, NIntra1768 is expressed at relatively high levels and both NIntra1768 and Su(H) are found in the nucleus. In the upper cell, NIntra1768 is expressed at relatively low levels and both NIntra1768 and Su(H) are found in the cytoplasm. When cells are doubly stained for DNA and NIntra1768, the two stains converge only when levels of NIntra1768 are high compared with Su(H) (data not shown). Su(H) was detected with a rat anti-Su(H) antibody and NIntra1768 with a mouse anti-Flag antibody.
of N\textsuperscript{pp114} in the soluble fraction (Fig. 5), perhaps phosphorylation is related to the release of cleaved intracellular N from the membrane. Alternatively, phosphorylation may promote nuclear translocation or association with Su(H), or both.

There is some salt extractable N\textsuperscript{pp114} associated with Su(H) in the membrane fraction. Finding the intracellular domain of N, which contains functional nuclear localization signals either in the membrane or cytoplasmic fractions, indicates that the cell contains mechanisms to restrain the nuclear entry of N cleavage products. Because it has been demonstrated that the cdc10 repeats of N mediate homodimerization (Matsumo et al. 1995; Roehl et al. 1996), newly produced intracellular forms of N may be retained by full-length forms of N at the membrane. This association might be particularly favored if, as believed, the receptor is presented at the cell surface as a dimer (Foster 1975; Portin 1975; Kelley et al. 1987; de Celis and Garcia-Bellido 1994). It is also conceivable that N\textsuperscript{pp114} is retained on the membrane by a complex of Su(H) and full-length N.

Su(H) may regulate nuclear entry of N

With respect to cytoplasmic retention of Su(H)/N\textsuperscript{pp114} complexes, regulation may come from Su(H) itself. We have shown that whereas coexpressing high levels of N\textsuperscript{intra} along with Su(H) in S2 cells results in both proteins translocating to nuclei, when low levels of N\textsuperscript{intra} are coexpressed along with Su(H) in S2 cells, there is retention of N\textsuperscript{intra} in the cytoplasm. This suggests that excess Su(H) can promote cytoplasmic localization of soluble, intracellular forms of N. Given that there are multiple binding sites for Su(H) in the cytoplasmic domain of N (Kato et al. 1997; Wettstein et al. 1997; S. Kidd, unpubl.), differences in subcellular localization could reflect the number of Su(H) molecules bound to N, with changes in stoichiometry resulting from increased levels of intracellular N in response to ligand. Because in vivo levels of Su(H) appear to be in excess to soluble N product, as there is sufficient Su(H) to bind to ectopically expressed N\textsuperscript{intra} and generate gain of function phenotypes (Lieber et al. 1993; Rebay et al. 1993; Struhl et al. 1993), the cytoplasmic retention we observe in Su(H)+ embryos is expected from the S2 cell studies. Further supporting our view that Su(H) can retain soluble N in the cytoplasm in vivo, we found that lowering the dose of Su(H) promotes nuclear localization of N\textsuperscript{intra} in embryos (Fig. 6E). We also find that lowering the dose of Su(H) increases the severity of the phenotype produced by ectopic expression of gain-of-function N proteins in transgenic flies: Whereas complete loss of Su(H) abolishes the ability of the E(spl) complex to respond to activated N (Bailey and Posakony 1995), lowering the Su(H) dose by one-half increases the lethality as well as the bristle loss observed in transgenic flies carrying N\textsuperscript{ALN,rpt5} under control of a heat shock promoter (T. Lieber, unpubl.). A priori, one would have predicted that lowering the dose of Su(H) would decrease the severity of gain-of-function N mutations. Lastly, it is possible that the subcellular distribution of Su(H)/N\textsuperscript{pp114} complexes is regulated by interaction with additional factors. For example, it has been shown that numb, a membrane-associated protein that is asymmetrically localized during division of sensory organ precursor cells in the peripheral nervous sys-
tem, is able to retain N\textsuperscript{intra} at the membrane and in the cytoplasm of S2 cells (Friese et al. 1996).

In the absence of Su(H), both N\textsuperscript{intra} and N appear to have undergone additional modification. In addition, many intermediate sized N proteins are missing (Fig. 2C). Lecourtois and Schweisguth (1998) and Schroeter et al. (1998) have suggested that the processed form of N is less stable in the absence of Su(H). Many proteins are targeted to the proteosome by ubiquitinylation. Perhaps the modification of N\textsuperscript{intra} and N we see in the absence of Su(H) is ubiquitinylation. Interestingly, phosphorylation has also been shown to target proteins to the ubiquitinylation machinery (King et al. 1996).

In addition to being required for the production of N\textsuperscript{pp114}, the N ligand Dl, when overexpressed, promotes accumulation of a hypophosphorylated N protein that has approximately the same mobility as N\textsuperscript{p1008} (Fig. 4A). Our fractionation studies also showed the presence of a hypophosphorylated protein of approximately the same size as N\textsuperscript{p1008} in this case associated with Su(H). This protein is retained in the membrane fraction under salt conditions that remove N\textsuperscript{pp114} suggesting that it is tightly associated with the membrane and may well span it (Fig. 5C). The extracellular domain of N has been shown previously to be cleaved at several positions (Blaumueller and Artavanis-Tsakonas 1997; Pan and Rubin 1997; Logeat et al. 1998). It has been proposed that the cleavage closest to the membrane is ligand dependent (Logeat et al. 1998). Such a cleavage product may correspond to the protein we described above.

Soluble N as a transcriptional transactivator

The work of Lecourtois and Schweisguth (1997) and Struhl and Adachi (1998) has shown genetically that the cytoplasmic domain of N has access to the nucleus. The most likely explanation for their results is that Drosophila N is proteolytically cleaved at the site described by Schroeter et al. (1998) to produce the fragment of N, N\textsuperscript{pp114}, that we have described in this paper. We have shown that when tethered directly to DNA via a bacterial DNA-binding domain, the cytoplasmic domain of N can activate transcription both in yeast and in vivo. Conversely, a viral activator fused to Su(H) can substitute for the functions of N mediated by its ability to activate transcription of m8, a natural target of N signaling, in embryos. Whereas maximal activation in yeast is seen with the entire cytoplasmic domain, in agreement with the results of Roehl et al. (1996) a truncated form of the cytoplasmic domain (N\textsuperscript{3792–2156}, Fig. 1) encompassing the cdc10 repeats does weakly activate and has a gain-of-function phenotype in embryos (T. Lieber, unpubl.). Smaller versions of the cytoplasmic domain (N\textsuperscript{1895–2156}) spanning just the cdc10 repeats are even weaker activators and when expressed in wild-type embryos do not have a gain-of-function phenotype (T. Lieber, unpubl.). Our data suggest that the prime function of the sequences downstream of the cdc10 repeats is to provide transactivator activity. In accord with this, the cytoplasmic domain of N has many features that are found in transcriptional activators (Lieber et al. 1993). Although it is possible that N indirectly confers activating ability on Su(H), given the finding of appropriately processed N proteins, which contain functional nuclear localization signals preferentially associated with Su(H), the simplest interpretation of our results is that one function of N is to bind to Su(H) and in the nucleus to directly act as its transcriptional transactivator. Recently it has been suggested that N activates transcription by disrupting the formation of a repressor complex between Su(H) and a histone deacetylase complex (SMRT/HDAC-1) (Kao et al. 1998). Our data suggest that rather than simply disrupting the Su(H)/SMRT/HDAC-1 complex, N\textsuperscript{pp114} plays a more active role of providing transactivator activity to Su(H).

One other class of membrane-bound transcription factors has been identified previously. The proteolysis of sterol regulatory element binding proteins (SREBPs) (for review, see Brown and Goldstein 1997) is regulated by sterols that accumulate in membranes. As N like molecules have been found in all multicellular organisms where they have been sought, N is an evolutionarily old protein. The existence of a transcription factor that spans the membrane with an extracellular domain capable of interacting with ligands and an intracellular domain that can enter nuclei and activate transcription would provide a simple means for transducing information from neighboring cells. Possibly, the only additional components required would be a protease capable of recognizing a conformational change induced in N on ligand binding resulting in its cleavage, and a second protease that would degrade the cytoplasmic domain in nuclei so that the signaling could be terminated.

Establishing a threshold for Notch signaling

The binary epidermal versus neural cell fate choice mediated by the N signaling pathway involves regulating groups of initially equivalent cells that express both ligand and receptor. Schroeter et al. (1998) have shown that in vertebrate cell culture, extremely low levels of nuclear N are sufficient for function, and our studies of the wild-type Drosophila embryo are consistent with this finding in that no N is detected in the nucleus either biochemically or by immunofluorescence. However, relatively abundant cleaved N associated with Su(H) is detected in the cytoplasm. Why should there be such a disparity between levels of soluble N in the cytoplasm and nucleus, and why shouldn't such a potent nuclear N signal favor saltatory cell fate decisions, with all cells composing an equivalence group assuming the same secondary cell fate? Uniform expression of ligand and receptor among interacting cells might also be expected to favor a salutary response.

Some of the puzzling aspects of N signaling are reminiscent of ultrasensitive systems such as the Xenopus oocyte system described by Ferrell and Machleder (1998), in which a continuously variable signal, progesterone, is converted into an all-or-none response, oocyte maturation. An ultrasensitive system exhibits little response to
low levels of stimulus but switches from off to on over a narrow range of stimulus concentration. We suggest that the cytoplasmic retention of Npp114/Su(H) complexes described in the present study may similarly reflect a mechanism in which the response to low levels of signal is damped. In such a model for N signaling, only high levels of signal result in sufficient cytoplasmic accumulation of Npp114/Su(H) complex to permit its nuclear entry.

In the Xenopus oocyte system, added ultrasensitivity is provided by a positive feedback loop. Earlier genetic studies have suggested that small differences in the expression of N and DI may also be amplified by positive feedback to generate robust intercellular differences in the expression of these proteins among cells derived from an equivalence group (Seydoux and Greenwald 1989; Heitzier and Simpson 1991; for review, see Greenwald 1998). By affecting the nuclear entry of N/Su(H) complex, which functions as a transcriptional activator, Su(H) would also be an element of such a feedback mechanism. We suggest that the N signal is initiated and maintained according to the relative amounts of N, DI, and Su(H). Together, these would determine the rate and duration of accumulation of N/Su(H) complex and the threshold at which it enters the nucleus.

Materials and methods

Constructs

N\textsuperscript{Intra\textsubscript{1768}} was expressed by cloning into a derivative of pUAST (Brand and Perrimon 1993) which contains the cactus initiation codon fused to a Flag epitope (Kidd 1992). To express the cytoplasmic domain of N by coupled in vitro transcription/translation (Promega), a derivative of N\textsuperscript{Intra\textsubscript{1768}} was made in which the region containing the last three introns of N was replaced with the corresponding segment of cDNA. N\textsuperscript{Intra\textsubscript{1790}} contains the first two amino acids of cactus followed by a 14 amino acid myc epitope (Xu and Rubin 1993), which was then fused to amino acid 1790 of N. hsN\textsuperscript{Intra\textsubscript{1790}} used in Fig. 2C is N\textsuperscript{Intra\textsubscript{1790}}, (Lieber et al. 1993).

N\textsuperscript{LexA} has amino acids 1–87 of LexA fused to the carboxyl terminus of N. N\textsuperscript{LEGF1-36-LexA} is a derivative of the above lacking the 36 EGF repeats. N\textsuperscript{Intra\textsubscript{1790}}–LexA has amino acids 1–87 of LexA fused to the carboxyl terminus of N\textsuperscript{Intra\textsubscript{1790}}. The LexA–β-galactosidase reporter has eight LexA operator sites (Ebina et al. 1983) upstream of a heat shock minimal promoter. This was then inserted in place of the GAL4–UAS region of pUAST (Brand and Perrimon 1993).

Myc-tagged Su(H) contains the first two amino acids of cactus followed by a 14 amino acid myc epitope that was then fused to amino acid 10 of Su(H) (Schweisguth and Posakony 1992). Myc-tagged Su(H)–VP16 has amino acids 19–105 of VP16 (Campbell et al. 1984) fused to the carboxyl terminus of myc Su(H).

With the exception of LexA–β-gal, all constructs were subcloned into pUAST (Brand and Perrimon 1993) for transformation into flies.

Yeast expression experiments were carried out as described by (Gyuris et al. 1993).

Fly stocks

The following fly stocks, w ovoD1 FRT101; hsFLP12, Sc/Cyo, ovoD1 FRT40A/Cyo (Chou and Perrimon 1996), h-GAL4, hs-GAL4 (Brand and Perrimon 1993) were obtained from A. Brand and N. Perrimon; Su(H) (FlyBase 1998) was obtained from the Bloomington Stock Center; Su(H)\textsuperscript{F38} FRT40A/Cyo (Schweisguth and Posakony 1994) was obtained from F. Schweisguth; DI\textsuperscript{10F} (Parks and Muskavitch 1993) were obtained from M. Muskavitch, UAS-DI\textsuperscript{10F} (Chou et al. 1993), hsFLP12; Sco/Cyo, ovoD1 FRT40A/Cyo (Chou and Perrimon 1996), h-GAL4, hs-GAL4 (Brand and Perrimon 1993) were obtained from A. Brand and N. Perrimon; Su(H) (FlyBase 1998) was obtained from the Bloomington Stock Center; Su(H)\textsuperscript{F38} FRT40A/Cyo (Schweisguth and Posakony 1994) was obtained from F. Schweisguth; DI\textsuperscript{10F} (Parks and Muskavitch 1993) were obtained from M. Muskavitch, UAS-DI\textsuperscript{10F}, (Doherty et al. 1996) was obtained from Y. Jan. N\textsuperscript{264–47} and DI\textsuperscript{10F} are described by (FlyBase 1998).

Antibodies

Antibodies were raised against histidine-tagged Su(H) as previously described (Kidd et al. 1986; Lieber et al. 1993). The remaining N antibodies (shown in Fig. 1) have been described previously (Lieber et al. 1993). Anti-LexA monoclonal antibody was from Clonetech. M5 anti-Flag antibody was from Kodak. c-Myc antibody was from Calbiochem. SYTOX Green used to label S2 cell nuclei was from Molecular Probes. Immunocytochemistry and immunofluorescence was as described previously (Lieber et al. 1993). Double labeling with RNA and antibody was as described by Apizaz and Frasch (1993).

Immunoprecipitations

Embryo extractions and immunoprecipitations were essentially as described by Kidd (1992). Between 300 μg and 1 mg of protein were used for immunoprecipitation with anti-Su(H) antibodies, one-fifth of this amount was used with anti-N antibodies. Immunoprecipitations were carried out overnight with protein A-Sepharose and GammaBind (Pharmacia) to collect rabbit and rat and mouse antibodies, respectively. After washing, the immunoprecipitates were treated with alkaline phosphatase (Boehringer Mannheim) as described previously (Kidd 1992) and electrophoresed without further washes. After blotting, N in rabbit anti-N and rat anti-Su(H) immunoprecipitates was detected with mouse anti-N, Su(H) in mouse anti-N immunoprecipitates was detected with rat anti-Su(H). Horseradish peroxidase (HRP) conjugated secondary antibodies were from Jackson. HRP activity was detected by the ECL system (Amersham).

Scanned autoradiographs were quantitated on a Macintosh computer with the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image).

Two procedures were used to produce subcellular fractions of embryos. The first, used for Figure 5A, was based on procedures for producing extracts for gel shifts (Andrews and Faller 1991). The second procedure, used for Figure 5, B and C, was as follows: Dechorionated embryos were extensively homogenized in 10 mM HEPES (pH 7.6), 100 mM KC1, 50 mM NaF, 2 mM EDTA, 2 mM EGTA, and 2 mM ammonium molybdate with protease inhibitors. After centrifuging the homogenate at 900g for 5 min, the resulting postnuclear supernatant was incubated on ice for 20 min either with no additional salt or an additional 0.4 or 0.8 mM KC1, and then centrifuged for 2 hr at 100,000g. The supernatants were adjusted to 0.5% Triton X-100 and to 400 mM KC1, and the pellets resuspended in the Triton lysis solution prior to immunoprecipitations.

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