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Signal transduction by activated mNotch: Importance of proteolytic processing and its regulation by the extracellular domain

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ABSTRACT **Previous studies imply that the intracellular domain of Notch1 must translocate to the nucleus for its activity. In this study, we demonstrate that a mNotch 1 mutant protein that lacks its extracellular domain but retains its membrane-spanning region becomes proteolytically processed on its intracellular surface and, as a result, the activated intracellular domain (mNotchIC) is released and can move to the nucleus. Proteolytic cleavage at an intracellular site is blocked by protease inhibitors. Intracellular cleavage is not seen in cells transfected with an inactive variant, which includes the extracellular lin-Notch-glp repeats. Collectively, the studies presented here support the model that mNotch1 is proteolytically processed and the cleavage product is translocated to the nucleus for mNotch1 signal transduction.**

Signaling through the Notch/Lin12 family of receptors regulates cell fate choice throughout development, but the molecular nature of the signaling pathway remains poorly understood. Although these receptors are transmembrane proteins activated by an emerging family of ligands (1), they may also be constitutively activated by truncation of the extracellular domain. Two forms of constitutively activated Notch proteins have been reported: deletions removing specific portions or all of the extracellular domain but retaining the transmembrane domain (2–5) and deletions resulting in an intracellular fragment of Notch (2, 4, 6, 7). The active untethered intracellular fragments of Notch family members encode nuclear localization sequences and are found in the nucleus. Recently, we showed that nuclear localization of the truncated intracellular protein, mNotchIC, is required for its ability to inhibit myogenesis in fibroblasts (6). Recent data demonstrate that mNotchIC interacts directly with KBF2/RBP-Jk in nuclear extracts to augment its ability to activate transcription of the HES-1 promoter. These experiments raise the question as to how endogenous mNotch1, a membrane-spanning protein, obtains access to the nucleus.

The existence of an *in vitro* assay for activated, membrane-spanning Notch1 molecules allows us to address the mechanism of Notch signaling. In this study, we compare the efficacy of membrane-spanning, epitope-labeled mNotch1 derivatives in inhibiting myogenesis to the proteolytic cleavage and subcellular localization of the resulting polypeptides. A mNotch1 derivative, lacking the extracellular domain but retaining the membrane-spanning domain, displays inhibitory activity on muscle-specific promoters and myogenesis (this work) and activates the HES-1 promoter (8). We now demonstrate that the membrane tether is proteolytically cleaved to release an activated intracellular fragment, which translocates to the nucleus, and that the cleavage is inhibited by protease inhibitors. These observations support a model for signal transduction in which the intracellular domain of Notch is released by proteolysis and translocates to the nucleus. Cleavage appears to be regulated by the extracellular domain, since mutant proteins containing the extracellular lin-Notch-glp (LNG) repeats show neither intracellular cleavage, nuclear localization, nor activity in either of our assays.

MATERIALS AND METHODS

Constructs and Assays. mNotch activity was quantified by measuring chloramphenicol acetyltransferase (CAT) activity in 3T3 cells following transient cotransfection with EMSV-MyoD, the MCKCAT reporter construct, and control or mNotch derivatives cloned into the CS2+ vector (9). A CMV-βGal construct was included as a transfection control. Transfections were done as described (6). βGal and CAT activity were detected by ELISA (5 Prime → 3 Prime). Percentage CAT activity was normalized to control experiments. Muscle cells were scored by myosin heavy-chain staining and scoring for myotubes (2–10% of cells, +++; <0.1%, −−−; cumulative results of multiple repeats were tabulated). Activation of the HES-1 promoter was measured as described (8). LNG mNotch and mNotchΔE were constructed by joining a PCR fragment encoding bases 1–63 at the 5′ end of mNotch to a *Ssp* I/*Xho* I fragment (residues 5260–6730) for mNotchΔE or a *Stu* I/*Xho* I fragment (residues 4493–6730) for LNGmNotch followed by a sequence encoding a hexameric Myc tag at the 3′ *Xho* I site. mNotchIC contains bases 5367–6730 with a 5′ myc epitope (6). The constructs ΔE, ΔE-M2, ICL, and IC used (8) contain the C terminus and one Myc-tag as described (10) and produced identical results in CAT assay as the C terminus deleted constructs used in this study. HAmNotchΔE was generated by inserting an oligonucleotide encoding the hemagglutinin (HA)-1 (11) peptide tag (M)YPYDVPDYA(L) following the signal sequence of the relevant mNotch clone. All sequence designations refer to mouse Notch (GenBank accession no. Z11886; ref. 12). mNotchΔE(Δ1757–1808) and mNotchΔE(Δ1769–1773) were generated by PCR (further details are available upon request). All constructs were sequenced.

Immunostaining. Frog embryos were injected and processed as described (6). Myc-tagged Notch was detected in frogs by the monoclonal anti-myc antibody (9E10; ref. 13) and alkaline phosphatase-conjugated anti-mouse secondary antibodies (see Fig. 2 *A* and *B*) HAmNotchΔE-transfected 3T3 cells were fixed with paraformaldehyde (4%) for 3 min and washed either with PBS (see Fig. 2*C*) or with 0.1% Triton X-100 in PBS (Fig. 2 *D* and *E*) for 1 min followed by indirect

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Abbreviations: LNG, lin-Notch-glp; CAT, chloramphenicol acetyltransferase; HA, hemagglutinin.

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immunofluorescence staining with 9E10 antibody or polyclonal anti-HA antibody (Babco, Emeryville, CA). Secondary antibodies were donkey fluorescein isothiocyanate anti-mouse and goat Cyanine-3 anti-rabbit (Jackson ImmunoResearch) antibodies.

Western Blots. For biochemical analysis, transfected 3T3 cells were grown for 48 hr following transfection with plasmid, lysed in hot (90°C) SDS sample buffer, and analyzed by SDS/PAGE. Proteins transferred to nitrocellulose were detected with the 9E10 monoclonal antibody and the ECL Western blot detection reagents (Amersham). The film was scanned (UMAX uc1260) and reproduced for publication with PHOTOSHOP (Adobe) and CANVAS (Denba) software.

Immunoprecipitation. Transiently transfected 3T3 cells grown on 60-mm dishes were washed three times in PBS and lysed in 300 μl of immunoprecipitation lysis solution [1% SDS/50 mM Tris·HCl, pH 7.5/100 mM NaCl/0.2 mM phenylmethylsulfonyl fluoride (PMSF)/0.5 μg of leupeptin per ml/1.0 μg of aprotinin per ml]. Chromosomal DNA was sheared by passing the extract several times through a 21-gauge needle. Before precipitating, the extracts were spun at 12,000 × *g* for 15 min at 4°C. Extracts were diluted with 1.2 ml of immunoprecipitation buffer (50 mM Tris·HCl, pH 7.5/100 mM NaCl/1% Triton X-100/0.5% deoxycholate/1% bovine serum albumin/0.02% sodium azide/0.2 mM PMSF/0.5 μg of leupeptin per ml/1.0 μg of aprotinin per ml). Protein A Sepharose CL-4B (Pharmacia) (25 μl) prebound with the monoclonal antibody 9E10 or 12CA5 was added and incubated at 4°C for 16 hr. Beads were washed three times with immunoprecipition buffer and three times with 50 mM Tris·HCl, pH 7.5/100 mM NaCl and boiled for 5 min in SDS sample buffer.

Immunoprecipitations for microsequencing were scaled up by transfecting multiple 100-mm dishes (2.5×10^8–1×10^9 cells). Plates were lysed in 500 μl and the lysates were pooled. The pooled extracts were placed in 50-ml conical centrifuge tubes in 10-ml aliquots and were precipitated with 40 ml of buffer and 50 μl of protein A beads prebound to 9E10. The washed precipitate was fractionated by SDS/8% PAGE and transferred onto PVDF (Problott; Applied Biosystems). Proteins were visualized by staining with Commassie blue R-250, and the appropriate bands were excised and destained for microsequencing on either model ABI 470A or model ABI 477A (Applied Biosystems).

Pulse–Chase. Forty-eight hours after transfection, cells were starved in methionine-free medium for 90 min. Cells were then pulse labeled for 10 min with 2 ml of medium containing 500 μCi of [^{35}S]methionine per ml (1 Ci = 37 GBq). Complete medium containing 10 μg of cycloheximide per ml was added and cells were incubated at 37°C for the times specified.

Inhibitors. Calpain I and calpain II inhibitors were obtained from Sigma. MG132 was generously provided by Myogenics (Cambridge, MA). All inhibitors were dissolved in dimethyl sulfoxide (DMSO) and added to the transiently transfected cell cultures 48 hr posttransfection (40 μg/ml; DMSO at a final concentration of 0.2%) for 1 hr of incubation. ^{35}S (100 μCi) was added for 3 hr followed by immunoprecipitation and analysis by SDS/PAGE.

RESULTS AND DISCUSSION

Membrane-Spanning Notch Mutants That Are Active in the Myogenic Inhibition Assay Release a Nuclear Localizing Fragment. To test whether membrane-spanning Notch mutants could be active in our assay and permit detection of their intracellular domains, we constructed mutants with a membrane tether and a Myc epitope tag replacing part of the C terminus (inserted at amino acid 2183). mNotchΔE has a deletion of the entire extracellular domain except for a signal sequence and 20 amino acids upstream of the transmembrane

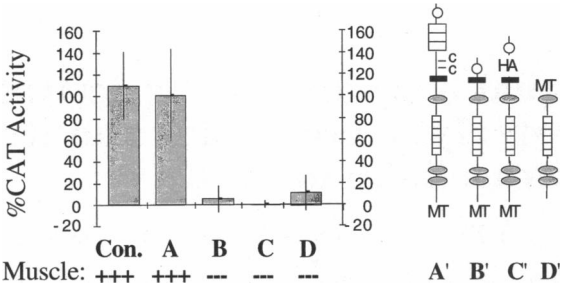

FIG. 1. Myogenic inhibition by mNotch derivatives in transiently transfected 3T3 cells (MyoD and MCKCAT in all transfections). Control (n = 7) and LNG mNotch cotransfected cells (A, A'; n = 9) show no myogenic inhibition. mNotchΔE (B, B'; n = 13), HAmNotchΔE (C, C'; n = 4), and mNotchIC (D, D'; n = 5) inhibit muscle formation and MCKCAT activation by MyoD. Stick figures show signal peptide (○), LNG repeats (□□□), conserved cysteines (cc), HA tag (HA), transmembrane domain (▮), 5′ and 3′ nuclear localization sequence (🛢), CDC 10 domain (□□□□□), and hexameric Myc tag (MT).

domain (Δ21–1703; Fig. 1B'). LNGmNotch contains an additional 256 amino acids, the LNG repeats (Δ21–1447; Fig. 1A'). These constructs were tested for myogenic inhibition in fibroblasts (6). mNotchΔE lowered the ability of MyoD to activate MCKCAT and blocked its ability to convert 3T3 cells to muscle (Fig. 1B). The overall activity of mNotchΔE was equivalent to the intracellular portion of mNotch (mNotchIC), with 90–99% inhibition of MyoD (ref. 6; Fig. 1 D and D'). In contrast, LNGmNotch had no effect on MyoD's ability to convert 3T3 cells into myocytes or to induce expression from MCKCAT (Fig. 1A).

Since the membrane-tethered mNotchΔE exhibited activity similar to that of the mNotchIC intracellular fragment, we wondered whether the intracellular domain of mNotchΔE became localized to the nucleus, as observed with mNotchIC (6, 10). Anti-Myc antibody staining of 3T3 cells transiently transfected with mNotchΔE revealed protein products in the nucleus, with exclusive nuclear staining in ≈10% of the transfected cells (Fig. 2E; see also ref. 8). Membrane staining of mNotchΔE was also detected (Fig. 2 B and D, arrow). However, none of the LNGmNotch transfected cells exhibited any nuclear staining. More dramatically, *Xenopus* embryos injected with mNotchΔE revealed nuclear staining in a large percentage of cells (Fig. 2B), while embryos injected with LNGmNotch showed only membrane staining (Fig. 2A). Similar results have been observed in *Drosophila*, using ΔECN Notch (33). Previously, we demonstrated that mNotchIC acts as an inhibitor of myogenesis in frog embryos (6). Injection of mNotchΔE produced perturbation of somite and muscle development, whereas LNGmNotch-injected embryos were unaffected (data not shown). The *Xenopus* homologue of mNotchΔE has also been demonstrated to behave as an activated receptor, producing alterations of cell fate in the neural tube and the eye on the injected side (5).

To establish that mNotchΔE polypeptide was inserted into the membrane, we constructed a double epitope-tagged derivative of mNotchΔE, HAmNotchΔE. This construct encodes a protein bearing a HA epitope tag positioned immediately after the signal peptide (Fig. 1C') in addition to its C-terminal Myc tag. Its activity in the myogenic inhibition assay was similar to that of mNotchΔE (Fig. 1C). In transfected, paraformaldehyde fixed, unpermeabilized fibroblasts, the HA epitope was detected at the extracellular surface (Fig. 2C). The myc epitope was found in the membrane and in vesicle-like bodies (Fig. 2C, arrows). Following Triton X-100 permeabilization (Fig. 2D) HA-positive cells also stain for myc

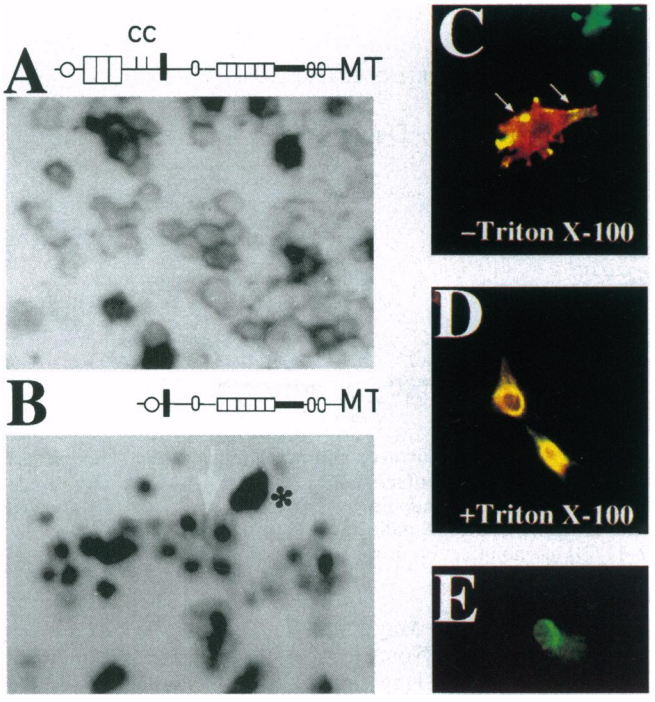

FIG. 2. Intracellular distribution of mNotch mutant proteins in *Xenopus* embryos and 3T3 fibroblasts. Ectodermal cells from *Xenopus* embryos (stage 25) injected with LNGmNotch (*A*) show membrane or cytoplasmic staining, while cells from mNotchΔE-injected embryos (*B*) show predominantly nuclear staining as well as membrane or cytoplasmic staining (*). mRNA was injected into *Xenopus* embryos (6) and mNotch products were detected with anti-Myc monoclonal antibody. (C) HAmNotchΔE-transfected fibroblasts double stained with anti-Myc and anti-HA antibodies reveal uniform HA staining (red) and punctate vesicular Myc staining. (*D*) HAmNotchΔE-transfected fibroblasts washed with Triton X-100 reveals HA and Myc immunoreactivity in membranes. (*E*) mNotchΔE-transfected 3T3 cell showing nuclear staining with the anti-Myc antibody.

(compare Fig. 2 *C* and *D*). In permeabilized cells, we detect ≈10% of cells in which Myc staining is exclusively nuclear (Fig. 2*E*). These data show that the N and C termini have different subcellular dispositions: HAmNotchΔE is inserted into the membrane, but fragments containing the C-terminal myc epitope are also found in a vesicular compartment of unknown nature (10, 14) and in the nucleus.

The Difference of Subcellular Localizations Can Be Explained by Proteolytic Processing. To determine whether proteolysis was occurring, we performed Western blot analysis and pulse–chase experiments of transiently transfected 3T3 cells with the tagged mNotch derivatives. When probed with an anti-Myc antibody, extracts of cells transfected with mNotchΔE revealed three fragments of an apparent molecular mass between 63 and 81 kDa (Fig. 3, lane A). To determine which fragment contained the N terminus in addition to the Myc-tagged C terminus, we examined HAmNotchΔE. A triplet of products was observed, the largest of which was 83 kDa, a size equivalent to the largest mNotchΔE band plus the added HA sequence. The apparent size of the two smaller products, however, remained unchanged (arrows in Fig. 3; compare lane A with lane C). Immunoprecipitation with the anti-HA antibody 12CA5 precipitated only the 83-kDa product (Fig. 3, lane D), indicating that the smaller fragments do not contain the HA epitope even though they contain the Myc tag. Since we find myc epitope-containing products in the nucleus, we conclude that the full-length molecule is membrane bound (83 kDa), and that the 70- to 63-kDa peptides are smaller fragments that can translocate to the nucleus. How processing of mNotchΔE is regulated in individual cells is not clear, since not

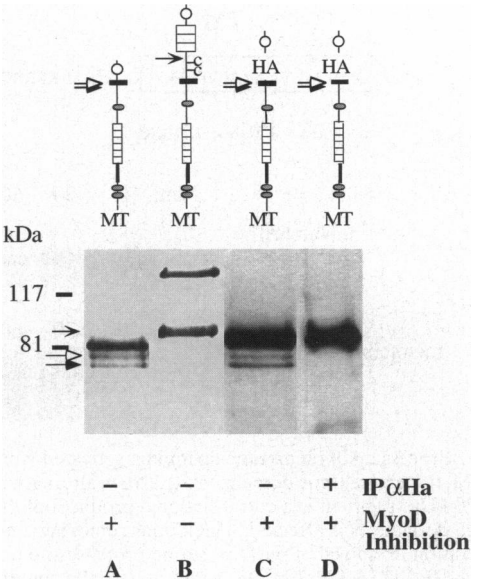

FIG. 3. Immunoblot analysis of mNotch products in fibroblasts. Extracts of fibroblasts transfected with mNotchΔE (lane A), LNGmNotch (lane B), and HAmNotchΔE (lanes C and D) were detected with anti-Myc antibody. In lane *D*, the extract from HAmNotchΔE-transfected cells was first immunoprecipitated with anti-HA monoclonal antibody 12CA5 (IPαHa) (11). Specific cleavage products of 75–63 kDa (arrows) are shown in lanes A and C. Activity in the myogenic inhibitory assay is indicated below the lanes. Arrows with the stick figures above the gel point to the site of processing.

all cells show accumulation of products in the nucleus to the same degree (see also ref. 8). In the frog ectoderm, it appears that nuclear transport of mNotchΔE fragments is occurring at a much higher degree than in transiently transfected 3T3 cells (Fig. 2).

To unequivocally determine the identity of the 63- to 81-kDa fragments, we microsequenced gel-purified peptides. The N terminus of the 70-kDa fragment of mNotchΔE is MYVAA, consistent with the sequence at amino acid 1726 of mNotch 1 (see Fig. 4). An N-terminal methionine raises the possibility of an alternative translation initiation. The internal M1726 is the first AUG in any frame following the Notch1 initiation methionine in mNotchΔE. This methionine and the methionine at position 1796 are conserved in mNotch1 from all species. Therefore, the activity of membrane-tethered Notch1 with large deletions of extracellular sequences could possibly be due to the proximity of an alternative translation initiation site downstream of the normal Notch1 translation start site. Such an initiation site would generate a protein that lacks the signal peptide, remains in the cytoplasm, and could translocate to the nucleus.

We introduced mutations that altered the methionine (M1726V or M1726A) to eliminate alternative initiation at this site. Western blots of M1726A and M1726V lack the 70-kDa polypeptide, whereas the 63-kDa fragment is unaffected (Fig. 4). Both mNotchΔEM1726V and mNotchΔEM1726A show inhibitory activity in myogenesis and stimulate HES-1 transcription; exclusive nuclear staining is detected in ≈10% of transfected cells (data not shown). This suggests that the 70-kDa alternative translation product and methionine-1726 are not required for activity.

To establish that the 63-kDa fragment represents a cleavage product formed during mNotchΔE signaling, pulse–chase experiments were performed. A short (10 min) pulse followed by a chase in the presence of cycloheximide labeled both the 81- and the 70-kDa fragments (Fig. 4*B*). The 63-kDa fragment accumulated only during the chase, requiring 30–60 min posttranslation in both mNotchΔE and mNotchΔEM1726V (Fig. 4*A*).

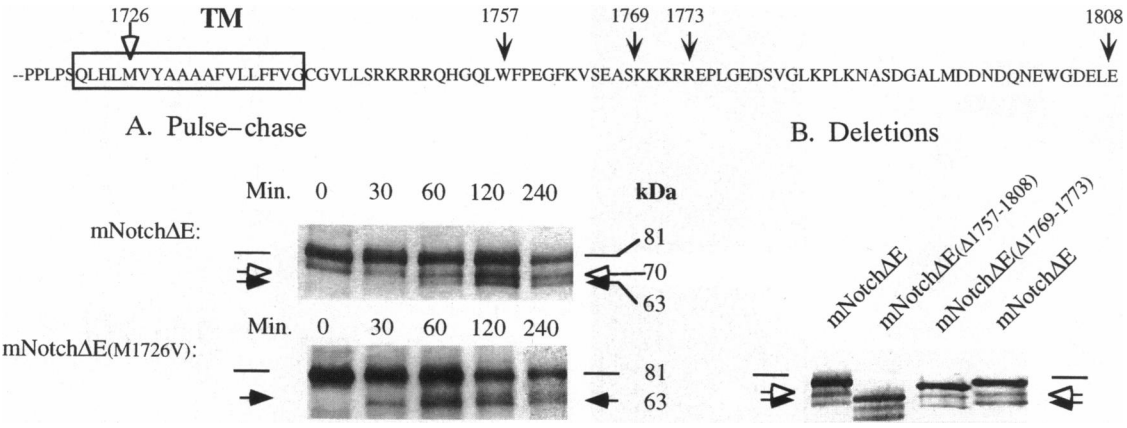

FIG. 4. Processing of membrane-spanning, activated Notch. Amino acid sequence of the region involved in processing and site of deletions are shown. TM, transmembrane domain; white arrow, alternative initiation site. (*A*) Pulse–chase analysis of mNotchΔE and mNotchΔEM1726V reveals that the 70-kDa fragment is a cotranslational product but the 63-kDa fragment is generated independently by proteolytic processing. (*B*) Mobility of mNotch derivatives is affected by deletions removing amino acids C-terminal to amino acid 1757, indicating that processing occurs N-terminal to that position. Removal of putative serine protease site [mNotchΔE(Δ1769–1773)] has no effect on processing or activity in our assays. Activity of mNotchΔE(Δ1757–1808) in our assays is partially compromised.

In pulse–chase experiments, the 70-kDa fragment is not present in mNotchΔEM1726V.

The exact site of cleavage in mNotchΔE that generates the 63-kDa product has not yet been determined. The cleavage site must lie between amino acids 1726 and 1757, as indicated by the molecular sizes of constructs with deletions distal to this region. Deletion at positions 1757–1808 or 1769–1773 resulted in size reduction of all fragments, indicating that these amino acids are included within the processed fragment and that the site of cleavage is N terminal to that deletion (Fig. 4*B*). The transmembrane domain extends to amino acid 1740, making cleavage upstream of amino acid 1740 unlikely, but we cannot rule out this possibility.

Generation of the 63-kDa Fragment Is Blocked by Protease Inhibitors. We tried several protease inhibitors in an effort to block the putative protease. The peptidyl aldehyde inhibitor *N*-Cbz-L-Leu-L-Leu-Leu-H (MG132), but not related inhibitors (calpain I and calpain II inhibitors), eliminated the 63-kDa cleavage product but not the 70-kDa translation product (Fig. 5, lanes A–E). Similar results were obtained with mNotchΔEM1726V where long exposure reveals complete disappearance of the 63-kDa fragment (lanes F–H). Collectively, these results clearly demonstrate that the 63-kDa fragment is a proteolytic product of the 81-kDa fragment.

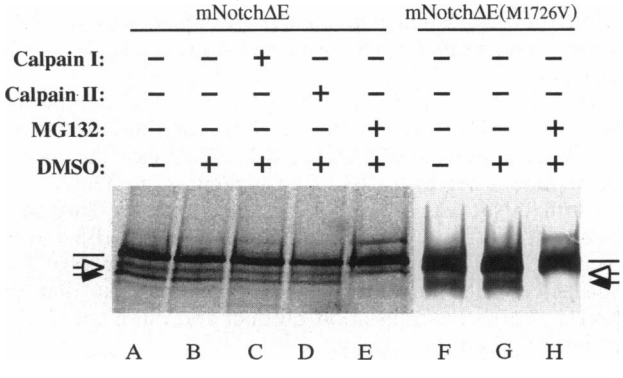

FIG. 5. Inhibition of the putative protease. Peptidyl aldehyde inhibitor MG132, but not related inhibitors (calpain I and calpain II inhibitors), eliminated the 63-kDa cleavage product but not the 70-kDa translation product of mNotchΔE (lanes A–E). In the presence of MG132, long exposure reveals complete disappearance of the 63-kDa fragment in cells transfected with mNotchΔEM1726V (lanes F–H).

The LNG Repeats May Regulate Intracellular Processing. The inactive LNGmNotch is also partially cleaved in transfected cells (Fig. 3, lane B), resulting in about half of the protein in the form of a shorter fragment (86 kDa). We isolated protein to identify the position of this cleavage site. Microsequencing of the N terminus of the 86-kDa cleavage product revealed the sequence ELDPMDI, which corresponds to extracellular amino acids 1655–1661. These amino acids are preceded by a typical serine protease site (RQRR), conserved in all Notch1 orthologues. The resulting inactive protein consists of the extracellular stalk with its conserved cysteines and the entire intracellular domain. These cysteine residues have apparent roles in preventing activation and possibly dimerization of *Drosophila* Notch (2, 15). It is likely that this fragment corresponds to the inactive 100/120-kDa fragment seen by others in cells expressing full-length TAN-1 or Notch, respectively (16, 17). The size differences between the 100/120-kDa fragments and our 86-kDa fragment are due to the removal of the C terminus in our LNGmNotch (see *Materials and Methods*). When inserted into the plasma membrane, the LNG repeats and the conserved cysteines appear to block intracellular processing. A similar construct, lacking the LNG repeats but containing the conserved cysteines [ΔECT+s(L)], is active as measured by oncogenic activity in bone marrow (17). These constructs, unlike LNGmNotch, seem to be processed further. In murine and human leukemia cells where biological activity of these constructs is demonstrated, smaller polypeptides that are presumably intracellular fragments are detected (ref. 17, see figure 6 and SUP-T1 extracts). Removal of the LNG repeats results in activation of full-length Notch (2), suggesting that they participate in maintaining the native receptor in an inactive state. Thus, it appears that the LNG repeats regulate both Notch activity and cleavage, consistent with a model where LNG repeats modulate oligomerization of Notch.

Implications. How mNotch exerts its inhibitory effects upon MyoD-induced transcription is unknown (6). The observation that mNotchIC localizes to the nucleus, forms a complex with KBF2/RBP-Jk, a *Su(H)* homologue capable of binding to promoter sequences, and activates downstream genes (8) provides a possible mechanism for its effect on myogenesis (ref. 18; R.K. and H.W., unpublished observations), and possibly other cell fate decisions (18) (Fig. 6*B*). We provide evidence to explain how a membrane-spanning protein can exert these activities in the nucleus. The data presented here suggest that intracellular proteolysis plays a role in the activation of membrane-tethered mNotch derivatives by produc-

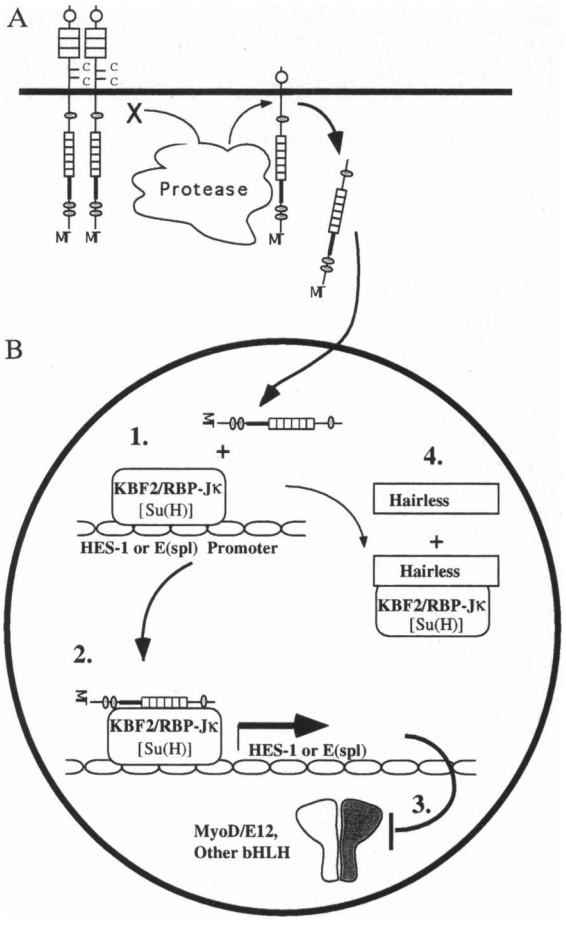

FIG. 6. Model for activation of signal transduction through mNotch. (*A*) Regulation of proteolytic cleavage of the intracellular domain by the extracellular domain. Sequences of the extracellular domain such as the LNG repeats and/or conserved cysteines may play a role in oligomerization of the transmembrane protein. An intracellular protease may distinguish oligomers from monomers and process only one form (depicted here as the monomer), releasing an active fragment capable of nuclear localization. In this model, ligand may activate mNotch by altering the state of oligomerization. (*B*) Control of transcription by mNotch fragments. (*1*) An active mNotch fragment interacts in the nucleus with KBF2/RBP-Jκ, a mammalian Su(H) homolog. This active complex stimulates transcription of downstream genes, such as HES-1 and possibly other E(spl) family members (19). (*2*) Transcription of HES-1 produces a basic helix–loop–helix (bHLH) protein that negatively regulates other cell type-specific bHLH proteins, such as MyoD (18). (*4*) In *Drosophila*, other molecules such as Hairless (20) also regulate Su(H) activity in the nucleus (20–22).

ing a stable proteolytic product that is capable of nuclear translocation. Only those membrane-spanning derivatives that are processed on the intracellular side produce inhibition of myogenesis and activation of the HES-1 promoter. Subsequent disposition of the cleaved intracellular fragment is unclear. The present studies finding nuclear localization of all activated mNotch species suggest that the ultimate disposition of an active fragment is in the nucleus. Other proteins containing CDC10/ANK repeats appear to have roles in transcriptional regulation by participating in a transcriptional complex (23–25). A similar mechanism of activation employing cleavage of a membrane-tethered transcription factor, the sterol regulatory element binding protein 1 (SREBP-1), has been recently reported (26).

Our observations of cleavage of mNotch and its nuclear translocation rely upon C-terminal epitope tagging of protein derivatives. The failure to detect the invertebrate family members Notch and glp-1 fragments in the nucleus (27, 28) by standard immunohistochemical techniques (2, 4, 10, 14, 27–29) raises questions as to the significance of nuclear localization for Notch1 activity during normal development. It is possible that techniques to improve the sensitivity of detecting the intracellular domain may be required to observe processing and translocation of the endogenous Notch/lin12 receptor family members. Immunoblot and immunohistochemistry assays of some human and murine tissues show that putative nuclear forms of Notch were found in transformed human cervical tissue, rat retina, and human leukemia cells, raising the possibility that Notch processing is indeed a physiological mechanism (16, 17, 29). Moreover, constructs used in these experiments lack PEST sequences thought to regulate protein degradation (30). The subsequent degradation of the 63-kDa fragment may have been slowed due to a lack of these sequences. In addition, rapid turnover is observed in nuclei overexpressing the intracellular fragment of Notch (2). This is consistent with speculation that intracellular peptides of wild-type Notch have extremely short half-lives and are not easily detected. However, it still remains to be proven that such processing takes place during ligand-dependent signaling.

How does ligand activate mNotch signaling? It has been shown in *Drosophila* that Notch sequesters Su(H) in the cytoplasm and ligand binding to Notch leads to its release (29, 31, 32). This model poses the problem as to what the distribution of Su(H) might be in the absence of Notch. The model predicts that both ligand-activated and null Notch lead to Su(H) translocation to the nucleus, a prediction that is inconsistent with the observed phenotypes (29, 31). One possible solution to this problem would be if the interaction of Su(H) and Notch led to a modification of Su(H), such that Su(H) was activated. Alternatively, if Notch fragments were translocated to the nucleus following ligand activation as we propose (see below), then the Notch–Su(H) complex would be positioned to activate or repress genes in the nucleus.

We propose that ligand binding to the extracellular domain may also regulate processing of Notch. Our data demonstrate that the presence of LNG repeats alters intracellular processing, and their removal activates mNotch1 (2). One possible model to account for the modulation of intracellular proteolysis by the extracellular domains would be if the extracellular domain regulated oligomer formation (ref. 14; see Fig. 6*A*). Oligomerization and the consequent close apposition of the intracellular surfaces may regulate access of an intracellular protease to the stalk region. Hypothetically, the interaction of ligand with Notch family members may then alter the oligomerization state of the molecules and lead to proteolytic cleavage, nuclear localization, and activation of Su(H) (8). Although we demonstrate the existence of such a mechanism only in active truncated mNotch mutants, we suggest that the endogeneous Notch family may undergo the same cleavage and nuclear localization to exert their effects upon cell fate and other processes.

This manuscript is dedicated to the memory of our mentor and friend, Hal Weintraub. We would like to thank Drs. R. Axel, D. Turner, A. Israel, and R. Cagan for valuable discussions during the course of this work. Special thanks to R. Cagan for reading the manuscript and making valuable comments. R.K. wishes to thank the Leukemia Society of America for their support. J.S.N. was supported by the National Institute of Neurological Disorders and Stroke, the March of Dimes, and the Colleen Giblin Foundation. This work was supported by the National Institutes of Health and the Howard Hughes Medical Institute.

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