THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 37, Issue of September 14, pp. 34371–34378, 2001

Printed in U.S.A.

Functional Interaction between SEL-10, an F-box Protein, and the Nuclear Form of Activated Notch1 Receptor*

Received for publication, February 12, 2001, and in revised form, June 22, 2001
Published, JBC Papers in Press, June 25, 2001, DOI 10.1074/jbc.M101343200

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The Notch signaling pathway is essential in many cell fate decisions in invertebrates as well as in vertebrates. After ligand binding, a two-step proteolytic cleavage releases the intracellular part of the receptor which translocates to the nucleus and acts as a transcriptional activator. Although Notch-induced transcription of genes has been reported extensively, its endogenous nuclear form has been seldom visualized. We report that the nuclear intracellular domain of Notch1 is stabilized by proteasome inhibitors and is a substrate for polyubiquitination *in vitro*. SEL-10, an F-box protein of the Cdc4 family, was isolated in a genetic screen for Lin12/Notch-negative regulators in *Caenorhabditis elegans*. We isolated human and murine counterparts of SEL-10 and investigated the role of a dominant-negative form of this protein, deleted of the F-box, on Notch1 stability and activity. This molecule could stabilize intracellular Notch1 and enhance its transcriptional activity but had no effect on inactive membrane-anchored forms of the receptor. We then demonstrated that SEL-10 specifically interacts with nuclear forms of Notch1 and that this interaction requires a phosphorylation event. Taken together, these data suggest that SEL-10 is involved in shutting off Notch signaling by ubiquitin-proteasome-mediated degradation of the active transcriptional factor after a nuclear phosphorylation event.

Notch receptors are implicated in alternative cell fate determination during invertebrate and vertebrate development (for review, see Refs. 1 and 2). The Notch gene, first identified in *Drosophila melanogaster*, encodes a 300-kDa type I integral membrane protein. Notch1, the most extensively studied of the four mammalian Notch molecules, contains 36 epidermal growth factor-like repeats and three Lin12/Notch repeats in its extracellular part; the intracellular part is composed of three putative nuclear localization signals (NLS)[1], six ankyrin repeats, and a C-terminal PEST domain.

Notch1 activation involves a series of proteolytic steps. A constitutive cleavage by a furin-like convertase first takes place in the *trans*-Golgi network and is required for cell surface expression of a functional receptor (3, 4). It generates a heterodimeric molecule made of the noncovalent association between a 200-kDa N-terminal ligand binding extracellular region and a 120-kDa fragment that includes the intracellular region, the transmembrane domain, and 69 amino acids of the extracellular region.

Ligand interaction leads to a second proteolytic step attributed to the metalloproteinase TACE (for *T*NF-α-converting *e*nzyme, also known as ADAM 17) (5, 6). A third cleavage releases the Notch intracellular domain by a yet unknown protease that shows strong similarities with the γ-secretase responsible for generation of the Aβ amyloid peptide from the amyloid precursor protein (7, 8). Presenilins have been described as key regulators of this last processing step (9–12). Although ligand binding is normally required for activation of the receptor, recent data suggest that the dissociation of the heterodimeric Notch1 receptor by ion chelators such as EDTA or EGTA mimics activation (13).

Once released through ligand binding (14) or through EDTA treatment (13), the Notch intracellular domain migrates to the nucleus, forms a complex with the DNA-binding subunit CSL (for CBF1/Su(H)/Lag1 also known as RBP-Jκ), and acts as a transcriptional modulator of Notch target genes (15–17). Some of these target genes such as *HES1* and *HES5* are transcription factors of the basic helix-loop-helix family (15, 18, 19).

Notch activity is strictly regulated by many intracellular modulators (Deltex, Numb, Mastermind; for review, see Ref. 1). Perturbation in Notch signaling often results in cancer and impaired development (20–25). Indeed, very low amounts of nuclear Notch are sufficient for CSL-dependent transcription, and the presence of nuclear Notch is often detected indirectly through its effects on transcription (26, 27). Although nuclear immunostaining for Notch1 has been detected in cervical carcinomas (21, 28), endogenous Notch nuclear fragments are virtually undetectable in normal cells. A rapid turnover of the nuclear form of Notch (referred to as N-IC) may explain this observation (29), and treatment of cells with a proteasome inhibitor such as lactacystin has been shown to allow its detection (10). The C-terminal PEST-like domain of Notch may contribute to instability and degradation of N-IC by the ubiquitin-proteasome pathway, thus preventing potentially deleterious transcription (30).

The ubiquitin-proteasome pathway is a tightly regulated process involved in intracellular protein degradation. It appears to play a key regulatory role in basic functions such as cell cycle regulation, cell growth and proliferation, differentiation, and development, and its substrates include transcrip-

* This work was supported in part by grants from the Association pour la Recherche contre le Cancer (to A. I.) and by TMR Grant CT 960026 from EC (to A. I. and A. C.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Recipient of a postdoctoral fellowship award from EC (Biomed program).

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[1] The abbreviations used are: NLS, nuclear localization signal(s); CSL, CBF1/Su(H)/Lag1; N-IC, nuclear form of Notch; EST, expressed sequence tag; HA, hemagglutinin; VSV, vesicular stomatitis virus; ALLN, *N*-acetyl-Leu-Leu-norleucinal; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate).

tional regulators to cell surface proteins (31). Ubiquitin is a 76-amino acids peptide that is covalently attached to substrate proteins by a complex cascade of enzymes: ubiquitin is first activated by the ubiquitin-activating enzyme E1; after activation, one of several E2 enzymes transfers ubiquitin from E1 to a member of the ubiquitin-protein ligase family, E3, to which the substrate is specifically bound. Successive rounds of conjugation end up in the attachment of polyubiquitin chains to the substrate, which is thus targeted for degradation by the proteasome. Only a few E3s have been identified so far, but they seem to belong to a rapidly growing family; and although one E1 and a few E2s seem to be sufficient to ubiquitinate most of the target proteins, each individual E3 seems to bind to a limited number of substrates. At least two large families of E3 enzymes have been identified in mammals: the HECT group (*H*omologous to *E*6-AP *C T*erminus), whose founding member, E6-AP, is required, together with the E6 protein of papillomavirus, to induce degradation of p53, and the SCF family (32, 33). This last group is represented by multiprotein complexes that contain at least four proteins: Skp1, Cul1 in metazoans or Cdc53 in yeast, Roc1/Rbx1/Hrt1, and an F-box protein. The F-box proteins can recognize different substrates through specific protein-protein interaction domains. The F-box domain interacts with Skp1, whereas the C terminus mediates substrate binding. A large number of F-box proteins have been identified (more than 100 in the *Caenorhabditis elegans* genome), suggesting the existence of a large number of substrate proteins. An important observation is that, in the case of the SCF complexes that have been studied in detail, substrate phosphorylation seems to be required for F-box protein binding (32, 33).

SEL-10, an F-box protein of the Cdc4 family, was isolated in a genetic screen for Lin12/Notch negative regulators in *C. elegans* (34). This protein carries seven WD40 repeats in its C-terminal region; in other complexes these motifs have been shown to interact specifically with the substrate. Coimmunoprecipitation of overexpressed proteins demonstrated that the intracellular domains of Lin12 and murine Notch4 interact with the worm SEL-10 protein (34).

In this study we show that mammalian SEL-10 binds to the C-terminal region of Notch1. More interestingly, binding and therefore degradation are restricted to the nuclear compartment and correlate with phosphorylation of the intracellular part of the receptor. A dominant-negative form of SEL-10 stabilizes this nuclear form and increases transcriptional activation of a reporter construct after activation of the receptor. These results strongly suggest that a SEL-10-containing complex is involved in the negative regulation of the Notch cascade by inducing the degradation of the intracellular form of the receptor.

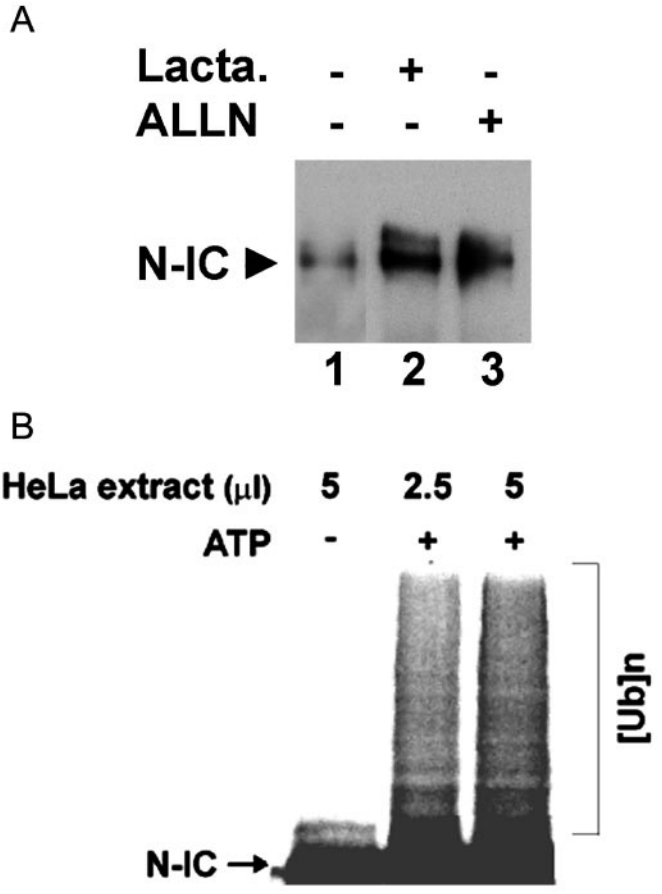

FIG. 1. **The intracellular form of Notch1 is a substrate for the ubiquitin-proteasome pathway.** *Panel A*, Western blot analysis of N-IC. 293T cells were transfected with expression vectors encoding N-IC. Cells were incubated with proteasome inhibitors for 90 min (50 μM lactacystin (*Lacta.*, *lane 2*) or 50 μM ALLN (*lane 3*)) before lysis, and 20 μg of each total cell extract was analyzed by SDS-polyacrylamide gel electrophoresis. *Panel B*, N-IC is ubiquitinated *in vitro*. Wheat germ-translated N-IC was incubated with ubiquitin and different volumes of HeLa cell extract as indicated, in the absence (−) or presence (+) of ATP. The *arrow* indicates unconjugated N-IC, slower migrating forms represent ubiquitin-conjugated N-IC (noted as *[Ub]n*).

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—293T cells and HeLa cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin. HeLa cells stably transfected with murine Notch1 cDNA, HeLaN1, were maintained as described previously (3). Cells were transiently transfected using the calcium phosphate coprecipitation procedure and harvested 48 h later.

Plasmids—All Notch1 constructs reported here were cloned into the pCS2+ vector. Notch1 constructs, NotchFL, LNG, N-ICΔCT, N-ICΔNLS, and N-ICΔNLS+NLS, have been described previously (7, 8, 15, 26, 35). The N-IC construct, which encodes the entire intracellular form of Notch1 from the γ-secretase cleavage site to the stop codon was constructed by cloning an *Eco*RV/*Spe*I fragment from NotchFL into *Eco*RV/*Xba*I-digested pCS2 N-ICΔCT.

hSEL-10 was amplified by polymerase chain reaction (primers 5′-CATGTATGTATGTGTGTCCCGAGAAGCGGTTTG and 3′-GTCCACATCAAAGTCCAGCAC) from the EST AI929793 (IMAGE 2519384) and cloned into the pcDNA3.1/V5-His-TOPO vector (Invitrogen). A polymerase chain reaction-amplified fragment from mouse EST AI747954 (IMAGE 2065067) using oligonucleotides GGAATTCCGCTCTAGAGCGACCTGCAGCTCGAGCATA and GGAATTCGATACAAACTGGAGACTAGGAGAA was digested and cloned into *Eco*RI/*Xba*I sites of a pCS2 HA-tagged plasmid. This construct represented the HA-tagged SEL-10ΔF (amino acids 262–627 of hSEL-10). VSV-tagged β-TrCP was a gift from S. Whiteside.

Antibodies—Anti-N-IC polyclonal antibody was described by Logeat *et al.* (3) and precipitated on protein A-Sepharose beads. Anti-HA (12CA5) and anti-VSV (P5D4) monoclonal antibodies were diluted 1/100 for immunoprecipitations and isolated on protein G-Sepharose. Myc-tagged N-ICΔNLS and N-ICΔNLS+NLS were immunoprecipitated with anti-Myc (9E10) monoclonal antibody.

Cell Extracts, Immunoprecipitations, and Immunoblots—Subconfluent 293T cells were transiently transfected with 250 ng of Notch1 derivatives and 4 μg of SEL-10ΔF or β-TrCPΔF when indicated. Total transfected DNA amount was equalized to 5 μg with pcDNA3 vector. Transfected cells grown on 60-mm dishes were washed in phosphate-buffered saline and lysed in 200 μl of lysis buffer (50 mM Tris-HCl, pH 8.0, 1% Nonidet P-40, 400 mM NaCl, and 1 mM EDTA) supplemented with 1× protease inhibitor mixture (Roche Molecular Biochemicals), 20 mM sodium fluoride, and 2 mM sodium orthovanadate as phosphatase inhibitors. After 20 min on ice, cell lysates were cleared at 14,000 rpm for 20 min at 4 °C, and protein concentration was determined by the Bradford method. Immunoprecipitations were carried out with the appropriate antibody in a 2-fold diluted lysis buffer. Subsequent steps of immunoprecipitations and immunoblots were done as described earlier (3). When mentioned, 50 μM *N*-acetyl-Leu-Leu-norleucinal (ALLN) or 50 μM lactacystin was added 90 min before cell lysis.

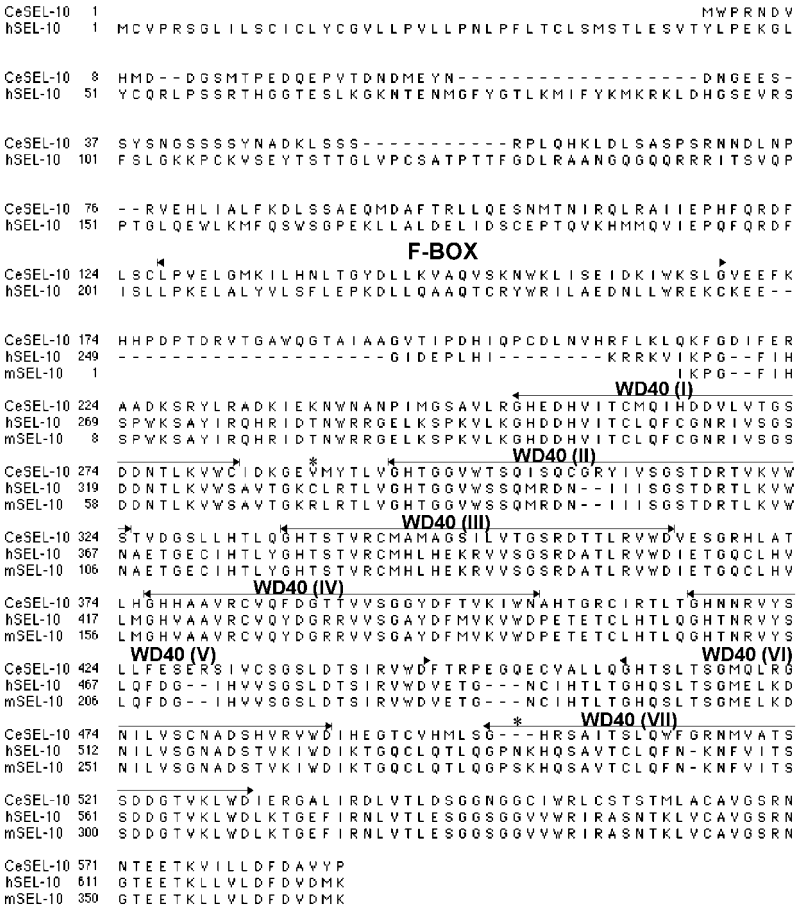

FIG. 2. **SEL-10 sequence alignments.** CeSEL-10 protein was aligned with full-length human SEL-10 (*hSEL-10*) and truncated mouse SEL-10 (*mSEL-10*) proteins using CLUSTALW. The F-box and WD40 domains are indicated above the sequences. Asterisks mark the two amino acid substitutions between human and murine sequences.

Luciferase Assay—Subconfluent HeLa or HeLaN1 cells were transfected with 500 ng of RBP-luciferase reporter construct (pGa981-6 (36)), 400 ng of EF1-β-galactosidase (provided by S. Mémet) along with the indicated amounts of SEL-10 or SEL-10ΔF. HeLa cells were transfected with the Notch1 derivatives, and total amounts of transfected DNA were equalized with empty pcDNA3 vector to 2 μg. After 48 h cells were harvested and luciferase activity measured in a luminometer (Berthold) and normalized according to β-galactosidase activity.

Metabolic Labeling—48 h after transfection, 293T cells were starved in methionine-free medium for 90 min. Cells were then pulse labeled for 15 min with medium containing 200 μCi of [^{35}S]methionine. Complete Dulbecco's modified Eagle's medium supplemented with 0.04% methionine was added, and cells were incubated at 37 °C for the times specified (see Fig. 6).

Orthophosphate Labeling—293T cells were transfected with N-IC or pcDNA3 as a control. 24 h after transfection, cells were washed twice in a phosphate-free medium and incubated for 2 h in phosphate-free RPMI supplemented with 10% phosphate-free fetal calf serum. Cells were labeled for 3 h with 500 μCi/ml $^{32}P_i$. Lysis and anti-Notch immunoprecipitation were carried out as described previously.

Phosphatase Treatment of Cell Lysates—Transfected 293T cell lysates were immunoprecipitated, and the immune complex was incubated in λ protein phosphatase buffer (50 mM Tris-HCl, 0.1 mM Na_2EDTA, 5 mM dithiothreitol, and 0.01% Brij) supplemented with 2 mM $MnCl_2$ at 30 °C for 30 min. Half of the sample was incubated without the enzyme; the other half was treated with 400 units of λ phosphatase (Biolabs). Samples were collected by centrifugation and resuspended in Laemmli buffer for SDS-polyacrylamide gel electrophoresis resolution on a 6.5% gel.

In Vitro Ubiquitin Conjugation Assay—cDNA encoding N-IC was translated *in vitro* in wheat germ extract in the presence of [^{35}S]methionine, and the ubiquitin conjugation assay was performed as described (37). The reaction mixture in a final volume of 12 μl contained the following components: 50 μg of HeLa cell extract to provide the various components of the ubiquitination machinery, 40 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, 2 mM dithiothreitol, 5 μg of ubiquitin, and ~25,000 cpm of the labeled substrate. To deplete endogenous ATP (*−ATP* in Fig. 1*B*), 20 mM 2-deoxyglucose and 0.2 μg of hexokinase were added. ATP-dependent conjugation was monitored in the presence of 2 mM ATPγS. 0.5 μg of ubiquitinaldehyde, a specific inhibitor of certain isopeptidases (38), was added to the reaction mixture to prevent deubiquitination. Reaction mixtures were incubated at 37 °C for 30 min, resolved via SDS-polyacrylamide gel electrophoresis, and analyzed with a PhosphorImager (Fuji, Tokyo, Japan).

RESULTS

Ubiquitin-Proteasome Degrades the Intracellular Form of Notch—293T cells transiently transfected with N-IC, a plasmid that encodes the entire intracellular form of Notch1, were treated with one of two proteasome inhibitors: lactacystin (Fig. 1*A*, *lane 2*) or ALLN (Fig. 1*A*, *lane 3*). Western blot analysis of N-IC revealed a strong stabilization of this protein in the presence of the two proteasome inhibitors. This result suggests that the proteasome-dependent degradation pathway is involved in the stability of the intracellular form of the Notch1 receptor.

To confirm this hypothesis, an *in vitro* ubiquitination experiment was performed. Data in Fig. 1*B* show that N-IC is conjugated with ubiquitin (indicated by *[Ub]n*) and that this conjugation is strictly ATP-dependent. Taken together these observations indicate that the activated form of Notch1 is probably degraded by the ubiquitin-proteasome pathway.

This degradation pathway requires a specific E3 ubiquitin ligase that interacts directly with the substrate and provides the specificity for the degradation reaction. As SEL-10, an F-box protein, was identified by a genetic screen for Notch negative regulators in *C. elegans* (34), we decided to investigate the role played by the mammalian counterpart of CeSEL-10 in the Notch signaling pathway.

Isolation of Human and Mouse SEL-10—Using a BLAST search for mammalian homologs of CeSEL-10 (39), two ESTs were found. Sequence analysis of mouse EST AI747954 re-

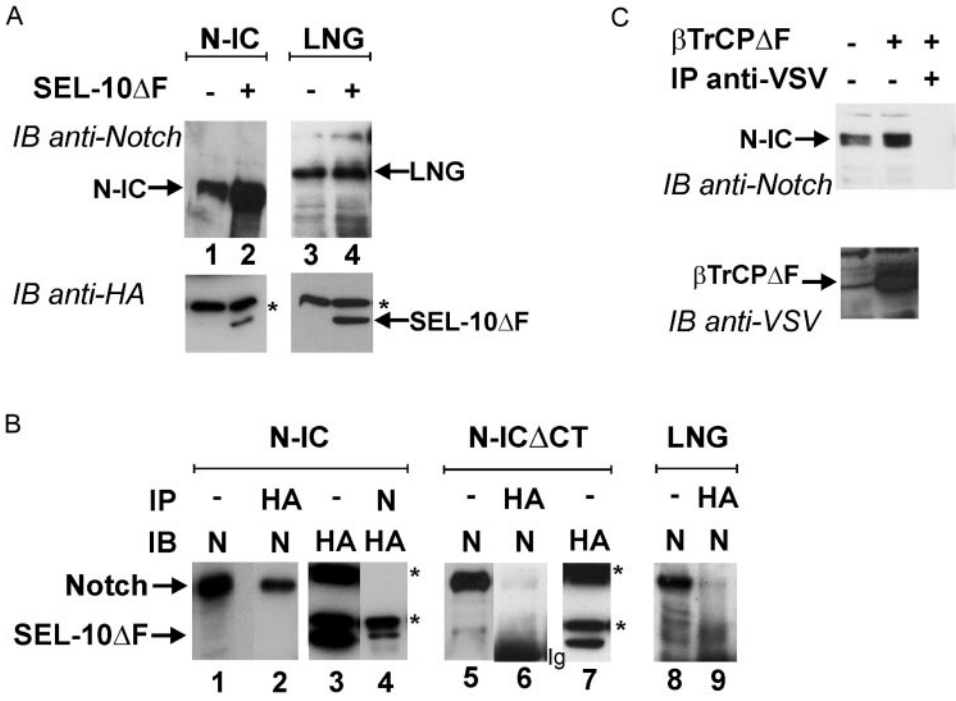

FIG. 3. **Specificity of SEL-10ΔF and Notch1 interactions.** *Panel A*, SEL-10ΔF stabilizes N-IC. 293T cells were transfected with expression vectors encoding different Notch1 derivatives: N-IC (*lanes 1* and *2*), LNG (*lanes 3* and *4*), with (+) or without (−) SEL-10ΔF. Cell extracts were analyzed directly by immunoblot with anti-Notch (*upper part*) or anti-HA (*lower part*). *Panel B*, SEL-10ΔF coimmunoprecipitates with N-IC. 293T cells were cotransfected with expression vectors encoding SEL-10ΔF and different Notch1 derivatives: N-IC (*lanes 1–4*), N-ICΔCT (*lanes 5–7*), and LNG (*lanes 8* and *9*). Cell extracts were analyzed by immunoblotting (*IB*) with the antibodies indicated above the *lanes*, directly (*lanes 1*, *5*, *7*, and *8*) or after immunoprecipitation (*IP*) with anti-HA (*lanes 2*, *6*, and *9*) or anti-Notch (*lane 4*) antibodies. *Panel C*, N-IC does not interact with β-TrCPΔF. Cotransfections were performed and analyzed as in *panel B* except that VSV-tagged β-TrCPΔF was used when indicated and immunoprecipitated (+) or not (−) with anti-VSV antibody. *Asterisks* indicate nonspecific bands; *Ig*, immunoglobulins.

vealed its homology with the C-terminal seven WD40 repeats of *C. elegans* SEL-10 (Fig. 2). The human EST, AI929793, isolated from fetal brain, contained the full-length open reading frame and included both the F-box and the seven WD40 repeats. The predicted protein was 627 amino acids long if the first in-frame methionine was considered (Fig. 2, *hSEL-10*). *In vitro* translation experiments yielded a protein of around 60 kDa (not shown).

Sequence analysis with the whole human genome (htgs, NCBI, NIH) revealed that the human SEL-10 gene localizes to the long arm of chromosome 4. The corresponding UniGene cluster, Hs.31945, contains more than 50 cDNAs expressed ubiquitously as assessed by their origins (16 different tissue sources of ESTs were mentioned in this cluster). This highly conserved protein shared 48% identity with CeSEL-10 and 56% identity when considering the WD40 repeats domain only. The N-terminal Leu-Pro amino acids required for F-box binding to the SCF complex have been conserved (32). Sequence comparisons of human and mouse WD40 repeats revealed only two amino acid substitutions (indicated by *asterisks*, Fig. 2). A *Drosophila* SEL-10, localized at the CG15010 locus, exhibited 78% identity with hSEL-10.

SEL-10 belongs to the Fbw family of F-box proteins (40), and this suggests its implication as part of a ubiquitin ligase of the SCF family. The F-box links the other members of the E3 complex through Skp1, whereas the WD40 repeats constitute the substrate recognition domain. Deleting the F-box domain enables recognition of the substrate without recruiting the rest of the ubiquitination complex, thus competing with the intact protein for substrate binding. A truncated SEL-10 protein was constructed from the mouse EST (SEL-10ΔF) and should represent a dominant-negative form of SEL-10.

Mammalian SEL-10 Interacts Specifically with N-IC—Different murine Notch constructs were cotransfected with HA-tagged SEL-10ΔF (Fig. 3). The level of N-IC was increased strongly when cotransfected with this molecule (Fig. 3*A*, compare *lanes 1* and *2*). This effect was not observed with the inactive membrane-anchored Notch LNG (Fig. 3*A*, compare *lanes 3* and *4*). Because these two Notch constructs are under the control of the same cytomegalovirus promoter, the increased amount of N-IC could not be attributed to a transcriptional effect of SEL-10ΔF.

We then investigated possible interactions between these proteins. Notch and SEL-10ΔF were coexpressed in 293T cells (Fig. 3*B*). Immunoprecipitation of one protein followed by Western blot analysis of the second revealed a strong interaction between N-IC and SEL-10ΔF (*lane 2*). However, association between SEL-10ΔF and Notch LNG or N-ICΔCT (an intracellular form of Notch1 deleted of the 349 C-terminal amino acids including the PEST domain (7)) was barely detectable (*lanes 6* and *9*). These results support the data shown in *panel A* and suggest that SEL-10ΔF associates with and stabilizes N-IC but that neither the transmembrane inactive Notch, LNG, nor the C-terminally deleted N-ICΔCT significantly interact with SEL-10ΔF. Therefore, SEL-10ΔF acts as a dominant-negative form of SEL-10 inducing N-IC stabilization, whereas full-length SEL-10 does not have this effect (data not shown).

To further assess the specificity of SEL-10-Notch interactions, we used another F-box protein, β-TrCP, of the same Fbw family (41). We cotransfected increasing amounts of β-TrCPΔF, a dominant-negative form of β-TrCP, with a given amount of N-IC. No accumulation of N-IC was observed even at very high concentrations of β-TrCPΔF (Fig. 3*C*). Immunoprecipitation of β-TrCPΔF did not pull down any Notch (Fig. 3*C*), nor did the anti-Notch antibody precipitate any associated β-TrCP (not shown).

As the association between SEL-10 and Notch seems to be

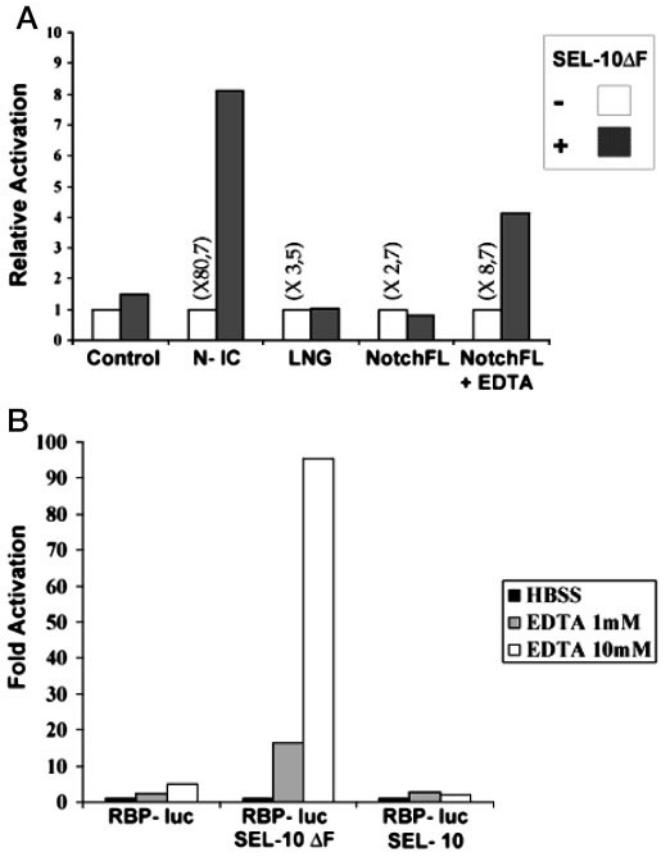

FIG. 4. **SEL-10ΔF enhances Notch1-induced transcriptional activation.** *Panel A*, HeLa cells were transfected with 500 ng of RBP-luciferase reporter gene along with 100 ng of different Notch derivatives as indicated below the histograms. One μg of control pcDNA3 plasmid (*blank bars*) or SEL-10ΔF (*dark bars*) was cotransfected in each case. In *lane NotchFL + EDTA* cells were incubated with 10 mM EDTA for 15 min 6 h before extraction. The luciferase activity was normalized to the value without SEL-10ΔF for each Notch construct. The *numbers* in *parentheses* indicate the actual transcriptional activation caused by each Notch construct compared with RBP-luciferase basal level. *Panel B*, HeLa cells stably transfected with Notch1 (HeLaN1) were transiently transfected with 500 ng of RBP-luciferase reporter gene alone or along with SEL-10ΔF or full-length SEL-10 constructs. Two days after transfection, cells were treated for 15 min with Hanks' balanced saline solution (*HBSS*) alone or containing 1 mM EDTA or 10 mM EDTA as indicated on the *right side* of the histogram. Cells were harvested 6 h after this treatment, and luciferase activity was measured and compared with the values without EDTA for each effector. Luciferase activity was normalized according to the β-galactosidase activity of the cotransfected EF1-β-galactosidase vector; each value corresponds to the average of at least four independent experiments.

specific, we determined whether SEL-10 could influence Notch-induced transcriptional activity.

SEL-10ΔF Enhances Notch-induced Transcriptional Activation—Notch transcriptional activity was first monitored by a reporter gene assay in transiently transfected HeLa cells. The reporter construct RBP-luciferase carries the luciferase gene under the control of a synthetic promoter containing 12 RBP-Jκ binding sites (36). The active Notch construct used here (Fig. 4*A*) is N-IC, which stimulates reporter gene activity 80-fold. No stimulation of the reporter gene was noticed with SEL-10ΔF (*Control lanes*). But when SEL-10ΔF was cotransfected with N-IC, transcriptional activation of the reporter gene was increased more than 8-fold. When inactive full-length (NotchFL) or LNG Notch constructs were used, no significant stimulation was observed, and addition of SEL-10ΔF had no effect. These experiments reveal that SEL-10ΔF enhanced the transcriptional activity of constitutively active Notch constructs only,

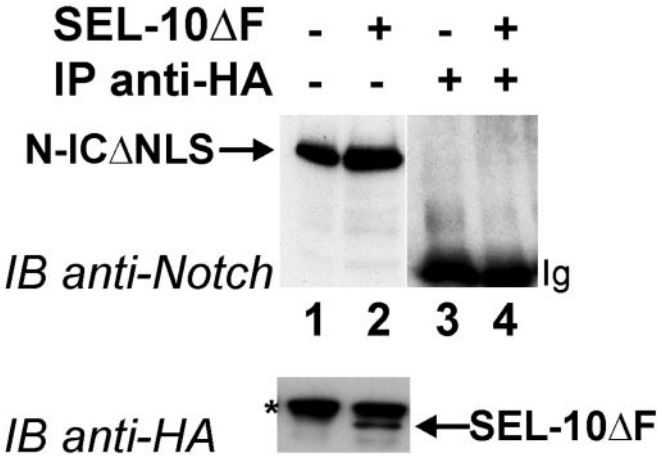

FIG. 5. **SEL-10ΔF does not interact with non-nuclear N-IC.** 293T cells were cotransfected with expression vectors encoding N-ICΔNLS with or without SEL-10ΔF. Cell extracts were analyzed by immunoblotting (*IB*) with the antibodies indicated on the *left*, directly (*lanes 1* and *2*) or after immunoprecipitation (*IP*; *lanes 3* and *4*) with anti-HA antibody. The *asterisk* corresponds to a nonspecific band; *Ig*, immunoglobulins.

but did not, on its own, induce any activation of the RBP promoter.

To mimic activation of the entire Notch1 receptor, HeLa cells transiently transfected with NotchFL and RBP-luciferase were treated with EDTA for 15 min; this treatment has been shown recently to induce the dissociation of the heterodimeric Notch1 receptor and to mimic activation (13). Notch-induced activation of the reporter gene was monitored 6 h later; an 8.7-fold stimulation of the reporter gene could be observed (Fig. 4*A*, *lane NotchFL + EDTA*). This activation was increased in a SEL-10ΔF dose-dependent manner (data not shown) up to 4-fold (Fig. 4*A*).

To measure the activity of SEL-10 under more physiological conditions, similar luciferase reporter gene assays were performed in stably Notch1-transfected HeLa cells, HeLaN1 (3). In these cells, EDTA induces a reproducible 2-fold increase in endogenous HES1 mRNA level as ascertained by Northern blot analysis (results not shown). When these cells were transfected with RBP-luciferase reporter plasmid no intrinsic stimulation was observed (Fig. 4*B*). But when these transfected cells were incubated with different concentrations of EDTA, a 2.4–5-fold activation was observed. This level was raised respectively to 16.4-fold (1 mM EDTA) and 95.5-fold (10 mM EDTA) when SEL-10ΔF was cotransfected with the reporter gene. As expected, when full-length SEL-10 was cotransfected with RBP-luciferase reporter gene no significant stimulation was detected; a small reproducible decrease (2.5-fold) in luciferase activity could even be observed at 10 mM EDTA (compare RBP-luc and RBP-luc/SEL-10 with 10 mM EDTA in Fig. 4*B*).

These experiments indicate that Notch activity is modulated by SEL-10 in a dose-dependent manner. As SEL-10ΔF seems to interact with the intracellular form of the Notch receptor, we investigated the subcellular localization of this interaction by using a non-nuclear form of N-IC.

SEL-10 Does Not Interact with a Non-nuclear Form of N-IC—The N-ICΔNLS protein carries a deletion of the three putative NLS and is predominantly localized to the cytoplasm (7). The level of N-ICΔNLS was not modified when cotransfected with SEL-10ΔF (Fig. 5, lanes 1 and 2), and immunoprecipitation of SEL-10ΔF did not pull-down any Notch (Fig. 5, *lanes 3* and *4*). Luciferase assays with N-ICΔNLS did not reveal any activation of the reporter gene, confirming previous results (7). Adding increasing amounts of SEL-10ΔF did not induce

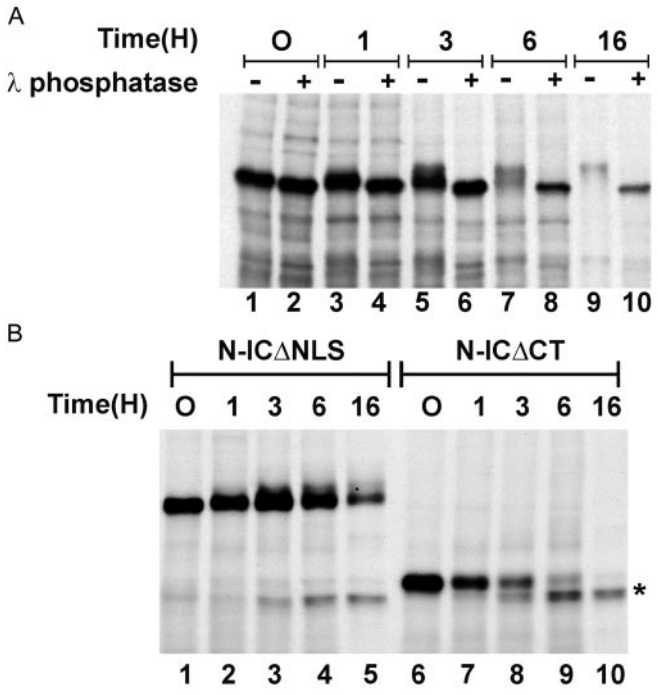

FIG. 6. **Pulse-chase analysis of N-IC, N-IC∆NLS, and N-IC∆CT.** 293T cells were transfected with expression vectors encoding N-IC (*panel A*) or the indicated N-IC derivatives (*panel B*). Cells were pulse labeled with [^{35}S]methionine for 15 min and chased for the indicated time (*H*, hours). Immunoprecipitated cell extracts were analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography (*panel A*, 7 days; *panel B*, 2 days). *Panel A*, Notch immunoprecipitates were treated (+) or not (−) with λ phosphatase. The *asterisk* in *panel B* indicates a nonspecific band.

any activation either (not shown). These results suggest that the Notch C-terminal domain is not sufficient for recognition of N-IC by SEL-10 (see Fig. 3*B*). As transfected SEL-10ΔF localizes to both cytoplasm and nucleus (not shown), the lack of interaction observed is probably caused by the absence of a specific modification of the N-IC protein when excluded from the nucleus.

So far, F-box proteins of the SCF family have been shown to bind only phosphorylated substrates. We therefore investigated whether the absence of interaction between N-ICΔNLS and SEL-10 might be caused by the lack of such event(s).

N-IC Is Extensively Phosphorylated—To study N-IC post-translational modifications and phosphorylation in particular, a pulse-chase radiolabeling experiment was performed in 293T cells transfected with N-IC (Fig. 6*A*). A progressive decrease in the mobility of N-IC was observed. λ phosphatase treatment of these species revealed at least two different phosphorylation events: a first phosphorylation was visible before 1 h of chase (first observed at 20 min, results not shown), and a second major shift occurred around 3 h. This latter band was still visible after 16 h.

When a similar pulse-chase experiment was performed on N-ICΔNLS-transfected cells, the second phosphorylation event did not occur (Fig. 6*B*). Interestingly, the N-ICΔCT protein showed no apparent phosphorylation.

When the N-ICΔNLS construct was modified by the addition of a NLS motif from SV40 (N-ICΔNLS+NLS), its nuclear translocation (7), its hyperphosphorylation after 3 h of chase (data not shown), and its association with SEL-10ΔF (Fig. 7, *lane 10*) were restored.

These phosphorylation events may therefore be crucial for SEL-10/Notch binding and thus account for the differences in interactions among N-ICΔNLS, N-ICΔCT, and N-ICΔNLS+NLS.

SEL-10 Only Binds to a Hyperphosphorylated Form of N-IC—To confirm the presence of N-IC phosphorylations, *in vivo* phosphate labeling experiments were carried out (Fig. 7, *lanes 1* and *2*). In the N-IC-transfected cells two discrete bands were observed, comigrating with the Notch immunoreactive bands seen by Western blot analysis (Fig. 7, compare *lane 1* with *lanes 3* and *4*).

To characterize the role of these phosphorylation events in Notch-SEL-10 interaction, 293T cells were cotransfected with N-IC and SEL-10ΔF. Treatment of immunoprecipitated Notch with λ phosphatase led to the replacement of the two bands (Fig. 7, *lane 4*) by a faster migrating band (*lane 5*) which represents the nonphosphorylated N-IC. When extracts of cells cotransfected with N-IC and SEL-10ΔF were immunoprecipitated with anti-HA, only the slowest migrating band of N-IC could be detected (Fig. 7, *lane 6*) and, here again, phosphatase treatment reduced it to the nonphosphorylated form (*lane 7*). The SEL-10-bound form likely corresponds to the second N-IC-phosphorylated form seen in *lane 1* and seems to result from a nuclear phosphorylation event. Indeed, no SEL-10 binding could be detected with N-ICΔNLS, but association was restored with the N-ICΔNLS+NLS slower migrating form (Fig. 7, *lane 10*), and N-ICΔNLS+NLS stabilization was observed with SEL-10ΔF (Fig. 7, *lane 9*).

This experiment suggests that only a specifically phosphorylated form of Notch associates with SEL-10.

Together with the pulse-chase analysis, these results show that, after its release from the membrane, N-IC is submitted to several phosphorylation events that give rise, in the nucleus, to a suitable substrate for degradation via the ubiquitin-proteasome pathway.

DISCUSSION

Notch signaling proceeds through a series of proteolytic steps that lead to the nuclear translocation of the intracellular part of the receptor. This molecule behaves as a transcriptional coactivator by associating with the DNA-binding subunit CSL (or RBP-Jκ). Previous data have indicated that the nuclear form of Notch is extremely difficult to detect, and this lack of detection has long been considered an argument against the processing model. The presence of nuclear forms of Notch have, however, been indirectly demonstrated through their transcriptional effects (26, 27, 42), although nuclear Notch immunoreactivity has been detected in cervical carcinomas (21, 28). Only recently has nuclear Notch immunoreactivity been detected in differentiating cortical neurons (43). This lack of detection suggests that the nuclear form of Notch is highly unstable. In *Drosophila*, a dominant-negative mutation of the β6 subunit of the proteasome was found to stabilize active forms of Notch (44). Indeed, proteasome inhibitors have been shown to have the same effect (Ref. 10 and our results), suggesting the involvement of the ubiquitin-proteasome pathway in this unstability. Recently, an F-box protein of the Cdc4 family, SEL-10, has been isolated in a genetic screen as a negative regulator of the Notch pathway in *C. elegans* (34) and has been shown to associate with the intracellular domain of *C. elegans* Lin12 and mammalian Notch4 in overexpression experiments. This F-box family of proteins is known to be part of multicomponent complexes called SCF, which are involved in the polyubiquitination of specific substrates, leading to their degradation by the proteasome. We therefore decided to determine whether mammalian SEL-10 plays a role in the unstability of the nuclear form of Notch1. We first cloned mouse and human SEL-10; the two mammalian proteins show more than 99% amino acid identity, and the human protein is 48% iden-

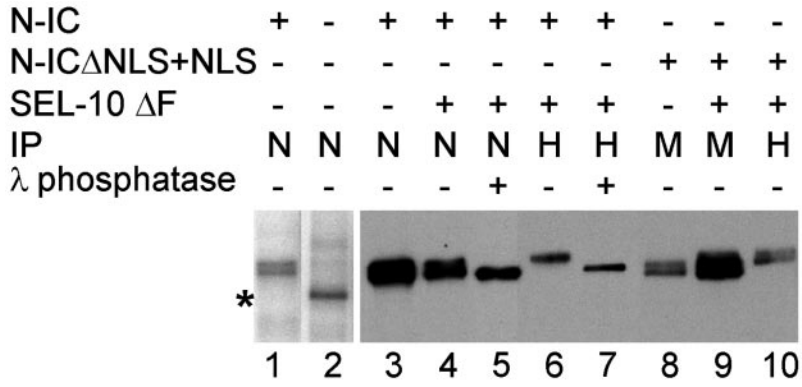

FIG. 7. **SEL-10 binds to a phosphorylated form of N-IC.** 293T cells were cotransfected with expression vectors encoding N-IC (*lanes 1* and *3–7*) or N-ICΔNLS+NLS (*lanes 8–10*) with (*lanes 4–7*, *9*, and *10*) or without SEL-10ΔF (*lanes 1–3* and *8*) as indicated. Control 293T cells were transfected with pcDNA3 alone (*lane 2*). *Lanes 1* and *2* represent anti-Notch immunoprecipitates from $^{32}P_i$ labeling. The *asterisk* indicates a nonspecific band that immunoprecipitates with anti-Notch antibody in control 293T cells (*lane 2*). In *lanes 3–10*, cell extracts were analyzed by immunoblotting with the anti-Notch antibody after immunoprecipitation with anti-Notch (*N*), anti-HA (*H*), or anti-Myc (*M*) antibodies. *Lanes 4* and *5* (− and + *phosphatase*) each represent half the amount of extract used in *lane 3*, explaining why the stabilizing effect of cotransfected SEL-10ΔF is not visible. However, this effect is clearly visible when comparing *lanes 8* and *9*.

tical to the *C. elegans* protein. The most conserved regions are the seven C-terminal WD40 repeats (56% identity), known to interact with the substrate. The N-terminal F-box interacts with the Skp1 subunit of the SCF complex and thus recruits the ubiquitination machinery.

We first demonstrated that the intracellular part of Notch1 can be polyubiquitinated in an ATP-dependent manner in a reconstituted *in vitro* assay that contains HeLa cell extract and ubiquitin. To confirm that SEL-10 does interact with Notch1, we cotransfected cells with N-IC and a derivative of SEL-10 deleted of the F-box, and we could indeed observe coimmunoprecipitation. But when N-IC was cotransfected with another Fbw protein, also deleted of the F-box, β-TrCP, no interaction could be detected. Interestingly, deletion of the C-terminal region of N-IC, including a putative PEST domain, almost completely abolished binding of SEL-10, suggesting that this region plays a major role in the interaction. An unexpected observation was that an inactive form of Notch1 (the LNG construct, a transmembrane derivative of Notch lacking 1,450 amino acids of the N-terminal region) did not interact with SEL-10, suggesting that SEL10 may only recognize nuclear and/or modified Notch1.

The next question was to determine whether SEL-10 plays a role during physiological activation of the Notch pathway. We took advantage of the SEL-10ΔF construct where the F-box has been deleted; as this type of construct binds to the substrate but not to the ubiquitination machinery, it can serve as a dominant-negative molecule and inhibit Notch1 ubiquitination. Cotransfection of a CSL/Notch-dependent reporter construct with different forms of Notch1 together with increasing amounts of SEL-10ΔF demonstrated a SEL-10ΔF-dependent increase in Notch-induced transcriptional activation. Although we demonstrated previously that Notch could be activated after coculture of Notch-expressing cells with Delta-expressing cells (19), we decided to use as a paradigm of Notch activation the treatment of Notch-expressing cells with EDTA (13). This treatment results in dissociation of the heterodimeric Notch molecule present at the cell surface and leads to the release of the intracellular part of the receptor and its nuclear translocation. One advantage of this technique is the reproducibility, the fact that 100% of the cells are theoretically stimulated and the possibility to carry out a precise time course analysis. In HeLa cells stably transfected with Notch1, EDTA induced transcriptional activation of Notch target genes, and this activation was strongly increased in the presence of SEL-10ΔF but not of full-length SEL-10. These results are in accordance with the postulated role of this molecule in Notch1-induced activation.

These data suggest that SEL-10 is involved in a degradation event that most likely takes place in the nucleus. To characterize this event in more detail, we used a derivative of N-IC where the putative NLSs have been deleted; this construct is mostly cytoplasmic and is inactive in our reporter gene assay and in a myogenesis inhibition test, an assay currently used to measure Notch activity (7). Interestingly, this construct no longer interacts with SEL-10ΔF, a molecule that is localized to cytoplasm and nucleus (data not shown). Therefore, this suggests that the lack of association is most likely caused by the lack of a nuclear specific modification of N-IC. Alternatively, the deletion of the three NLS motifs might have also deleted the relevant modification sites. Several groups have reported the existence of phosphorylated forms of Notch (45–48). To clarify this issue we carried out a pulse-chase analysis of N-IC in transfected 293T cells and could observe at least two phosphorylation events: one early event was visible at 1 h, and the second one only took place after 3 h. Interestingly, only the first modification was visible when the N-ICΔNLS was used, but adding a NLS to this molecule restored the second phosphorylation event as well as SEL-10 binding. The two phosphorylation events probably occur in the C-terminal part of N-IC because we do not observe them when analyzing the N-ICΔCT molecule (which is also localized to the nucleus). The phosphorylation patterns of these mutant molecules suggest that (i) the second phosphorylation event takes place in the nucleus and might be required for the interaction with the ubiquitination machinery; and (ii) the reason for the lack of phosphorylation of the N-ICΔNLS construct resides in its exclusion from the nucleus and not in the deletion of the relevant phosphorylation sites.

These results suggest a model according to which, following activation, the intracellular form of Notch is subjected to two phosphorylation events that depend upon its C-terminal region, the second one taking place in the nucleus and being required for targeting the molecule to the proteasome via SEL-10 binding and polyubiquitination. Since submission of the present study, Floyd *et al.* (49) demonstrated that the nuclear ubiquitin-proteasome system could account for the degradation of short lived nuclear proteins. In our case, the nuclear phosphorylation event appears crucial for Notch ubiquitination. We are currently trying to identify the phosphorylation sites as well as the responsible kinases.

It has been shown recently that *C. elegans* SEL-10 interacts with the SEL-12 protein, an ortholog of mammalian presenilins (50), and that loss of SEL-10 can suppress the egg-laying defect

phenotype associated with reduced SEL-12 activity. The authors also suggest that a PEST-like region of SEL-12 is involved in this interaction. Therefore the F-box protein, SEL-10, might play a role in at least two steps of the Notch signaling cascade. Experiments are in progress to characterize the role played by SEL-10 in presenilin stability. A recent report hypothesized that proteasome-mediated degradation might regulate the intracellular concentration of transcriptional activators (51) and that binding or recruitment to the DNA is an important element of this degradation. This apparently also applies to Notch, and we are currently testing whether mutating the region of interaction between N-IC and CSL somehow stabilizes the former molecule.

Recently another E3 ubiquitin ligase has been suggested to be involved in the degradation of Notch through the proteasome (52). The mammalian protein Itch is a member of the HECT family and has been shown to be able to ubiquitinate Notch and to bind to the N-terminal portion of the intracellular region of the molecule. Suppressor of Deltex, a *Drosophila* ortholog of Itch, had been shown previously to be a negative regulator of the Notch pathway (53). Are Itch and SEL-10 both involved in Notch degradation? Interestingly, Itch does not require the PEST domain to bind Notch, suggesting that it is probably involved in a different event. Besides, the authors use a membrane-attached constitutive Notch derivative and inhibit the proteasome with the drug MG132. That this drug is known to inhibit the γ-secretase-like cleavage that is required for nuclear translocation of N-IC suggests that Itch can bind to a membrane-attached form of Notch, irrespective of signaling. However, it is unclear whether a modification is required for this interaction to take place. It is therefore possible that two different E3 complexes control the stability of Notch, one in the cytoplasm, probably independently of signaling events, and a second one in the nucleus, following signaling.

Acknowledgements—We thank R. Kopan (St. Louis, MO) for the kind gift of the N-ICΔNLS and N-ICΔNLS+NLS constructs and T. Honjo (Kyoto, Japan) for the pGa981-6 plasmid.

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