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.

Notch 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 TNF-α-converting enzyme, 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-
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 (Homologous to E6-AP C Terminus), 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. Immunoprecipitation 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.

**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 calcium phosphate coprecipitation procedure and harvested 48 h later.

**Plasmids**—All Notch1 constructs reported here were cloned into the pcDNA3+ vector. Notch1 constructs, NotchFL, LNG, N-IC, were is gift of S. Whiteside. 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-ICANLS and N-ICANLS+-NLS were immunoprecipitated with anti-Myc (9E10) monoclonal antibody.

**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 untagged N-IC, slower migrating forms represent ubiquitin-conjugated N-IC (noted as [Ub]), slow migras forms represent ubiquitin-conjugated N-IC (noted as [Ub]).

**Cell Extracts, Immunoprecipitations, and Immunoblots**—Subconfluent 293T cells were transiently transfected with 250 ng of Notch1 derivatives and 4 μg of SEL-10ΔP or β-TrCPΔ 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 1X 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.
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**Results**

Ubiqutin-Proteasome Degradates 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. 1A, lane 2) or ALLN (Fig. 1A, 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. 1B 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 AI747952.
revealed 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, predicted protein was 627 amino acids long if the first in-frame methionine was considered). Sequence analysis with the whole human genome (htgs, NCBI, NIH) revealed that the human SEL-10 gene localizes to 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 conserved protein shared 48% identity with CeSEL-10 and 56% identity when considering the WD40 repeats domain only.

**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. 3A, compare lanes 1 and 2). This effect was not observed with the inactive membrane-anchored Notch LNG (Fig. 3A, compare lanes 1 and 2). 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. 3B). 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. 3C). Immunoprecipitation of β-TrCPΔF did not pull down any Notch (Fig. 3C), nor did the anti-Notch antibody precipitate any associated β-TrCP (not shown).

As the association between SEL-10 and Notch seems to be
activity was normalized to the value without SEL-10 F for each Notch construct. The value without SEL-10 F was observed, and addition of SEL-10 F had no effect. These experiments reveal that SEL-10 F enhanced the transcriptional activity of constitutively active Notch constructs only, 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. 4A, lane NotchFL + EDTA). This activation was increased in a SEL-10 F dose-dependent manner (data not shown) up to 4-fold (Fig. 4A).

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. 4B). 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-10 F 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. 4B).

These experiments indicate that notch activity is modulated by SEL-10 in a dose-dependent manner. As SEL-10 F 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 notch. The SEL-10 Does Not Interact with a Non-nuclear Form of notch—the N-IC-NLS protein carries a deletion of the three putative NLS and is predominantly localized to the cytoplasm (7). The level of N-IC-NLS was not modified when cotransfected with SEL-10 F (Fig. 5, lanes 1 and 2), and immunoprecipitation of SEL-10 F did not pull-down any notch (Fig. 5, lanes 3 and 4). Luciferase assays with N-IC-NLS did not reveal any activation of the reporter gene, confirming previous results (7). Adding increasing amounts of SEL-10 F did not induce
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Fig. 6. Pulse-chase analysis of N-IC, N-I\textsuperscript{C\textunderscore}\text{NLS}, and N-I\textsuperscript{C\textunderscore}\text{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 \(^{35}\text{S}\)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 \(\lambda\) 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. 3B). 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-I\textsuperscript{C\textunderscore}\text{ANLS} 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. 6A). A progressive decrease in the mobility of N-IC was observed. \(\lambda\) 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-I\textsuperscript{C\textunderscore}\text{ANLS}-transfected cells, the second phosphorylation event did not occur (Fig. 6B). Interestingly, the N-I\textsuperscript{C\textunderscore}\text{CT} protein showed no apparent phosphorylation.

When the N-I\textsuperscript{C\textunderscore}\text{ANLS} construct was modified by the addition of a NLS motif from SV40 (N-I\textsuperscript{C\textunderscore}\text{ANLS\textplus\textsuperscript{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-I\textsuperscript{C\textunderscore}ANLS, N-I\textsuperscript{C\textunderscore}ACT, and N-I\textsuperscript{C\textunderscore}ANLS\textplus\textsuperscript{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-10\textsuperscript{AF}. Treatment of immunoprecipitated Notch with \(\lambda\) 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-I\textsuperscript{C\textunderscore}ANLS, but association was restored with the N-I\textsuperscript{C\textunderscore}ANLS\textplus\textsuperscript{NLS} slower migrating form (Fig. 7, lane 10), and N-I\textsuperscript{C\textunderscore}ANLS\textplus\textsuperscript{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-J\textkappa). 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 \(\beta 6\) 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 instability. 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 instability 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-
tical to the \textit{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 communoprecipitation. 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-10F, 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-IC-NLS 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 \textit{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 be involved in the degradation of Notch through the proteasome-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 complexes control the stability of Notch, one in the cytoplasm, probably independently of signaling events, and a second one in the nucleus, following signaling.

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