c-Cbl Binding and Ubiquitin-dependent Lysosomal Degradation of Membrane-associated Notch1*

Regulation of Notch1 activity is critical for cell fate decisions and differentiation of skeletal myoblasts. We have employed the skeletal myoblast cell line C2C12 to study posttranslational regulation of Notch1 protein levels during myogenesis. Although the major degradation pathway of the activated intracellular Notch1 fragment appears to involve ubiquitination and degradation by the 26 S proteasome, we provide evidence for an alternative catalytic pathway where the endogenous, transmembrane form of Notch1 is targeted to the lysosomal compartment. Immunoprecipitation analysis revealed ubiquitin-dependent accumulation of transmembrane Notch1 protein after treatment with the lysosomal inhibitor chloroquine but not after treatment with various proteasome inhibitors. This finding was supported by the observation that the transmembrane form of Notch1 was tyrosine-phosphorylated and specifically co-precipitated with the ubiquitin ligase c-Cbl. Our data suggest a regulatory mechanism down-regulating Notch1 protein levels already at the cellular surface, possibly with consequences for Notch-dependent signal transduction during terminal differentiation processes.

A critical cellular signaling pathway important for decisions whether to differentiate or not consists of the evolutionary highly conserved members of the transmembrane Notch receptor family. Generation of the mature Notch receptor at the cell surface and eventually Notch1 activation itself requires at least three critical proteolytic cleavage steps. The 300-kDa Notch1 polypeptide precursor, respectively, is first processed by a furin-like protease activity in the Golgi apparatus yielding an N-terminal extracellular fragment and a C-terminal transmembrane fragment (N\textsubscript{TM}). These two subunits associate to form the heterodimeric, mature receptor at the cellular surface (1, 2). Signaling mediated by Notch-dependent pathways involves the interaction of the receptors with specific cell surface ligands of the Delta or Jagged families. It could be shown that the intracellular portion of Notch1 via a yet unknown mechanism (5, 6).

Studies of Notch1 function can be addressed particularly during formation of skeletal muscle structures, where myoblast cells coordinate through differentiation stages to give rise to fused myotubes, whereas other cells of the same origin remain undifferentiated to maintain a population of precursor cells. It has been reported that MyoD and MEF2c activity required for myogenesis can be inhibited by activated Notch1 via a yet unknown mechanism (5, 6). Tight down-regulation of the activity of proteins and transcription factors in particular is often achieved via ubiquitination and subsequent degradation, preferentially in the proteasome. Ubiquitination is the result of multienzyme processes, involving protein complexes of ubiquitin-activating and ubiquitin-conjugating enzymes and typically require ubiquitin-protein ligases, like c-Cbl, Itch, or Sel-10, that mediate the substrate specificity (reviewed in Refs. 7 and 8). Genetic and biochemical evidence in invertebrates has suggested that proteasomal degradation of activated forms of Notch may be required for the cessation of Notch signaling (9–11). In human Jurkat cells, cotransfection experiments indicated an interaction of the Hect-type ubiquitin ligase Itch with ectopically expressed Notch1 proteins (12).

Here we present data addressing a posttranslational regulation mechanism of endogenous Notch1 in the course of vertebrate myoblast differentiation. We provide evidence for the targeting of cell membrane-associated endogenous Notch1 (N\textsubscript{TM}) to the lysosomal protein degradation pathway. Furthermore, we demonstrate that posttranslational modifications of Notch1 include specific tyrosine phosphorylation and interaction with the ubiquitin-ligating protein c-Cbl as well as mono-ubiquitination. Thus, for the first time our data indicate a proteasome-independent degradation mechanism of Notch1 possibly down-regulating the activity of its membrane-bound form in differentiating cells.

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¶ The abbreviations used are: N\textsubscript{TM}, C-terminal transmembrane fragment; N\textsubscript{IC}, intracellular form of Notch 1; GST, glutathione S-transferase; PBS, phosphate-buffered saline; HA, hemagglutinin; USAB, a ubiquitin-specific antibody.
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EXPERIMENTAL PROCEDURES

Cell Culture—The mouse skeletal myoblast line C2C12 was grown in Dulbecco’s modified Eagle’s medium/Glutamax (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum, sodium pyruvate, and penicillin/streptomycin at 7.5% CO₂. For passaging, cells were kept at low densities, and medium was exchanged every other day. To induce terminal differentiation and fusion, cells were grown to confluency and then treated with a serum containing 2% horse serum. Extracts for Western blot analysis and immunoprecipitations were grown in parallel and prepared at indicated time points.

COS1 cells were grown in Dulbecco’s modified Eagle’s medium/Glutamax supplemented with 10% heat-inactivated fetal calf serum, sodium pyruvate, and penicillin/streptomycin at 7.5% CO₂.

Northern Blot Analysis—Total RNA was extracted from C2C12 cells stimulated to undergo fusion by serum withdrawal for 0–7 days. RNA was isolated using the RNAeasy protocol (Qiagen) according to the manufacturer’s instructions. 10 µg of total RNA was separated on 1% agarose gels and ethidium bromide-stained. RNAs were subsequently transferred overnight to a nylon membrane. Hybridization was carried out using the non-radioactive DIG System for Nucleic Acid Labeling and Detection (Roche Molecular Biochemicals) and an in vitro synthesized, digoxigenin-labeled murine Notch1-specific antisense probe (base pairs 5328–6958) as described in the manufacturer’s protocol.

Transient Transfections—Cells were plated at a density of 1.5 × 10⁵ cells in 6-well plates 24 h prior to transient transfections. A total of 5 µg of the indicated plasmid DNAs was transiently transfected using the SuperFect Reagent (Qiagen) according to the manufacturer’s instructions. 48 h post-transfection cells were washed twice in cold PBS, lysed for 15 min in 5 µg/ml EDTA, containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and the protease inhibitors aprotinin (15 µg/ml) (Sigma), leupeptin (5 µg/ml) (Sigma), pepstatin (10 µg/ml) (Sigma), Pefabloc (500 µg/ml) (Roche Molecular Biochemicals), and sodium orthovanadate (1 mM) (Sigma). Lysates were cleared by centrifugation and either subjected to Western blot analysis or to immunoprecipitations.

To generate the GST-Notch1 expression plasmid, the intracellular domain of TAN1 (human Notch1) was PCR-amplified using the vector construct pGCT-16 (16) as template. The PCR fragment was cloned to the 3’ end of the GST tag into the eukaryotic expression vector pEBG.

EDTA Treatment of C2C12 Cells—Confluent C2C12 cells, kept for 48 h in fusion medium, were washed with PBS and incubated for 15 min with 5 µM EDTA in PBS at 37°C. Control cells were incubated in PBS alone. The stimulation was followed by recovery for either 30 min or 4 h in fusion medium. Subsequently, cells were treated for Western blot analysis and immunoprecipitations as described below.

Proteasome and Lysosome Inhibition—Confluent C2C12 were kept for 48 h in fusion medium before proteasomal and lysosomal inhibitors were added for various stimulation periods. Inhibitors were resuspended either in Me₆SO or PBS and, if not indicated otherwise, were used as follows: proteasome inhibitor I at 3 μM final; MG132 at 5 μM final; clasto-lactacytin β-lactone at 2 μM final (all from Calbiochem). Chloroquine (Sigma) was used at a final concentration of 100 μM. Controls were either performed with equivalent volumes of Me₆SO for the proteasome inhibitors or with equivalent volumes of PBS for chloroquine stimulations.

Western Blot Analysis and Immunoprecipitation—Whole cell extracts were prepared from equivalent amounts of unstimulated and stimulated cells using a lysis buffer containing 1× PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and the protease inhibitors aprotinin (15 µg/ml) (Sigma), leupeptin (5 µg/ml) (Sigma), pepstatin (10 µg/ml) (Sigma), Pefabloc (500 µg/ml) (Roche Molecular Biochemicals), and sodium orthovanadate (1 mM) (Sigma). 5× Laemmli buffer, containing β-mercaptoethanol, was added to the lysates, and equal volumes were loaded per lane. Proteins were separated on 8% denaturing SDS-PAGE gels and were transferred to polyvinylidene difluoride membranes (Schleicher & Schuell). After blocking, the membranes were incubated with the indicated primary antibodies as follows: goat anti-Notch1, rabbit anti-c-Clu, mouse anti-phosphotyrosine (PY99; Santa Cruz Biotechnology) and monoclonal mouse anti-GST (Santa Cruz Biotechnology). The monoclonal mouse anti-HA antibody was a kind gift of Victor Wixler, Erlangen, Germany. After incubation for 2 h on ice in 50 µl of protein G-Sepharose beads (Pierce) were added to each sample and incubated overnight at 4°C with gentle agitation. Beads were washed four times, resuspended in 1× SDS loading buffer, and loaded immediately on denaturing 8% SDS-PAGE gels. Proteins were transferred and visualized as described above.

Preparation of Subcellular Fractions Enriched for Endosomal and Lysosomal Compartments—C2C12 cells were grown to confluency as described above and shifted for 48 h to Dulbecco’s modified Eagle’s medium containing 2% horse serum. Cells were washed 3 times with PBS and lysed in 2 ml of cold homogenization buffer (0.25 m sucrose; 25 mM KCl; 5 mM MgCl₂; 10 mM Hepes-NaOH, pH 7.4, supplemented with protease inhibitors) per 20-cm plate. After disruption of the cells in a Teflon-glasm homogenizer (Potter-Elvehjem), nuclei, membranes and other heavy organelles were pelleted by a 10-min centrifugation step at 1000 × g. Supernatants were then centrifuged at 3000 × g for 10 min, and the supernatants were removed again and centrifuged at 17,000 × g for 10 min to harvest the lysosome-enriched fraction. Pellets from the 1000 and 17,000 × g centrifugation steps were resuspended in RIPA buffer and analyzed on denaturing SDS-PAGE gels as described.

RESULTS

Notch1 Protein Levels Are Regulated During Differentiation of Myogenic Cells—Myogenic cells in culture are capable of proliferating in the presence of rich nutrients and undergo terminal differentiation and fusion after a shift to low serum levels. To analyze changes in Notch1 expression and protein levels during myoblast differentiation, C2C12 myoblasts were cultured in 10% serum, and terminal differentiation was induced by a shift to medium containing 2% horse serum. Although Notch1 mRNA (∼8.0 kb) was detectable up to 7 days after induction of terminal differentiation with a maximum at day 2 (Fig. 1A), the amount of N¹, representing a 120-kDa protein, appeared to be regulated more significantly (Fig. 1B). Western blot analysis with a Notch1-specific antibody revealed that Notch1 protein increased from low levels at day 0 and 1 to highest levels ∼48 h after induction of terminal myoblast differentiation via low serum cultivation. On days 3 and 4 Notch1 levels decreased slightly and reached low to undetectable levels within 7 days of terminal myoblast differentiation (Fig. 1B).

To demonstrate that the Notch1-specific signal shown in Fig. 1B represented the transmembrane anchored N¹ fragment, C2C12 cells were treated with 5 µM EDTA. EDTA treatment has been shown to mimic Notch-ligand binding, causing the release of the activated intracellular N¹C fragment (17). Using Notch1 specific antibodies in a Western blot demonstrated the emergence of a smaller 110-kDa protein fragment, when cells were treated with EDTA followed by a 30 min of recovery in medium (Fig. 2, lanes 5 and 7). This 110-kDa fragment, representing the activated N¹C protein, is not detectable when cells were incubated in PBS only (Fig. 2, lanes 1–4). The 110-kDa band disappeared when cells were allowed to recover for 4 h after EDTA treatment, very likely reflecting the rapid degradation of N¹C (Fig. 2, lanes 6 and 8). This analysis indicates that the Notch1 protein detectable in our experiments represented membrane-associated 120-kDa N¹M fragments.

N¹M Fragments Are Associated with a Ubiquitinated Protein Complex in C2C12 Cells—During terminal differentiation of the myogenic cell line C2C12, Notch1 protein levels were significantly reduced. As shown in Fig. 1B, Notch1 protein increased at early stages of myoblast fusion with the highest levels at day 2 and declined at later time points of differentiation to low to undetectable levels. Therefore, the question was
addressed whether N\textsuperscript{TM} is regulated posttranslationally via ubiquitination and subsequent proteolytic degradation. For this purpose immunoprecipitation assays were performed using antibodies specific for ubiquitin and Notch1 to determine whether endogenous N\textsuperscript{TM} is an integral part of ubiquitinated protein complexes or even ubiquitinated itself. As shown in Fig. 3A, lane 1, N\textsuperscript{TM} fragments can be precipitated and detected specifically by using a polyclonal Notch1 antibody. We were also able to pull down N\textsuperscript{TM} protein, employing a ubiquitin-specific antibody (USAB) in immunoprecipitation experiments (Fig. 3A, lane 2). The appearance of a single Notch1 band from lysates precipitated with USAB may either indicate mono-
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A. IP: m α-GST + - - - - - + +
   m α-HA - + - + - - - +

Transf.: GST N-IC - + - - - - - -
   HA-Ubi - + + + + + + +

WB: m α-GST

IP: DMSO: 1 3 6 hrs
   c-Lactacystin -L.: 1 3 6 hrs

GST N-IC 150 kDa

B. IP: rb α-N + - - - - - - -
   rb α-Ubi - + + + + + + +

Transf.: GST N-IC - + - - - - - -
   HA-Ubi - + + + + + + +

GST N-IC 150 kDa

FIG. 4. Ubiquitination of ectopic N1C in transiently transfected COS1 cells. To test ubiquitination of Notch1 in an independent experimental system, COS1 cells were transiently transfected with HA-tagged ubiquitin (HA-Ubi) and GST-tagged N1C expression vectors (GST-IC) either alone or in combination (lanes 1–8). Whole cell lysates of double transfected (Transf.1) cells were loaded in lane 9. The lanes indicate the ~150 kDa, GST-N1C-specific signal. A, cellular lysates of the differentially transfected COS1 cells were prepared and subjected to immunoprecipitations (IP) using anti-HA or anti-GST-specific antibodies. Western blot (WB) analysis was conducted using a mouse monoclonal GST-specific antibody (m α-GST). B, lysates of transfected COS1 cells were used for immunoprecipitations, using Notch1 (rb α-N) or ubiquitin-specific (rb α-Ubi) antibodies. Western blot analysis was conducted using a mouse monoclonal HA-specific antibody (m α-HA). Note: because the final amounts of transfected plasmid DNAs were matched in each experiment, lane 8 received half the amount of HA-tagged ubiquitin plasmid when compared with lane 4. This is reflected by the strength of the signal in the Western blot analysis.

of 150 kDa, whereas a mono-ubiquitinated Notch1-GST is only slightly larger, ~158 kDa. As shown in Fig. 4, the specific conjugation of HA-tagged ubiquitin to the GST-N1C could be verified in this experimental system. Specific signals for the GST-tagged Notch1 protein were detected only in COS1 cells transfected with GST-N1C alone or together with the HA-ubiquitin expression vector. Most importantly, as shown in Fig. 4A, lane 8, the GST-N1C corresponding band also appears when lysates from cotransfected cells were precipitated with the HA tag antibody and detected with an anti-GST antibody, indicating the conjugation of HA-ubiquitin to GST-N1C. Using a Notch1 antibody for precipitation of cotransfected COS1 cells, the positive reaction of the HA-tag specific antibody in the subsequent Western blot analysis confirms this finding (Fig. 4B, lane 7). The single band at ~150 kDa corresponding to GST-N1C in Fig. 4A, lane 8, as well as in Fig. 4B, lane 7, may indicate covalent coupling of a mono-ubiquitin subunit to a significant portion of the intracellular Notch1 domain.

N1M Fragments Are Targeted to a Chloroquine-sensitive Degradation Pathway—It was reported previously (18) that poly-ubiquitin chains, consisting of at least four ubiquitin polypeptides, target proteins for specific degradation by the proteasome. However, mono-ubiquitination was described for different transmembrane receptor proteins and discussed as a regulatory mechanism for the localization and activity of proteins (14, 19, 20). In particular, it is believed that mono-ubiquitination serves as a signal to trigger the internalization of membrane-bound proteins and to regulate the activity of components of the endocytotic/lysosomal machinery (reviewed in Ref. 21). To test our hypothesis that N1M is targeted either to the proteosomal or to the endosomal/lysosomal pathway of protein degradation in the course of terminal differentiation of myoblasts, confluent C2C12 cells were incubated in fusion medium for 2 days to enhance endogenous Notch1 synthesis. The cells were subsequently treated for various hours with different proteasomal inhibitors. Neither treatment with the highly specific proteasome inhibitor clasto-lactacystin β-lactone (c-Lacta β-L., lanes 4–6) nor with the proteasome inhibitor-1 nor with MG132 (data not shown) revealed significant accumulation of overall or USAB-precipitable N1M species. Remarkably, the inhibitor concentrations used were sufficient to induce enrichment of ubiquitinated protein species in general, as shown in Western blot analysis (data not shown). In addition, no accumulation of N1M fragments was detected during longer incubation times with these inhibitors (data not shown).

To investigate whether Notch1 might be targeted to the lysosomal/endosomal machinery of protein degradation, analogous experiments were performed, incubating C2C12 cells with the lysosomal inhibitor chloroquine for various time points (Fig. 5B). Noticeably, whereas the overall N1M levels appear not to change during incubation with chloroquine (Fig. 5B, lanes 4–6), a USAB-precipitable N1M subfraction appears to be strongly enriched within 6 h of chloroquine treatment (Fig. 5B, lanes 10–12), indicating that N1M is posttranslationally regulated by a chloroquine-sensitive pathway. Control incubation of C2C12 cells with PBS resulted in unchanged levels of N1M protein (Fig. 5B, lanes 1–3) and no enrichment of USAB-pre-
c-Cbl Coprecipitates with N\textsuperscript{TM} in C2C12 Myoblasts—It was reported previously (22) that various transmembrane receptor proteins are internalized by a c-Cbl-mediated receptor sorting which involves covalent attachment of ubiquitin, tyrosine phosphorylation events, and subsequent lysosomal degradation, thereby controlling the fate and signaling capacity of growth factor receptors. Detailed analysis of the protein motifs within the intracellular portion of the Notch1 protein revealed the presence of two potential c-Cbl-docking sites at the C terminus of the Notch1 protein, close to the PEST domain, mediating protein stability and turnover rates of proteins. The consensus sequences for c-Cbl-docking sites were reported to span the amino acid sequences YQGLP and YSSSP. Additionally, using the NetPhos 2.0 prediction program, the second potential c-Cbl-docking site (YSSSP) is predicted to become phosphorylated after activation by ligand binding. Furthermore, tyrosine phosphorylation is also a prerequisite for c-Cbl binding (24, 25). To investigate whether Notch1 is phosphorylated after activation by ligand binding, we treated C2C12 cells with the lysosomal inhibitor chloroquine (Fig. 8). This interaction strengthens the evidence that endogenous Notch1 and c-Cbl physically associated with NTM after stimulation with chloroquine (Fig. 7B). In this context, it is important to note that c-Cbl and N\textsuperscript{TM} have a nearly identical molecular mass of 120 kDa. The treatment of C2C12 cells with the lysosomal inhibitor chloroquine for 1, 3, and 6 h after the induction of terminal differentiation for 48 h showed increasing amounts of c-Cbl protein physically associated with N\textsuperscript{TM} after stimulation with chloroquine (Fig. 8). This interaction strengthens the evidence that the cell membrane-associated Notch1 (N\textsuperscript{TM}) can be targeted to the endosomal pathway of protein degradation, possibly mediated by the ubiquitinating enzyme c-Cbl and the attachment of ubiquitin residues.

**Notch1 Is Tyrosine-phosphorylated and Accumulates Upon Chloroquine Treatment of C2C12 Cells**—As described previously (25), receptor proteins, which are targeted to the endosomal protein degradation machinery, are often tyrosine-phosphorylated posttranslationally by a chloroquine-sensitive pathway, thereby leading to lysosomal degradation rather than being targeted to the proteasomal degradation machinery. In addition, using the NetPhos 2.0 Prediction Program, a potential tyrosine phosphorylation site (marked with a bold circle) was identified within the C-terminal c-Cbl docking motif.
Confluent C2C12 cells were induced to differentiate by serum withdrawal for 2 days and were subsequently treated for 1, 3, or 6 h with either the inhibitor chloroquine (100 μM) or an equivalent volume of PBS. A, cellular lysates were subjected to immunoprecipitation (IP) analysis with a phosphotyrosine-specific mouse monoclonal antibody (m α-P-Tyr). Immunoprecipitated proteins were resolved on denaturing 8% SDS-PAGE gels, and blots were developed with a goat anti-Notch1-specific polyclonal antibody (gt α-N). The left panel represents control-treated cells (lanes 1–3), and the right panel (lanes 4–6) shows chloroquine-treated cells. Lines indicate the 120-kDa NTM fragment. The asterisk indicates a band corresponding to the 300–330-kDa precursor form of Notch1. B, cellular lysates were subjected to immunoprecipitation analysis with a phosphotyrosine-specific mouse monoclonal antibody (m α-P-Tyr). Immunoprecipitated proteins were resolved on denaturing 8% SDS-PAGE gels, and blots were developed with a rabbit anti-c-Cbl antibody (rb α-c-Cbl). Lanes 1–3 represent control-treated cells, and lanes 4–6 show chloroquine-treated cells. WB, Western blot.

Also tyrosine-phosphorylated under these conditions, we applied a phosphotyrosine-specific polyclonal antibody (Fig. 9A). Only after treatment of terminally differentiating C2C12 cells with the endosomal inhibitor chloroquine an accumulation of tyrosine-phosphorylated NTM protein can be observed (Fig. 9A, lanes 4–6), whereas in lysates of PBS-stimulated C2C12 cells no tyrosine-phosphorylated NTM protein is evident (Fig. 9A, lanes 1–3). Confirming results were obtained in a reciprocal analysis when lysates of chloroquine-treated C2C12 cells were immunoprecipitated with Notch1-specific antibodies and analyzed in Western blots using the monoclonal phosphotyrosine-specific antibody (data not shown).

In addition, we analyzed whether c-Cbl is tyrosine-phosphorylated also and whether the levels of phosphorylated c-Cbl protein are regulated after treatment with the lysosomal inhibitor chloroquine. As shown in Fig. 9B, c-Cbl is tyrosine-phosphorylated, but the levels remain unchanged after treatment with either PBS (Fig. 9B, lanes 1–3) or chloroquine (Fig. 9B, lanes 4–6). From these data, it is conceivable that the NTM fragment becomes specifically tyrosine-phosphorylated. This feature might be recognized by the ubiquitin-protein ligase c-Cbl leading to ubiquitination and subsequent targeting of Notch1 to the endosomal/lysosomal protein degradation machinery. This may contribute to the termination of Notch1-mediated signal transduction in the course of terminal differentiation of myoblast cells.

**Discussion**

Ubiquitination and subsequent degradation of cellular proteins, especially of receptors and transcription factors, serves as a major mechanism to regulate their activity. Several lines of evidence from the literature have suggested a connection between Notch and the ubiquitin/proteasome-dependent degradation pathway. In Drosophila melanogaster, the E3 class ubiquitin-protein ligase **Suppressor of deltex** has been shown to impact the stability of Notch and the processing of its target genes.
interact specifically with Notch and is capable of suppressing its activity (10). Furthermore, dominant-negative mutations in different proteasome subunits do affect cell fate decisions in Drosophila with an enhancement of Notch signaling activity (11). Finally, Itch, a mouse ortholog of Suppressors of deltex interacts specifically with Notch1 in mice (12). In addition, Gupta-Rossi et al. (26) demonstrated very recently that the mammalian ubiquitin-ligase SEL-10 could bind to an activated form of Notch1 protein, overexpressed in 293T and HeLa cells. Nevertheless, these studies rather suggest a role for ubiquitination and proteasomal degradation of activated Notch, possibly taking place in the nucleus.

To address the biological relevance of ubiquitination for the stability of the transmembrane Notch1 protein during terminal differentiation processes in myogenesis, we have employed the myoblastic cell line C2C12 that allows the analysis of Notch-dependent differentiation leading from mononucleated precursor cells to multinucleated, terminally differentiated myotubes. We were interested whether the endogenous Notch1 receptor protein is regulated at the cellular periphery before being activated and translocated into the nucleus.

Recently, various experimental settings have demonstrated that ubiquitination of certain transmembrane receptors, particularly of receptor tyrosine kinases, serves as a signal for their targeting to the endosomal/lysosomal compartment where they may undergo proteolytic degradation (22, 27–29). Here, ubiquitination is not only discussed in the context of protein degradation but also in connection with the internalization and trafficking of integral receptors where mono-ubiquitination itself can serve as an internalization signal (15, 20). Our results of the transient cotransfection experiments in Fig. 4 indicate such mono-ubiquitination of the ectopically expressed and GST-tagged version of the Notch1 protein. In this context, it is noteworthy that internalization of transmembrane receptors may not necessarily require ubiquitination of the receptor itself but may also depend on associated proteins of the ubiquitination machinery, which mediate the critical signal for receptor internalization and degradation (30).

To distinguish between lysosomal and proteasomal pathways of degradation, we were using either the lysosomal inhibitor chloroquine, which blocks endosomal protein degradation by inhibiting the acidification of the lysosomes or various proteasome inhibitors. The latter included the highly specific proteasome inhibitor clasto-lactacystin β-lactone. Incubation with proteasome inhibitors did not result in significant changes of NTM protein levels (Fig. 5A). Only treatment of C2C12 cells with the lysosomal inhibitor chloroquine leads to a significant accumulation of ubiquitin-associated NTM protein (Fig. 5B, lanes 10–12). These findings provide strong evidence that NTM fragments are targeted preferentially to the lysosomal/endosomal machinery of protein degradation rather than to the 26S proteasome-mediated proteolytic pathway. In our hands and as described previously (31–33), proteasomal inhibitors as well as chloroquine are capable of blocking differentiation and fusion of myogenic cells or increasing cell death rates after prolonged incubation. Nevertheless, short term exposure to these substances has been proven to be sufficient in many cellular systems, leading to the accumulation of otherwise degraded proteins (31–33)³.

The proto-oncogenic ubiquitin-ligase c-Cbl (34) plays a pivotal role in the context of ubiquitination and targeting of receptors to the endosomal/lysosomal pathway (22, 29). The 120-kDa c-Cbl polypeptide chain contains a phosphotyrosine recognition domain, an SH2 motif, and a RING finger ubiquitin ligase domain that recruits E2 ubiquitin-conjugating enzymes (35). c-Cbl acts as a negative regulator for several growth factor receptors, including the receptors for the epidermal growth factor, the platelet-derived growth factor, and the colony-stimulating factor-1 (22, 29, 36). It was reported that c-Cbl plays a key role for their ubiquitination and degradation in the course of endosomal/lysosomal sorting, thereby representing a key mechanism for the removal of these receptors from the cell surface. Consequently, this may reduce the potential of receptor-mediated signaling within the cell. The notion of c-Cbl-dependent modulation of Notch1 function is strongly supported by the presence of two potential docking sites for c-Cbl (23, 24) within the C-terminal part of the intracellular portion of the Notch1 protein. Furthermore, we have shown that c-Cbl specifically interacts with Notch1, and we propose that this interaction is mediated via binding of c-Cbl to a tyrosine-phosphorylated form of the NTM protein. Interaction of c-Cbl with target receptors apparently occurs in response to their tyrosine phosphorylation and requires phosphorylation of c-Cbl itself (25).

It is apparent from Fig. 9A that the full-length prepro-form of Notch1 appears to be constitutively tyrosine-phosphorylated. This may indicate that tyrosine-specific phosphorylation events take place very early after the synthesis of the full-length precursor Notch1 protein, even before the receptor is present on the cell surface. It is tempting to speculate that phosphorylation patterns of the full-length Notch1 precursor protein might be required for specific proteolytic processing events resulting in the formation of an active Notch1 receptor complex consisting of N-terminal extracellular fragment and NTM and the formation of the activated intracellular form of Notch1 (NICD). Alternatively, phosphorylation may be a requirement for specific intracellular trafficking of the Notch precursor protein. Further analysis of this particular observation will be addressed in our ongoing research.

Based on these data we postulate a novel mechanism for the regulation of Notch1 activity during terminal differentiation of myoblast cells. Lysosomal degradation appears to be an important regulatory mechanism to ensure a timely limited activity of the developmental essential protein Notch1. Dysregulation of Notch1 activity may result in formation of tumors as it was described in the hematopoietic cell lineage where a translocation of the intracellular portion of Notch1 leads to constitutive activity of the protein and to the formation of acute lymphocytic T cell leukemias (16).

Removal of Notch1 from the cell surface by endocytosis may allow cells to regulate their predisposition to respond to Notch ligands present in their vicinity. Endocytosis of Notch1 may regulate both the extent of a receptor-mediated response and the specificity of the response. For example, it was described that a short duration of signaling of receptor tyrosine kinases in the cell line PC12 promotes proliferation, whereas a longer duration of the signal results in differentiation (37). Thus, changing the signaling kinetics and the signaling magnitude of receptor proteins might be an important parameter in the regulation of cellular responses. In this regard, it is of special interest whether the regulation of Notch1 observed in C2C12 myoblasts represents a cell type-specific feature or whether cessation of Notch1 function via ubiquitination and endosomal degradation is also detectable in other systems. Future research should be addressed to characterize this system in a broader range of cells and tissues.

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³ B. M. Jahn, I. Dittert, S. Beyer, K. v. d. Mark, and W. Bielke, unpublished observations.
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