

Degradation of the Id2 developmental regulator: targeting via N-terminal ubiquitination

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Received 19 December 2003

Abstract

Degradation of cellular proteins via the ubiquitin–proteasome system (UPS) involves: (i) generation of a substrate-anchored polyubiquitin degradation signal and (ii) destruction of the tagged protein by the 26S proteasome with release of free and reusable ubiquitin. For most substrates, it is believed that the first ubiquitin moiety is conjugated to a ϵ -NH₂ group of an internal Lys residue. Recent findings indicate that for several proteins, the first ubiquitin moiety is fused, in a linear manner, to the free α -NH₂ group of the protein. Here, we demonstrate that the inhibitor of differentiation (or inhibitor of DNA binding) 2, Id2, that downregulates gene expression in undifferentiated and self-renewing cells, is degraded by the UPS following ubiquitination at its N-terminal residue. Lysine-less (LL) Id2 is degraded efficiently by the proteasome following ubiquitination. Fusion of a Myc tag to the N-terminal but not to the C-terminal residue of Id2 stabilizes the protein. Furthermore, deletion of the first 15 N-terminal residues of Id2 stabilizes the protein, suggesting that this domain serves as a recognition element, possibly for the ubiquitin ligase, E3. The mechanisms and structural motives that govern Id2 stability may have important implications to the regulation of the protein during normal differentiation and malignant transformation.

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The ubiquitin–proteasome system (UPS) targets for specific degradation numerous cellular regulatory proteins, including oncoproteins and tumor suppressors, transcriptional activators and inhibitors, ER and cell surface receptors, cell cycle regulators, and abnormal/misfolded/mutated proteins. Formation of ubiquitin conjugates requires the sequential action of three enzymes: (i) the ubiquitin-activating enzyme, E1, (ii) one of several ubiquitin-carrier proteins, E2s (known also as ubiquitin-conjugating enzymes, UBCs), and (iii) a member of the ubiquitin–protein ligase family, E3, to which the substrate is specifically bound. E3s catalyze the last step in the conjugation reaction, covalent attachment of ubiquitin to the substrate. Therefore, they play an important role in the proteolytic process as they endow the system with the high specificity towards its

numerous substrates. Successive conjugation of additional activated ubiquitin moieties to the previously attached ones generates the polyubiquitin chain that serves as a degradation signal for the proteasome (for recent reviews on ubiquitin-mediated proteolysis, see, for example [1–3]).

In most cases, the first ubiquitin molecule is transferred to a ϵ -NH₂ group of an internal Lys residue of the target protein. However, targeting of the myogenic transcription factor MyoD [4], the Epstein–Barr virus (EBV) latent membrane proteins 1 (LMP1 [5]) and 2A [6], the human papillomavirus 16 (HPV 16) E7 oncoprotein [7], and p21^{waf1/cip1} [8] appears to involve initial ubiquitination at the free N-terminal residue followed by synthesis of a “canonical,” Lys⁴⁸-polymerized, ubiquitin chain anchored to Lys⁴⁸ of the N-terminally conjugated ubiquitin. Thus, unlike many known substrates of the UPS, degradation of these proteins does not require any internal Lys residue. It is important to note,

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however, that all these proteins contain internal lysine residues, but nevertheless appear to use their N-terminal residue as the ubiquitin chain acceptor.

N-terminal ubiquitination is a novel pathway, distinct from the N-end rule pathway [9]. In the latter, the N-terminal residue serves as a recognition and binding motif to the ubiquitin ligase, E3 α , however, ubiquitination occurs on an internal lysine(s). In contrast, in the N-terminal ubiquitination pathway, modification occurs on the N-terminal residue, whereas recognition of the E3 probably involves a downstream motif. It should be mentioned that in yeast, using the model fusion protein ubiquitin-Pro- β -galactosidase, a new proteolytic pathway was described, designated the ubiquitin fusion degradation (UFD) pathway [10]. The stably fused ubiquitin moiety (that in the exceptional case when Pro is the linking residue is not removed by ubiquitin C-terminal hydrolases) functions as a degradation signal, where its Lys⁴⁸ serves as an anchor for the polyubiquitin chain. This pathway involves several enzymes, UFD 1–5, some of which appear to be unique and are not part of the “canonical” UPS. It is possible that N-terminal ubiquitination is the most upstream event in the UFD pathway that was discovered using a stable chimeric ubiquitin–protein model substrate: N-terminal ubiquitination, in contrast, is the native mode of ubiquitination that provides substrates to the UFD pathway.

The helix–loop–helix (HLH) family of transcriptional regulators plays key roles in a wide array of developmental processes. They can be divided into different subgroups based on their structure, expression pattern, and DNA-binding activity [11]. The E proteins are ubiquitously expressed and they can bind DNA as homodimers or as heterodimers with tissue-specific basic HLH transcription factors. Nearly all HLH proteins possess a region of highly basic residues adjacent to the HLH domain, which facilitates binding to DNA sequences that contain the canonical ‘E-box’ recognition motif [12]. However, a distinct subfamily of HLH proteins, the inhibitors of differentiation or inhibitors of DNA binding (Id) proteins, lacks the basic DNA-binding domain [13]. The four mammalian Id proteins, Id1–Id4, form transcriptionally inactive heterodimers, primarily with E proteins, and thereby prevent the E proteins from forming functional heterodimers with tissue-specific bHLH proteins. Hence, Id proteins act as dominant-negative regulators of bHLH proteins and repress transcription [12,13].

Id proteins negatively regulate gene expression during cell development and differentiation, and play complex roles in mammalian cell fate determination. In general, their expression is highest in undifferentiated, self-renewing cell populations, and is downregulated as cells exit cell cycle and differentiate terminally [14–17]. Id proteins are essential for embryogenesis and for cell cycle progression [18]. Quiescent cells express low/un-

detectable levels of Id gene products. However, following mitogenic stimulation, Id expression is rapidly induced. After an initial decline, Id expression is sustained throughout the G1 phase and is further upregulated as cells enter the S phase [18]. Id proteins function in cell cycle control by binding to important cell cycle regulators other than bHLH proteins. Thus, they bind, for example, to the Retinoblastoma tumor suppressor (pRB) and abolish its growth-suppressing activity [19]. In light of the many roles Id proteins play, it was not surprising to find that their dysregulation has been implicated in the pathogenesis of tumor growth, vascularization, invasiveness, and metastasis [14–16].

Regulation of the level and activity of the Id proteins provides the cell with an important mechanism to control the balance between active and inactive dimers, allowing fine tuning of the activities of various bHLH transcription factors. Id proteins can be regulated at multiple levels, i.e., transcriptional regulation, post-translational modification, and protein stability. It was reported that Id2 and Id3 are phosphorylated in late G1 ([20,21]; see Discussion). Id3 is a short-lived protein and is degraded by the UPS [22]. Here, we demonstrate that Id2 is a novel substrate of the ubiquitin pathway that is targeted for degradation via N-terminal ubiquitination.

Materials and methods

Materials

Materials for SDS-PAGE and Bradford reagent were from Bio-Rad. Mixture of L-[³⁵S]methionine/cysteine for metabolic labeling, L-[³⁵S]methionine for in vitro translation, M.W. markers, and DEAE-dextran were purchased from Amersham-Pharmacia Biotech. Tissue culture sera and media were from Sigma. Antibodies against Id2 and His tag were from Santa Cruz and Qiagen, respectively. *N*-Carbonyloxy-Leu-Leu-Leucinal (MG132), clasto-lactacystin β -lactone, and protease inhibitor cocktail I were from Calbiochem. Ubiquitin, dithiothreitol (DTT), adenosine-5'-triphosphate (ATP), phosphocreatine, creatine phosphokinase, [Tris(hydroxymethyl)amino-methane] (Tris buffer), and 2-deoxyglucose were from Sigma. Methylated ubiquitin and ubiquitin aldehyde were from Affiniti-Research. Hexokinase, adenosine-5'-*O*-(thiotriphosphate) (ATP γ S), immobilized Protein A, and Fugene 6 transfection reagent were from Roche Molecular Biochemicals. Reagents for enhanced chemiluminescence were from Pierce. Wheat germ extract-based transcription–translation coupled kit (TNT) was from Promega. Restriction and modifying enzymes were from New England Biolabs. Oligonucleotides were synthesized by Sigma.

Methods

Cell lines. Cos7/5 cells grown at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) were transiently transfected with the indicated cDNAs using either the DEAE-dextran method [23] or the Fugene 6 transfection kit. A31N and A31N-ts20 E1 mutant cells [24] grown at 32°C in DMEM supplemented with 10% FCS were transiently transfected with a cDNA coding for WT Id2 using the Fugene 6 kit.

Plasmids and construction of mutant cDNAs. WT Id2 cDNAs were subcloned from pCDNA3.1 into the *Bam*HI site of the mammalian

expression vectors pCS2 and pCS2 + MT (for expression of 6× Myc-tagged Id2s). These vectors were used for both *in vitro* translation (under the control of SP6 RNA polymerase) and expression in cells. In the case of 6× Myc tagging, PCR products were digested with *Xho*I and *Xba*I for N-terminal tagging and with *Bam*HI for C-terminal tagging, and ligated into the pCS2 + MT vector. In-frame insertion of 1× Myc tag in the N- or C-terminus of Id2 was carried out using the pCDNA3.1 vector and the appropriate PCR primers. PCR products were digested with *Bam*HI and *Xba*I for N-terminal tagging and with *Hind*III and *Kpn*I for C-terminal tagging. Lysine-less (LL) Id2 was generated via site-directed mutagenesis using the QuickChange kit (Stratagene). Deletion of the first 15 (Δ 15) N-terminal amino acid residues of Id2 was carried out using PCR and specific primers. PCR products were digested with *Bam*HI and *Xba*I, and ligated into the pCDNA3.1 vector. Sequences of all constructs were confirmed using an automatic sequencing system (ABI 310).

Preparation of crude cell extract. HeLa cell extract was prepared by hypotonic lysis as described [25].

Conjugation and degradation of Id2 in a cell-free reconstituted system. The different Id2 cDNAs were translated *in vitro* in the presence of [³⁵S]methionine using wheat germ coupled transcription–translation extract and SP6 RNA polymerase. Conjugation and degradation assays in cell-free reconstituted systems were carried out as described [26]. Briefly, reaction mixtures contained in a final volume of 12.5 μ l: 50 μ g HeLa cell extract proteins, 5 μ g ubiquitin, and ~25,000 cpm of *in vitro* translated and labeled Id2. Reactions were performed in the presence of 0.5 mM ATP and an ATP-regenerating system (10 mM phosphocreatine and 5 μ g phosphocreatine kinase), or ATP γ S (5 mM) as indicated. For depletion of ATP, 0.5 μ g hexokinase and 20 mM deoxyglucose were added. When indicated, the chain terminator methylated ubiquitin (MeUb [27]) was added at 2.5 μ g. Conjugation assays contained, in addition, 0.5 μ g of the isopeptidase inhibitor ubiquitin aldehyde (UbAl [28]). Degradation reactions were carried out at 37 °C for 2 h, whereas conjugation assays were incubated at 37 °C for 1 h. Reactions were terminated by the addition of sample buffer and resolved by SDS–PAGE (15%). Id2 and Id2–ubiquitin conjugates were visualized by PhosphorImager (Fuji, Japan).

Stability of proteins *in vivo*. Cellular stability of Id2 proteins was monitored in a pulse-chase labeling and immunoprecipitation experiments as described [25]. When using the A31N and A31N-ts20 E1 mutant cells [24], the cells were either shifted to 39 °C or incubation was continued at 32 °C, as indicated, for the night before the experiment and throughout the experiment. For labeling, cells were washed and incubated in a medium that lacks methionine and cysteine for 1 h, and labeled (pulse) for additional 30 min with a mixture of [³⁵S]methionine and cysteine (0.1 mCi/ml). Chase was carried out for the indicated times in a complete medium containing 2 mM methionine and cysteine. The proteasome inhibitors, MG132 (20 μ M) or clasto-lactacystin β -lactone (10 μ M), were added 30 min prior to the beginning of labeling and were present throughout the experiment. Following labeling, cells were harvested at the indicated times, lysed, and the labeled proteins were precipitated using anti-Id2 antibody. Immune complexes were collected using immobilized protein A. Following SDS–PAGE (15%), proteins were visualized by a PhosphorImager.

Protein concentration. Protein concentration was determined according to Bradford [29] using BSA as a standard.

Results

Id2 is conjugated to ubiquitin and degraded in an ATP-dependent manner in a cell-free system

To study the mechanisms that underlie the degradation of Id2, we reconstituted a cell-free system for

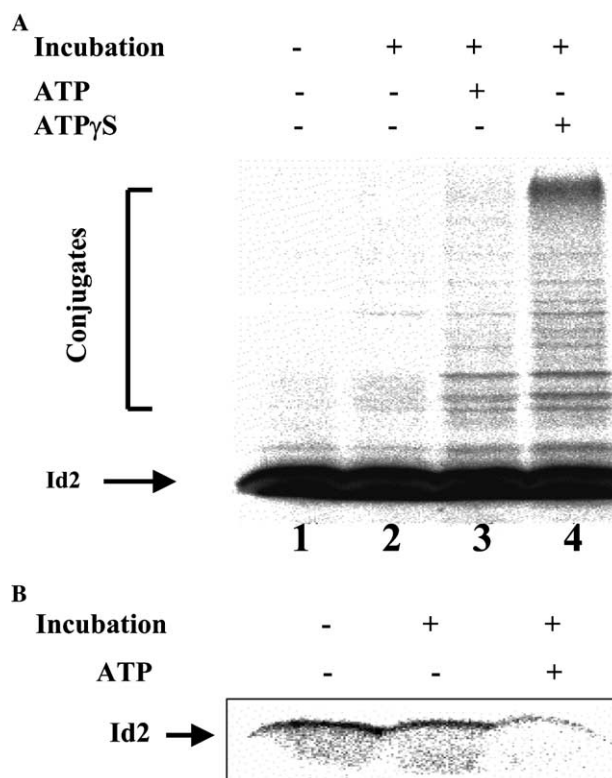


Fig. 1. Id2 is conjugated to ubiquitin and degraded *in vitro* in an ATP-dependent manner. (A) Id2 is conjugated to ubiquitin. Conjugation of *in vitro* translated and [³⁵S]methionine-labeled Id2 was carried out in a cell-free reconstituted system that contained HeLa cell extract as described under Materials and methods. ATP or ATP γ S was added as indicated. (B) Degradation of Id2 in a cell-free system requires ATP. Degradation of *in vitro* translated and labeled Id2 was monitored in a cell-free reconstituted system that contained HeLa cell extract as described under Materials and methods. ATP was added as indicated. Following incubation, reactions were resolved and visualized as described under Materials and methods.

conjugation and degradation of Id2. As can be seen in Fig. 1A, Id2 is conjugated efficiently in crude HeLa cell extract (used as a source for ubiquitin system enzymes) in the presence of ATP or ATP γ S. The latter nucleotide can support the activity of E1 (in which the α - β bond is utilized), but not the activity of the 26S proteasome that requires cleavage of the β - γ bond [30]. Also, it cannot catalyze phosphorylation. As can be clearly seen, ATP γ S stimulates polyubiquitination more efficiently than ATP (compare lane 4 to lane 3), suggesting that phosphorylation of Id2 is not required for its modification by ubiquitin (see Discussion). As can be seen in Fig. 1B, degradation of Id2 *in vitro* requires ATP, suggesting that it is mediated by the UPS, as both ubiquitination and proteasomal degradation require energy.

Degradation of Id2 in cells is mediated by the proteasome

Since in a cell-free system Id2 is ubiquitinated and degraded in an ATP-dependent manner, it was impor-

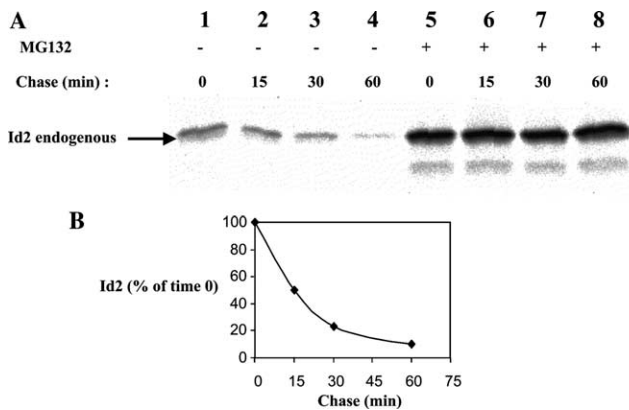


Fig. 2. Id2 is short-lived and its degradation is mediated by the 26S proteasome. (A) Proteasome inhibitors stabilize cellular Id2. The stability of Id2 was monitored in a pulse-chase labeling and immunoprecipitation experiment in the absence (lanes 1–4) or presence (lanes 5–8) of the 26S proteasome inhibitor MG132 as described under Materials and methods. (B) Half-life of Id2 is ~15 min. Data from three independent experiments, similar to the one depicted in (A), were quantified and plotted as percentage of the signal at time 0.

tant to test whether it is subjected to proteasomal degradation. To that end, we followed the stability of the protein in cells in the absence or presence of the specific proteasome inhibitor MG132. As can be seen in Fig. 2A, Id2 is short-lived (lanes 1–4) and its degradation is mediated by the proteasome: addition of a proteasomal inhibitor stabilized the protein (lanes 5–8). Measurement of its stability revealed that the half-life of the protein in Cos7/5 cells is ~15 min (Fig. 2B).

Degradation of Id2 requires a functional E1

To establish a causative linkage between ubiquitination and degradation, which are not necessarily linked, we monitored the stability of Id2 in A31N-ts20 cells that harbor a mutant temperature-sensitive E1 [24]. As can be clearly seen in Fig. 3, in the parent A31N cell, Id2 was degraded efficiently at both the permissive and non-permissive temperatures. In striking contrast, in

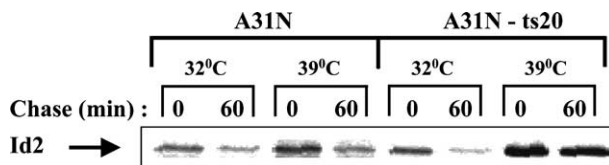


Fig. 3. Stabilization of Id2 at the restrictive temperature in the ubiquitin-activating enzyme, E1, ts mutant cell A31N-ts20. A31N or A31N-ts20 cells were transiently transfected with a vector (pCDNA3.1) that contains the Id2 cDNA. Sixteen hours before the experiment, cells were either shifted to 39°C or incubation was continued at 32°C. Cells were labeled with [³⁵S]methionine, chased with unlabeled medium, and harvested and lysed as described under Materials and methods. Id2 was immunoprecipitated and the labeled protein was resolved via SDS-PAGE and visualized as described under Materials and methods.

A31N-ts20 cells, degradation of the protein was efficient at the permissive temperature, but was essentially arrested at the non-permissive temperature, thus demonstrating that a functional E1 is essential for its degradation.

Lysine-less Id2 is degraded by the ubiquitin system

To establish the possible involvement of the N-terminal residue of Id2 in its ubiquitination, we replaced all nine Lys residues with Arg and generated a lysine-less (LL) Id2 protein (Id2 K2,12,26,45,47,57,58,61,129R). The stability of the LL protein was monitored in cells using a pulse-chase labeling and immunoprecipitation experiment. As can be seen in Fig. 4A, the LL protein was degraded almost as efficiently as the WT protein (compare to Fig. 2A, lanes 1–4). Also, similar to the WT protein, degradation of LL Id2 is mediated by the proteasome: addition of MG132 or clasto-lactacystin β-lactone stabilizes the LL Id2 (Fig. 4B; compare to Fig. 2A, lanes 5–8).

At that point it was important to demonstrate whether LL Id2, similar to the WT protein, is also ubiquitinated. As can be seen in the experiment depicted in Fig. 5, incubation of labeled LL Id2 in a cell-free reconstituted system generates a polyubiquitin chain in the presence of ATPγS (lane 7). In addition, we used the methylated derivative of ubiquitin (MeUb) that can modify the target protein only once and serves as a chain terminator [27]. As can be clearly seen in Fig. 5, both the WT and the LL proteins generate a mono-ubiquitin adduct (lanes 4 and 8, respectively). As we have reported for MyoD [4], LMP1 [5], and E7 [7], the amount of

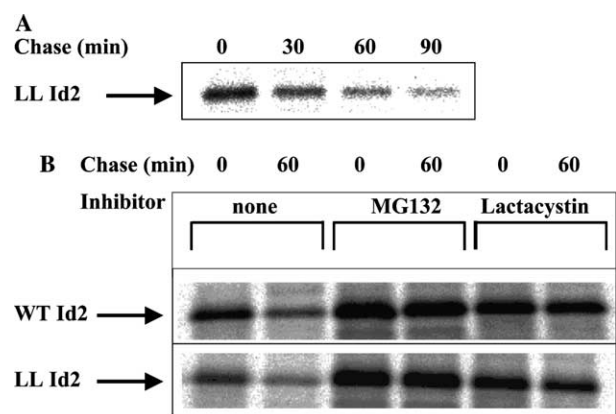


Fig. 4. Lysine-less (LL) Id2 is rapidly degraded by the proteasome. (A) LL Id2 is short-lived in cells. Degradation of LL Id2 in Cos7/5 cells was monitored in a pulse-chase labeling and immunoprecipitation experiment as described under Materials and methods. (B) Degradation of Id2 is inhibited by proteasome inhibitors. Stability of WT and LL Id2s was monitored in a pulse-chase labeling and immunoprecipitation experiment in the absence or presence of the 26S proteasome inhibitors MG132 or lactacystin as indicated and as described under Materials and methods.

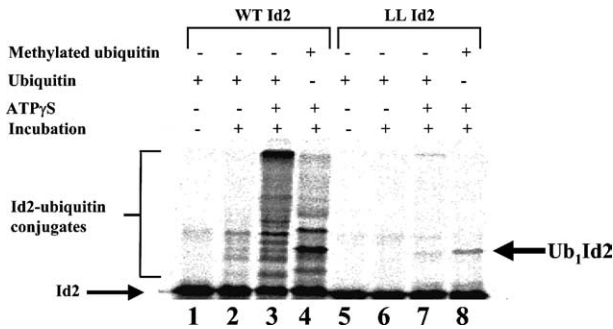


Fig. 5. LL Id2 is conjugated to ubiquitin in vitro in an ATP γ S-dependent manner. Conjugation of in vitro translated and [35 S]methionine-labeled WT (lanes 1–4) and LL (lanes 5–8) Id2s was monitored in a cell-free reconstituted system that contained complete HeLa cell extract and ubiquitin or methylated ubiquitin as indicated and as described under Materials and methods. ATP γ S was added as indicated. Following incubation, reactions were resolved via SDS-PAGE and proteins were visualized by PhosphorImager. Multiply ubiquitinated (Id2–ubiquitin conjugates) as well as monoubiquitinated (Ub $_1$ Id2) Id2s are marked.

ubiquitin adducts formed is lower in the case of the LL mutant, suggesting that in the WT protein, internal Lys residues are also targeted and may play a role, though not an essential one, in the proteolytic process (see Discussion).

Blocking of the N- but not of the C-terminus of Id2 inhibits its degradation

We have previously shown that degradation of WT MyoD [4], LMP1 [5], and HPV-16 E7 [7] can be blocked following fusion of a Myc tag to the N-terminus of these proteins. For the E7 protein, we have shown that N-terminal tagging also inhibits conjugation of the protein, and that the effect was specific, i.e., fusion of tag to the C-terminus of the protein did not affect neither its conjugation nor its degradation [7]. A recent report demonstrated that p21 was also stabilized following N-terminal Myc tagging [8]. We speculated that N-terminal ubiquitination occurs on a specific residue that must be free and resides within a defined context of the N-terminal domain. Downstream positioning of the N-terminal residue along with the entire N-terminal domain that is caused by the fused tag will abolish conjugation and degradation (see Discussion). We tested these notions for Id2 as well. As can be seen in Fig. 6A, N-terminally Myc-tagged Id2 is significantly more stable than its C-tagged counterpart. The half-life of the N-terminally tagged protein is similar to that of the WT protein. Importantly, even a single Myc tag is sufficient to stabilize Id2 (Fig. 6B). This new finding is not surprising, as even in this fusion protein, the N-terminal residue and domain are not accessible to ubiquitin, and possibly to the ligase (see below).

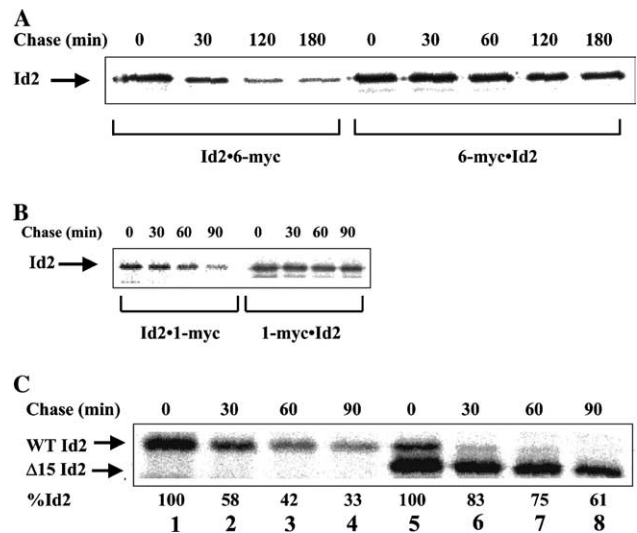


Fig. 6. N-terminal but not C-terminal tagging as well as truncation of a short N-terminal fragment stabilize cellular Id2. (A) Effect of 6 \times Myc tag extension on Id2 stability. Degradation of Id2 with a 6 \times Myc tag fused to its N- or C-terminal residue was monitored in a pulse-chase labeling and immunoprecipitation experiment in transiently transfected Cos7/5 cells as described under Materials and methods. (B) Effect of 1 \times Myc tag terminal extension on Id2 stability. Degradation of Id2 with 1 \times Myc tag fused to its N- or C-terminal residue was monitored as described under Materials and methods and under (A). (C) Effect of truncation of a 15 residue N-terminal fragment on Id2 stability. Cos7/5 cells were transiently transfected with a vector (pCDNA3.1) that contains cDNA that codes for either WT Id2 (lanes 1–4) or for an Id2 that lacks the first 15 amino acid residues (Δ 15 Id2; lanes 5–8). Degradation of the Id2 proteins was monitored in a pulse-chase labeling and immunoprecipitation experiment as described under Materials and methods and under (A). Band intensities were quantified and data are presented as percentage of the signal at time 0.

The N-terminal domain of Id2 is essential for its degradation

We have demonstrated that deletion of the first 12 N-terminal residues of EBV LMP1 [5], 11 N-terminal residues of HPV-16 E7 [7], and 15 N-terminal residues of MyoD stabilizes the proteins in vitro (MyoD) and in vivo (data for MyoD are unpublished). To study if, similar to these proteins, the N-terminal domain of Id2 also plays a role in governing the stability of the protein, we deleted the first 15 N-terminal residues of WT Id2. As can be seen in Fig. 6C, the deletion of the first 15 residues stabilizes the protein (compare lanes 5–8 to lanes 1–4; note the quantification of the data).

Taken together, the results strongly suggest that the N-terminal domain and its position within the protein play an important role(s) in governing the stability of Id2. The combined findings from all four proteins, MyoD, LMP1, HPV-16 E7, and now Id2, indicate that the signal is constituted of a relatively long segment. This raises the hypothesis that it serves not only as an anchor for ubiquitination, but has an additional function. It can serve, for example, as a binding site for

another component of the UPS such as the ubiquitin ligase E3 (see Discussion).

Discussion

Ubiquitin-mediated degradation of multiple key regulatory proteins is involved in the regulation of many basic cellular processes. Here, we demonstrate that Id2 is a substrate for the ubiquitin system. It is ubiquitinated (Fig. 1A) and degraded in an ATP-dependent manner in a cell-free reconstituted system (Fig. 1B). Interestingly, ATP γ S, the non-hydrolyzable analog of ATP, was able to promote efficient conjugation. This analog can be used by E1 (that utilizes the β - γ bond of ATP), but not by the proteasome. Importantly, it cannot be used by kinases, suggesting that phosphorylation of Id2 is not required for its ubiquitination. It is important to note that Id2 is phosphorylated by CDK2 on Ser5 during the transition from G0 to S, towards late G1 [20,21]. Phosphorylation is accompanied by a decrease in the level of the protein, and therefore, it is tempting to link between the two modifications, phosphorylation and ubiquitination/degradation. Yet, the finding that ATP γ S supports ubiquitination, along with the finding that S5A Id2 mutant is equally sensitive to both ubiquitination and degradation *in vitro* (not shown), rules out, at least in our systems, the hypothesis that phosphorylation may signal ubiquitination and degradation. Id2 is extremely unstable and has a half-life of \sim 15 min in Cos7/5 cells (Fig. 2B, lanes 1–4). More direct examination of the role of the ubiquitin system in the degradation of Id2 revealed that the process is mediated by the proteasome; inhibition of proteasomal function stabilized the protein (Fig. 2B, lanes 5–8). Furthermore, degradation of Id2 requires a functional E1 (Fig. 3). Taken together, these findings strongly suggest that Id2 is targeted for degradation by the proteasome following its ubiquitination.

To dissect the mechanisms that underlie the recognition and degradation of Id2, it was important to study whether a specific Lys residue is essential for formation of the polyubiquitin chain, or whether the first ubiquitin residue is attached to the free N-terminal NH₂ group as is the case for MyoD, EBV LMP1, and LMP2A, HPV-16 E7, and p21 (see above). To that end, we replaced all nine Lys residues in positions 2, 12, 26, 45, 47, 57, 58, 61, and 129 with Arg. The LL Id2 was degraded in cells as efficiently as the WT protein (Fig. 4A), and its degradation was mediated by the proteasome (Fig. 4B). Importantly, incubation of labeled LL Id2 in a cell-free reconstituted system in the presence of ubiquitin and ATP γ S generates a polyubiquitin chain (Fig. 5, lanes 7 and 8). However, the efficiency of the conjugation of LL Id2 was much lower than that of the WT protein (compare lanes 7 and 8 to lanes 3 and 4). A similar observation was noted also for MyoD [4], LMP1 [5],

and E7 [7]. This finding demonstrates that the internal Lys residues are also targeted by ubiquitin, yet they may not be essential in the proteolytic process. The implication of this finding is important, as it demonstrates that ubiquitination, at least for some proteins, must occur on a specific residue, and when it occurs on other(s), the conjugate is not recognized efficiently by the proteasome (see also below). This is not always the case, as in cyclin B [31], and in the α chain of the T cell receptor cytosolic tail [32], for example, any internal Lys residue, even artificially inserted, can serve as a ubiquitin acceptor. In some of the N-terminally ubiquitinated proteins, such as in MyoD, removal of all internal Lys affects stability, although in a minor way, suggesting that they may modulate proteolysis [4]. We also used the methylated derivative of ubiquitin that can modify the target protein only once and thus serves as a chain terminator [27]. Utilization of this derivative enabled us to see more clearly the formation of the mono-ubiquitin adduct of Id2 (Fig. 5, Ub₁Id2) which is similar to the one generated with the WT protein. Since no lysine residues were available, it seems reasonable to assume that the first ubiquitin residue is attached to the free N-terminal group, as is the case for MyoD, LMP1, LMP2A, E7, and p21.

To further test the role of the N-terminal residue as a ubiquitination anchor, we fused a 6 \times or a single Myc tag to either the N- or the C-terminal residue of WT Id2. As can be seen in Figs. 6A and B, only N-terminal tagging but not C-terminal tagging stabilized the protein in cells. The effects of Myc tagging are even more striking if one takes into consideration the existence of six additional (or one, dependent on the tag fused) lysine residues in the tag. It appears therefore that ubiquitination at the N-terminal residue is required for recognition of the protein by the proteasome. For HPV-16 E7 we have observed that N-terminal tagging decreases also the ubiquitination, suggesting that the internal lysine residues in the tag are weak targets for modification by ubiquitin [7]. In contrast, Bloom and colleagues [8], using N-terminally Myc-tagged p21, demonstrated that the protein is stabilized, yet efficiently ubiquitinated. However, they have not demonstrated whether ubiquitination occurs on an internal Lys of the tag or at the N-terminal residue of the tag. The finding that N-terminally HA-tagged LL p21 is ubiquitinated [8] raises the possibility that ubiquitination of the tagged protein occurs on the N-terminal residue, as the HA tag does not contain any Lys residue.

Because in most substrates of the ubiquitin pathway the targets of ubiquitination are internal lysines, attachment of ubiquitin to the N-terminal residue is probably sequence- and protein specific. The simple notion is that the Myc tag acts to stabilize the target protein by blocking the access of ubiquitin. Yet, the tag can act via an additional/different mechanism. To study

the role of the entire N-terminal domain in the proteolytic process, we deleted the first 15 N-terminal amino acid residues which resulted in stabilization of the protein. Our previous results indicated that deletion of the first amino acid residues stabilized LMP1 [5], E7 [7], and MyoD (unpublished), suggesting that in all these proteins the N-terminal domain plays an additional role beyond “providing” the N-terminal ubiquitination site. It can serve, for example, as a binding site for the ubiquitin ligase, E3, and the tagging may interfere sterically with the association between the ligase and its substrate. Also, it can “provide” the flexible arm needed for the N-terminal residue to serve as a ubiquitin acceptor. These hypotheses are currently testable. It should be noted, however, that we could not find sequence homology between the N-terminal regions of MyoD, LMP1, LMP2A, E7, p21, and Id2, but such functional motifs or epitopes may be generated following folding of the protein rather than at the primary sequence level.

Acknowledgments

The authors thank Dr. Eyal Bengal (Faculty of Medicine, Technion, Haifa, Israel) for the WT Id2 cDNA clone. A.C. is an Israel Cancer Research Fund (ICRF) USA Professor and is supported by grants from CapCure Israel—Centers of Excellence Program, the Israel Science Foundation founded by the Israeli Academy of Sciences and Humanities—Centers of Excellence Program, and the German–Israeli Project Cooperation (DIP). Infrastructural equipment was purchased with support of the Wolfson Charitable Fund Center of Excellence for studies on *Turnover of Cellular Proteins and its Implications to Human Diseases*. A.L.S. is supported by the NIH.

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