The Tumor Suppressor Protein p16<sup>INK4a</sup> and the Human Papillomavirus Oncoprotein-58 E7 Are Naturally Occurring Lysine-less Proteins That Are Degraded by the Ubiquitin System

DIRECT EVIDENCE FOR UBIQUITINATION AT THE N-TERMINAL RESIDUE*

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Conjugation of ubiquitin to an internal lysine is the initial step in the degradation of the majority of the substrates of the ubiquitin system. For several substrates, it has been shown that the first ubiquitin moiety is conjugated to the N-terminal residue. In all these substrates, however, the internal lysines also played a role in modulating their stability. To better understand the physiological significance of this novel mode of modification, it was important to identify proteins in which degradation is completely dependent on N-terminal ubiquitination. Also, although the experimental evidence for N-terminal ubiquitination is rather strong, nevertheless, it has remained indirect. Here we demonstrate that an important group of proteins that are targeted via N-terminal ubiquitination are the naturally occurring lysine-less proteins such as the human papillomavirus (HPV)-58 E7 oncoprotein and the cell cycle inhibitor and tumor suppressor p16<sup>INK4a</sup>. For these proteins, the only residue that can be targeted is the N-terminal residue. Interestingly, p16<sup>INK4a</sup> is degraded in a cell density-dependent manner. Importantly, we provide evidence for the first time direct evidence for N-terminal ubiquitination. Analysis of tryptic digest of the ubiquitin conjugate of HPV-58 E7 revealed a fusion peptide that is composed of the C-terminal domain of ubiquitin and the N-terminal domain of E7. With the abundance of native lysine-less proteins, among which are important viral and cell regulators, this novel mode of protein targeting has implications for both physiological and pathophysiological processes.

Ubiquitin modification of cellular proteins plays important roles in a variety of basic cellular processes. In many cases, modification by ubiquitin signals proteins for degradation by the 26 S proteasome. Conjugation of ubiquitin to the target substrate involves activation of ubiquitin by the ubiquitin-activating enzyme E1 followed by its transfer to a member of the ubiquitin carrier protein E2 family of enzymes. In most studied cases, E2 transfers the activated ubiquitin either directly or indirectly to an ε-NH₂ group of an internal lysine residue in the substrate that is specifically bound to E3, a member of the ubiquitin-protein ligase family of proteins. Subsequent conjugation of additional activated ubiquitin molecules to previously attached molecules generates the polyubiquitin chain that serves as a degradation signal for the 26 S proteasome (reviewed in Ref. 1).

For several proteins, however, it was suggested that the first ubiquitin is conjugated to the N-terminal residue rather than to an internal lysine (reviewed in Ref. 2). Replacement of all the lysine residues in the muscle-specific transcription factor MyoD (3) only slightly affected its ubiquitination and degradation, suggesting that an alternative ubiquitin docking site must exist. Degradation of the lysine-less (LL) protein in cells was inhibited by proteasome inhibitors, and inhibition was accompanied by accumulation of ubiquitin-MyoD conjugates. In vitro, LL MyoD was polyubiquitinated, and this modification was required for its degradation; proteolysis was inhibited by the ubiquitin chain terminator methylated ubiquitin (MeUb), a ubiquitin derivative in which all internal lysines are modified, and therefore it cannot polymerize (4). In agreement with the results obtained with the LL MyoD, selective chemical modification of all internal lysines of MyoD by guanidination affected proteolysis only slightly. In striking contrast, selective modification of its N-terminal group by carbamylation almost completely blocked degradation. Fusion of a Myc tag to the N-terminal residue of WT MyoD stabilized the protein both in vivo and in vitro (3). These findings rule out the possibility that

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The abbreviations used are: E1, ubiquitin-activating enzyme; E2, ubiquitin carrier protein; E3, ubiquitin-protein ligase; LL, lysine-less; MeUb, methylated ubiquitin; WT, wild type; HPV, human papillomavirus; E7, HPV oncoprotein; LMP, latent membrane protein; Id, inhibitor of differentiation (or inhibitor of DNA binding); HA, hemagglutinin; NOLL, naturally occurring lysine-less protein; TEV, tobacco etch virus; MG132, N-carboxyamido-L-leucyl-L-leucyl-L-leucinal; ATP-S, adenosine 5′-γ-thiotriphosphate; MS, mass spectrometry; MS/MS, tandem mass spectrometry.
substitution of all the internal lysine residues “forced” ubiquitination at the N-terminal residue that would not have otherwise occurred. N-terminal tagging probably stabilizes MyoD by blocking access of ubiquitin to the specific ubiquitination site at the N terminus; however, it can also affect recognition of the substrate by the E3 by altering the structure of the N-terminal domain. Taken together, these results strongly suggest that the first event in ubiquitin-mediated degradation of MyoD involves fusion of ubiquitin to the N-terminal residue. Additional ubiquitin moieties are then added to Lys48 of the previously conjugated moiety (3).

Using a similar set of experiments, seven additional proteins have recently been identified that possibly traverse the N-terminal ubiquitination pathway: (i) the human papillomavirus-16 (HPV-16) oncoprotein E7 (5), a potent transforming protein (see also below); (ii) the latent membrane protein 1 (LMP1) (6) and (iii) LMP2A (7) of the Epstein-Barr virus, two multiple transmembrane-spanning proteins involved in the regulation of B cell receptor-mediated signal transduction and viral activation from latency; (iv) the cell cycle-dependent kinase inhibitor p21 (8, 9); (v) the extracellular signal-regulated kinase 3 (ERK3) (9); and the inhibitors of differentiation (or inhibitors of DNA binding) (vi) (10) and (vii) Id1 (11), two pre-proliferative helix-loop-helix proteins that lack the basic DNA-binding domain. These two proteins repress transcription by generating transcriptionally inactive heterodimers, primarily with E proteins, which act as dominant negative regulators of basic helix-loop-helix proteins. It has been recently reported that hydroxymethylglutaryl-CoA reductase, the first critical enzyme in the cholesterol biosynthetic pathway, may also be ubiquitinated on the N terminus (12). This finding, however, and the essentiality of the modification for degradation of the protein still need further substantiation. The case of p21 attracted special attention because it was shown earlier that a mutant lysine-less p21 is still degraded by the proteasome without prior ubiquitination. This finding suggested that, similar to ornithine decarboxylase, p21 is also degraded in a manner that is ubiquitin-independent yet proteasome-dependent (13). The new findings suggest that this may not be the case, and the protein must be ubiquitinated probably on its N-terminal residue prior to its recognition and degradation by the 26 S proteasome.

It should be noted that although the internal lysine residues are not essential for degradation of MyoD, they nevertheless play a modulating role. Thus, the LL MyoD is ubiquitinated less efficiently then its WT counterpart, and its degradation is slowed by ~2-fold (3).

Is there a direct evidence for N-terminal ubiquitination of a native cellular substrate? Apparently there is not. Although all the different and independent lines of experimental evidence presented in the various studies strongly suggest that ubiquitination occurs on the N-terminal residue, the only direct evidence will be the demonstration of a fusion peptide between the C-terminal domain of ubiquitin and the N-terminal domain of the substrate. Such a fusion fragment has not yet been identified. For p21 it has been shown that ubiquitination must occur in the N-terminal domain (8) (see also under “Discussion”), and a fusion peptide has been identified for N-terminally HA-tagged p21 (see Ref. 9, which was published in parallel with the on-line version of this study), but direct evidence for modification of the N-terminal residue of the native protein is still missing.

The physiological significance of N-terminal ubiquitination is not yet clear because all of the studied proteins contain internal lysines that can still be ubiquitinated. An important group of potential substrates for N-terminal ubiquitination is that of naturally occurring lysine-less proteins (NOLLPs). Because these proteins cannot use the “canoncial” lysine conjugation pathway, to be targeted by the ubiquitin system they must use an alternative site for their tagging. Searching the data base, we were able to identify 178 eukaryotic NOLLPs of which 15 are human; in addition, we have identified 111 viral NOLLPs. Here we report on two of these proteins, the human tumor suppressor p16INK4a and the viral oncoprotein HPV-58 E7. p16INK4a is a well characterized tumor suppressor that acts as a specific inhibitor of D-type cyclin-dependent kinases (reviewed in Ref. 14). Its expression enhances the growth-suppressive function of the retinoblastoma protein, thus arresting cells in early G1. In contrast, inactivation of p16INK4a has been implicated in the deregulation of the cell cycle and, therefore, in the pathogenesis of different tumors such as malignant melanoma (15). Although the biochemical properties and the interactions of p16INK4a with other cell cycle regulators have been studied extensively, little is known about its regulation. It is generally believed that the ability of p16INK4a to inhibit Cdk4 is regulated only by the abundance of the proteins (16). Recently, it was reported that p16INK4a is phosphorylated (17), the only known post-translational modification that this protein undergoes; however, the role of this modification has remained obscure. The E7 oncoprotein of the high risk HPV is a potent transforming protein that can immortalize various human cell types either alone or, in higher efficiency, in cooperation with HPV-E6. It has been associated etiologically with the pathogenesis of human uterine cervical cancer (reviewed in Refs. 18 and 19).

Here we provide evidence that both p16INK4a and HPV-58 E7 are degraded by the ubiquitin system. Importantly, we have identified the fusion peptide between the C-terminal domain of ubiquitin and the N-terminal domain of HPV-58 E7, thus providing for the first time direct evidence for the attachment of ubiquitin to the N-terminal residue of a target substrate. Interestingly, we demonstrate that p16INK4a is ubiquitinated and degraded only in sparse cells and is stable in dense cells.

EXPERIMENTAL PROCEDURES

Materials

Materials for SDS-PAGE and the Bradford reagent were from Bio-Rad. A mixture of [3H]methionine and [35S]methionine (see also below), and a fusion peptide have been identified for N-terminally HA-tagged p21 (see Ref. 9, which was published in parallel with the on-line version of this study), but direct evidence for modification of the N-terminal residue of the native protein is still missing.

Method

Identification of NOLLPs in the Protein Data Base—The UniProt data base (www.uniprot.org) was scanned (in January 2004) for full-length lysine-less proteins. Short (less than 50 residues) and prokaryotic entries were filtered out. Plasmids and Construction of Mutant cDNAs—HPV-58 E7 cDNA was amplified by PCR from a plasmid vector containing the complete HPV-58 genome (kindly received from Dr. Toshihiko Matsukura, Laboratory of Tumor Viruses, National Institute of Infectious Diseases, Japan) and subcloned into M13mp19 by the quick in vitro method of Kunkel et al. (20). C-terminal mutations were introduced into the cloned fragment using the QuikChange Kit (Stratagene).

Method

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Tokyo, Japan) and subcloned into the Xhol-XbaI site of the mammalian expression vector pRI332+ upstream of the cytomegalovirus and SP6 promoters. This vector was used for expression of the protein in COS-7 cells and for in vitro translation. For expression in Escherichia coli, the cDNA was amplified by PCR and subcloned into the Ndel-BamHI site of the bacterial expression vector pT7-7. A His$_6$ tag was added at the C terminus into the BamHI site by PCR. A mutant HPV-58 E7-His in which we substituted the Arg residue in position 2 with His (HPV-58 E7-His R2H) was generated by site-directed mutagenesis. A TEV protease cleavage site (Glu-Asn-Leu-Tyr-Phe-Gln- Gly-Ser) was inserted by PCR into HPV-58 E7-His (cloned into the pT7-7 vector) after either the first (Met, designated HPV-58 E7-TEV1) or the 21st (Asp, designated HPV-58 E7-TEV21) residue. All sequences were confirmed using the 3100 automatic sequencer.

Expression and Purification of Reconstituant Proteins—Rosetta™ (DE3)lysE cells (Novagen) induced by isopropyl-b-D-thiogalactopyranoside were used for bacterial expression of HPV-58 E7. Purification of His$_{10}$-tagged HPV-58 E7-R2H and HPV-58 E7-TEV1 and TEV-21 proteins was performed by using immobilized nickel-nitrilotriacetic acid (Qiagen) according to the manufacturer’s instructions.

Cell Lines and Transfection—COS-7 and HeLa cells were grown at 37°C in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 2 mM l-glutamine, and antibiotics (penicillin/streptomycin). Transient transfections were carried out using the jetPEI™ transfection reagent according to the manufacturer’s instructions, and cells were harvested after 24–48 h.

Stability of Proteins in Cells—Cellular stability (t$_{1/2}$) of HPV-58 E7 was monitored using a cycloheximide chase. At time 0, cells were harvested or further incubated for 3 h in the presence of cycloheximide (100 μg/ml). The proteasome inhibitor MG132 (20 μM) was added 1 h prior to the addition of cycloheximide and was present throughout the experiment. Cells were lysed in radioimmune precipitation assay buffer (50 mM Tris, pH 7.6, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, and protease inhibitor mixture set I), and protein concentration was determined according to the Bradford method (21) using bovine serum albumin as a standard. Equal amounts of protein were resolved via SDS-PAGE (18%), and proteins were visualized using Coomassie Blue staining. Cellular stability of p16 was monitored in a pulse-chase labeling and immunoprecipitation experiment. HeLa cells expressing endogenous p16 were incubated for 1 h in a methionine- and cysteine-free medium followed by a 1-h labeling (pulse) with 0.17 mCi/ml of [35S]methionine and cysteine mixture (P30-MIX, Amersham Biosciences). Decay of the labeling (chase) was monitored in a complete medium containing 2 mM methionine and 2 mM cysteine for the indicated time periods. The proteasome inhibitor MG132 (20 μM) was added for the last 30 min of the labeling period and was present throughout the experiment. After labeling, cells were harvested and lysed, and the labeled proteins were precipitated with anti-p16 antibody. Immune complexes were collected using immobilized protein A. Following SDS-PAGE (15%), proteins were visualized using a PhosphorImager.

Demonstration of p16 Native Ubiquitin Conjugates in Cells—Ubiquitin conjugates of p16 were detected in cells using a lysis procedure that inhibits the activity of ubiquitin proteases as follows. HeLa cells expressing endogenous p16 were incubated for 3 h in the presence or absence of 20 μM proteasome inhibitor MG132. Following incubation, the cells were lysed in a buffer containing 1% SDS, 1 mM EDTA, and protease inhibitor mixture set I, all of which were dissolled in phosphate-buffered saline and heated to 95°C. The lysates were then boiled for 5 min, sheared by a syringe equipped with a 25-gauge needle, boiled again for 1 min, and centrifuged for 5 min at room temperature for 20,000 × g. The supernatant was diluted 2-fold with a buffer containing 2% Triton X-100, 0.5% deoxycholate, and protease inhibitor mixture set I in 1% bovine serum albumin. p16 was immunoprecipitated using anti-p16 antibody and immobilized protein A. The immune complexes were resolved via SDS-PAGE (15%), and after Western blot, proteins were visualized using an anti-ubiquitin antibody or anti-p16 antibody.

In Vitro Translation of HPV-58 E7—The HPV-58 E7 cDNA was translated in the presence of [35S]methionine using wheat germ-coupled transcription-translation-extract and SP6 RNA polymerase according to the manufacturer’s instructions.

Conjugation and Degradation Assay—Conjugation and degradation of recombinant cell-free systems were performed essentially as described previously (3). Briefly, reaction mixtures contained crude HeLa cell extract (50 μg of protein), 5 μg of ubiquitin, and in vitro translated HPV-58 E7 (~25,000 cpm) in a final volume of 12.5 μl. Degradation reactions were carried out in the presence of either 0.5 mM ATP and an ATP-regenerating system (10 μM phosphocreatine and 0.5 μg of creatine phosphokinase) or 0.5 μg of creatine phosphokinase and 10 mM 2-deoxyglucose to deplete endogenous ATP. Conjugation reactions were carried out in the presence of the isopeptidase inhibitor ubiquitin aldehyde (0.5 μg) and either ATP-γS or 0.5 μg of hexokinase and 10 mM 2-deoxyglucose to deplete endogenous ATP. 5 μg of MeUb were added where indicated. Reactions were incubated for 1 h at 37°C and terminated by the addition of 7.0 μl of 3-fold concentrated sample buffer. After boiling, reaction mixtures were resolved using SDS-PAGE (15%), and gels were dried and exposed to a PhosphorImager screen.

Ubiquitination at the N-terminal Residue as Demonstrated by TEV Proteolysis—151-MeUb was prepared using the chloramine-T method as described previously (22). Conjugation of bacterially expressed C-terminally His$_{10}$-tagged HPV-58 E7-TEV1 and -TEV21 (~1 μg) proteins was carried out in a cell-free reconstituted system containing crude HeLa cell extract and 151-MeUb (~2.0 × 10$^5$ cpm/2 μg) (added instead of WT ubiquitin) as described above. ATP-γS was added as indicated. Following incubation, the free proteins and the conjugates were isolated using Ni$_{2+}$-agarose beads. Cleavage by the TEV protease was carried out at 30°C for 2 h using the Ni$_{2+}$-immobilized E7 as a substrate in the presence of TEV buffer (1 mM dithiothreitol and 1% Triton X-100) and in the presence or absence of 10 units of the TEV protease. Reactions were resolved using SDS-PAGE (18%), and proteins were visualized by PhosphorImager.

Conjugation and Isolation of HPV-58 E7 for Analysis via Mass Spectrometry—151-MeUb (~40 μg) of bacterially expressed and purified C-terminally His$_{10}$-tagged HPV-58 E7-R2H were incubated in a 500-μl scale-up in vitro ubiquitination reaction that also contained MeUb as described above. Following conjugation, 500 μl of dilution buffer (20 mM Tris, pH 7.2, 150 mM NaCl, 20 mM imidazole) was added, and the His-tagged protein was purified using Ni$_{2+}$ affinity chromatography. Following SDS-PAGE (15%), proteins were visualized using Coomassie Blue staining (SeeBand Forte). Bands representing free and conjugated HPV-58 E7 R2H were excised from the gel and analyzed by MS.

In-gel Proteolysis and Mass Spectrometry Analysis—Excised gel bands (see above) were reduced in-gel (with 10 mM dithiothreitol and incubation at 60°C for 30 min), alkylated (using 10 μl iodoacetamide at room temperature for 30 min), and digested with trypsin (overnight at 37°C using modified trypsin (Promega) at 1:100 enzyme to substrate ratio). The resulting tryptic peptides were resolved by reverse-phase chromatography on 0.1 × 300-mm fused silica capillaries (J&W Scientific) packed with POROUS R2–10 reversed phase material (Applied Biosystems). The peptides were eluted with a linear 50 min gradient of 5–95% acetonitrile, 0.1% formic acid in water at a flow rate of 0.4 μl/min. Mass spectrometry was performed by an ion-trap mass spectrometer (LCQ-DecaXP, Finnigan, San Jose, CA) in a positive ionization mode using repetitively full MS scan followed by collision-induced dissociation of the three most dominant ions selected from the first scan. The identity of the peptides was further confirmed using tandem mass spectrometry. Following MS (7000, Applied Biosystems). The MS data were analyzed using the Sequest software.2

RESULTS

The NOLLP HPV-58 E7 Is Targeted by the Ubiquitin-Proteasome System in Vitro and in Vivo—Once HPV-58 E7 had been identified as a NOLLP (Fig. 1A), it was important to examine whether it is degraded by the ubiquitin system. As can be seen in Fig. 1B, HPV-58 E7 is polyubiquitinated in a cell-free system, and the reaction requires energy. The finding that the modification is dependent on ATPγS, a derivative of ATP that can activate E1 via cleavage of the βγ bond but cannot release ubiquitin, demonstrated that ubiquitination does not require phosphorylation. Since ubiquitination does not necessarily signal the target protein for degradation, it was important to demonstrate that, in this case, it serves as a recognition motif for the 26 S proteasome and leads to destruction of the protein. As can be seen in the experiment described in Fig. 1C, degradation of HPV-58 E7 is dependent on the presence of both ATP and ubiquitin. Addition of the chain terminator MeUb competed with the endogenous ubiquitin in the HeLa extract and inhibited the degradation of E7 significantly. Addition of

J. Eng and J. Yates, unpublished data.
excess WT ubiquitin that competes with the derivatized ubiquitin for activation by E1 alleviated the inhibition, and degradation resumed.

Next we examined whether the degradation of HPV-58 E7 is carried out by the proteasome in cells. As can be seen in Fig. 2, the protein is degraded in cells and has a half-life of ~20 min. Addition of MG132, an inhibitor of the proteasome, completely blocked degradation.

The NOLLP p16INK4a Is Targeted by the Ubiquitin-Proteasome System in Vivo and Its Degradation Is Cell Density-dependent—We next examined whether the degradation of another NOLLP, the tumor suppressor protein p16INK4a (Fig. 3A), is mediated by the proteasome following its ubiquitination. Our initial experiments revealed that the protein is stable in non-synchronized cells. To examine whether the level of p16 is regulated during the cell cycle, we used a double thymidine block (23) to arrest HeLa cells in G1/S phase. We could not observe any oscillations in the abundance of p16 during the cell cycle (data not shown). This finding was consistent with the observation that the level of the highly related cell cycle inhibitor p19INK4d changes dramatically during the cell cycle and in correlation with its very short half-life (24), whereas p16INK4a is a much more stable protein (26). Several groups have reported up-regulation of p16INK4a in response to contact inhibition of growth (see for example Refs. 25–27). We decided therefore to examine whether the ubiquitin-proteasome system is involved in the cell density-dependent regulation of p16INK4a.

Indeed, we noted that the steady-state level of p16 changes with cell density; that is, the sparser the cells, the lower the level of the protein (Fig. 3B). This finding could be attributed to density-dependent alteration in the transcription of the mRNA or translation of the protein, or to a change in the stability of the message or of the protein itself. To test the possibility that the increase in the steady-state level of p16 seen at high cell density is the result of alteration in the stability of the protein, we monitored its $t_{1/2}$ in sparse and confluent cells. As clearly seen in the experiment described in Fig. 3C, p16 is unstable in sparse cells, having a half-life of ~2 h, and its degradation is completely blocked by MG132. Thus, the low steady-state level of p16 observed in sparse cells may result, at least partially, from rapid degradation of the protein. Therefore, we examined whether there is a correlation between ubiquitination of the protein and its destabilization. The experiment described in Fig. 3D demonstrates that this is indeed the case; whereas in confluent cells we could not detect any conjugates of p16, in sparse cells and following the addition of MG132, specific conjugates of p16 could easily be detected.

Ubiquitin Is Fused to the N-terminal Domain of HPV-58 E7—As noted, Bloom et al. (8) provided evidence that ubiquitin is fused to the N-terminal domain of p21. We now corroborate their finding, providing evidence that ubiquitin is fused to the N-terminal domain of a different protein, HPV-58 E7. We generated two species of the protein that contain the 8-amino acid TEV protease cleavage site either 21 amino acid residues after the iMet or immediately after the iMet. A His6 tag attached to the C-terminal residue of the protein facilitated its purification from the conjugation reaction mixture prior to the addition of the TEV protease and its buffer. The prediction from this experiment was that if ubiquitin is indeed attached to the N-terminal domain of HPV-58 E7, TEV protease-catalyzed cleavage would generate an extended ubiquitin molecule that would also contain the respective N-terminal domain of HPV-58 E7 (21 or 1 residue, respectively, depending on whether the substrate of the reaction is HPV-58 E7-TEV21 or -TEV1) and the six amino acids derived from the TEV cleavage site (for the expected extended ubiquitin derivative cleaved from the HPV-58 E7-TEV21, see Fig. 4A). Indeed, as seen from the experiment shown in Fig. 4B(i), lane 8, TEV cleavage of HPV-58 E7-TEV21 yielded a ubiquitin derivative that is larger by ~3 kDa than its WT counterpart. In the case of HPV-58 E7-TEV1, we also obtained an extended ubiquitin derivative, yet the addition of the seven amino acids was not sufficient to discern it from the WT protein, even not following resolution of the Ni$^{2+}$-precipitated mixture in several gel systems. Consequently, we were able to observe only an increase in the amount of the labeled ubiquitin in the TEV-treated mixture compared with the untreated one (compare lane 3 with lane 2 in Fig. 4B(ii), and see quantified data in Fig. 4B(iii)). As ex-
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Fig. 3. Degradation $\text{p16}^{\text{INKA}}$ is cell density-dependent. A, the amino acid sequence of $\text{p16}^{\text{INKA}}$, which is a NOLLP, is shown. B, the steady-state level of $\text{p16}^{\text{INKA}}$ in cells is cell density-dependent. The indicated number of HeLa cells was plated on a 60-mm dish. After 18 h, the cell density was estimated by light microscopy. The cells were lysed, and equal amounts of protein were resolved via SDS-PAGE, blotted onto nitrocellulose membrane, and visualized using anti-p16 antibody as indicated, as described under “Experimental Procedures.” C, degradation of $\text{p16}^{\text{INKA}}$ in sparse and confluent HeLa cells is shown. (i), endogenous $\text{p16}^{\text{INKA}}$ stability was monitored in 100 or 15% confluent HeLa cells using a pulse-chase labeling and immunoprecipitation experiment as described under “Experimental Procedures.” (ii), quantitative analysis of the data is shown for sparse cells in (i). Quantities are relative to the amount of protein at time 0. D, conjugation of $\text{p16}^{\text{INKA}}$ in HeLa cells is shown. 100 or 20% confluent (see B) HeLa cells were treated with MG132 for 2 h as indicated. Following lysis, $\text{p16}^{\text{INKA}}$ was immunoprecipitated, and the immune complexes were resolved via SDS-PAGE, blotted onto nitrocellulose membrane, and visualized using either anti-ubiquitin or anti-p16 antibody (as indicated), as described under “Experimental Procedures.” Ig denotes the heavy chain of the immunoprecipitating (IP) antibody as detected by the secondary antibody.

expected in both HPV-58 E7-TEV21 and -TEV1, TEV cleavage resulted in a significant decrease in the amount of the monoubiquitin conjugate of the HPV-58 E7 (compare the amount of the conjugate in Fig. 4B(i)), lane 8, with that in lane 7, and Fig. 4B(ii), lane 3, and see also quantified data in Fig. 4B(iii)). Also, generation of the extended ubiquitin moiety was completely dependent on the presence of the TEV site in the E7 molecule. As expected, TEV did not affect at all an E7 molecule that did not contain the appropriate cleavage site (Fig. 4B(i), lanes 1–4). Interestingly, generation of both poly- and monoubiquitinated HPV-58 E7-TEV1 was much less efficient than that of HPV-58 E7-TEV21, suggesting that the integrity of the N-terminal domain is important for conjugation of ubiquitin to the N-terminal residue. We have suggested that the N-terminal domain plays a role in the N-terminal ubiquitination pathway because deletion of a short stretch of amino acids from the N-terminal domain of HPV-16 E7 (5), LMP1 (6), and Id2 (10) stabilized the proteins. Notably, in the two experiments presented in Fig. 4B(i) and (ii), polyubiquitinated species of the HPV protein are generated (see top of the two gels). This is because the cell extract used to generate the conjugate also contains free ubiquitin, which is not competed out efficiently by the added MeUb.

Ubiquitin Is Fused to the N-terminal Amino Acid of HPV-58 E7—Addition of the TEV cleavage site (as described above) or of any other specific protease site or a tag (8, 9) and isolation of an extended ubiquitin moiety still leave the possibility that modification by ubiquitin can occur on any of the residues of the added amino acids, including the N-terminal, but would not occur on the native substrate (see under “Discussion”). Although a modification by ubiquitin on any non-lysine residue would be novel, the chemistry of such a modification on an amino acid of the inserted proteolytic site or the inserted tag would not be different from the chemistry on any other similar residue along the native protein. Thus, the isolation of such an extended ubiquitin derivative only narrows the ubiquitin attachment site to a shorter segment in the N-terminal domain where the site or tag was added but does not constitute direct evidence for ubiquitination on the N-terminal residue. Only the identification of a fusion peptide between the C-terminal domain of ubiquitin and the native N-terminal domain of the target protein will constitute such evidence. To isolate such a fusion peptide, we substituted the arginine residue in position 2 in HPV-58 E7 with histidine, generating HPV-58 E7-R2H. Otherwise, tryptic digestion of the ubiquitin conjugate would yield a tetrapeptide, GG-MR, in which the GG would be derived from the ubiquitin moiety of the conjugate (following cleavage in the $7^R \downarrow GG^{76}$ site), and the MR would be derived from the HPV-58 E7 moiety of the conjugate (following cleavage in the $1^MR \downarrow G^3$ site). Such a small peptide would be difficult if not impossible to identify and analyze via MS. Thus, we substituted Arg$^2$ with His, which now allows tryptic cleavage in HPV-58 E7 only after Arg$^2$, generating an 11-mer $7^R \downarrow GG^{76}$, $1^\text{MHGNNTPLR}^9 \downarrow$ or $\text{GGMHGNTPLR}$ (Fig. 5A). We assumed that it would be easier to identify the 11-mer, which was indeed the case. Thus, we generated the mono-ubiquitin con-
jugate, purified and resolved it on a gel (Fig. 5B), and subjected it to tryptic digestion. MS analysis of the tryptic peptides resulted in identification of a specific, energy-generated peptide, with a molecular weight identical to the expected fusion peptide (Fig. 5C(i)). MS/MS analysis of this peptide revealed with high certainty that this is, indeed, the fusion peptide (Fig. 5C(ii)).

DISCUSSION

Strong experimental evidence supports the notion that MyoD, p21, the extracellular signal-regulated kinase 3, the Epstein-Barr virus LMP1 and LMP2A, HPV-16 E7, Id1 and Id2, and possibly hydroxymethylglutaryl-CoA reductase are degraded following attachment of the first ubiquitin moiety to their N-terminal moiety. However, for some of the substrates that were tested (MyoD (3), HPV-16 E7 (5), LMP1 (6), and Id2 (10)), it has been shown that their internal lysine residues play an important role in modulating their stability, probably by also undergoing ubiquitination. Thus, it has become important to identify a group of proteins the degradation of which will be completely dependent on ubiquitination at the N-terminal residue. At least theoretically, such a group may be the NOLLPs. A search of the protein data base has revealed that ~300 proteins do not contain any lysine residue; and almost 200 of them are eukaryotic, of which ~20 are human and ~100 are of viral origin. We elected to study the mechanisms that underlie the degradation of two of these proteins, the human tumor suppressor and cell cycle regulator p16INK4a and the viral oncopogene HPV-58 E7.

As seen in Figs. 1 and 2, HPV-58 E7 is conjugated by ubiquitin followed by its degradation. In vitro, we demonstrate that the protein is polyubiquitinated (Fig. 1B), and the modification is necessary for its degradation by the system; it was inhibited by the chain terminator MeUb, and the inhibition was alleviated by addition of excess WT ubiquitin (Fig. 1C). In cells, the protein was rapidly degraded, and the degradation was completely inhibited by MG132, an inhibitor of the 26 S proteasome (Fig. 2).

As for p16, we demonstrate that the protein is degraded only in sparse cells and is stable in confluent cells (Fig. 3, B and C). In correlation with this finding is the observation that ubiquitination of the protein also occurs only in sparse cells, thus strongly linking ubiquitination to degradation of the protein (Fig. 3D). The mechanisms that signal p16 for degradation only in sparse cells and stabilize the protein in confluent cells are still obscure. The only known post-translational modification that the protein undergoes is phosphorylation, yet this modifi-
cation has not been associated with its stability (17). A surprising finding in that context is that confluence affected HeLa cells, which are malignantly transformed. Typically, contact inhibition is observed in primary mortal cells. It is possible that HeLa cells still retain some properties of primary cells but are evading cell cycle arrest that should have resulted from increased p16 via other mechanisms. Such a mechanism may be, for example, deregulation of the retinoblastoma protein by HPV-E7 protein.

Another important yet unsolved problem has been the lack of direct evidence for N-terminal ubiquitination. Although the accumulating experimental evidence strongly suggests that modification occurs on the N-terminal residue, this has not been shown directly. Interestingly, even for the canonical ε-NH₂ group internal lysine ubiquitination, only in a handful of cases it has been shown directly (via chromatographic and Edman degradation or via mass spectrometric analysis) that ubiquitin is bound to a specific internal lysine residue (see, for example, Refs. 28 and 29). In most cases studied, and there are not many, the assumption that an internal lysine serves as the polyubiquitin chain anchor has been based on mutational analyses. Bloom et al. (8) brought us a bit closer, showing that ubiquitination of p21 must occur on a residue in its N-terminal domain. They conjugated N-terminally His-tagged ubiquitin to N-terminally HA-tagged p21 that contained a Factor X proteolytic site immediately after the HA tag (His-ubiquitin conjugated to HA-Factor X site-p21). Treatment of the p21 ubiquitin conjugate with Factor X released a smaller species of p21 (that lacks the HA tag-Factor X site) and His-ubiquitin-HA tag-Factor X site, thus demonstrating that the HA tag, which was previously part of p21, has become now part of the Factor X-cleaved ubiquitin. Using a TEV protease cleavage site inserted into HPV-58 E7 protein, we now provide similar evidence (Fig. 4). Thus, cleavage of the ubiquitin conjugate of the protein that contains the TEV site generated an extended ubiquitin molecule because the site is cleaved C-terminally to its sixth amino acid. When the site was inserted after the first 21 residues of E7, a ubiquitin derivative, larger by ~3 kDa (27 residues), was generated. When the site was inserted immediately following the iMet, the derivative was larger by ~0.8 kDa (7 residues). The most likely conclusion that can be derived from these two experiments is that the ubiquitin moiety was fused to the N-terminal residue of the target protein. However, it is still possible that ubiquitin modifies a non-lysine residue such as

![Diagram](image_url)
Ser, Thr, Cys, or Tyr, generating an ester or a thiol ester bond. It should be noted, however, that heating the conjugate of HPV-58 E7 in an alkaline pH or in a high concentration of dithiothreitol or β-mercaptoethanol did not affect the conjugate, ruling out the formation of high pH or high SH-sensitive bonds (data not shown). Thus, the evidence provided by the two experiments clearly limits an unlikely non-peptide (and probably non-ester or non-thiol ester) bond ubiquitination to a much narrower zone in the N-terminal domain of p21 or E7. Coulombe et al. (9) brought us even closer in a study that was published in parallel to the on-line version of this one. They were able to isolate a fusion peptide between the C-terminal domain of ubiquitin and the N-terminal domain of HA-tagged p21 that also contained, downstream from the tag, a stretch of residues derived from the N-terminal domain of the native substrate but without the iMet (which was removed during the construction of the tagged protein). All these results, however, do not provide direct evidence for ubiquitination on the native N-terminal residue of a native cellular protein. The only direct evidence for such a modification would be the demonstration of a fusion peptide between the C-terminal domain of ubiquitin and the N-terminal domain of an unmodified target substrate. We now provide such evidence. Mass spectrometric analysis of tryptic digest of the isolated mono-ubiquitin adduct of HPV-58 E7 revealed a peptide of 11 amino acids, GG-MHGNNPTLR (Fig. 5). It should be noted that WT HPV-58 E7 contains an Arg residue in position 2. It was necessary to substitute this Arg with His because otherwise the digesting enzyme, trypsin, would have generated a tetrapeptide, GG-MR, that would have been difficult if not impossible to identify in the MS analysis. MS/MS analysis of the 11-mer verified its internal sequence.

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Note Added in Proof—After this manuscript was submitted, Kuo and colleagues published data demonstrating that an additional NOLLP, p19Ref, is degraded following its ubiquitination at the N-terminal residue (Kuo, M. L., den Besten, W., Bertwistle, D., Roussel, M. F., and Sherr, C. J. (2004) Genes Dev. 18, 1862–1874)

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