The Tumor Suppressor Protein p16\(^{INKa}\) 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 lysine 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, neverthless, 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\(^{INKa}\). For these proteins, the only residue that can be targeted is the N-terminal residue. Interestingly, p16\(^{INKa}\) is degraded in a cell density-dependent manner. Importantly, we provide 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\(^1\) 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 \(\epsilon\)-NH\(_2\) 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 lysine residues 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 carbamoylation almost completely blocked degradation. Fusion of a Myc\(_\ddagger\) 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...

\(\ddagger\) 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; NOLLP, naturally occurring lysine-less protein; TEV, tobacco etch virus; MG132, N-carbobenzoxy-l-leucyl-l-leucyl-leucinal; ATP-S, adenosine 5’-\(\gamma\)-thiotriphosphate; MS, mass spectrometry; MS/MS, tandem mass spectrometry.

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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 “canonical” 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 L-35S-labeled methionine and cysteine for metabolic labeling, [35S]methionine for in vitro translation, and prelabeled molecular weight markers were obtained from Amersham Biosciences. Ubiquitin, dihydroxyacetone phosphate, ATP, phosphocreatine, creatine phosphokinese, 2-deoxyglucose, isopropyl-β-D-thiogalactopyranoside ampicillin, and Tris buffer were from Sigma. ATP-S and immobilized protein A- and G-agarose were from Roche Applied Science. Methylation ubiquitin was from Affiniti-Research (Bioulom, Ltd.). All tissue culture reagents were purchased from Biological Industries (Bet Haemek, Israel) or from Sigma. Protein staining solution (SeeBand Forte) was from Gene Bio-Application. Antibodies against E7 and p16 (ED-17 and sc-468, respectively) were from Santa Cruz Biotechnology. Anti-His antibody was from Qiagen. Anti-ubiquitin polyclonal antibody was raised in rabbits as described previously (20). N-Carboxbenzoxyl-l-leucyl-l-leucyl-leucinal (MG132) and protease inhibitor mixture set I were from Calbiochem. Wheat germ extract-based transcription-translation-coupled kit (T7-VIP) was from Promega. ECL reagent was from Pierce. Recombinant tobacco etch virus (TEV) protease was from Invitrogen. Restriction and modifying enzymes were from New England Biolabs and Roche Applied Science. Oligonucleotides were synthesized by Sigma JetPEITM transfection reagent was from Polyplus Transfection. All other reagents were of high analytical grade.

Methods

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 plink322 vector containing the complete HPV-58 genome kindly received from Dr. Toshihiko Matsukura, Laboratory of Tumor Viruses, National Institute of Infectious Diseases,
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Tokyo, Japan) and subcloned into the XhoI-Xbal site of the mammalian expression vector pCS2+ 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 NdeI-BamHI site of the bacterial expression vector pT7-7. A His-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 310 per migration osmotic sequenator (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 (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 μM). 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 (15%). The gel was blotted onto nitrocellulose membrane, and proteins were detected by ECL following incubation with anti-E7 antibody and an appropriate secondary antibody.

Cellular stability of p16 was monitored in a pulse-chase labeling and immunoprecipitation experiment. HeLa cells expressing endogenous p16 were incubated in 1 h in a methionine- and cysteine-free medium following by a 1-h labeling (pulse) with 0.17 mCi/ml of [35S]methionine and cysteine mixture (PPO-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. Immunocomplexes were collected using immobilized protein A. Following SDS-PAGE (15%), proteins were visualized by a PhosphorImager.

Demonstration of p16TagUb 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 dissolved 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 5 min, and centrifuged for 5 min at room temperature at 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 stained with 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 assays 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 mM phosphocreatine and 0.5 μM of creatine phosphokinase) or 0.5 μg of hemoglobin 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·S or 0.5 μM 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 via SDS-PAGE (15%), and gels were dried and exposed to a PhosphorImager screen.

Ubiquitination at the N-terminal Residue as Demonstrated by TEV Proteolysis—[125I]MeUb was prepared using the chloramine-T method as described previously (22). Conjugation of bacterially expressed C-terminal His-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 125I-MeUb (~2 × 10^5 cpm/μg) (added instead of WT ubiquitin) as described above. ATP·S was added as indicated. Following incubation, the free proteins and the conjugates were isolated using Ni2⁺-agarose beads. Cleavage by the TEV protease was carried out at 30 °C for 2 h using the Ni2⁺-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 via SDS-PAGE (18%), and proteins were visualized by a PhosphorImager.

In-vivo 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 mM iodoacetamide at room temperature for 30 min), and digested with trypsin (overnight at 37 °C using modified trypsin (Promega) at a 1:100 enzyme to substrate ratio). The resulting tryptic peptides were resolved by reverse-phase chromatography on a 0.1 × 300-mm fused silica capillaries (J&W Scientific) packed with POROS 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 MS scan. The identity of the peptides was further confirmed using MALDI-TOF (matrix-assisted laser desorption ionization time-of-flight) MS (4700, Applied Biosystems). The MS data were analyzed using the Sequest software.2

RESULTS

The NOLLHPV-58 E7 Is Targeted by the Ubiquitin-Proteasome System In Vitro and In Vivo—Once HPV-58 E7 had been identified as a NOLLHP (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 ATP·S, a derivative of ATP that can activate E1 via cleavage of the β-γ bond but cannot release the γ-phosphate, demonstrates that ubiquitination does not require phosphorylation. Moreover, 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 terminus MeUb competed with the endogenous ubiquitin in the HeLa extract and inhibited the degradation of E7 significantly. Addition of

2 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 p16^{INK4a} 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 p16^{INK4a} (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 weather 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 p19^{INK4d} changes dramatically during the cell cycle and in correlation with its very short half-life (24), whereas p16^{INK4a} is a much more stable protein (26). Several groups have reported up-regulation of p16^{INK4a} 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 p16^{INK4a}.

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,
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Fig. 3. Degradation p16K is cell density-dependent. A, the amino acid sequence of p16K, which is a NOLLP, is shown. B, the steady-state level of p16K 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 either anti-ubiquitin or anti-p16 antibody (as indicated), as described under “Experimental Procedures.” C, degradation of p16K in sparse and confluent HeLa cells is shown. (i), endogenous p16K 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 p16K in HeLa cells is shown. 100 or 20% confluent (see B) HeLa cells were treated with MG132 for 2 h as indicated. Following lysis, p16K was immunoprecipitated, and the immune complexes were resolved via SDS-PAGE, blotted onto nitrocellulose membrane, and subjected to Western blot analysis using anti-p16 antibody 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 mono-ubiquitin 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, with that in lane 2, 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(ii), lanes 1–4). Interestingly, generation of both poly- and mono-ubiquitinated 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 RGG76 site), and the MR would be derived from the HPV-58 E7 moiety of the conjugate (following cleavage in the MR G3 site). Such a small peptide would be difficult if not impossible to identify and analyze via MS. Thus, we substituted Arg2 with His, which now allows tryptic cleavage in HPV-58 E7 only after Arg2, generating an 11-mer "RGG76 site), and the MR would be derived from the HPV-58 E7 moiety of the conjugate (following cleavage in the MR G3 site). 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jugate, purified and resolved it on a gel (Fig. 5B), and subjected it to trypsic digestion. MS analysis of the trypsic 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 p16<sup>INK4a</sup> and the viral oncogene 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 /H9280-NH2 group internal lysine ubiquitination, only in a handful of cases has it 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, 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 /H110113 kDa (27 residues), was generated. When the site was inserted immediately following the iMet, the derivative was larger by /H110110.8 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
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 joined with His because otherwise the digesting enzyme, trypsin,
The Tumor Suppressor Protein p16\textsuperscript{INK4a} 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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