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Preservation of Caspase-3 Subunits from Degradation Contributes to Apoptosis Evoked by Lactacystin: Any Single Lysine or Lysine Pair of the Small Subunit Is Sufficient for Ubiquitination

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ABSTRACT

Procaspase-3 (p32) is processed by upstream caspases to p12 and p20 subunits, which heterodimerize. Concomitant with formation of the active heterotetramer, p20 is autoprocessed to p17. Treatment of HL-60 cells with lactacystin, a selective inhibitor of the proteasome, exponentially increased caspase-3-like hydrolytic activity and induced apoptosis but had little or no effect on the activity of upstream caspase-8, caspase-9, or granzyme B. Lactacystin treatment decreased the p32 zymogen and evoked the accumulation of the p17 and p12 subunits. Treatment of transfected human retinoblast 911 cells with a proteasome inhibitor evoked the accumulation of epitope-tagged p12, p17, and p20 but had no effect on p32 zymogen. This result suggests that caspase-3 subunits, in contrast to the zymogen, are unstable because of degradation by the ubiquitin-proteasome system. Ubiquitin conjugates of p12 and p17 accumulated in cells that were cotransfected with p12 and a caspase inactive mutant of p17. Substitution of arginine for all eight lysines of p12 almost abolished its ubiquitination. Any single lysine or lysine pair was sufficient for p12 ubiquitination. Lactacystin treatment of HL-60 cells induced proteolytic processing of the X-linked inhibitor of apoptosis (XIAP) and decreased full-length XIAP, which is known to have ubiquitin-protein ligase activity for active caspase-3. These findings indicate that caspase-3 subunits can be degraded by the ubiquitin-proteasome system and suggest that lactacystin induces apoptosis in part by disabling the ubiquitin-protein ligase function of XIAP and by stabilizing active caspase-3 subunits.

Apoptosis is a cell-autonomous death process that depends on intracellular signal-transducing networks for the disassembly of the cell (Hengartner, 2000). The cardinal enzymes of the apoptosis pathway are caspases, a family of cysteine proteases that process substrates at specific aspartate residues (Earnshaw et al., 1999). Active caspases are heterotetramers composed of two large and two small subunits. Caspases are expressed as zymogens composed of three sequential domains (from the amino to the carboxyl terminus): prodomain, large subunit, and small subunit. Thus, caspase activation involves two processing events, one that removes the prodomain and the other that cleaves the zymogen between the large and small subunits. In the case of caspase-3, the zymogen is initially processed between the small and large subunits, usually by initiator caspase-8 or -9 or by granzyme B (Quan et al., 1996; Li et al., 1997; Atkinson et al., 1998; Stennicke et al., 1998), which generates the 12-kDa small subunit and a processing intermediate composed of the 3-kDa prodomain and the 17-kDa large subunit. The second processing step generates the large subunit by removal of the prodomain, which occurs by interior intramolecular autoprocessing (Han et al., 1997; Deveraux et al., 1998).

Caspase functions are subject to modulation by a family of inhibitor of apoptosis proteins (IAPs) (Deveraux and Reed, 1999). IAP proteins, such as X-chromosome linked IAP (XIAP), cIAP1, and cIAP2 contain a carboxy-terminal RING finger motif, a type of zinc-binding motif. The IAPs suppress apoptosis in part by directly binding and inhibiting various

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ABBREVIATIONS: IAP, inhibitor of apoptosis protein; XIAP, X-linked inhibitor of apoptosis protein; Ub, ubiquitin; Lacta, lactacystin; zIEALal, benyzloxycarbonyl-Ile-Glu(OtBu)-Ala-Leucinal; PARP, poly(ADP-ribose) polymerase; Ac-DEVD-amc, *N*-acetyl-Asp-Glu-Val-Asp-amino-4-methylcoumarin; Ac-LEHD-amc, *N*-acetyl-Leu-Glu-Hisl-Asp-amino-4-methylcoumarin; Ac-IETD-amc, *N*-acetyl-Ile-Glu-Thr-Asp-amino-4-methylcoumarin; Suc-Leu-Leu-Val-Tyr-amc, succinyl-Leu-Leu-Val-Tyr-amino-4-methylcoumarin; TNF-α, tumor necrosis factor-α; DMSO, dimethyl sulfoxide; His_6, hexahistidine; FBS, fetal bovine serum; PBS, phosphate-buffered saline; BSA, bovine serum albumin; PAGE, polyacrylamide gel electrophoresis; UPS, ubiquitin-proteasome system; HA, hemagglutinin.

caspases and by blocking zymogen processing (Deveraux et al., 1998; Deveraux and Reed, 1999). Although XIAP interacts with other proteins besides caspase-3, such as processed caspase-9 and Smac/DIABLO (Du et al., 2000; Verhagen et al., 2000; Srinivasula et al., 2001), Silke et al. (2002) have shown that XIAP can at least partially inhibit cell death by blocking caspase-3 alone. Three reports have suggested that IAPs suppress apoptosis by a mechanism involving ubiquitination and degradation of caspase proteins. Attachment of a chain of four or more ubiquitins (Ubs) (a conserved 76 amino acid protein) to a protein serves as a recognition signal for the proteasome, an ATP-driven protein disassembly system composed of the 26S compartmentalized multicatalytic protease (Hershko and Ciechanover, 1998; Pickart, 2000). Yang et al. (2000) reported that recombinant cIAP1 and XIAP have Ub-protein ligase activity and can mediate their own ubiquitination and degradation in vivo in transfected 293 cells. Huang et al. (2000) demonstrated that cIAP2 promotes the ubiquitination of caspase-3 and caspase-7 in vitro in reconstituted systems. Recently, Suzuki et al. (2001) showed that cotransfection of XIAP decreased the level of an inactive mutant of "reverse caspase", an unprocessed form of p32 that has a reversed order of the p12 and p17 subunits. The inactive mutant of reverse caspase-3 was used to avoid induction of apoptosis and because unprocessed reverse caspase-3 is active without the separation of the two subunits (Srinivasula et al., 1998).

Inhibition of the proteasome has been shown to induce apoptosis in HL-60 cells (Drexler, 1997) and in other cell types, such as Madin-Darby canine kidney cells (Bush et al., 1997), U937 cells (Imajoh-Ohmi et al., 1995), Molt-4 cells (Shinohara et al., 1996), and mouse lymphoma RVC cells (Tanimoto et al., 1997). Although the biochemical mechanisms of apoptosis evoked by proteasome blockade remain to be determined, current reports have implicated accumulation of the tumor suppressor p53 (Lopes et al., 1997; Wagenknecht et al., 1999; Chen et al., 2000a), heat shock proteins (Bush et al., 1997), p27Kip1 (Drexler, 1997), or proapoptotic proteins Bax (Li and Dou, 2000) and Bid (Breitschopf et al., 2000). A plausible mechanism that has not been tested, however, is the preservation of active caspase subunits from degradation by the Ub-proteasome system. Here, we report evidence that the highly selective proteasome inhibitor lactacystin (Lacta) (Fenteany et al., 1995) induces apoptosis in human leukemia HL-60 cells by stabilizing active caspase-3 subunits. Ubiquitination of the p12 or p17 caspase-3 subunits has not been reported in eukaryotic cells. We show here that ubiquitin conjugates of p12 and p17 subunits of caspase-3 accumulated in cells that were cotransfected with p12 and a mutant of p17 that lacks caspase activity. Degradation of the p12 subunit by the proteasome seems to be particularly efficient because p12 was difficult to detect in transfected cells, even after affinity purification, unless the proteasome was blocked. Lacta treatment of HL-60 cells decreased XIAP, the most abundantly expressed IAP in HL-60 cells (Tamm et al., 2000), which is expected to contribute to the stabilization of active caspase-3 during the induction of apoptosis by proteasome blockade.

Materials and Methods

Reagents. Lacta was prepared as described previously (Omura et al., 1991) and generously provided by S. Omura (Kitasoto Institute, Tokyo, Japan). *N*-Benzyloxycarbonyl-Ile-Glu(OtBu)-Ala-Leucinal (zIEALal) and Suc-Leu-Leu-Val-Tyr-amc were from Bachem Biosciences (King of Prussia, PA). Horseradish peroxidase-conjugated immunogloblin to the hemagglutinin epitope tag (F-7) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Monoclonal antibodies to the Xpress and V5 epitope tags were from Invitrogen (Carlsbad, CA). Anti-poly(ADP-ribose) polymerase (PARP) and XIAP (H62120) monoclonal antibodies were from BD Biosciences (San Jose, CA). Recombinant porcine tumor necrosis factor-α (TNF-α) was from R&D Systems (Minneapolis, MN). Fluorogenic substrates Ac-DEVD-amc, Ac-LEHD-amc, and Ac-IETD-amc and the caspase inhibitor *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone were purchased from Enzyme Systems Products (Livermore, CA) and dissolved in DMSO. Ni^{2+}-nitrilotriacetic acid resin was from QIAGEN (Valencia, CA), and pGEM markers were from Promega (Madison, WI).

Plasmids. pRSC plasmid containing the CPP32 cDNA (Srinivasula et al., 1998) was used as the polymerase chain reaction template to produce cDNAs encoding p12, p17, p20, p29, and p32 with *Bam*HI and *Eco*RI restriction sites. The cDNAs were cloned into the corresponding restriction sites of pcDNA4HisMax to generate the indicated cDNAs with amino-terminal His_6 and Xpress epitope tags. Site-directed mutagenesis of p12 and p17 was done with the QuikChange method as described by the manufacturer (Stratagene, La Jolla, CA). C/A p17 has alanine substituted for the active site cysteine 163. A pcDNA3.1 plasmid encoding a tandem repeat of Ub with an amino-terminal hemagglutinin epitope tag was prepared by subcloning the *Eco*RI/*Not*I restriction fragment from a previously described HA-Ub pBluescript SK plasmid (Treier et al., 1994). Each of the constructions was validated by sequencing, carried out on double-stranded DNA with dye-terminator chemistry and an ABI Prism 377 automated sequencer (Applied Biosystems, Foster City, CA).

Cell Culture, Transfection, and Treatment with Proteasome Inhibitors. HL-60 cells were purchased from American Type Culture Collection (Manassas, VA) and grown in RPMI 1640 medium containing 10% fetal bovine serum (FBS), 100 units/ml penicillin G, and 0.1 mg/ml streptomycin. The medium was diluted with fresh medium three times per week, and cell density was kept below a million per milliliter. The cells were collected by centrifugation, and 1×10^7 cells were incubated in 1 ml of RPMI 1640 medium containing 10% FBS. Lacta was added to some cells from a 1000-fold concentrated stock solution in DMSO. Caspase activity, proteasome activity, and Western blot analysis were performed as described below.

The 911 line of adenovirus-transformed human embryonic retinoblasts was grown in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium containing 10% FBS, 100 units/ml penicillin G, and 0.1 mg/ml streptomycin (Fallaux et al., 1996). Cells were grown and incubated in a humidified atmosphere with 5% CO_2, 95% air at 37°C. 911 cultures (100-mm diameter) were transfected by a modified calcium phosphate method (Chen and Okayama, 1987; Fallaux et al., 1996; Jordan et al., 1996). Transfection efficiency was monitored with pcDNA4HisMaxLacZ plasmid (Invitrogen) and histochemical staining with the β-galactosidase substrate X-Gal (Cepko et al., 1995). Twenty-four hours after starting transfection, the cultures were rinsed once with room temperature PBS, and 10 ml of fresh culture medium was added. Forty-eight hours after starting transfection, the volume of the culture medium was reduced to 5 ml. Some cultures were treated with 20 μM zIEALal, which was added from a thousand-fold concentrated stock solution in DMSO. zIEALal was used for the transfection experiments because of the limited supply and prohibitive cost of Lacta. Four or 6 h after the addition of zIEALal, the cells were rinsed once with ice-cold PBS and lysed with 1 ml of ice-cold guanidine lysis solution that contained 6 M guanidine HCl, 50 mM sodium phosphate buffer, pH 8.0, 0.3 M NaCl, and 5 mM imidazole. The lysate was centrifuged at 16,000*g* at 4°C for 30 min, and the protein concentration of lysate was determined by the Brad-

ford method with BSA as a standard (Bio-Rad, Hercules, CA). His_6-tagged proteins were affinity purified and subjected to Western blot analysis as described below.

Purification of His_6-Tagged Proteins. Denaturing conditions were used to minimize copurification of noncovalently associated proteins and the degradation or deubiquitinated of protein-Ub conjugates, essentially as described previously (Chen et al., 2000b). To affinity purify the His_6-tagged proteins, the cultures (usually three per condition) were lysed with 1 ml of guanidine lysis buffer (described above). The lysate was homogenized with a 26-gauge needle and centrifuged for 30 min at 16,000*g* at 4°C in a Microfuge. The lysate was continuously rotated for 1 h at 4°C with 40 μl of 50% (v/v) cobalt affinity resin (Talon; BD Biosciences Clontech, Palo Alto, CA). The resin was washed twice with lysis buffer, twice with lysis buffer lacking guanidine, and twice with lysis buffer lacking NaCl and guanidine. Proteins were extracted from the resin with twice concentrated, SDS-PAGE sample solution containing 0.2 M imidazole, pH 6.8, and incubated for 5 min in a boiling water bath. Proteins were fractionated by SDS-PAGE (10% gel), electrophoretically transferred to a polyvinylidene difluoride membrane, and subjected to Western blot analysis as described previously (Smith et al., 2000).

Affinity purification of the complex of His_6-tagged p12 with C/A p17 lacking the His_6 tag was done under nondenaturing conditions. The nondenaturing lysis buffer contained 50 mM sodium phosphate buffer, pH 8.0, 0.3 M NaCl, 5 mM imidazole, 0.4 M guanidine HCl, 0.2% Nonidet NP-40, 1 mM Pefabloc with SC protector, 25 μM acetyl-Leu-Leu-norleucinal, and 2 μg/ml leupeptin. The lysate (1.5 ml) was homogenized with a 26-gauge needle and rotated with 60 μl of cobalt affinity resin (Talon; BD Biosciences Clontech) for 1 h at 4°C. The resin was washed twice with lysis buffer, twice with wash buffer that contained 50 mM sodium phosphate buffer, pH 8.0, 0.3 M NaCl, 5 mM imidazole, and twice with wash buffer lacking NaCl. Proteins were extracted from the resin as described for affinity purification under denaturing conditions.

Western Blot Analysis. After the incubation in the presence or absence of Lacta, HL-60 cells were collected by centrifugation (10 min, 100*g*) and lysed with 0.1 ml of >90°C SDS buffer that contained 1% (w/v) SDS, 10 mM Tris-HCl, pH 7.4, 2 mM EDTA, and 2 mM EGTA. Protein concentration was determined by the bicinchoninic acid method (Pierce Chemical, Rockford, IL) with BSA as a standard. Lysate proteins (30 μg) were fractionated by SDS-PAGE (10% gel), electrophoretically transferred to a polyvinylidene difluoride membrane, and subjected to Western blot analysis as described previously (Smith et al., 2000). Each Western blot is representative of at least three experiments.

Caspase Activity, DNA Fragmentation, and Cell Viability. The cells were rinsed twice with ice-cold PBS and suspended in a buffer containing 20 mM Tris-HCl, pH 8.0, 1 mM EGTA, 1 mM dithiothreitol, and 10 μg/ml each of leupeptin, aprotinin, and pepstatin. The cells were disrupted by three freeze-thaw cycles, and the lysate was centrifuged at 4°C for 30 min at 16,000*g*. The supernatant was assayed for protein by the Bradford method (Bio-Rad, Hercules, CA) with BSA as a standard, and 0.1 mg was used to assay caspase activity with 50 μM Ac-DEVD-amc, Ac-IETD-amc, or Ac-LEHD-amc as substrate for caspase-3, -8, and -9, respectively. Fluorescence caused by the production of amino-4-methylcoumarin was continuously recorded at 440 nm (excitation at 380 nm) at 37°C in 2 ml of buffer that contained 20 mM Tris-HCl, pH 8.0, and 2 mM $MgCl_2$.

Genomic DNA was extracted from HL-60 cells with a kit from Stratagene and fractionated on a 1.5% agarose gel in the presence of Tris-borate, pH 8.0, and ethidium bromide. Cell viability was estimated by hemocytometer counting of total and trypan blue-excluding cells. Results are mean ± S.E.M. (n = 3).

Proteasome Activity. Chymotrypsin-like activity of the proteasome was assayed essentially as described previously (Dick et al., 1997). HL-60 cells were suspended in 1 ml of fresh culture medium containing 10% FBS and incubated with or without 20 μM Lacta for 1 h. The cells were rinsed twice with ice-cold PBS and suspended in homogenization buffer that contained 20 mM Tris-HCl, pH 8.0, 1 mM ATP, 1 mM EGTA, 1 mM EDTA, and 5 mM β-mercaptoethanol. The cells were disrupted with a Dounce homogenizer (50 strokes with a tight-fitting pestle). Homogenates were centrifuged at 16,000*g* for 30 min at 4°C, and the supernatant was assayed for protein by the Bradford method with BSA as standard. Chymotrypsin-like activity of the proteasome was assayed at 37°C with 50 mM Suc-Leu-Leu-Val-Tyr-amc as substrate in 2 ml of buffer that contained 20 mM Tris-HCl, pH 8.0, and 2 mM $MgCl_2$. The rate of amino-4-methylcoumarin production was determined by continuously recording fluorescence at 440 nm (380-nm excitation).

Results

Lacta Activates Caspase-3-Like Hydrolytic Activity and Induces Apoptosis in HL-60 Cells. Lacta is a highly selective inhibitor of the 26S proteasome (Fenteany et al., 1995). Lacta treatment exponentially increased Ac-DEVD-amc hydrolysis, a measure of caspase-3-like hydrolytic activity, from 1 to 8 h (Fig. 1). At 4 and 8 h, caspase-3-like activity was 8- and 38-fold that of untreated cells, respectively (Fig. 1A). Treatment of HL-60 cells with 20 μM Lacta for 1 h essentially abolished the chymotrypsin-like hydrolytic activity of the 26S proteasome, which was determined with Suc-Leu-Leu-Val-Tyr-amc as substrate. Chymotrypsin-like activity was 1.24 ± 0.09 nmol/min · mg in untreated cells and undetectable in the treated cells.

In contrast to the profound effect of the Lacta treatment on caspase-3-like hydrolytic activity, it had no effect on caspase-8-like activity. Caspase-8-like activity, which was assayed with Ac-IETD-amc, was unaffected by the 8 h of Lacta treatment (Fig. 1B). Because Ac-IETD-amc is also efficiently hydrolyzed by granzyme B (Thornberry et al., 1997), these results suggest that the Lacta treatment failed to activate caspase-8 or granzyme B. The Lacta treatment (8 h) modestly increased caspase-9-like hydrolytic activity, assayed with Ac-LEHD-amc, to 1.6 times that of untreated cells (Fig. 1B). In contrast to Lacta, treatment with TNF-α plus cycloheximide for 8 h increased caspase-8-like and caspase-9-like hydrolytic activities by 2.3 and 3.9 times (Fig. 1B). Shorter Lacta treatments (4 h) had no effect on either caspase-8 like- or caspase-9-like activities (L. Chen and J. Smith, unpublished observations).

Lacta treatment for 4 to 8 h progressively changed cell morphology from spheroid to irregular with large protrusions (L. Chen and J. Smith, unpublished observations). At 4 h of Lacta treatment, cell viability was unchanged compared with the untreated cells (98 ± 3%) but at 8 and 24 h of Lacta treatment cell viability decreased to 72 ± 6 and 33 ± 2%, respectively. In addition to caspase-3 like hydrolytic activity, two additional hallmarks of apoptotic cell death were documented. A 5-h incubation with 10 μM Lacta induced DNA fragmentation and produced the 86-kDa carboxyl-terminal fragment of the DNA damage recognition protein PARP (Fig. 2). Treatment of the cells with lower concentrations of Lacta (1 or 3 μM) produced much less of the PARP fragment and less DNA fragmentation than the treatment 10 μM Lacta (Fig. 2). In agreement with the lesser effects of the lower Lacta concentrations on PARP processing and DNA fragmentation, treatment with 1 or 3 μM Lacta for 1 h only partially inhibited the chymotrypsin-like activity of the proteasome (L. Chen and J. Smith, unpublished observations).

Lacta Decreases Caspase-3 Zymogen and Evokes the Production of Active Caspase-3 Subunits. Treatment of HL-60 cells with 20 μM Lacta from 4 to 8 h progressively decreased caspase-3 zymogen (p32) and increased active caspase subunits (p17 and p12) as determined by Western blot analysis (Fig. 3). Longer film exposures were required to detect the active caspase-3 subunits, which overexposed and obscured the changes in the p32 zymogen (Fig. 3B). Untreated cells (1 or 8 h) lacked detectable p12 and p17 subunits but, interestingly, had a small amount of the 20-kDa protein, which seems to be an intermediate in caspase-3 processing (Fig. 3B). The 20-kDa band (p20) decreased progressively as the duration of the Lacta treatment increased concomitant with the increase in p17 (Fig. 3B). The observed reciprocal changes in p20 and p17 bands produced by the Lacta treatment are in agreement with the two-step sequential model of caspase-3 zymogen processing to active subunits (Han et al., 1997; Deveraux et al., 1998).

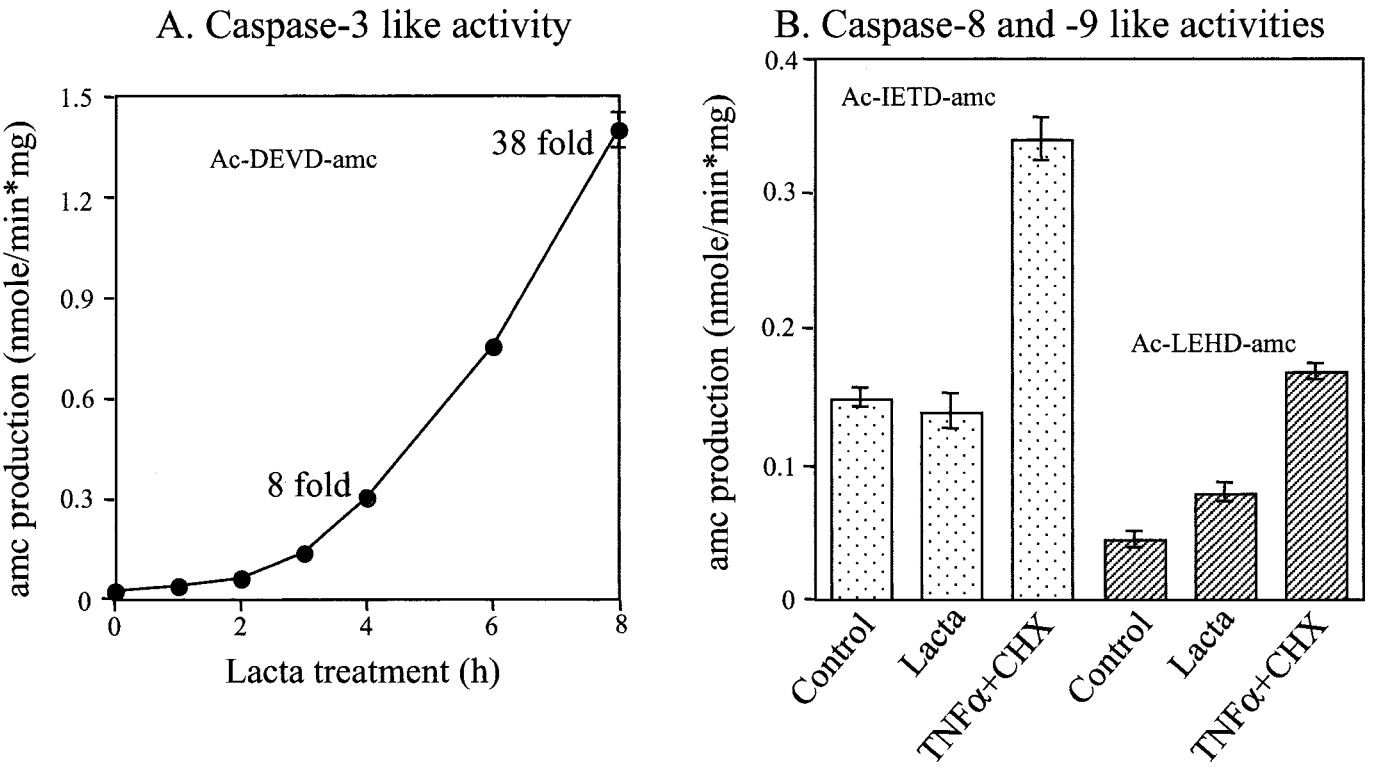

Fig. 1. Effect of Lacta treatment on caspase hydrolytic activities of HL-60 cells. A, cells were treated with 20 μM Lacta for 1 to 8 h in RPMI 1640 medium containing 10% FBS. Cell lysates (0.1 mg of protein) were assayed for caspase-3-like activity by continuous recording of 7-amino-4-methylcoumarin fluorescence produced by hydrolysis of Ac-DEVD-amc. B, cells were treated with 20 μM Lacta or 25 ng/ml TNF-α plus 10 μg/ml cycloheximide (CHX) for 8 h in RPMI 1640 medium containing 10% FBS. Cell lysates (0.2 mg) were assayed for caspase-8-like (Ac-IETD-amc) or caspase-9-like (Ac-LEHD-amc) activities. Results are mean ± S.E.M. (n = 3).

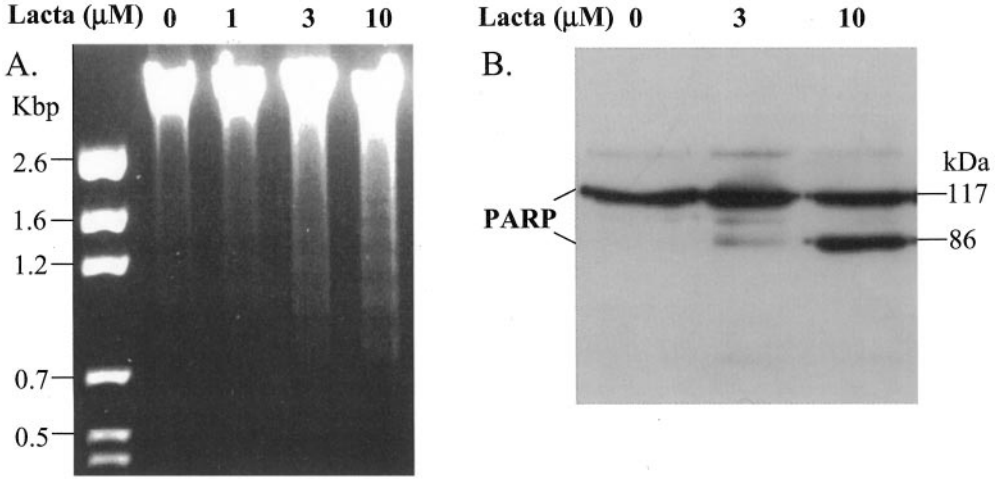

Fig. 2. Treatment of HL-60 cells with Lacta induced DNA fragmentation and the proteolytic processing of PARP. The cells were incubated in RPMI 1640 medium containing 10% FBS and treated with 3 or 10 μM Lacta for 5 h as indicated. A, genomic DNA was isolated and 10-μg samples were size fractionated on a 1.5% agarose gel with pGEM DNA standards in the presence of ethidium bromide. B, cells were rinsed with PBS, and proteins were extracted with SDS sample solution, fractionated by SDS-PAGE, and subjected to Western blot analysis with a monoclonal antibody to PARP.

Proteasome Inhibition Increases the Levels of Caspase-3 Polypeptides in Transfected Cells. Because Lacta treatment of HL-60 cells predominantly increased caspase-3-like hydrolytic activity and the levels of active caspase-3 subunits (Figs. 1 and 3), we decided to determine whether caspase-3 subunits were degraded by the Ub-proteasome system (UPS). The 911 adenovirus helper line of human embryonic retinoblasts was transiently transfected with pcDNA4HisMax plasmids encoding the following caspase-3 polypeptides: p12, p17, p20, p29, and p32. The p20 and p32 polypeptides included the 3-kDa amino-terminal prodomain, which was absent from p29. Each of the peptides had amino-terminal Xpress epitope and His_6 tags. Two days after the start of the transfection, some cultures were treated with a potent peptidyl aldehyde proteasome inhibitor, zIEALal, for 4 h. The cells were lysed and His_6-tagged peptides were affinity purified under denaturing conditions. Figure 4A shows an anti-Xpress immunoblot of the affinity-purified polypeptides. Treatment with the proteasome inhibitor markedly increased accumulation of p12, p17, and p20 but had no effect on the levels of p29 or p32 (Fig. 4A). The p12 polypeptide was not detectable in the untreated cells (Fig. 4A). These results suggest that p12 is the least stable of the caspase-3 polypeptides. Because they were all expressed from the pcDNA4HisMax vector with amino-terminal His_6 and Xpress epitope tags, the rates of transcription and translation of the caspase-3 cDNAs are probably similar.

Longer film exposures of the anti-Xpress immunoblot showed that the His_6Xress-tagged p20 was present in cells that were transfected with His_6Xpress-tagged p32, and His_6Xpress-tagged p17 accumulated in cells that were transfected with His_6Xpress-tagged p29 (Fig. 4B). The zIEALal treatment increased the accumulation of the p20 and p17 species in cells that were transfected with p32 and p29, respectively (Fig. 4B). These findings support the view that there is a spontaneous conversion of caspase-3 zymogen to active subunits in untreated cells and that proteasome blockade increased accumulation of the active subunits. It is noteworthy that 911 cells, in contrast to HL-60 cells (Figs. 1 and 4), require a prolonged zIEAL treatment (16–24 h) to activate caspase-3 (L. Chen and J. Smith, unpublished observations). Because proteasome blockade increased the accumulation of p12, p17, and p20 polypeptides, they are probably unstable because of degradation by UPS. Conversely, the lack of effect of the proteasome inhibitor on p29 and p32 suggest that they are relatively stable compared with the subunits.

Accumulation of Ub Conjugates of Caspase-3 Subunits after Inhibition of the Proteasome. Fig. 5 shows that mono-, di-, and tri-Ub conjugates of the p12 subunit of caspase-3 accumulated after the treatment of transfected 911 cells with the proteasome inhibitor zIEALal. Note that the 911 cells were cotransfected with hemagglutinin (HA) epitope-tagged ubiquitin (HA-Ub) and His_6 and Xpress epitope tagged p12. After the 4-h treatment of the cells with the proteasome inhibitor, the cells were lysed under denaturing conditions, and the His_6 tagged p12 species (non-Ub-conjugated and Ub-p12 conjugates) were affinity purified using a metal chelate resin. Western blot analysis with antibodies to the HA or the Xpress epitope tag showed that protein bands with the predicted apparent molecular masses of mono-, di-, and tri-Ub-p12 conjugates were immunostained by both antibodies (Fig. 5).

Little p12 accumulated in the 911 cells in the absence of proteasome blockade (no zIEALal) (Fig. 5), in agreement with the experiment shown in Fig. 4, which was done without cotransfection with HA-Ub. This result is particularly significant because p12 was affinity purified from one (Fig. 4) or three (Fig. 5) 100-mm-diameter cultures of 911 cells. 911 cells were chosen for these experiments because they can be transfected by the calcium phosphate method to a relatively high efficiency (>50%). Because inhibition of the proteasome dramatically increased the accumulation of p12 (Figs. 4 and

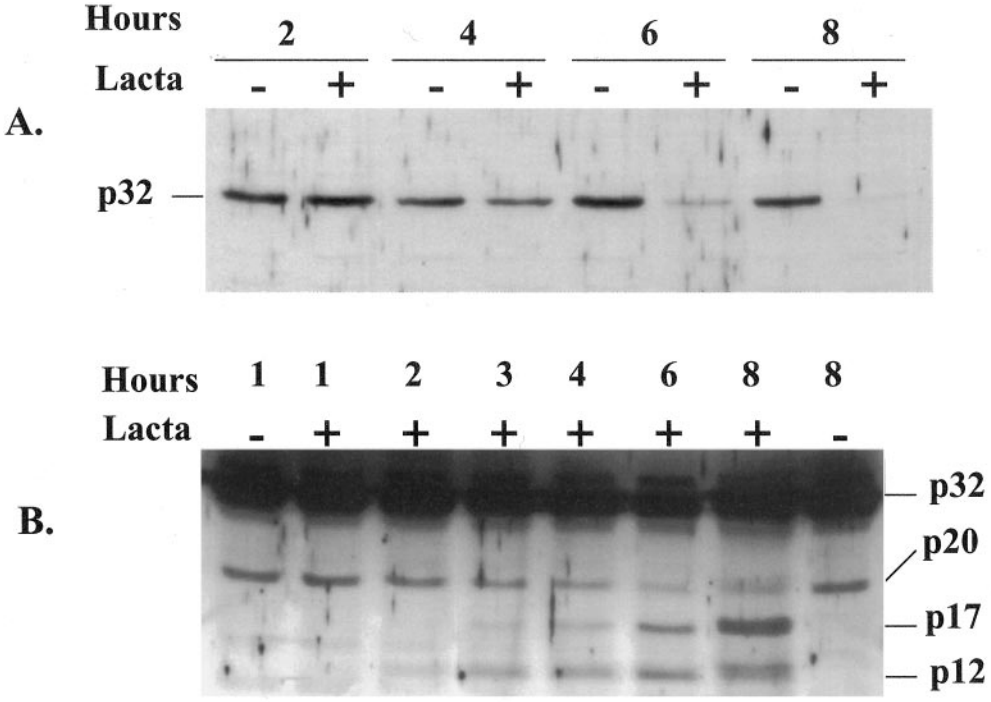

Fig. 3. Treatment of HL-60 cells with Lacta induced the proteolytic processing of procaspase-3 to subunits. The cells were treated for the indicated interval with 20 μM Lacta in RPMI 1640 medium containing 10% FBS, collected by centrifugation, and lysed with >90°C SDS sample solution. Proteins (30 μg) were fractionated by SDS-PAGE and subjected to Western blot analysis with a rabbit polyclonal antibody to p32 (H-277). The films were exposed for a short (A) or long (B) interval to detect p32 or active subunits (p12, p17, and p20), respectively.

5), the low level of p12 expression probably results from the efficient ubiquitination and degradation of the protein. In contrast to p12, the p17 subunit accumulated to a significant level in the absence of proteasome inhibition (Figs. 4 and 6). Treatment of the cells with the proteasome inhibitor increased the accumulation of p17 as expected for a protein

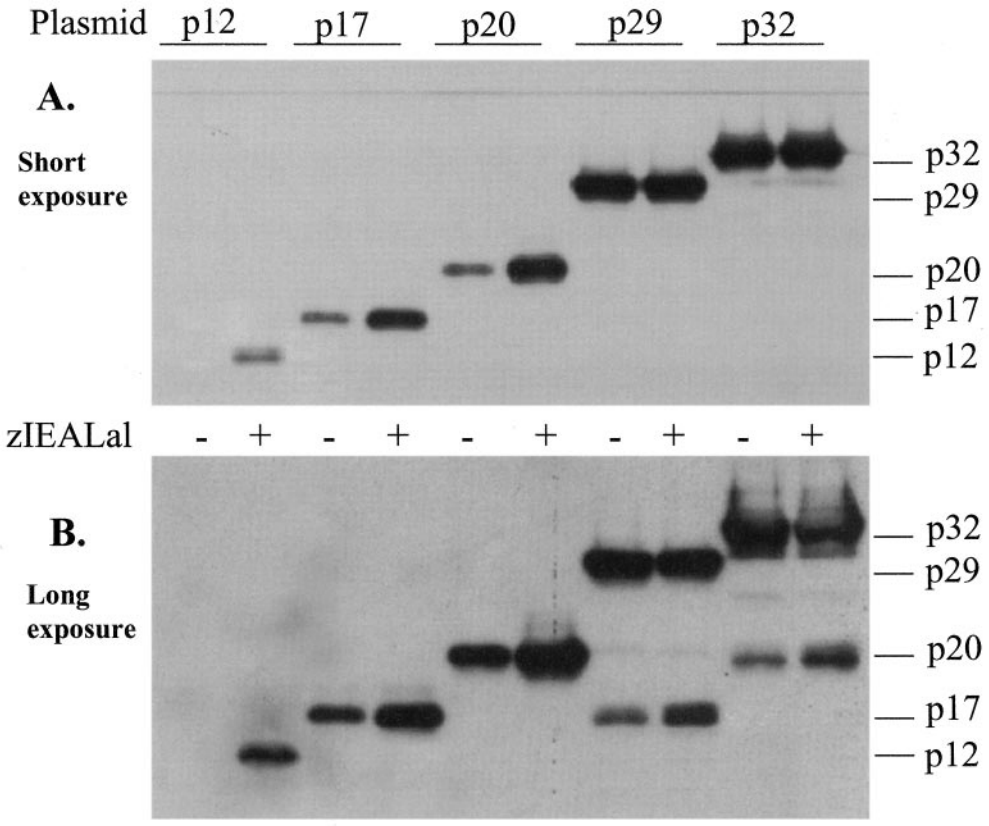

Fig. 4. Treatment of transfected 911 cells with a proteasome inhibitor increased the levels of caspase-3 polypeptides but not caspase-3 zymogen. 911 cultures (one per condition) were transfected with a plasmid encoding the indicated Xpress- and His_6-tagged caspase-3 subunit (p12, p17, or p20) or precursor form (p29 or p32). Two days after starting transfection, the indicated cultures were treated for 4 h with 20 μM zIEALal to inhibit the proteasome. Proteins were extracted and affinity purified under denaturing conditions (Chen et al., 2000b), fractionated by SDS-PAGE, and subjected to Western blot analysis with anti-Xpress antibody.

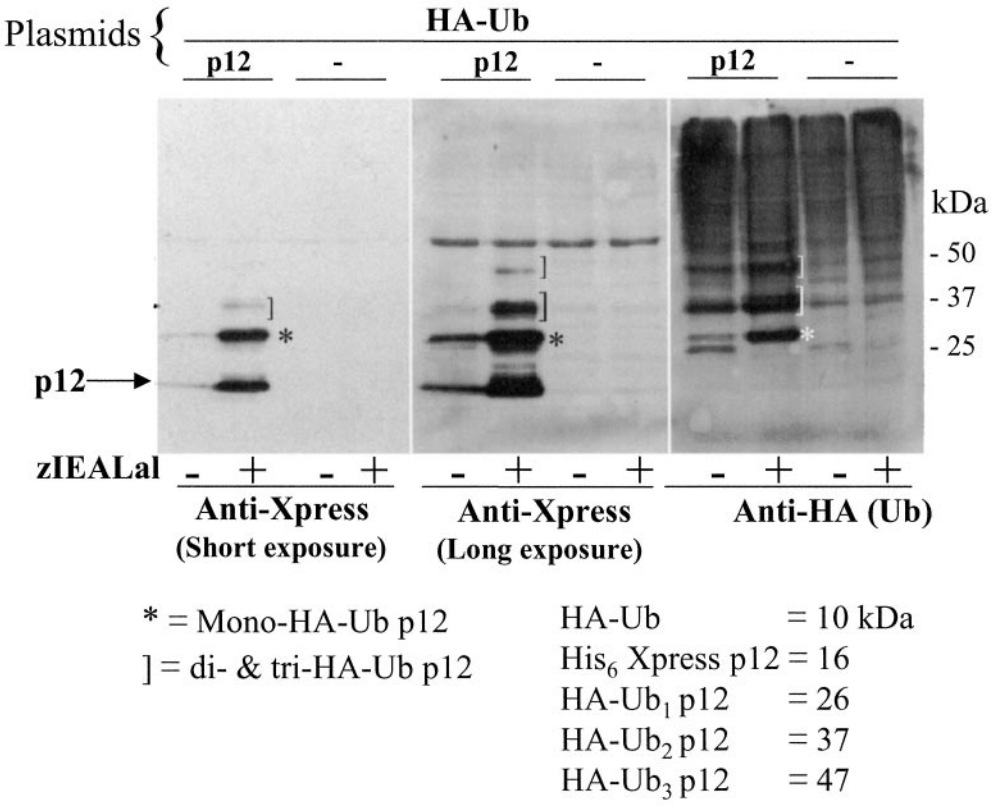

Fig. 5. Accumulation of mono-, di-, and tri-ubiquitinated p12 species after the treatment of transfected 911 cells with a proteasome inhibitor. 911 cultures (three per condition) were cotransfected with plasmids (7.5 μg each) encoding HA-tagged Ub and the indicated Xpress- and His_6-tagged p12 or empty vector (pcDNA3.1), which is indicated by the dash (−). Two days after starting transfection, the indicated cultures were treated for 4 h with 20 μM zIEALal to inhibit the proteasome. Proteins were extracted with buffer containing 6 M guanidine HCl, affinity purified under denaturing conditions, fractionated by SDS-PAGE, and subjected to Western blot analysis with an anti-Xpress or anti-HA antibody.

that is degraded by UPS. Note that a p17 mutant with cysteine 163 replaced by alanine (C/A p17) was used for these experiments because some cells were cotransfected with both caspase-3 subunits. If the active site cysteine 163 of p17 were not mutated, the two subunits would be expected to form active caspase-3, which would kill the cells. Mutation of the active site cysteine of p17 does not affect heterodimerization of the caspase subunits (Riedl et al., 2001).

The goal of the p12 and C/A p17 cotransfection experiment was to express both subunits so they could heterodimerize. C/A p17 was present in excess relative to p12 (Fig. 6). Hence, most of the p12 would be expected to be present as a heterodimer with C/A p17. Interestingly mono-, di-, and tri-Ub-p12 conjugates accumulated after the treatment with the proteasome inhibitor zIEALal in the cells that were cotransfected with both of the caspase-3 subunits (Fig. 6), as observed with cells transfected with p12 alone (Fig. 5). In contrast to the experiment shown in Fig. 5, the di- and tri-Ub-12 conjugates were detected by anti-HA staining but not by anti-Xpress staining (Fig. 6, compare middle and right), which may be because of differences in the level of p12 expression. This finding suggests that the p12 subunit of caspase-3 can be conjugated to Ub when p12 is dimerized with p17, which is known to occur when procaspase-3 is processed to active subunits. To determine whether p12 was in fact present as a heterodimer with p17, the following protein interaction experiment was done (Fig. 7).

Coexpressed Caspase-3 Subunits Complex with One Another. The cells were cotransfected with His_6-tagged p12 and C/A p17 that lacked the His_6 tag. The results of this experiment show that C/A p17, which lacked the His_6 tag, coaffinity purified with His_6-tagged p12 (Fig. 7, lane 1). The complex between the two caspase subunits was dissociated under denaturing conditions (Fig. 7, lane 2). The other lanes of Fig. 7 are controls. Lane 5 shows that p17 without a His_6 tag did not bind nonspecifically to the metal affinity resin. Lanes 6 and 7 show that cells transfected with either His_6-tagged p17 or p17 lacking the His_6 tag expressed similar amounts of p17, as determined by immunoprecipitation and Western blot analysis with antibody to the Xpress epitope tag.

Replacement of the Eight Lysine Residues of p12 with Arginine Stabilizes p12 and Prevents the Formation of Ub-p12 Conjugates. Ub is conjugated to a target protein by formation of an isopeptide bond between the carboxyl group of glycine 76 (the carboxyl terminus) and the epsilon amino group of one or more lysine residues of the target protein (Hershko and Ciechanover, 1998). The p12 subunit of caspase-3 has eight lysines. The effect of replacing all eight lysine residues of p12 with arginine was determined. Figure 8 shows that p12 with no lysines (No K p12) accumulated in the transfected cells without blockade of the proteasome (no zIEALal). In contrast to p12 lacking lysine, wild-type p12 accumulated only after treatment with the proteasome inhibitor (Fig. 8), as also shown in Figs. 4 to 6. Importantly, the treatment of the cells with the proteasome inhibitor had no effect on the accumulation of p12 lacking lysine, which suggests that it was not degraded by UPS.

Formation of Ub-p12 Conjugates Can Occur via Any Lysine or Lysine Pair of p12. Fig. 9 shows that any single lysine (or a lysine pair in the case of lysine 224 and 229 or lysine 259 and 260) is sufficient for the formation of a mono-Ub-p12 conjugate. For example, after transfection of the cells with p12 with only lysine 186 (K186 only p12) or only lysine

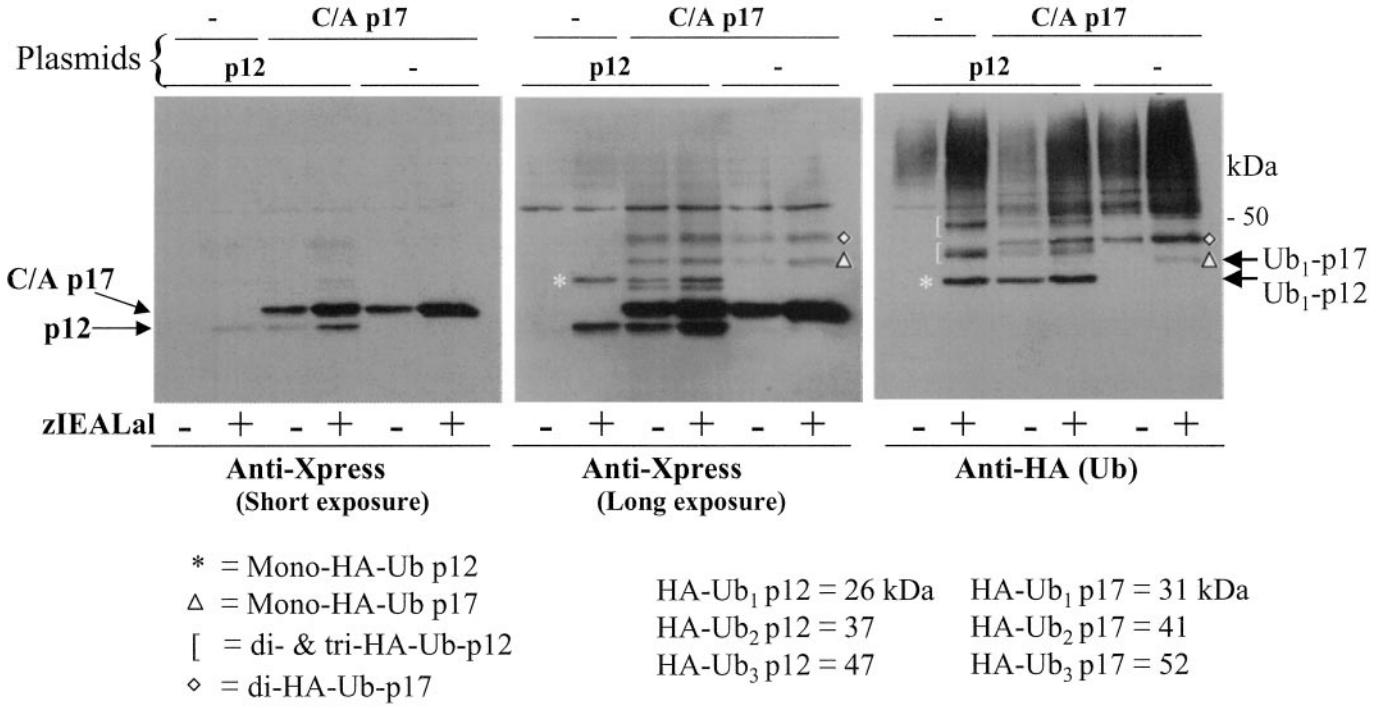

Fig. 6. Accumulation of mono-, di-, and tri-, and multiubiquitinated p12 and C/A p17 species after cotransfection of 911 cells with p12 and/or C/A p17 caspase-3 polypeptide and proteasome blockade. 911 cultures (three per condition) were cotransfected with the indicated His_6-Xpress-tagged p12 and/or His_6-Xpress-tagged p17 polypeptide and HA-Ub (7.5 μg each). Two days later, the indicated cultures were treated for 6 h with 20 μM zIEALal. His_6-tagged proteins were affinity purified under denaturing conditions and subjected to Western blot analysis with an antibody to the Xpress- or HA-epitope tag. C/A p17 has an alanine substituted for active site cysteine 163 to inactivate the caspase function.

210 or 242 (K210 only p12 or K242 only p12), mono-Ub-p12 accumulated. For this experiment, the cells were cotransfected with the indicated p12 mutant, C/A p17, and HA-tagged Ub, and they were treated with the proteasome inhibitor to induce the accumulation of each p12 mutant and the mono-Ub conjugate of each p12.

Note that the cells expressed a similar level of C/A p17 under all conditions, but the different p12 mutants accumulated to different extents (Fig. 9). For example, p12 with lysine 271 only (K271 only p12) accumulated to an appreciable extent (similar to wild-type p12), but little Ub-K271 only p12 accumulated in the cells after the treatment with the proteasome inhibitor. Therefore, it seems that the p12 with only lysine 271 does not form Ub conjugates as readily as

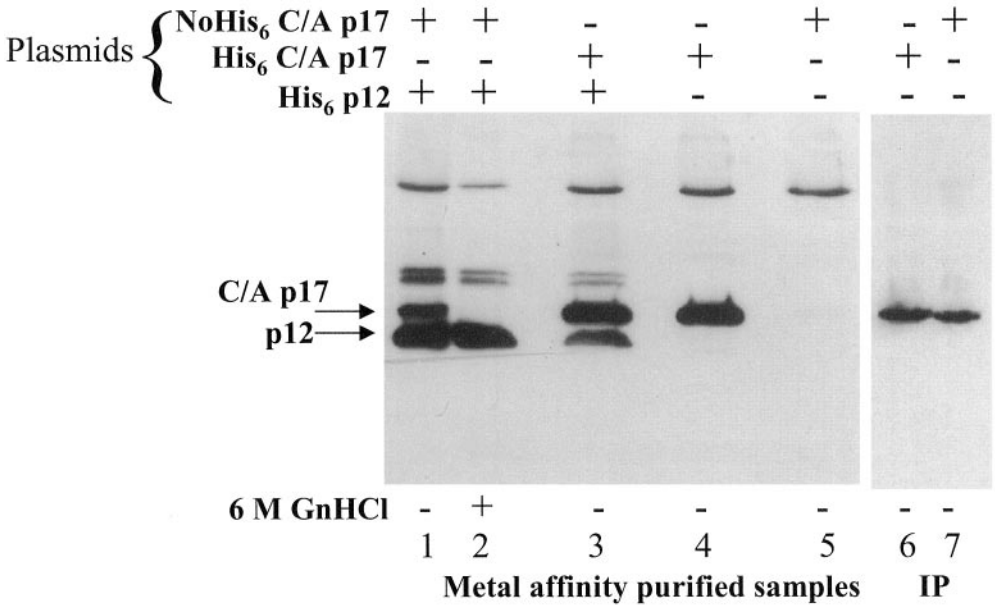

Fig. 7. C/A p17 lacking a hexahistidine tag coaffinity purified with His_6-tagged p12 under nondenaturing conditions. 911 cultures (five per condition) were cotransfected with 7.5 μg of HA-Ub and either 10 μg of His_6-Xpress-tagged p12 or empty vector (pcDNA4HisMax). Additionally, the cultures were cotransfected with 5 μg of either His_6-Xpress-tagged C/A p17 or Xpress-tagged C/A p17 lacking the His_6 tag as indicated. Two days later, the cultures were treated for 6 h with 20 μM zIEALal. His_6-tagged proteins were affinity purified and subjected to Western blot analysis with antibody to the Xpress epitope tag. Affinity purification was done under nondenaturing conditions, except for the sample shown in lane 2, which was washed with 6 M guanidinium HCl, to dissociate the p12 complex with C/A p17. C/A p17 with or without a His_6 tag was immunoprecipitated (IP) with 4 μg of antibody to the Xpress epitope to determine the relative levels of expression of C/A p17 with or without the His_6 tag. Immunoprecipitates were subjected to Western blot analysis with horseradish peroxidase-conjugated antibody to the Xpress tag.

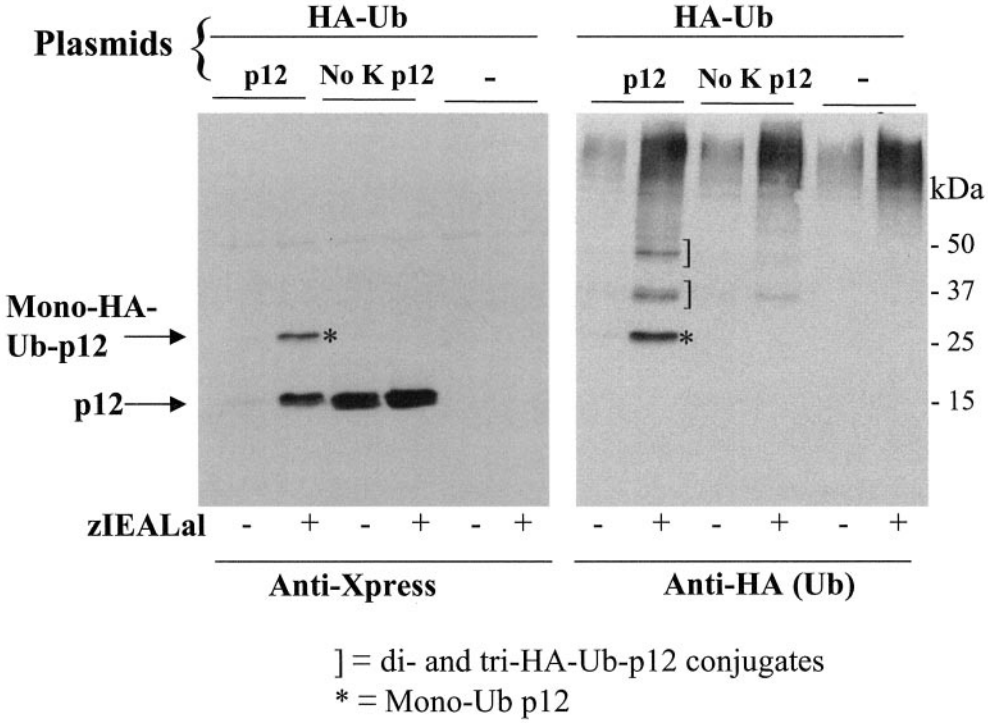

Fig. 8. Substitution of arginine for all eight lysines of p12 abolished its ubiquitination and stabilized the protein. 911 cultures (three per condition) were cotransfected with 10 μg of HA-Ub and 10 μg of wild-type p12, No K p12 with all eight lysines replaced by arginine or empty vector as indicated. Two days later, the indicated cultures were treated for 6 h with 20 μM zIEALal. His_6-tagged proteins were affinity purified under denaturing conditions and subjected to Western blot analysis with an antibody to the Xpress- or HA-epitope tag.

certain other p12 mutants. For example, p12 with only lysine 224 and 229 accumulated to a small extent, but the amount of the Ub-K224, 229 only p12 conjugate that accumulated was similar to that formed with K271 only p12 (Fig. 9). Hence, these results suggest that there is no absolute specificity for a single lysine or lysine pair for conjugation to Ub, although there may be a preference for one or more lysines.

Lacta Evokes Proteolytic Processing of XIAP and Disappearance of Full-Length XIAP in HL-60 Cells. XIAP has a carboxyl-terminal RING domain and was recently shown to catalyze its own ubiquitination and to have a Ub-protein ligase function (Yang et al., 2000). Caspases have been shown to regulate the function of XIAP by cleavage at $SESD_{242}A$, which essentially divides the protein in half and subverts the antiapoptotic barrier posed by XIAP (Deveraux et al., 1999). Consequently, proteasome blockade may increase or decrease XIAP, depending on whether degradation by the Ub-proteasome or caspase processing of XIAP has the predominant effect. Figure 10 shows the effect of Lacta treatment for 2 to 8 h on XIAP in HL-60 cells, which is the predominant IAP in these cells (Tamm et al., 2000). Treatment with Lacta for 6 or 8 h markedly decreased 50-kDa XIAP and evoked accumulation of the 30-kDa carboxyl-terminal fragment of XIAP. The general caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (20 μM) prevented the Lacta-induced conversion of full-length XIAP to the 30-kDa fragment (L. Chen and J. Smith, unpublished observations). Thus, caspase processing of XIAP was the dominant effect produced by proteasome blockade in HL-60 cells. Caspase processing of XIAP would subvert its antiapoptotic function by separating the E2 binding RING domain from the caspase-3 binding BIR2 domain (Yang et al., 2000; Riedl et al., 2001).

Discussion

This report shows for the first time that caspase-3 subunits can be ubiquitinated in vivo after cotransfection of cells with p12 and a p17 mutant. The p17 mutant (C163A), which is known to heterodimerize with p12 (Riedl et al., 2001), was used to prevent the formation of active caspase-3 and cell death. Mono-, di-, and tri-Ub conjugates of p12 and p17 accumulated after proteasome blockade (Figs. 5, 6, and 8). The subunits formed a stable complex in the transfected cells because p17 coaffinity purified with p12 (Fig. 7). Previously, mono-Ub conjugates of caspase-3 and caspase-7 subunits were produced in vitro using a reconstitution system that included cIAP2 as the Ub protein ligase (Huang et al., 2000). Monoubiquitination is known to play a role in protein sorting in the endosome and in protein trafficking (Garcia-Higuera et al., 2001; Hicke, 2001). For example, attachment of a single Ub to the Fanconi anemia protein, FANCD2, targets it to nuclear foci in response to DNA damage (Garcia-Higuera et al., 2001). Thus, monoubiquitination of caspase-3 subunits may have a function that is distinct from that of multiubiquitination, which targets proteins to the proteasome (Hershko and Ciechanover, 1998; Pickart, 2000). Treatment of HL-60 cells with Lacta, a highly selective proteasome inhibitor (Fenteany et al., 1995), evoked the processing of procaspase-3 to active subunits, exponentially increased caspase-3-like hydrolytic activity, and induced apoptosis (Figs. 1–3). Surprisingly, untreated HL-60 cells seemed to have a small amount of the p20 intermediate form of caspase-3, but no detectable active subunits (p12 and p17) (Fig. 3). The presence of p20 without p12 in HL-60 cells would indicate that p12 is rapidly degraded, which agrees with our ubiquitination results in transfected 911 cells (Figs. 4–6). Because proteasome blockade evoked the accumulation of active caspase subunits and Ub conjugates of the subunits in cells that were cotransfected with p12 and p17 (Fig. 6), it is likely that they are degraded by UPS.

The small (p12) subunit was particularly difficult to detect, even after affinity purification from large cultures of transfected cells, unless they were treated for several hours with a proteasome inhibitor (Figs. 4–8). This finding suggests that p12 is especially unstable by comparison with the other sub-

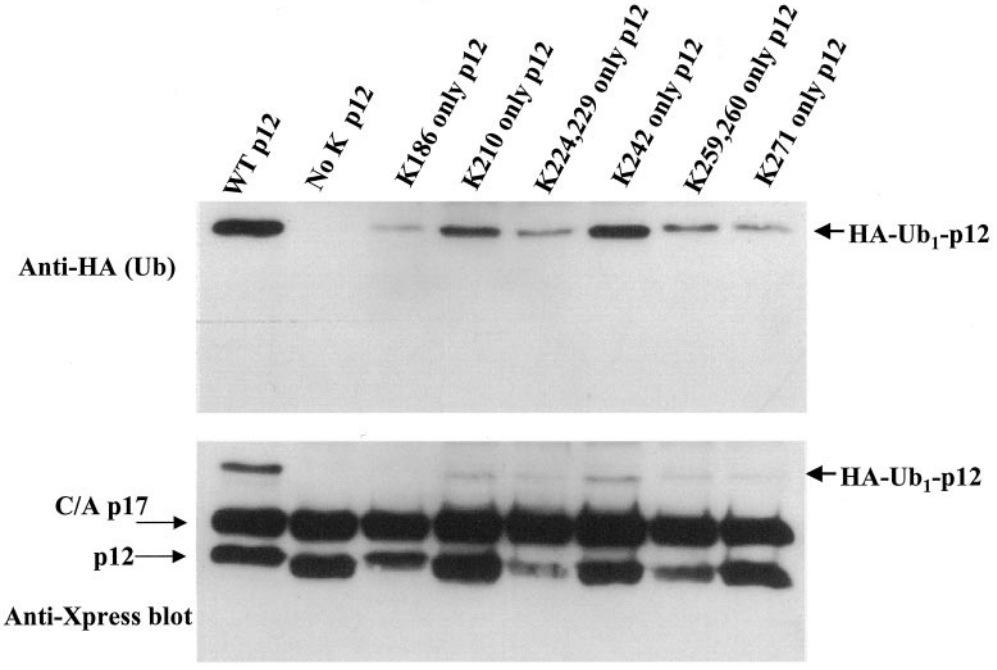

Fig. 9. Any single lysine (K186, K210, K242, or K271) or a pair of lysines (K224,229; K259,260) of p12 is sufficient for the formation of a mono-ubiquitin conjugate. 911 cultures (three per condition) were cotransfected with 7.5 μg of HA-Ub, 5 mg of C/A p17, and 10 μg of either wild-type p12, No K p12 with all eight lysines replaced by arginine, or p12 with only a single lysine or a pair of lysines as indicated. Two days later, the cultures were treated for 6 h with 20 μM zIEALal. His_6-tagged proteins were affinity purified under denaturing conditions and subjected to Western blot analysis with an antibody to the Xpress- or HA-epitope tag.

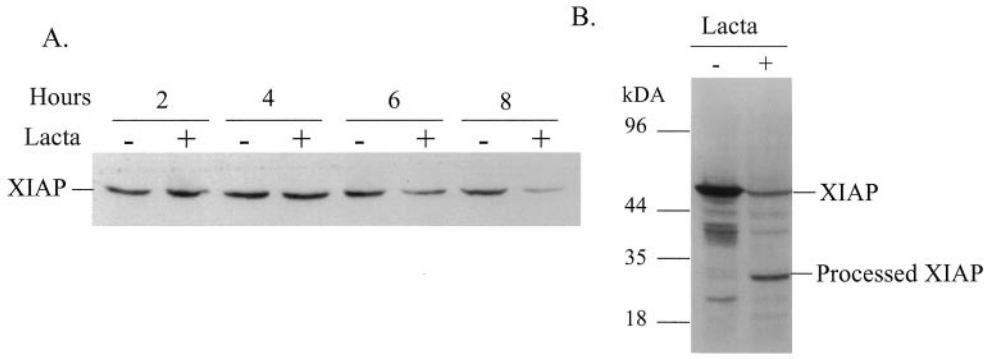

Fig. 10. Treatment of HL-60 cells with Lacta induced the proteolytic processing of XIAP. The cells were treated with 20 μM Lacta for the indicated interval (A) or for 8 h (B) in RPMI 1640 medium containing 10% FBS. Proteins were extracted with >90°C SDS sample solution, fractionated by SDS-PAGE (10% gel), and subjected to Western blot analysis with a monoclonal antibody to XIAP. A longer film exposure (B) was necessary to observe the proteolytically processed carboxyl-terminal XIAP band.

units (p17 or p20). Unfortunately, p12 is so unstable that it is not possible to measure its half-life directly in this system. Importantly, replacement of all eight lysines of p12 with arginine stabilized the protein and essentially abolished its ubiquitination (Fig. 8), as expected for Ub conjugation. This result and the fact that proteasome blockade dramatically increased the accumulation of the p12 protein suggests that UPS is the major pathway for eliminating caspase-3 subunits from mammalian cells.

p32 seems to be more stable than active caspase-3 subunits in the transfected cells because proteasome blockade failed to increase its accumulation or that of procaspse-3 lacking the prodomain (p29) (Fig. 4). The p3 prodomain clearly is not sufficient to confer stability on procaspase-3 because proteasome blockade increased the accumulation of p20 similarly to p17 but had no effect on p29 or p32 (Fig. 4). Ubiquitination of some proteins occurs at a specific lysine, and arginine substitution abolishes or markedly decreases ubiquitination, for example, as recently shown for the G protein α subunit in yeast (Marotti et al., 2002). Interestingly, analysis of p12 mutants showed that any single lysine or lysine pair was sufficient for Ub conjugation (Fig. 9). Apparently, ubiquitination of some proteins lacks specificity such that replacement of one p12 lysine with arginine leads to ubiquitination of another one, which has been shown to occur for other natural ubiquitination substrates (for review, see Pickart, 2001). At present, there is no known basis for the paradoxical finding that mono-Ub-p12 accumulates yet any lysine (or lysine pair) of p12 can be ubiquitinated. One untested idea is that ligation of the first Ub switches Ub conjugation from p12 to Ub itself (Pickart, 2001).

HL-60 cells predominantly express XIAP and relatively little of cIAP1 or cIAP2 (Tamm et al., 2000). XIAP is a potent inhibitor of caspase-3 hydrolytic activity and a caspase-3 substrate (Deveraux et al., 1999). Lacta treatment induced the disappearance of full-length XIAP and evoked accumulation of the 30-kDa fragment (Fig. 10), which is characteristic of caspase processing of XIAP (Deveraux et al., 1999). Caspase processing of XIAP would be expected to abolish its E3 Ub-protein ligase function toward active caspase-3 because processing divorces the BIR2 and flanking segment that specifically binds caspase-3 from the BIR3-RING segment that is essential for E3 Ub-protein ligase activity (Yang et al., 2000; Riedl et al., 2001; Suzuki et al., 2001). The RING domain of cIAP2, however, was sufficient to promote monoubiquitination of caspase-3 and caspase-7 in vitro (Huang et al., 2000). Possibly under the in vitro ubiquitination conditions, the concentrations of the caspase and the RING finger domain circumvented the requirement for the caspase-3 binding BIR2 and flanking segment. Further studies are needed to identify additional protein components of the XIAP E3 Ub-protein ligase to reconstitute a ligase complex with physiological properties (Pickart, 2001). Ub-protein ligases other than an IAP may act on caspase-3 subunits in mammalian cells.

The exponential increase in caspase-3 hydrolytic activity may be a distinctive feature of proteasome blockade (Fig. 1A) because treatment of HL-60 cells with etoposide produced a linear increase in caspase-3 activity (L. Chen and J. Smith, unpublished observations). The following two factors may contribute to the exponential change in caspase activity: 1) the preservation of active caspase-3 from degradation by the proteasome, and 2) caspase processing of XIAP has been shown to circumvent the barrier it poses to apoptosis (Deveraux et al., 1999). Lacta treatment had no effect on caspase-8 or granzyme B-like hydrolytic activity at 4 or 8 h and only slightly increased caspase-9 like activity at 8 h (Fig. 1B) but not at all at 4 h (L. Chen and J. Smith, unpublished observations). Thus, proteasome blockade may predominantly activate effector caspase-3 in HL-60 cells. Autoprocessing of procaspase-3 has been demonstrated in vitro and in vivo in response to synthetic peptides containing the arginine-glycine-aspartate (RGD) motif (Buckley et al., 1999). Procaspase-3 itself contains an RGD motif and a potential RGD-binding motif, aspartate-aspartate-methionine, near the site of processing to produce p12 and p17 subunits. More work is needed to determine whether caspase-3 activation after proteasome blockade occurs by autoprocessing and/or by a feedback loop where caspase-3 can process caspase-9.

Adventitious caspase activation must occur on a routine basis, which if unchecked would amplify and result in unintended cell death. The present results implicate a novel IAP function for suppressing inadvertent cell death, namely, catalyzing the ubiquitination of active caspase-3 subunits. Although the reversible inhibition of activated caspase by an IAP could be sufficient to temporarily suppress apoptosis, the E3 Ub-protein ligase function of IAP may be essential for the prevention of unintended apoptosis. Ub conjugation and rapid elimination of active caspase subunits would raise the apoptotic threshold and provide a fail-safe check on death

signaling. Although much progress has been made with respect to the regulation of upstream caspase-8 and caspase-9 by Smac/DIABLO, cytochrome *c*, and Apafs (Du et al., 2000; Green, 2000; Liu et al., 2000; Srinivasula et al., 2000, 2001; Verhagen et al., 2000; Wu et al., 2000), there is still relatively little understanding of the regulation at the level of active caspase turnover. For example, ubiquitination is a dynamic reversible process that involves a family of enzymes that deubiquitinate target proteins (Wilkinson, 2000), and nothing is known about deubiquitination of caspase-3. The observation that both subunits of active caspase-3 are ubiquitinated and unstable because of degradation by UPS opens a new avenue for modulating apoptotic cell death.

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