Rapid Deubiquitination of Nucleosomal Histones in Human Tumor Cells Caused by Proteasome Inhibitors and Stress Response Inducers: Effects on Replication, Transcription, Translation, and the Cellular Stress Response[†]

Edward G. Mimnaugh,*[,‡] Hou Yu Chen,[§] James R. Davie,[§] Julio E. Celis,[||] and Len Neckers[‡]

Department of Cancer and Cell Biology, Medicine Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892, Department of Biochemistry and Molecular Biology, The University of Manitoba, Winnipeg, Manitoba, Canada R3E OW3, and Danish Centre for Human Genome Research, University of Aarhus, DK-8000 Aarhus C, Denmark

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ABSTRACT: The proteasome inhibitors, lactacystin and *N*-acetyl-leucyl-leucyl-norlucinal, caused a rapid and near-complete loss of ∼22−23-kDa ubiquitinated nucleoproteins, which we have identified as monoubiquitinated nucleosomal histones H2A and H2B by immunological and two-dimensional electrophoretic techniques. In human SKBr3 breast tumor cells, depletion of monoubiquitinated histones by the proteasome inhibitors coincided with the accumulation of high molecular weight ubiquitinated proteins in both nucleoprotein and cytosolic fractions and decreased unconjugated ubiquitin in the cytosol, without changes in the nonubiquitinated core histones. Unconjugated ubiquitin was not detected in isolated tumor cell nuclei. A similar loss in monoubiquitinated histones occurred in cells harboring a defective, temperature-sensitive mutation of the ubiquitin-activating E1 enzyme, after these cells were elevated from 33 °C to the non-permissive temperature of 39 °C. DNA replication and RNA transcription were decreased by the proteasome inhibitors most strongly after 90% of the ubiquitin had been removed from ubiquitinated histones H2A and H2B, suggesting a relationship between the nucleosomal histone ubiquitin status and the processing of genetic information. Interestingly, although both proteasome inhibitors caused a generalized decrease in methionine incorporation into proteins, they strongly induced the synthesis of the hsp72 and hsp90 stress proteins. Finally, treating cells with heat-shock at 43 °C, with stress response-provoking chemicals or with several other proteasome inhibitors caused ubiquitinated proteins to accumulate, depleted free ubiquitin, and concomitantly decreased nucleosomal monoubiquitinated histones. These results suggest that deubiquitination of nucleosomal histones H2A and H2B may play a previously unrecognized role in the cellular stress response, as well as in the processing of chromatin, and emphasize the important role of the proteasome in cellular homeostasis.

The assembly of DNA and histones into nucleosomes presents an obvious topological barrier for polymerase complexes and transcription factor and transcription activator access to the DNA, and this obstacle must be overcome before replication and transcription occurs (Suditsky et al., 1995; Svaren & Horz, 1993; Van Holde et al., 1992). Reversible post-translational modification of the amino-terminal tails of the four nucleosomal core histones by acetylation, phosphorylation, and ubiquitination causes allosteric changes in the nucleosome structure that may play important regulatory roles in the processing of genetic information [for reviews, see Davie (1996), Lee et al. (1993), and Varga-Weisz and Becker (1995)]. Acetylation and ubiquitination of nucleosomal histones H2A and H2B destabilize the association of the histone H2A−H2B dimers with histone H3−H4 tetramers within the nucleosomal octamer and weaken the intimate interactions of the core histone tails with DNA (Bradbury, 1992; Lee et al., 1993). Indeed, much higher levels of acetylated (Lee et al., 1993) and ubiquitinated histones (Davie & Murphy, 1990) have been found in actively transcribing euchromatin regions of the genome than in quiescent heterochromatin, suggesting an obligatory role for these molecular modifications in the regulation of transcription. Although the consequences of histone acetylation in transcription regulation are currently being elucidated (Davie, 1996; Patterton & Wolffe, 1996), the physiological consequences of nucleosomal histone ubiquitination and deubiquitination cycles remains ill-defined. This is partly due to the fact that experimental manipulation of the ubiquitin status of histones has been difficult, as there are no known specific inhibitors of the histone ubiquitin-conjugating or histone ubiquitin-cleaving enzymes.

While characterizing the ubiquitin-dependent degradation of c-ErbB$_2$ receptor tyrosine kinase (Mimnaugh et al., 1996), we noted that a prominent ∼22−23-kDa protein doublet observed on anti-ubiquitin immunoblots of crude nucleoprotein fractions was rapidly lost after cells were exposed to lactacystin (LC),[1] a specific and potent inhibitor of the 26S proteasome (Fenteany et al., 1995). This observation was

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* Corresponding author. Telephone: (301) 496-8050. Telefax: (301) 435-4655. E-mail: edmim@box-e.nih.gov.

[‡] National Institutes of Health.

[§] The University of Manitoba.

[||] Universityof Aarhus.

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[1] Abbreviations: ALLnL, *N*-acetyl-leucyl-leucyl-norlucinal; DCI, 3,4-dichloroisocoumarin; hsp72, 72-kDa heat-shock protein; hsp90, 90-kDa heat-shock protein; LC, lactacystin; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; uH2A, monoubiquitinated histone H2A; uH2B, monoubiquitinated histone H2B; u-p23, ubiquitinated 23 kDa nucleoprotein.

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paradoxical because proteasome inhibition causes an accumulation of ubiquitinated proteins that otherwise are rapidly degraded (Ciechanover et al., 1984; Finley & Chau, 1991; Hershko & Ciechanover, 1992; Jennissen, 1995). In the present study, we identify the proteasome inhibitor-sensitive, ubiquitinated 22−23-kDa nucleoprotein doublet (u-p23) as monoubiquitinated histones H2A (uH2A) and H2B (uH2B), and we show that deubiquitination of these two nucleosomal core histones strongly represses replication and transcription. Additionally, although the proteasome inhibitors markedly decreased overall protein translation, the synthesis of hsp72 and hsp90 stress proteins was induced concomitantly with proteasome inhibition. These observations imply that nucleosomal histone monoubiquitination−deubiquitination cycles may participate in the regulation of the cellular stress response.

MATERIALS AND METHODS

Tumor Cells. Mycoplasma-free SKBr3 human breast tumor cells were obtained from the American Type Culture Collection, Rockville, MD; temperature-sensitive ts-20 and ts-A1S9 cells were obtained from Dr. Linda Wolff, NCI, NIH, and Dr. Jonathan Yewdell, NIAID, NIH, respectively. Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, and 10 mM HEPES, pH 7.5, under standard tissue culture conditions, except the temperature-sensitive cells were propagated at 33 °C instead of 37 °C. Sub-confluent cells, exponentially growing in 80 or 145 cm^2 plates, were treated with the proteasomal inhibitors LC and *N*-acetyl-leucyl-leucyl-norlucinal peptide (ALLnL) at several concentrations and for various times; control cells received equivalent volumes of DMSO (0.1% v/v, or less). Other treatments included sodium arsenite, cadmium chloride, cycloheximide, geldanamycin, aclacinomycin A, MG-132, 3,4-dichloroisocoumarin (DCI), and heat-shock (43 °C for up to 5 h).

Lymphocyte Isolation and Treatments. Lymphocytes separated from heparinized peripheral human blood using Ficoll-Paque according to the manufacturer's instructions (Pharmacia Biotech) were resuspended in RPMI 1640 medium and dispensed into 80 cm^2 tissue culture plates at 10^7 cells/plate. After a 2-h incubation at 37 °C, the lymphocytes were exposed to 10 μM LC, 100 μM ALLnL, or 0.5% v/v DMSO for an additional 4 h and washed by centrifugation twice in cold phosphate-buffered saline, and the cell pellets were lysed directly into 200 μL of reducing-SDS loading buffer (0.1 M dithiothreitol, 2% w/v SDS, 80 mM Tris, pH 6.8, 10% v/v glycerol) (Laemmli, 1970). Aliquots representing 2.5×10^6 lymphocytes were analyzed for ubiquitinated histones.

Immunoreagents and Chemicals. Antibodies used were as follows: rabbit polyclonal anti-ubiquitin, mouse monoclonal anti-hsp72, and goat anti-rabbit antibody linked to horseradish peroxidase (Sigma, St. Louis, MO); rabbit anti-mouse IgG_1 (Cappel, Durham, NC), horseradish peroxidase-conjugated sheep anti-mouse, and horseradish peroxidase-conjugated donkey anti-rabbit antibodies (Amersham Life Science, Arlington Heights, IL); and anti-ubiquitinated histone H2A antibody E6C5 (a gift from Dr. Julio E. Celis, Danish Centre for Human Genome Research, Denmark). LC was purchased from Dr. E. J. Corey, Harvard University, Cambridge, MA. The proteasome inhibitor, MG-132, was from CalBioChem; all other chemicals used in this study, including ALLnL, were purchased from Sigma Chemical Co. [^{3}H]Uridine, [^{3}H]thymidine, and [^{35}S]methionine radiochemicals were from ICN Pharmaceuticals (Irvine, CA).

Crude Nucleoprotein Fraction and Histone Extraction. Exponentially growing control and treated tumor cells were washed twice in ice-cold phosphate-buffered saline and then lysed on ice with TNESV lysis buffer (50 mM Tris-HCl, pH 7.5, 1% Nonidet P-40 detergent, 2 mM EDTA, 100 mM NaCl, 10 mM orthovanadate) with added protease inhibitors: 1 mM phenylmethanesulfonyl fluoride, 20 μg/mL leupeptin, and 20 μg/mL aprotinin. After the lysates were centrifuged at 14000*g* for 20 min at 4 °C, the supernatant fractions were removed, and the crude nuclear pellets (mostly NP-40 detergent-insoluble chromatin) were mechanically resuspended in TNESV lysis buffer with protease inhibitors, briefly sonicated, assayed for protein concentration, and then diluted into reducing-SDS loading buffer. In some experiments, intact cells and nucleoprotein pellets were dissolved directly into reducing-SDS loading buffer. In other experiments, cells were disrupted with a Dounce homogenizer, and intact nuclei were isolated in nuclei-isolation buffer (100 mM KCl, 10 mM Tris, pH 7.5, 5 mM sodium butyrate, 10 mM iodoacetamide, and 0.1% v/v NP-40 with 1 mM phenylmethanesulfonyl fluoride and 0.1 μg/mL aprotinin and leupeptin. Histones were subsequently extracted with 0.4 N sulfuric acid and extensively dialyzed and lyophilized, as described in detail elsewhere (Davie & Murphy, 1990; Nickel et al., 1989). Protein concentrations were determined by the bicinchoninic acid method using bovine serum albumin as the standard (Smith et al., 1985).

Immunoblot Analysis for Ubiquitinated Proteins and Unconjugated Ubiquitin. After samples were fractionated by 15% SDS−PAGE, proteins were electrotransferred to Protran nitrocellulose membranes (Schleicher & Schuell, Keene, NH), and the membranes used for anti-ubiquitin immunoblotting were autoclaved submerged in deionized water for 20 min to ensure complete denaturation of the bound ubiquitinated proteins (L. Guarino, personal communication).

For unconjugated ubiquitin detection, tumor cells were rinsed with and then rapidly scraped into ice-cold phosphate-buffered saline, boiled for 20 min to inactivate ubiquitin-cleaving enzymes, then chilled on ice, and centrifuged at 14000*g* for 10 min. Aliquots (10−25 mg) of the resulting supernatant fractions were resolved by 18% SDS−PAGE; after electrophoresis, gels were equilibrated at room temperature for 30 min in 63 mM Tris, pH 6.8, 2.3% SDS, and 5% 2-mercaptoethanol to completely denature free ubiquitin in order to facilitate its overnight electrotransfer (50 V) to nitrocellulose at 4 °C in 25 mM cyclohexylaminopropane sulfonic acid, pH 10, and 20% methanol alkaline transfer buffer. The membranes were air-dried and then autoclaved for 20 min submerged in deionized water prior to blocking and immunoblotting for ubiquitin. In some experiments, isolated nuclear and cytosolic fractions from tumor cells were subjected to the same procedure.

After membranes were blocked with 5% fat-free dry milk in 10 mM Tris, pH 7.5, 50 mM NaCl, and 2.5 mM EDTA buffer, antigens were immunodetected with appropriate antibodies followed by horseradish peroxidase-linked secondary antibodies. Visualization was by chemiluminescence

(Bobrow et al., 1989), using a luminol-based commercial kit (Pierce, Rockford, IL). Exposed X-OMAT AR films (Kodak) were developed and scanned (Microtek Scanmaker III), and the images were captured and processed with a Macintosh computer using Adobe Photoshop 3.0 software and quantified using image analysis software (NIH Image 1.59).

Two-Dimensional Urea Gel Analysis of Ubiquitinated Histones. Acid-extracted histones from nuclei isolated from control and ALLnL-treated cells were separated in the first dimension on AUT gels [acetic acid−6.7 M urea−0.375% (w/v) Triton X-100−15% polyacrylamide] followed by SDS−15% polyacrylamide gel electrophoresis in the second dimension and were electrotransferred onto nitrocellulose. To visualize ubiquitinated histones, the membranes were immunochemically stained with rabbit anti-ubiquitin antibody and then goat anti-rabbit antibody linked to horseradish peroxidase (Sigma) followed by ECL (enhanced chemiluminescence) detection (Amersham) (Delcuve & Davie, 1992). Aliquots of histone extracts were also separated by one-dimensional AUT gel electrophoresis and stained with Coomassie brilliant blue dye to display the overall histone profile and also by 15% SDS−PAGE for anti-ubiquitin immunoblotting after electrotransferring proteins to membranes. A distinctive feature of the monoubiquitinated histones is that they often migrate as doublets in the second dimension SDS gel (Nickel et al., 1989).

Transcription, Replication, and Protein Synthesis. To assess the effects of the proteasome inhibitors on the rates of RNA and DNA synthesis, cells were grown overnight in 96-well plates, then treated with 10 μM LC or 100 μM AllnL, followed immediately, or 4 h later, by 1.0 μCi of [^{3}H]uridine or [^{3}H]thymidine added to each well. At various times, cells were mechanically harvested by aspiration onto glass fiber filters and washed sequentially several times with water and then ethanol, and RNA- and DNA-incorporated radioactivity were measured by scintillation counting (BetaScan-96, Packard). The effects of LC and ALLnL on protein synthesis were measured by exposing cells to the proteasome inhibitors simultaneously with or 4 h before [^{35}S]-methionine (1274 Ci/mmol, 100 μCi/mL) was added for a 2-h pulse. Cells were lysed, the crude chromatin pellet and soluble lysate fractions were isolated by centrifugation, and aliquots were counted for radioactivity directly and after TCA precipitation. Following separation of labeled proteins by SDS−PAGE, gels were fixed with 10% acetic acid−25% methanol−5% glycerol, enhanced with Enlightening (Dupont), and dried; the radioactive protein bands were visualized by autoradiography.

Statistical Analysis. Where appropriate, data were analyzed by Student's *t*-test (Snedecor, 1956), and differences between mean values at $P < 0.05$ were considered to be significant.

RESULTS

Lactacystin and ALLnL Decrease the Amount of u-p23 in SKBr3 Cells. The effect of proteasome inhibitors on the quantity of the ubiquitinated 22−23-kDa nucleoprotein was investigated by incubating cells with several concentrations of LC or ALLnL for various times prior to lysing cells, isolating nucleoprotein pellet and cytosolic fractions, and separating proteins by 15% SDS−PAGE. Following electrotransfer to nitrocellulose, the membranes were autoclaved, before probing with an anti-ubiquitin antibody. As shown in Figure 1, the proteasome inhibitors diminished the amount of the u-p23 in a concentration and time-dependent fashion (Figure 1, panels A−D). No u-p23 signal was observed when the primary antibody was omitted (not shown). The extent of u-p23 depletion by 10 μM LC after 4 h was roughly equivalent to that caused by 100 μM ALLnL, which correlates with the approximately 10-fold difference in the potency of these two chemicals to inhibit the proteasome and to promote the accumulation of high molecular weight ubiquitinated proteins in cells (Adams & Stein, 1996; Fenteany et al., 1995). u-p23 was found exclusively in the nucleoprotein fraction from both control and proteasome inhibitor-exposed cells, indicating that it did not redistribute from the nucleoprotein pellets into the soluble fraction of lysates after LC or ALLnL treatments (not shown). In samples from LC or ALLnL-treated cells where the level of u-p23 was low or in underexposed control samples, the u-p23 band clearly appeared as a doublet, suggesting that it was composed of two or more individual proteins (Figure 1, compare panels A−D). The specificity of the loss of u-p23 from cells treated with LC for 4 h is illustrated in the Coomassie brilliant blue-stained gel showing nucleoprotein and soluble fractions in which only minor changes in the overall pattern of proteins from control and LC-treated cells were detected (Figure 1, panel C).

At this point, we suspected that the identities of the LC-sensitive ubiquitinated 22−23-kDa proteins in the doublet band were monoubiquitinated histones H2A and H2B, based on their exclusive localization in the crude chromatin pellets and on the similarity of the apparent molecular weights and doublet character observed on SDS−PAGE gels to authentic ubiquitinated histone H2A (uH2A) (22.5 kDa) and ubiquitinated histone H2B (uH2B) (22.3 kDa).

Heat-Shock Decreases u-p23 Protein. Heat-shock treatment has been shown to decrease the cellular level of monoubiquitinated nucleosomal histones (Davie & Murphy, 1990; Deveraux et al., 1990). Therefore, we incubated SKBr3 cells at 43 °C for 4 h to determine whether the u-p23 band could be altered by heat-shock. We found that the elevated temperature significantly diminished the u-p23 band when compared to normothermic controls kept at 37 °C (Figure 2, panel A). This result is consistent with the likelihood that the ubiquitinated 22−23-kDa protein doublet was nucleosomal uH2A and uH2B.

The u-p23 kDa Band Contains Immunoreactive Monoubiquitinated Histone H2A. The anti-ubiquitin polyclonal antisera we used to detect u-p23 in the previously described experiments recognizes the ubiquitin moiety of protein conjugates; however, it has no specificity for the partner protein of mono- or multi-ubiquitinated complexes. To immunologically validate the possibility that the 22−23-kDa nucleoprotein band contained uH2A, we probed membranes with a mouse monoclonal antibody specific for monoubiquitinated H2A (E6C5) (Vassilev et al., 1995). This antibody recognized the u-p23 band from crude chromatin fractions and in histones extracted from isolated nuclei from control cells, and the intensity of the E6C5 antibody-probed u-p23 band was greatly diminished by ALLnL treatment (Figure 2, panel B). These data confirmed that the proteasome inhibitor-sensitive ubiquitinated 22−23-kDa nucleoprotein doublet band contained uH2A.

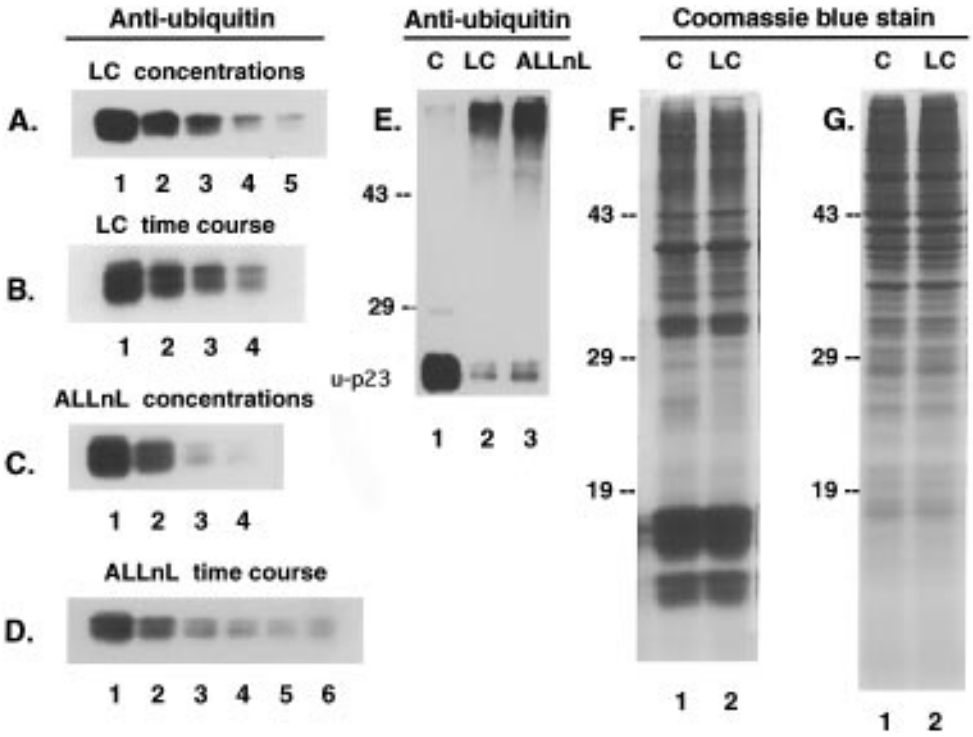

FIGURE 1: Proteasome inhibitors, LC and ALLnL, deplete a ubiquitinated 22−23-kDa cellular nucleoprotein doublet. SKBr3 tumor cells were treated with either LC or ALLnL at various concentrations for up to 4 h (control cells were treated with dimethyl sulfoxide); cells were lysed with TNESV plus protease inhibitors; equal amounts of nucleoprotein and lysate supernatant fractions were resolved by 15% SDS−PAGE, transferred to nitrocellulose membranes, and immunoblotted for ubiquitinated proteins. In panel A, cells were treated with 0, 1, 2, 6, and 10 μM LC for 3 h (lanes 1−5); in panel B, cells were exposed to 6 μM LC for 0, 1, 2, and 4 h (lanes 1−4). Note that the u-p23 protein appears as a doublet that is more easily observed after LC treatment. Panel C shows the effect of 0, 10, 50, and 100 μM ALLnL for 4 h on the level of u-p23 (lanes 1−4); panel D displays the time course of the loss of u-p23 resulting from exposure to 50 μM ALLnL for 0, 1, 2, 4, 6, and 18 h (lanes 1−6). LC was approximately 10 times as potent as ALLnL in depleting u-p23 from cells. A 4-h treatment with 10 μM LC or 100 μM ALLnL caused the accumulation of ubiquitinated high molecular weight proteins concomitant with the disappearance of u-p23 (panel E, lanes 1−3). Coomassie brilliant blue-stained gels containing nucleoprotein fractions (panel F) or lysate supernatant fractions (panel G) from control and LC-treated (10 μM, 4 h) cells show that the depleting effect of LC appeared to be specific for the ubiquitinated 22−23-kDa protein doublet, because essentially all other stain-detectable protein bands are equivalent in both the control and the LC treated samples. The same result was observed after ALLnL (not shown).

Inactivation of ts-Mutant Ubiquitin-Activating E1 Enzyme Causes Loss of u-p23. To determine whether the status of u-p23 was related to the ability of cells to conjugate proteins to ubiquitin, we used ts-20 (Hirschberg & Marcus, 1982; Kulka et al., 1988) and ts-A1S9 cells (Thompson et al., 1970), which have temperature-sensitive mutations in the E1 ubiquitin-activating enzyme. It has been previously reported that uH2A levels decline in these two mutated cell lines after the defective E1 enzyme becomes inactivated at the non-permissive temperature (Cox et al., 1995). When ts-20 and ts-A1S9 cells were shifted from 33 to 39 °C, a temperature that inactivates E1 but does not provoke the heat-shock stress response, the u-p23 signal was decreased to a level comparable to that caused by ALLnL or LC (Figure 2, lower panels D and E, compare lanes 2 and 3). In control A1S9 cells containing the active, wild-type E1 enzyme, the level of u-p23 was unaltered by elevating the incubation temperature from 33 to 39 °C, although treating the wild-type cells with ALLnL quickly depleted u-p23 (not shown). These data provide additional evidence that the u-p23 doublet contained uH2A and possibly uH2B.

Proteasome Inhibitors Deplete u-p23 in Human Lymphocytes. Proteasome inhibitor-caused loss of u-p23 (presumably uH2A) might be a phenomenon unique to transformed tumor cells grown in culture. To determine whether normal cells responded to proteasome inhibitors in a fashion similar to tumor cells, we investigated the effects of LC and ALLnL treatment on u-p23 levels in freshly-isolated, unstimulated, human peripheral blood lymphocytes. Following a 4-h exposure of lymphocytes to ALLnL or LC, we found that the amount of u-p23 was decreased to 37% and 28%, respectively, of the control level (Figure 2, panel F). Although not shown in this figure, the proteasome inhibitors potently increased the accumulation of an array of ubiquitinated proteins in lymphocytes—most conspicuously those above 30-kDa mass—exactly as observed in the SKBr3 tumor cells. These results indicate that proteasome inhibitors deplete u-p23 in normal human cells under the same conditions as in tumor cells.

Two-Dimensional Gel Analysis of Ubiquitinated Histones Extracted from Isolated Tumor Cell Nuclei. It remained possible that the proteasome inhibitors could have diminished uH2A and uH2B by causing a general depletion of histones rather than by specifically altering the ubiquitin status of the core histones. To rule-out this possibility, the histones were extracted with sulfuric acid from nuclei isolated from control and ALLnL-treated cells, equal amounts of histones were separated by 15% AUT one-dimensional gel electrophoresis, and the gel was stained with Coomassie brilliant blue dye. ALLnL treatment did not alter the amounts of acid-extractable nucleosomal core histones: H2A, H2B, H3, and H4 or linker histone H1 (Figure 3, panel A). This result indicates that uH2A and uH2B were not diminished because nucleosomal histones were depleted and that only a relatively

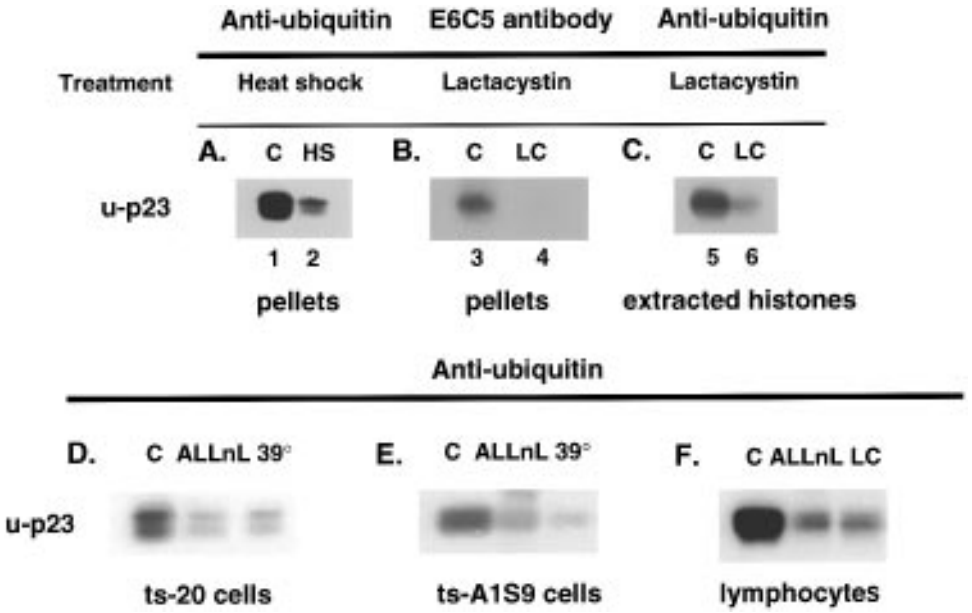

FIGURE 2: Ubiquitinated 22−23-kDa doublet most likely contains uH2A. The level of u-p23 detected by anti-ubiquitin immunoblotting was decreased more than 75% by thermal stress (44 °C for 4 h) (panel A, lanes 1 and 2). We therefore evaluated the possibility that u-p23 might be a ubiquitinated histone by probing blots with an antibody that specifically detects ubiquitinated histone H2A (E6C5). This antibody recognized the 23-kDa doublet band from nucleoprotein samples from control SKBr3 cells, and the E6C5 antibody-visualized 23-kDa band was nearly completely eliminated from cells exposed to 10 μM LC for 4 h (panel B, lanes 3 and 4). Panel C shows an anti-ubiquitin immunoblot of purified histones acid-extracted from nuclei isolated from control and LC-treated (10 μM for 4 h) cells. A prominent ubiquitinated 23-kDa band was recognized in the histone extracts by anti-ubiquitin immunoblotting, and this band was diminished by LC treatment (lanes 5 and 6). Cells with a temperature-sensitive mutation in the gene coding for the ubiquitin-activating E1 enzyme (ts-20, panel D; ts-A1S9, panel E) were treated with either ALLnL (100 μM for 4 h) or exposed to the nonpermissive temperature of 39 °C, which indirectly prevents the ubiquitination of proteins. Notably, in both cases, the u-p23 band was greatly diminished as compared to u-p23 from the control cells incubated at 33 °C. The effects of 100 μM ALLnL and 10 μM LC (4 h exposure) on the level of u-p23 in unstimulated human peripheral blood lymphocytes is shown in panel F.

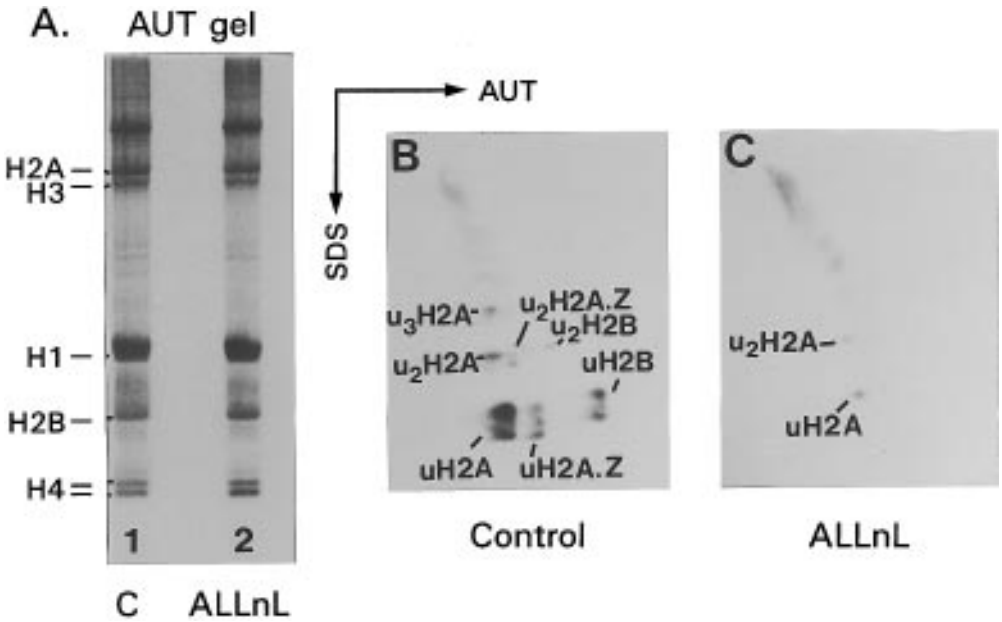

FIGURE 3: Two-dimensional AUT−SDS gels of extracted histones show that ALLnL treatment diminished several ubiquitinated histones. Nuclei were isolated from control and ALLnL-treated cells (100 μM for 4 h), and histones were sulfuric acid-extracted and subjected to one- and two-dimensional acetic acid−urea−Triton-X 100 (AUT) gel electrophoresis. In panel A, the various histones were separated on a one-dimensional AUT gel, and Coomassie brilliant blue-staining shows that ALLnL treatment did not alter the steady-state concentrations of any of the major histones. The extracted histones were then separated by two-dimensional AUT−SDS gel electrophoresis, transferred to nitrocellulose membranes, and probed with the anti-ubiquitin antibody (panel B, control; panel C, ALLnL-treated). Based upon their known mobility in this two-dimensional system, various ubiquitinated histones are identified: uH2A, monoubiquitinated histone H2A; u_2H2A, diubiquitinated histone H2A, etc. ALLnL treatment caused the loss of ubiquitin from the most prominent monoubiquitinated H2A histone as well as from ubiquitinated H2B and the lesser monoubiquitinated and multi-ubiquitinated histones. These results confirm the identification of the 22−23-kDa band as ubiquitinated histones H2A and H2B.

small proportion of the histones were ubiquitinated, on the order of 5−10%, as previously reported (Davie & Nickel, 1987; Wu et al., 1981). When the sulfuric acid-extracted histones were separated by 15% SDS−PAGE, transferred

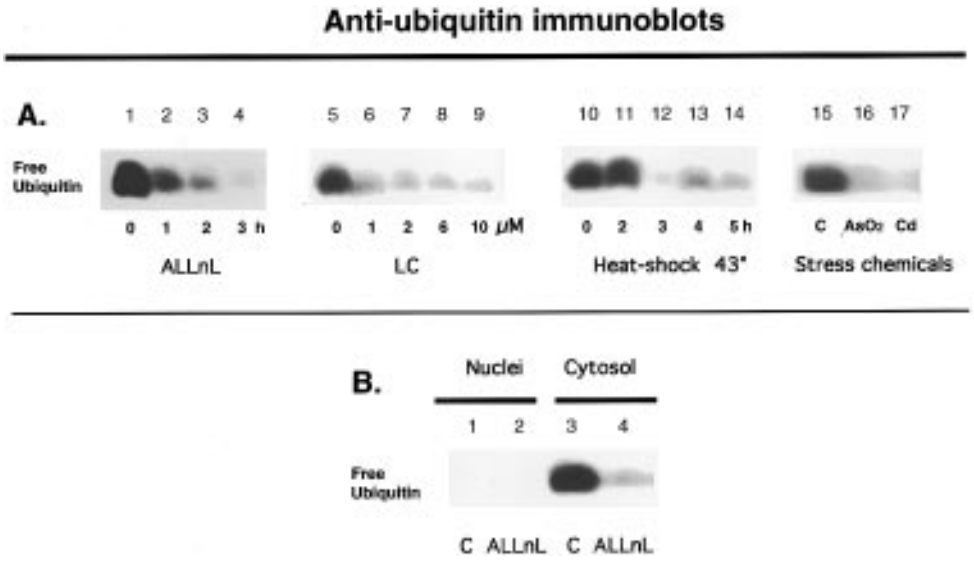

FIGURE 4: Exposing cells to proteasome inhibitors, heat-shock, and chemical stress decreases unconjugated ubiquitin. (Panel A) Tumor cells were treated with 100 μM ALLnL for various times (lanes 1−4), with LC at the indicated concentrations for 3 h (lanes 5−9), heat-shocked at 43 °C from 2 to 5 h (lanes 10−14), or exposed to 100 μg/mL sodium arsenite (AsO_2) or 100 μg/mL cadmium chloride (Cd) for 4 h (lanes 15−17, "C" indicates the control sample) prior to scraping the cells and quickly boiling them to inactivate ubiquitin-cleaving enzymes. Free ubiquitin in 25-μg samples was isolated on 18% SDS−PAGE gels, transferred to nitrocellulose in alkaline cyclohexylaminopropane sulfonic acid−methanol transfer buffer, pH 10, and detected by anti-ubiquitin immunoblotting. Free ubiquitin migrated with an apparent molecular mass of 5.5−6.0 kDa in SDS gels and coincided exactly with the location of purified ubiquitin standards run on the same gels. (Panel B) Nuclei (lanes 1 and 2) and cytosol (lanes 3 and 4) were isolated from control (C) and 100 μM ALLnL-treated tumor cells and boiled, and then 25-μg aliquots were processed as described in panel A in order to measure free ubiquitin in the two cellular compartments. Unconjugated ubiquitin is conspicuously absent in the nuclear fraction (lanes 1 and 2), and a 10 times longer film exposure did not visualize a free ubiquitin band.

to membranes, and immunoblotted with the anti-ubiquitin antibody, ALLnL-sensitive, ubiquitinated 22−23-kDa histones were detected that were identical in mobility to the u-p23 doublet bands from crude nucleoprotein pellets (Figure 2, compare panels B and C).

The extracted histones were then resolved by two-dimensional AUT−SDS gel electrophoresis, transferred to nitrocellulose membranes, and probed with the polyclonal anti-ubiquitin antibody to visualize ubiquitinated histones. Based on the known mobility of ubiquitinated histone standards in this system, panel B of Figure 3 clearly shows uH2A as the predominant ubiquitinated histone, with smaller amounts of uH2B and the uH2A.Z variant and only trace quantities of di- and tri-ubiquitinated histones. ALLnL treatment (100 μM for 4 h) depleted uH2A and also decreased uH2B, uH2A.Z, and the polyubiquitinated histones to nearly undetectable levels, showing that the proteasome inhibitors caused deubiquitination of all of the ubiquitinated histone isoforms (Figure 3, panel C). Most importantly, these results identify the ubiquitinated, 22−23-kDa, proteasome inhibitor-sensitive nucleoproteins as uH2A and uH2B.

Proteasome Inhibitors and Heat-Shock Deplete Cellular Free Ubiquitin. The apparent redistribution of ubiquitin from nucleosomal uH2A and uH2B to high molecular weight nonhistone proteins after cells were exposed to proteasome inhibitors or to heat-shock suggests the possibility that unconjugated ubiquitin may become depleted when cells are exposed to noxious conditions. To test this hypothesis, we treated tumor cells with ALLnL, LC, heat-shock at 43 °C, or the stress response-provoking chemicals, sodium arsenite and cadmium chloride; quickly boiled the cells to inactivate ubiquitin-cleaving isopeptidases (or lysed cells directly into hot SDS-reducing loading buffer); separated proteins on 18% SDS−polyacrylamide gels, and measured unconjugated ubiquitin by immunoblotting. We found that cellular free ubiquitin and purified ubiquitin standards failed to electrotransfer efficiently from gels immersed in the commonly-used Tobin Tris-glycine−SDS−methanol transfer buffer, so instead we used an alkaline cyclohexylaminopropane sulfonic acid−20% methanol buffer, which transferred unconjugated ubiquitin from gels more effectively. Shown in Figure 4A, total cellular free ubiquitin was decreased more than 50% by 100 μM ALLnL within 1 h, and after a 3-h exposure to ALLnL, free ubiquitin was nearly undetectable. LC also effectively diminished free ubiquitin in a concentration-dependent fashion, markedly consuming unconjugated ubiquitin within 3 h in cells exposed to 1 μM LC (Figure 4A). Several other proteasome inhibitors (MG-132, aclacinomycin A, and DCI) also depleted free ubiquitin; in contrast, cycloheximide and geldanamycin, agents that do not decrease uH2A or uH2B, failed to change unconjugated ubiquitin levels (data not shown). Heat-shocking cells also decreased free ubiquitin by 3 h, although the level of free ubiquitin appeared to rebound slightly with longer exposure at 43 °C (Figure 4A). Sodium arsenite and cadmium chloride also mimicked the heat-shock and proteasome inhibitor-caused depletion of cellular free ubiquitin (Figure 4A).

Ubiquitinated proteins accumulated in both the crude chromatin pellet and in the supernatant fraction from cells challenged with the proteasome inhibitors. This observation prompted us to examine the level of unconjugated ubiquitin in nuclear and cytosolic fractions from control and ALLnL-treated cells. Although free ubiquitin was easily measured in the soluble fraction from control cells and was found to be sensitive to depletion by ALLnL, surprisingly, unconjugated ubiquitin was undetectable in nuclei that had been rapidly isolated from either control or ALLnL-treated cells (Figure 4B). Likewise, no unconjugated ubiquitin was found

in nuclei lysed directly into reducing SDS-loading buffer (not shown). This implies that the free ubiquitin measured in boiled whole cell preparations shown in Figure 4A was exclusively cytosol-derived and that the nuclear pool of ubiquitin must be covalently attached to histones and nonhistone proteins via isopeptide bonds or linked to ubiquitin-activating enzymes via thioester bonds.

Effects of Lactacystin and ALLnL on DNA and RNA Synthesis. Previous studies have established that actively transcribing genes have a higher content of ubiquitinated nucleosomal histones (Davie & Murphy, 1990; Levinger & Varshavsky, 1982), and when transcription is inhibited, the level of nucleosomal uH2B specifically decreases (Davie & Murphy, 1990, 1994). We therefore investigated the effects of LC and ALLnL on the incorporation of [^{3}H]uridine into the mRNA of SKBr3 tumor cells. The results of two separate experiments are shown, where LC was either added to cells simultaneously with [^{3}H]uridine (Figure 5, panel A) or added to cells 4 h before [^{3}H]uridine (Figure 5, panel B). For comparison, the effects of ALLnL pretreatment are also shown in panel B. Note that LC did not significantly decrease the rate of [^{3}H]uridine incorporation into mRNA until 4 h had elapsed and that the maximal inhibition of mRNA synthesis under these conditions was about 30%. This result suggests that inhibition of transcription by LC did not begin until a time when most of the ubiquitin was removed from uH2A and uH2B. Following pretreatment of cells with either LC or ALLnL for 4 h prior to adding [^{3}H]uridine, mRNA synthesis was inhibited by 40 and 46%, respectively (Figure 5, panel B). These results suggest that proteasome inhibitor-caused deubiquitination of uH2A and uH2B may have caused structural changes in the nucleosomes, which interfered with the transcription of at least a subset of genes.

The inhibition by LC of [^{3}H]thymidine incorporation into cellular DNA is shown in Figure 5, panel C. When LC was added simultaneously with [^{3}H]thymidine, DNA synthesis was inhibited by approximately 30%. In contrast, when cells were pretreated with LC for 4 h before adding [3H]-thymidine, the rate of DNA synthesis was inhibited by more than 70%. Essentially identical results were obtained following pretreatment of cells with ALLnL.

Lactacystin and ALLnL Treatment Markedly Decreased the Synthesis of Most Cellular Proteins and Induced the Synthesis of hsp72 and hsp90. We next investigated the effects of the two proteasome inhibitors on the incorporation of [^{35}S]methionine into cellular proteins. Following a 4-h pretreatment with either 10 μM LC or 100 μM ALLnL, cell-associated [^{35}S]methionine-derived radioactivity after a 2-h [^{35}S]methionine pulse was decreased 26% by LC and 44% by ALLnL as compared to the control level. TCA-precipitable radioactivity remained proportional in both control and inhibitor-treated cells (~58% of total cell-associated radioactivity, not shown). Following SDS−PAGE separation of equal amounts of nucleoprotein pellet and supernatant fraction proteins from lysed cells, autoradiography of dried gels revealed a generalized decrease in the labeling of most proteins from cells treated with either LC or ALLnL (Figure 6, panels A and C, bands indicated with arrow heads). Interestingly, however, autoradiograms of the supernatant fractions from LC and ALLnL-treated cells showed two prominent protein bands that were more intensely labeled than in control cells (Figure 6, panels A and B). This pattern of a generalized decrease in protein synthesis with selective induction of certain proteins is reminiscent of the cellular heat-shock response.

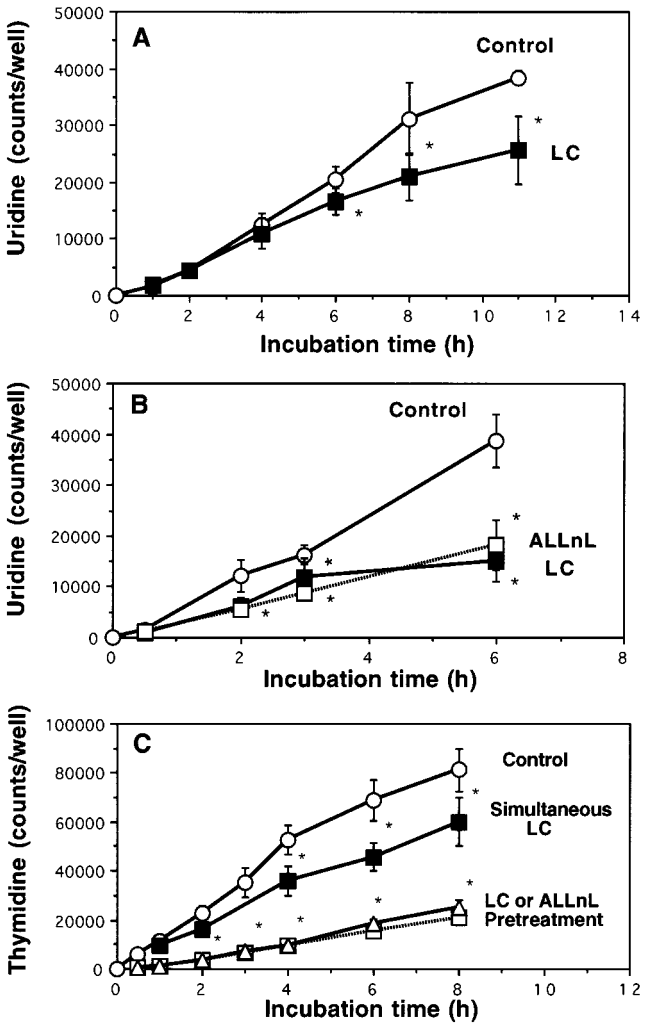

FIGURE 5: Effects of LC and ALLnL on DNA replication and RNA transcription. SKBr3 tumor cells were exposed to [^{3}H]uridine and LC at time zero, and the incorporation of [^{3}H]uridine into cellular RNA was assessed at various times after harvesting cells onto glass fiber filters and counting the radioactivity by solid-phase scintillation counting. Panel A shows [^{3}H]uridine incorporation in control (open circles) and LC-treated (closed squares) cells. In panel B, control cells (open circles) and cells pretreated with 10 μM LC (closed squares) or 100 μM ALLnL (open squares) for 4 h were exposed to [^{3}H]uridine (5 μCi/mL), and RNA synthesis was measured at various times afterward. In panel C, the incorporation of [^{3}H]-thymidine (5 μCi/mL) into cellular DNA is shown in control (open circles), in cells treated simultaneously with LC and [^{3}H]thymidine (closed squares), and in cells pretreated for 4 h with 10 μM LC (open triangles) or 100 μM ALLnL (open squares) prior to adding [^{3}H]thymidine. Inhibition of RNA and DNA synthesis by the proteasome inhibitors was strongest following the 4-h pretreatments with the proteasome inhibitors. Asterisks indicate statistically significant differences from control values at $P < 0.05$.

The apparent molecular weights of the two proteins in the SDS gels that were induced by LC and ALLnL suggested that they might be the stress proteins hsp72 and hsp90 (Figure 6, panel B). In fact, it has recently been reported that ALLnL treatment specifically induces hsp72 (Zhou et al., 1996). To investigate the possibility that LC and ALLnL induced stress proteins in the tumor cells used in this study, radiolabeled proteins were separated by SDS−PAGE, transferred to nitrocellulose membranes, and immunoblotted for hsp72 and hsp90 stress proteins. The radioactive bands on the membrane were then visualized by autoradiography. The bands

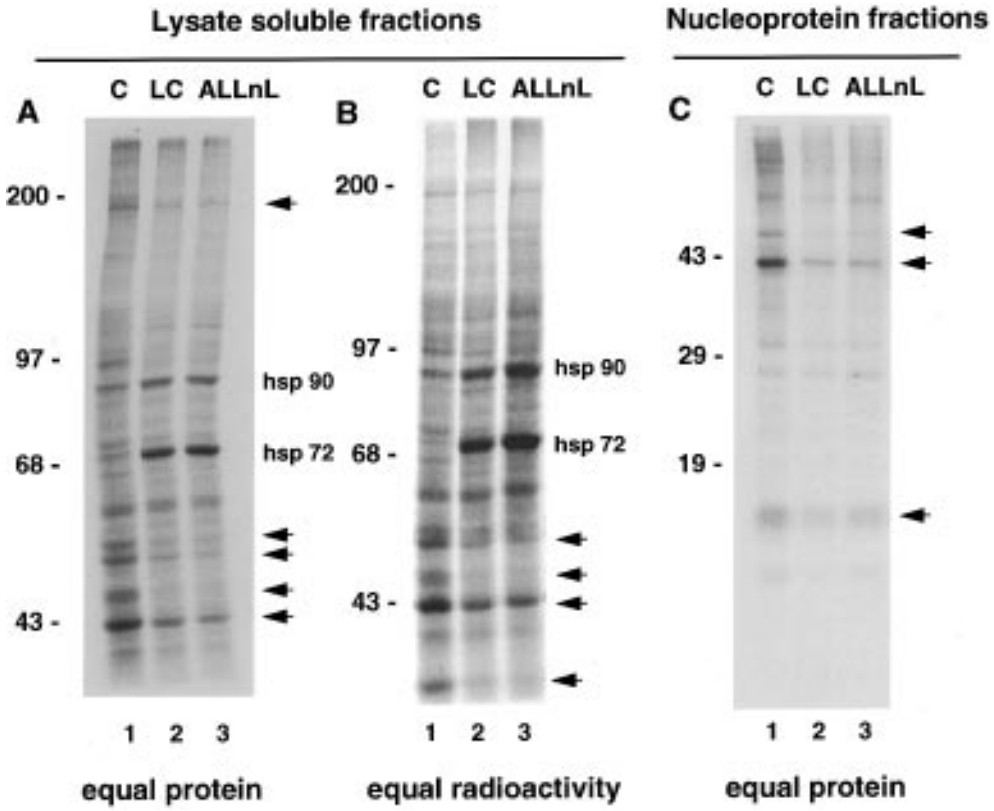

FIGURE 6: LC and ALLnL inhibit the synthesis of many proteins but induce the synthesis of stress proteins hsp72 and hsp90. Cells were pretreated with 10 μM LC or 100 μM ALLnL for 4 h prior to pulsing with [^{35}S]methionine for an additional 2 h. Nucleoprotein and soluble lysate fractions were subjected to 15% SDS−PAGE to resolve proteins, the gels were dried, and newly-synthesized radioactive proteins were visualized by autoradiography. In panel A, the autoradiogram was from a gel with equal amounts of soluble fraction protein (25 μg) from control, LC, and ALLnL-treated cells. In panel B, soluble lysate fractions with equal radioactivity (3.6×10^6 cpm) were electrophoresed and autoradiographed. Panel C shows the autoradiogram of nucleoprotein fractions (equal protein, 25 μg) from control LC and ALLnL-treated cells separated by 15% SDS−PAGE. The arrows indicate various protein bands that were diminished by treating cells with the proteasome inhibitors. Both LC and ALLnL induced the synthesis of the hsp72 and hsp90 stress-response proteins, and these are indicated at their respective locations. hsp72 and hsp90 were identified by immunoblotting with appropriate antibodies following transfer of radiolabeled proteins to nitrocellulose membranes from a separate undried gel. Immunoblotted hsp72 and hsp90 superimposed with the radioactive bands that were increased by LC and ALLnL treatments.

corresponding to hsp72 and hsp90 detected by immunoblotting exactly corresponded to the radioactive 70- and 90-kDa bands that were strongly induced by LC and ALLnL treatments (Figure 6, panels A and B). Although this experiment does not constitute proof, it strongly supports our interpretation that the synthesis of hsp72 and hsp90 was induced by LC and ALLnL. At the same time, the proteasome inhibitors caused a generalized decrease in the synthesis of many other proteins, and the synthesis of most proteins appeared to be unchanged. When autoradiograms of gels containing equal amounts of radioactivity from control and proteasome inhibitor-treated cells (Figure 6, panel B) were scanned and quantified, radiolabeled hsp72 was increased more than 3-fold by LC and 4-fold by ALLnL. The hsp90 band was increased approximately 2-fold by each of the proteasome inhibitors.

Deubiquitination of Nucleosomal uH2A and uH2B is a Component of the Cellular Stress Response. Because the proteasome inhibitors caused deubiquitination of uH2A and uH2B and induced the synthesis of stress-response proteins, we investigated whether the deubiquitination of uH2A and uH2B might also occur in cells exposed to stress response-inducing treatments. Following thermal shock at 43 °C or chemically-induced stress with sodium arsenite or cadmium dichloride, the level of monoubiquitinated histones in the isolated nucleoprotein fractions was markedly decreased (Figure 7, panel A). Simultaneously, ubiquitinated high molecular weight proteins were increased in both nucleoprotein and cytosolic fractions, suggesting a close correlation between the two phenomena (Figure 7, panels A and B). Treating cells with aclacinomycin A, which has recently been shown to inhibit the chymotrypsin activity of the proteasome (Figueiredo-Pereira et al., 1996), or with the proteasome inhibitors MG-132 and 3,4-dichloroisocoumarin similarly decreased uH2A and uH2B levels verifying that proteasome inhibition leads to depletion of u-histones (Figure 7, panel A). In contrast, inhibiting protein synthesis with cycloheximide or treating cells with geldanamycin, a novel agent which promotes the degradation of several proteins in tumor cells (Mimnaugh et al., 1996; Schulte et al., 1995; Whitesell et al., 1994), had no effect on the status of monoubiquinated histones, indicating that deubiquitination of uH2A and uH2B did not occur as a consequence of a general cytotoxic insult or drug-induced protein degradation (Figure 7, panel A). Heat-shock, chemical stress response-inducing agents and the additional proteasome inhibitors all decreased uH2A and uH2B levels and simultaneously increased high molecular weight ubiquitinated proteins in both nucleoprotein and supernatant fractions, with the exception of aclacinomycin, which increased ubiquitinated proteins solely in the supernatant fraction. None of these various treatments caused monoubiquitinated histones to redistribute from the nucleo-

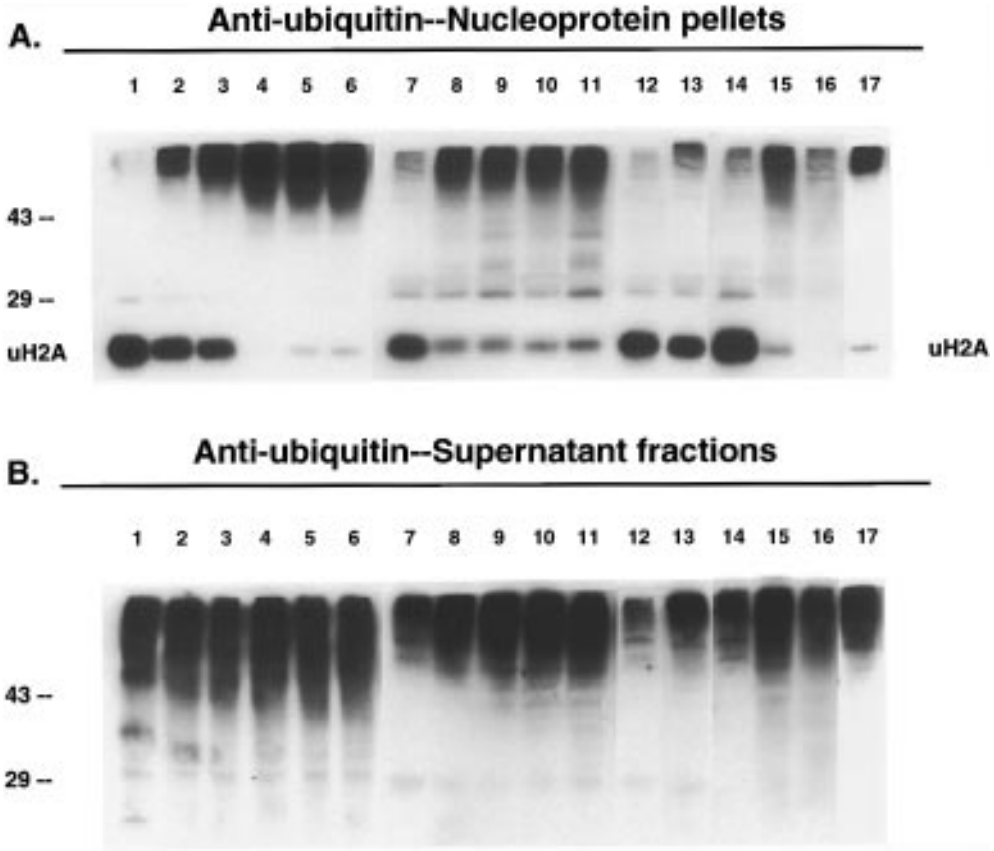

FIGURE 7: Treating cells with thermal stress or stress response-inducing chemicals decreases nucleosomal uH2A and uH2B. SKBr3 cells were exposed to thermal shock at 43 °C for various times or to chemical agents for 2−4 h prior to harvesting cells by lysis, separating the nucleoprotein and soluble lysate fractions by SDS−PAGE, and immunoblotting for ubiquitinated proteins. Panel A shows the decreased relative amounts of uH2A (and uH2B) and increased ubiquitinated high molecular weight proteins from nucleoprotein fractions; panel B shows ubiquitinated proteins from the corresponding supernatant fractions. The treatments were as follows: lanes 1−6, heat-shock (43 °C) for 0, 1, 2, 3, 4, and 5 h; lane 7, control (0.1% DMSO); lanes 8 and 9, 100 μg/mL sodium arsenite for 2 and 4 h, respectively; lanes 10 and 11, 100 μg/mL cadmium dichloride for 2 and 4 h, respectively; lane 12, 100 μM cycloheximide for 4 h; lane 13, 20 μM geldanamycin for 2 h; lane 14, a separate control (0.1% DMSO); lane 15, 50 μM MG-132 for 4 h; lane 16, 20 μM aclacinomycin A for 4 h; lane 17, 50 μM DCI for 4 h. Note that these additional proteasome inhibitors—but not cycloheximide and geldanamycin—decreased uH2A and uH2B levels.

protein fraction to the soluble fraction of cells, ruling out any artifactual loss of uH2A and uH2B from nucleoprotein pellet fractions (Figure 7, compare panels A and B). These results suggest that the deubiquitination of uH2A and uH2B may be a hitherto unrecognized and important component of the general cellular stress response to both heat and noxious chemicals.

DISCUSSION

LC, ALLnL, and several other proteasome inhibitors rapidly and nearly completely deplete the 22−23-kDa ubiquitinated nucleoproteins from SKBr3 tumor cells, and we have identified the u-p23 protein doublet as uH2A and uH2B, the most prominent monoubiquitinated proteins in mammalian cells. To our knowledge, this is the first report describing the modification of the ubiquitin status of nucleosomal histones by treating cells with proteasome inhibitors.

How do LC and ALLnL cause nucleosomal histone deubiquitination? Inhibition of the proteasome stabilizes ubiquitinated proteins that are normally targeted for rapid degradation by the ubiquitin-dependent proteasome pathway and causes those proteins to accumulate in cells. The stabilized ubiquitinated proteins appear to function as a ubiquitin sink in both the nucleus and the cytosol, eventually consuming the free ubiquitin pool. Even newly-synthesized ubiquitin would be quickly "locked" onto cytosolic proteins in cells with non-functioning proteasomes, and little or no replacement ubiquitin would be expected to translocate to the nucleus. The monoubiquitin moiety of uH2A and uH2B is in dynamic equilibrium and turns over rapidly (Seale, 1981); consequently, in the presence of proteasome inhibitors or under conditions of extensive protein damage such as heat-shock, the massive conjugation of ubiquitin to cellular proteins eventually consumes free ubiquitin and results in the redistribution of ubiquitin away from nucleosomal uH2A and uH2B. This hypothesis is supported by the observations that heat-shock as well as the stress response-inducing chemicals, arsenite and cadmium and the proteasome inhibitors markedly increased the cellular content of ubiquitinated proteins, overwhelmed the capability of the proteasome to digest those denatured proteins, depleted the cellular cytosolic pool of unconjugated ubiquitin, and concomitantly promoted deubiquitination of uH2A and uH2B. Similarly, inactivation of the ubiquitin-activating E1 enzyme in ts-mutant cells at the nonpermissive temperature unbalanced the histone ubiquitination−deubiquitination cycling and depleted uH2A and uH2B just as effectively as if the free ubiquitin had been consumed. In contrast, exposing cells to lethal concentrations of cycloheximide or geldanamycin did not cause the

accumulation of high molecular weight ubiquitinated proteins, did not deplete free ubiquitin, and did not decrease uH2A and uH2B. It should be noted that ALLnL treatment (and most likely the other proteasome inhibitors as well) depleted the uH2A.Z isoform and a small amount of di- and tri-uH2A, indicating that the removal of ubiquitin was not specific to uH2A and uH2B, although uH2A and uH2B are the most abundant of the ubiquitinated histones. Moreover, proteasome inhibitor-caused histone deubiquitinatin was not specific to transformed tumor cells because exactly the same phenomenon occurred in normal human lymphocytes. These collective observations imply that whenever free ubiquitin becomes limiting, the ubiquitinated histones rapidly undergo deubiquitination, regardless of the mechanism of free ubiquitin depletion.

Approximately 10% of nucleosomal histone H2A and 2% of histone H2B are constitutively monoubiquitinated in interphase cells (Cook & Chock, 1988; Goldknopf & Busch, 1977). This situation is dynamic, however, because ubiquitin is enzymatically cleaved from histones in condensed chromatin at metaphase (Matsui et al., 1979), and histones are reubiquitinated when cells return to anaphase (Mueller et al., 1985), implicating an important role for nucleosomal histone deubiquitination in mitotic chromatin remodeling. Additionally, in individual nucleosomes, uH2A and uH2B exist in a dynamic equilibrium where the ubiquitin moiety constantly exchanges as a consequence of cycling on and off (Seale, 1981; Wu et al., 1981). The ubiquitin status of histones H2A and H2B is balanced by a family of ubiquitin-conjugating enzymes (Haas & Bright, 1985), by ubiquitin-cleaving isopeptidases (Anserson et al., 1981; Kanda et al., 1986) and by the availability of free ubiquitin. We believe it is unlikely that proteasome inhibitor-caused depletion of uH2A and uH2B resulted from inhibition of E2 ubiquitin-conjugating enzymes or as a consequence of activating ubiquitin-histone cleavage isopeptidases. A hypothetical LC or ALLnL-caused change in these enzyme activities would have to be extremely specific for nucleosomal monoubiquitinated histones as substrates, because blocking proteasome protease activity LC and ALLnL clearly generates high levels of ubiquitinated nonhistone proteins in cells, indirectly indicating that ubiquitinating E1, E2, and E3 enzymes retain activity in the presence of proteasome inhibitors. Although we cannot absolutely rule out the possibility that some of the agents used in this study caused uH2A and uH2B cleavage by activating isopeptidases, the wide diversity of the chemical structures and properties of the agents makes it unlikely. Furthermore, there is no evidence that heat-shock or stress-response chemicals can activate ubiquitin-cleaving isopeptidases, yet heat-shock, arsenite, and cadmium diminished the level of monoubiquitinated nucleosomal histones, which would appear to rule out isopeptidase activation as a mechanism of histone deubiquitination.

It is noteworthy that LC added simultaneously with [^{3}H]-uridine failed to appreciably inhibit transcription until 4 h had elapsed, the length of time required for the near-complete deubiquitination of uH2A and uH2B. In contrast, pretreatment of cells with LC for 4 h before adding [^{3}H]uridine almost immediately slowed the rate of uridine incorporation into RNA by 40%, suggesting that the deubiquitination of uH2A and uH2B preceded proteasome inhibitor-caused transcription inhibition. Transcriptionally active regions of chromatin are known to be highly enriched with uH2A-containing nucleosomes (Levinger & Varshavsky, 1982), and conversely, uH2B (but not uH2A) is deficient in nucleosomes when transcription is blocked by actinomycin D or by the polymerase II inhibitor, 5,6-dichloro-1-ribofuranylbenzimidazole, (Davie & Murphy, 1994), suggesting that the ubiquitin status of nucleosomal histones is dependent upon ongoing transcription, perhaps as a consequence of polymerase complex advancement along the DNA strand (Davie & Murphy, 1994). However, in other studies, histone monoubiquitination was found to be independent of the rate of RNA synthesis, indicating that ubiquitination of histones may not be a direct consequence of alterations in transcription (Davie & Murphy, 1994; Ericsson et al., 1986).

In the progressive nucleosome displacement model of transcription (Van Holde et al., 1992; Workman & Buchman, 1993), the H2A-H2B dimer is released or at least loosened from the core histone octamer by RNA polymerases as they advance along the DNA strand and approach the face of the nucleosome. The ubiquitination of histones H2A and H2B tails could facilitate the action of polymerases and transcription factors (Cook & Chock, 1988) because H2A and H2B ubiquitination is thought to relax the nucleosome structure by weakening the interactions of the histone tails with either DNA or accessory nonhistone proteins (Van Holde et al., 1992). If the H2A−H2B dimer fails to become ubiquitinated, it may resist displacement from the nucleosome histone core, and transcription would necessarily be impeded. Exactly how deubiquitination uH2A and uH2B may be linked to gene silencing is presently unknown, but it is likely that conformational changes resulting from the deubiquitination of uH2A and uH2B stabilize the nucleosomes and shift chromatin toward a more ordered, condensed structure (Davie, 1996). Such a change in the structure of chromatin could prevent advancing polymerases from dissociating or loosening the H2A−H2B dimers from the core nucleosome octamer or prevent interactions of transcription factors with DNA, causing transcription repression (Becker, 1995). Interestingly, uH2A rapidly disappears from condensed chromatin in cells undergoing apoptosis (Marushige & Marushige, 1995), and nucleosomal uH2A (and presumably uH2B) is negligible in nearly transcriptionally-silent mature erythrocyte chromatin (Goldknopf et al., 1980). The observation that [^{3}H]uridine incorporation into mRNA is only partially blocked by the proteasome inhibitors suggests that the effects of histone deubiquitination on mRNA synthesis may be selective, perhaps because nucleosomes appear to occupy the 5′ coding region of only a subset of genes (Barsoum & Varshavsky, 1985; Levinger & Varshavsky, 1982). We plan to investigate this possibility using the technique of differential display of mRNA expression to assess whether there are pleiotrophic effects of proteasome inhibitors on gene expression.

LC added to cells simultaneously with [^{3}H]thymidine decreased DNA synthesis by ∼40%; however, DNA synthesis was more potently inhibited (>70%) when cells were pretreated with LC for 4 h before adding [^{3}H]thymidine, mirroring what we observed with RNA synthesis. The 2-fold greater inhibition of DNA synthesis after deubiquitination of histones had occurred implies that a crucial relationship may exist between the ubiquitin status of H2A and H2B, the physical structure of the nucleosome, and the regulated initiation and progression of DNA replication. Deubiquitination of nucleosomal histones in tsFT5 cells with a mutant

thermolabile ubiquitin-activating E1 enzyme slightly precedes the cessation of DNA synthesis when those cells are heated to the non-permissive temperature, suggesting that DNA replication in those cells is influenced by the ubiquitin status of histones (Mori et al., 1993). In contrast, we should point out that ALLnL exposure for 12 h has been reported to cause cell-cycle arrest at the G1/S boundary in CHO cells simultaneous with prevention of cyclin B degradation (Sherwood et al., 1993); however, chromatin condensation was also noted during ALLnL-induced mitotic arrest of CHO cells. We hypothesize that chromatin condensation subsequent to the loss of ubiquitin from nucleosomal histones H2A and H2B may be a mechanism by which proteasome inhibitors decrease DNA replication. However, it is possible that proteasome inhibitors diminished RNA and DNA synthesis indirectly by promoting the ubiquitination of transcription factors and critical nonhistone proteins involved in replication, inactivating them or preventing them from translocating to the nucleus. Whether proteins like p53, c-Jun, and NF-kB maintain normal function after they become ubiquitinated but fail to be degraded when the proteasome is inhibited by LC or ALLnL is an interesting question.

If deubiquitination of nucleosomal monoubiquitinated histones selectively alters gene expression, one would predict differential protein synthesis as the outcome. Although overall [^{35}S]methionine incorporation into newly synthesized proteins after LC or ALLnL treatment was decreased, several [^{35}S]methionine-radiolabeled proteins, including hsp72 and hsp90, were strongly induced by the proteasome inhibitors. If this selective stimulation of protein synthesis is associated with the deubiquitination of uH2A and uH2B, alterations in gene expression caused by histone deubiquitination may not be indiscriminate, and this raises the possibility that certain genes may respond to and be regulated by nucleosomal histone ubiquitination−deubiquitination cycles.

Although it is difficult to understand how deubiquitination of uH2A and uH2B induces the synthesis of stress-response proteins and, at the same time, decreases the synthesis of many other cellular proteins, it has been known for some time that exactly the same effects on protein synthesis occur when cells are heat-shocked (Lindquist, 1986; Schlesinger, 1986). Recently, it was reported that ALLnL and LC specifically and reversibly induced hsp72 more than 9-fold in HepG2 cells, and this induction could be blocked by either actinomycin D or cycloheximide (Zhou et al., 1996). These investigators hypothesized that proteasome inhibition prevented the degradation of a yet unidentified, normally unstable protein that interacted with (and presumably activated) HSF1, perhaps causing its trimerization. This finding prompted us to compare several biochemical effects of proteasome inhibitors with the effects of heat-shock and chemical agents known to strongly evoke the cellular stress response. The common consequences of these various treatments include (1) accumulation of ubiquitinated high molecular weight proteins, (2) depletion of unconjugated ubiquitin, (3) decreased ubiquitination of nucleosomal uH2A and uH2B, and (4) induction of hsp72 and hsp90. The uniformity of these observations of diverse treatments strongly suggest a common interactive mechanism centered around ubiquitin. Indeed, Finley and Varshavsky (1985) have suggested the heat-shock response and ubiquitin-dependent proteasomal degradation of damaged and misfolded proteins perform essential and complementary functions in protecting cells from noxious environments. And fitting nicely into this complementary scheme is the fact that ubiquitin itself is a heat-shock-inducible protein (Bond & Schlesinger, 1985). Perhaps the ubiquitin status of the nucleosomal histones indirectly regulates the expression of hsp72 and hsp90 or other proteins by modifying chromatin structure in the vicinity of their genes and interfering with transcription factor or repressor access. Given the unusual situation that approximately 50% of nucleosomes in proximity to the uninduced hsp72 gene contain uH2A (Levinger & Varshavsky, 1982), deubiquitination of nucleosomal uH2A and uH2B in this disproportionally ubiquitinated region of the genome may specifically remodel those particular nucleosomes and influence the binding of heat-shock factors to their response elements or modulate the action of other regulatory proteins such as GAGA, which helps to activate genes, including the hsp72 gene (Shopland et al., 1995; Tsukiyama et al., 1994).

Altering the ubiquitin status of nucleosomal histones H2A and H2B through the use of proteasome inhibitors and/or cellular stress response-provoking agents may be a useful strategy to uncover the functional significance of this novel and reversible molecular modification and to elucidate whether it plays an important regulatory role in the ordered processing of genetic information.

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