THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 270, No. 5, Issue of February 3, pp. 2344–2351, 1995
Printed in U.S.A.

Proteolysis in Cultured Liver Epithelial Cells during Oxidative Stress

ROLE OF THE MULTICATALYTIC PROTEINASE COMPLEX, PROTEASOME*

(Received for publication, July 22, 1994, and in revised form, October 7, 1994)

Tilman Grune‡, Thomas Reinheckel§, Minakshi Joshi, and Kelvin J. A. Davies¶

From the Department of Biochemistry and Molecular Biology, The Albany Medical College, Albany, New York 12208

Exposure to various forms of mild oxidative stress significantly increased the intracellular degradation of both "short-lived" and "long-lived," metabolically radiolabeled, cell proteins in cultures of Clone 9 liver cells (normal liver epithelia). The oxidative stresses employed were bolus H_2O_2 addition; continuous H_2O_2 flux; the redox cycling quinones, menadione and paraquat; and the aldehydic products of lipid peroxidation, 4-hydroxynonenal, malonyldialdehyde, and hexenal. In general, exposure to more severe oxidative stress produced a concentration-dependent decline in intracellular proteolysis, in some cases to below baseline levels. Oxidatively modified "foreign" proteins (superoxide dismutase and hemoglobin) were also selectively degraded, in comparison with untreated foreign proteins, when added to lysates of Clone 9 liver cells. As with intracellular proteolysis, the degradation of foreign proteins added to cell lysates was greatly increased by mild oxidative modification, but depressed by more severe oxidative modification. The proteinase activity was recovered in >300-kDa cell fractions, and inhibitor profiles and immunoprecipitation studies indicated that the multicatalytic proteinase complex, proteasome, was responsible for most of the selective degradation observed with mild oxidative stress; up to approximately 95% for intracellular proteolysis and 65–80% for degradation of foreign modified proteins. Seven days of daily treatment with an antisense oligodeoxynucleotide, directed against the initiation codon region of the proteasome C2 subunit gene, severely depressed the intracellular levels of several proteasome subunit polypeptides (by Western blot analysis), and also depressed the H_2O_2 induced increase in intracellular proteolysis by approximately 95%, without significantly affecting baseline proteolytic rates. Extensive studies revealed only small or no increases in the overall capacity of oxidatively stressed cells to degrade oxidatively modified protein substrates; a finding supported by both Western blot and Northern blot analyses which revealed no significant increase in the levels of proteasome subunit polypeptides or mRNA transcripts. We conclude that mild oxidative stress increases intracellular proteolysis by modifying cellular proteins, thus increasing their proteolytic susceptibility. In contrast, severe oxidative stress diminishes intracellular proteolysis, probably by generating severely damaged cell proteins that cannot be easily degraded (*e.g.* cross-linked/aggregated proteins), and by damaging proteolytic enzymes. We further conclude that the multicatalytic proteinase complex proteasome is responsible for most of the recognition and selective degradation of oxidatively modified proteins in Clone 9 liver cells.

Over the past several years a wide series of publications from this laboratory (1–21) and other groups (22–32) have reported a relationship between protein oxidation and proteolysis. Such studies have been conducted with erythrocytes and reticulocytes from rabbits, cows, and human beings (1–7, 9–11, 13–17, 19, 21), with *Escherichia coli* (1, 9, 10, 14, 21), with isolated mitochondria *in vitro* (12, 14), with rat muscles *in vitro* (1, 14), with primary cultures of rat hepatocytes (31, 32), and with purified proteins and proteases *in vitro* (1–32). These studies have all concluded that proteins are inherently susceptible to oxidative damage, and that oxidative damage alters proteolytic susceptibility. Our own studies have consistently demonstrated that relatively low-level oxidative damage alters proteolytic degradation, whereas extensive oxidative damage causes decreased proteolysis due to cross-linking, aggregation, and decreased solubility (1–21).

Both our group (1–21) and that of Stadtman and his colleagues (25–32) have suggested that increased degradation of mildly oxidized proteins is a normal function of intracellular proteolytic systems, whereas inability to degrade extensively oxidized proteins may contribute to certain disease states and aging.

In bacteria a series of proteolytic enzymes appear to conduct the degradation of oxidatively modified proteins (9, 10). In primary hepatocytes (31, 32), and erythrocytes and reticulocytes (1–7, 9–11, 13–17, 19–21) strong evidence has been presented for the role of the multicatalytic proteinase complex, proteasome; initially referred to as macroxyproteinase by this group (13–15). Evidence has also been provided for a mechanism of proteolytic recognition and degradation by bacterial proteases (30) and erythrocyte/reticulocyte proteasome (1–5, 13, 16, 17, 20, 21), based on partial unfolding and exposure of (previously shielded) hydrophobic amino acid R groups, as a result of protein oxidation.

A recent publication by Dean *et al.* (33), however, has seriously questioned whether increased degradation of mildly oxidized proteins is, indeed, a widespread property of eucaryotic cells and organisms. Dean *et al.* (33) question whether terminally differentiated red blood cells (RBC)[1] are a suitable model

* This research was supported in part by National Institutes of Health, National Institute of Environmental Health Sciences Grant ES-03598 (to K. J. A. D.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Supported by Deutsche Forschungsgemeinschaft Grant Gr 1240/1-1.

§ Supported by the Hans-Böckler-Stiftung.

¶ Senior author to whom all correspondence and reprint requests should be addressed: Chairman, Dept. of Biochemistry & Molecular Biology, The Albany Medical College, New Scotland Ave., A-10, Albany, NY 12208. Tel.: 518-262-5364; Fax: 518-262-5689.

[1] The abbreviations used are: RBC, red blood cells; Hb, hemoglobin; s-LLVY-MCA, succinyl-leucine-leucine-valine-tyrosine-MCA (a fluoro-

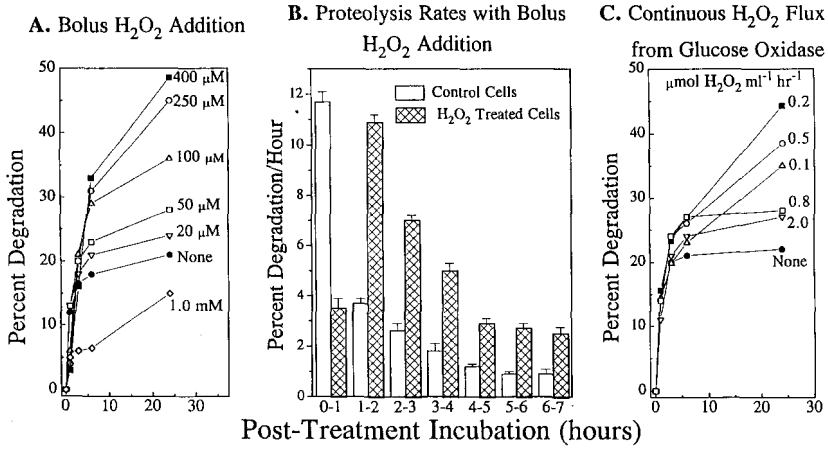

FIG. 1. **Degradation of short-lived cellular proteins in hydrogen peroxide-treated Clone 9 liver cells.** Cell proteins were metabolically labeled with [^{35}S]Met/Cys for 2 h, and then exposed for 30 min in phosphate-buffered saline plus 5.0 mM glucose to either bolus H_2O_2 addition (*panels A* and *B*), or to a continuous H_2O_2 flux generated by 0.14–2.77 μg/ml glucose oxidase (*panel C*), as described under "Materials and Methods." After treatment, or "sham-treatment" for control cells, the culture plates were washed twice with phosphate-buffered saline, replenished with culture medium, and incubated for 0–24 h at 37 °C. Protein degradation was measured, at the indicated times, following addition of 20% (w/v) trichloroacetic acid, to lyse the cells and precipitate remaining intact proteins. Percent protein degradation was measured by liquid scintillation in the trichloroacetic acid supernatants as: (acid soluble counts − background counts) ÷ (total counts initially incorporated − background counts) × 100. *Panel A* reports cumulative proteolysis over a 24-h period following bolus H_2O_2 treatment. *Panel B* reports actual rates of protein degradation measured at 1-h intervals during the first 7 h after bolus H_2O_2 treatment. *Panel C* shows the effects of a 30-min continuous H_2O_2 flux (generated by 0.14–2.77 μg/ml glucose oxidase and 5.0 mM glucose) on cumulative protein degradation over a 24-h post-treatment period. All values are means of eight independent experiments, for which S.E. (in *panels A* and *C*) were always less than 10%.

for other eucaryotic cell types, and note that the hypothesis has not been thoroughly tested in other mammalian cells. Additionally Matthews *et al.* (34) have questioned the proposed role of proteasome in degrading oxidatively modified proteins.

We undertook the present investigation with two major goals: first, to test the hypothesis that low-level oxidative stress can result in increased intracellular proteolysis, in dividing mammalian cells: and second, to test the possible involvement/importance of proteasome in this process. Since liver cells are continuously exposed to oxidative stress through the metabolism of numerous endogenous and exogenous toxicants, at least two reports have suggested the degradation of oxidatively modified proteins by proteasome in primary cultures of hepatocytes (31, 32), and mammalian proteasome has been most extensively studied in liver cells (35, 36), we selected an immortalized liver cell culture line for our studies.

MATERIALS AND METHODS

Cells and Cell Culture—Clone 9 liver cells (normal rat liver epithelia) were obtained from American Tissue and Cell Culture (ATCC CRL 1439). The cells were cultured in 90% Ham's F-12K medium, supplemented with 10% fetal bovine serum. Cells were initially plated at a density of 2 × 10^4 cells/cm^2, grown for 3 days, and then replated at a density of 2 × 10^4 cells/cm^2 for a further 2 days of growth before oxidative stress studies.

Proteolysis Measurements—Proteolysis studies involved either the degradation of metabolically radiolabeled cellular proteins, or the degradation of radiolabeled "foreign" proteins or peptides during incubation with (non-labeled) cell lysates. Metabolic radiolabeling of intracellular proteins was achieved during either a 2-h labeling procedure or a 16-h labeling procedure (followed by a 2-h "cold chase"). Cell proteins were metabolically radiolabeled by incorporation of a [^{35}S]methionine/cysteine mixture (14) using subconfluent monolayers of cells in methionine/cysteine-free minimal essential Eagle's medium. After 2 or 16 h of exposure the labeling mixture was removed and the cell monolayers were washed twice with phosphate-buffered saline containing excess unlabeled methionine/cysteine (as a "chase"). The 2-h labeling procedure was considered to generate mostly "short-lived" radiolabeled proteins, whereas the 16-h labeling procedure was considered to generate predominantly "long-lived" radiolabeled cell proteins. The degradation of metabolically labeled cell proteins was assessed by release of acid-soluble counts from, previously acid-precipitable, cell proteins.

The degradation of radiolabeled foreign proteins, and of foreign peptides was assessed following addition of these substrates to unlabeled cell lysates. Cells were lysed by a 1-h incubation in water, supplemented with 1.0 mM dithiothreitol. Membrane debris, nuclei, organelles, and unlysed cells were then removed by centrifugation at 14,000 × *g*.

Exposure of Clone 9 Liver Cells to Oxidative Stress—Subconfluent monolayers of cells (3 × 10^5 cells/cm^2) were exposed to bolus additions of H_2O_2, paraquat (methylviologen), menadione (vitamin K_3), hexenal, malonyldialdehyde, and 4-hydroxynonenal, or to continuous H_2O_2 production by the enzyme glucose oxidase. Exposures were conducted for 30 min at 37 °C, in phosphate-buffered saline (pH 7.4). After exposure to oxidative stress, cells were washed twice and then incubated in tissue culture medium (90% Ham's F-12K medium, supplemented with 10% fetal calf serum) for proteolysis measurements.

RESULTS

Oxidative Stress Affects the Degradation of Short-lived Cell Proteins—The cumulative degradation of short-lived proteins (those labeled during a 2-h [^{35}S]Met/Cys incorporation) was strongly affected by exposure of Clone 9 liver cells to H_2O_2 (Fig. 1). Proteolysis gradually increased with H_2O_2 concentrations from 20 μM to 0.4 mM, by as much as 150% (Fig. 1*A*). At the high H_2O_2 concentration of 1.0 mM, however, protein degradation actually decreased by as much as 50% (Fig. 1*A*). Optimal H_2O_2 concentrations for increased proteolysis after 6–24 h incubation ranged from 0.25 to 0.4 mM. At these same concentrations, however, a decrease in proteolysis actually appeared to occur in the first hour of incubation after H_2O_2 treatment (Fig. 1*A*). To further investigate this phenomenon we next measured actual rates of protein degradation for 7 h after H_2O_2 treatment. As shown in Fig. 1*B*, treatment of cells with 0.4 mM H_2O_2 caused a transient depression of proteolytic rates in the first hour after exposure, but thereafter proteolysis was significantly increased over basal rates.

Since H_2O_2 exposures *in vivo* are more likely to involve sources of continuous H_2O_2 production, rather than a bolus addition, we also tested the effects of continuous H_2O_2 production by the enzyme glucose oxidase. As shown in Fig. 1*C*, continuous exposure to H_2O_2 production, over a 30-min glucose oxidase treatment, caused a generalized increase in subsequent proteolysis at all H_2O_2 exposure rates tested. Proteolysis

peptide substrate for proteolysis); H_2O_2, hydrogen peroxide; $O_2^{\cdot-}$, superoxide anion radical.

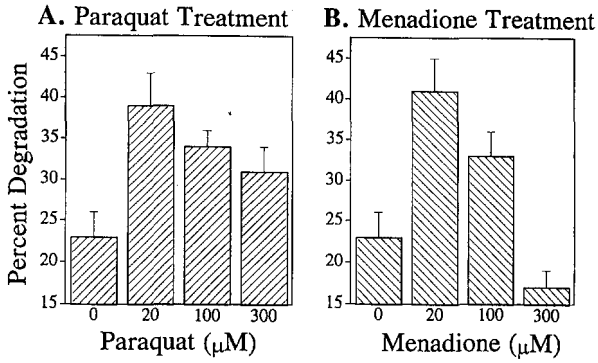

FIG. 2. **Degradation of short-lived cellular proteins in cells exposed to redox cycling quinones.** Clone 9 liver cells were exposed, or sham-exposed to paraquat (*panel A*) or to menadione (*panel B*) for 30 min. Protein degradation was measured at 24 h of incubation after exposure. All exposure conditions and proteolysis measurements were performed as described in the legend to Fig. 1. Values reported are means ± S.E. of at least six independent determinations.

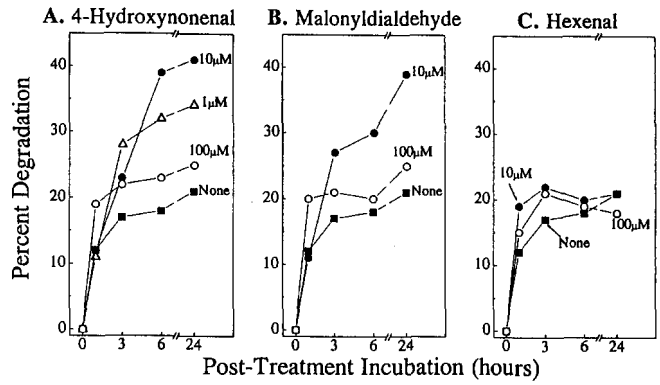

FIG. 3. **Degradation of short-lived cellular proteins in cells exposed to aldehydic products of lipid peroxidation.** Clone 9 liver cells were exposed, or sham-exposed to 4-hydroxynonenal (*panel A*), to malonyldialdehyde (*panel B*), or to hexenal (*panel C*) for 30-min periods. All exposure conditions and proteolysis measurements were conducted as described in the legend to Fig. 1. The 4-hydroxynonenal was the kind gift of Prof. Hermann Esterbauer (University of Graz, Austria). Malonyldialdehyde was prepared as described previously (37) and *trans*-2-hexenal was obtained from Sigma. All three lipid aldehydes were extensively purified prior to use. Values reported in *panels A-C* are means of at least eight independent determinations, for which S.E. were always less than 10%.

began to increase at 50 nmol of $H_2O_2 \cdot ml^{-1} \cdot 30\ min^{-1}$, peaked at 100 nmol of $H_2O_2 \cdot ml^{-1} \cdot 30\ min^{-1}$, and gradually decreased again at the higher exposures of 250, 400, and 1,000 nmol of $H_2O_2 \cdot ml^{-1} \cdot 30\ min^{-1}$. Since the cumulative H_2O_2 exposures during the 30-min treatments of Fig. 1*C* were similar to the bolus H_2O_2 additions of Fig. 1, *A* and *B*, it is interesting to note that no depression of proteolysis below control rates was observed at any of the continuous H_2O_2 flux rates tested. Importantly, no significant loss of cell viability, as judged by trypan blue exclusion, occurred during any of the experiments shown in Fig. 1, *A-C* (data not shown).

We next tested for effects of the superoxide ($O_2^{\cdot -}$) generating agents paraquat and menadione on the degradation of short-lived proteins in Clone 9 liver cells (Fig. 2). Protein degradation was maximally increased by treatment of cells with 20 μM paraquat (Fig. 2*A*) or 20 μM menadione (Fig. 2*B*). Higher concentrations of either agent again resulted in decreased proteolysis; in the case of 300 μM menadione, to below control levels.

Membrane lipid peroxidation is a well known outcome of cellular oxidative stress, and lipid oxidation products are able to react with other cellular components; including proteins. To model possible lipid peroxidation effects on protein integrity and stability we incubated Clone 9 liver cells with the aldehydic lipid peroxidation products 4-hydroxynonenal, malonyldialdehyde, and hexenal (Fig. 3). At the low concentrations of 1 or 10 μM, both 4-hydroxynonenal (Fig. 3*A*) and malonyldialdehyde (Fig. 3*B*) caused increased degradation of short-lived cellular proteins (by as much as 100%), but proteolysis returned toward baseline levels following 100 μM treatments with either agent. Hexenal caused only a small increase in proteolysis during the first 3 h after treatment and this trend actually reversed from 6 to 24 h post-treatment (Fig. 3*C*).

Oxidative Stress Affects the Degradation of Long-lived Cell Proteins—The degradation of long-lived proteins (those labeled during a 16-h [^{35}S]Met/Cys incorporation followed by a 2-h chase) was generally increased by exposure to low (100 μM) H_2O_2 concentrations (Fig. 4*A*) or low (20 μM) paraquat concentrations (Fig. 4*B*). Higher concentrations of H_2O_2 (0.4–1.0 mM) or paraquat (0.3 mM) produced smaller increases in proteolysis. Interestingly, no overall decrease in proteolysis to below baseline control levels, and no transient decrease in proteolysis during the first hour of H_2O_2 exposure, occurred in our studies of long-lived proteins (Fig. 4, *A* and *B*), although such effects were observed with oxidatively stressed short-lived proteins (Figs. 1 and 2*B*).

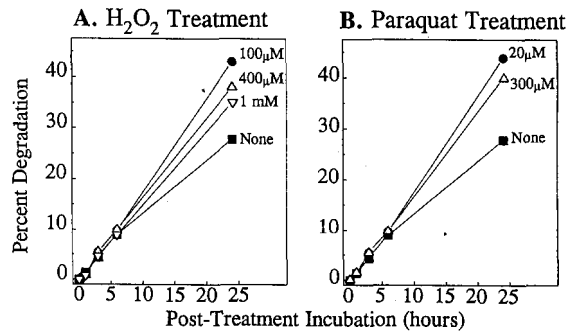

FIG. 4. **Degradation of long-lived cellular proteins in cells exposed to hydrogen peroxide or paraquat.** Cell proteins were metabolically labeled with [^{35}S]Met/Cys for 16 h, and then chased with excess unlabeled Met/Cys for 2 h. After this 18-h period, cells were either exposed or sham-exposed, to H_2O_2 (*panel A*) or paraquat (*panel B*) for 30 min, washed, and then incubated for a further 0–24 h in culture medium for measurements of protein degradation. All H_2O_2 (bolus) and paraquat exposure conditions, and all proteolysis measurements, were performed as described in the legends to Figs. 1 and 2. Values in both panels are means of at least six independent determinations, for which S.E. were always less than 10%.

Degradation of Exogenous Protein Substrates in Cell Lysates—Our working model to explain the results of Figs. 1–4 is that cellular proteins are mildly damaged by exposure to low-level oxidative stress, and degraded. We also propose that severe oxidative stress generates more extreme forms of modified proteins, including cross-linked and/or aggregated proteins, that are poor substrates for proteolysis. An alternate explanation would be that mild stress activates or induces cellular proteases (to degrade even normal proteins) and severe stress inactivates proteases. To begin testing these hypotheses we prepared lysates from both untreated and H_2O_2-treated cells, and incubated them with the radiolabeled foreign proteins, superoxide dismutase or hemoglobin (Hb).

Undamaged superoxide dismutase and Hb were degraded at low rates during incubation with untreated Clone 9 liver cell lysates, but prior oxidative modification (by H_2O_2) of these foreign proteins increased their proteolytic susceptibility by up to 5–8-fold (Fig. 5*A*). When the Clone 9 cells were pretreated by exposure to 0.25 or 0.4 mM H_2O_2, or to 20 or 300 μM paraquat (as per Figs. 1 and 3) before lysis, essentially the same differ-

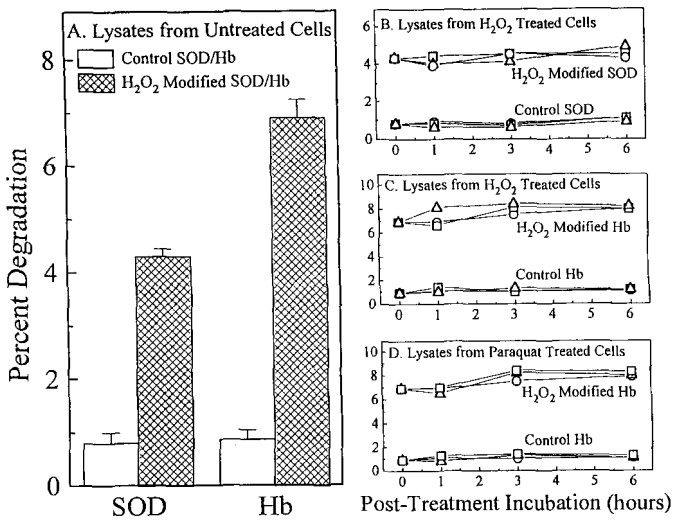

FIG. 5. **Degradation of H_2O_2 modified foreign superoxide dismutase and hemoglobin added to lysates of control and oxidatively stressed Clone 9 liver cells.** Tritium-labeled superoxide dismutase (*SOD*) and hemoglobin were added to centrifuged lysates of unlabeled Clone 9 liver cells for measurements of proteolysis over a 6-h period. A proteolysis buffer consisting of 50 mM Tris-HCl (pH 7.8), 20 mM KCl, 5 mM MgOAc, and 0.5 mM dithiothreitol was used, as previously (14). Both foreign protein substrates were radiolabeled by reductive methylation with [^{3}H]formaldehyde and sodium cyanoborohydride as described by Jentoft and Deanborn (38), and then extensively dialyzed. The tritiated superoxide dismutase and Hb were either undamaged, or were oxidatively modified immediately prior to the experiment by exposure to 15 mM H_2O_2 for 2 h, as described previously (13, 15). Percent superoxide dismutase or Hb degradation was determined, as described previously (14), by liquid scintillation in supernatants of trichloroacetic acid precipitated lysates, by the formula: (acid-soluble counts − background counts) ÷ (initial acid precipitable counts − background counts) × 100. *Panel A* reports the selective degradation of H_2O_2-modified superoxide dismutase and Hb (in comparison with undamaged superoxide dismutase and Hb) during a 2-h incubation of these protein substrates with centrifuged lysates from untreated Clone 9 liver cells. *Panels B-D* report the degradation of both H_2O_2 modified and undamaged superoxide dismutase and Hb during incubation with centrifuged lysates from control, H_2O_2-treated, and paraquat-treated Clone 9 liver cells. The 30-min H_2O_2 or paraquat cell treatments were conducted as described in the legends for Figs. 1 and 2. In *panels B* and *C* the H_2O_2 cell pretreatments were as follows: ○, none; Δ, 0.25 mM; □, 1.0 mM. In *panel D* the paraquat pretreatment of cells was as follows: ○, none; Δ, 20 μM; □, 300 μM. Both the bovine RBC superoxide dismutase and the bovine Hb were obtained from Sigma. Values reported in all four panels are means of at least four independent determinations for which S.E. (not shown in *B*, *C*, and *D*) were always less than 10%.

ence in degradation of control and oxidatively modified superoxide dismutase and Hb was again observed (Fig. 5, *B*, *C*, and *D*). It would, thus, appear that oxidative stress damages cellular proteins, making them more (or less) susceptible to proteolysis, rather than activating the proteolytic machinery of the cell.

Importance of Proteasome in the Intracellular Degradation of Oxidatively Modified Proteins—Our previous work with red blood cells indicated that the 20 S multicatalytic proteinase complex, proteasome, is responsible for at least 70% of the increased proteolysis observed with H_2O_2 exposure (16, 20, 21). Since proteasome is ubiquitously distributed in eucaryotes we decided to test its possible involvement in the degradation of oxidized proteins in Clone 9 liver cells.

Our first experiment was to compare the inhibitor profile of Clone 9 cell lysates, RBC lysates, and purified RBC proteasome, conducting the degradation of oxidatively modified superoxide dismutase and Hb. As shown in Table I the degradation of oxidized superoxide dismutase and Hb was similarly inhibited by serine reagents (phenylmethanesulfonyl fluoride and diisopropyl fluorophosphate), sulfhydryl reagents (*N*-ethylmaleimide), and transition metal chelators (EDTA and 8-hydroxyquinoline) in all three test systems. The lysosomal proteolysis inhibitor leupeptin and ATP-Mg^{2+} both caused only minor inhibition in all cases.

TABLE I
Inhibitor profile for the degradation of H_2O_2-modified superoxide dismutase and hemoglobin

Clone 9 liver cell lysates were prepared, as described under "Materials and Methods," from untreated cells. RBC lysates and purified RBC proteasome were prepared as previously described (13–17, 19, 21). Tritium-labeled bovine superoxide dismutase and Hb were prepared, and H_2O_2 modified, as described in the legend to Fig. 5. H_2O_2-modified [^{3}H]superoxide dismutase and [^{3}H]Hb were incubated with cell lysates or purified proteasome for 6 h, at 37 °C, as described in the legend to Fig. 5. Percent inhibition of proteolysis values given above are averages of at least six experiments for each protein substrate, with each inhibitor; for which S.E. values were always less than 10%. Since no significant differences were observed in the inhibition profiles of superoxide dismutase and Hb, the results for both proteolytic substrates have been averaged to save space. The inhibitors used were as follows: 5.0 mM PMSF (phenylmethanesulfonyl fluoride), 5.0 mM DFP (diisopropyl fluorophosphate), 10.0 mM NEM (*N*-ethylmaleimide), 10.0 mM EDTA, 1.0 mM 8-hydroxyquinoline, 0.1 mM leupeptin, and 5.0:5.0 mM ATP/Mg^{2+}.

Inhibitor	% Inhibition		
	Clone 9 liver cell lysates	RBC lysates	RBC proteasome
PMSF	84	86	81
DFP	80	84	85
NEM	64	72	76
EDTA	44	51	42
8-Hydroxyquinoline	46	49	53
Leupeptin	14	9	6
ATP/Mg^{2+}	22	18	15

We next separated Clone 9 liver cell lysates into fractions of less than 300 kDa and fractions of greater than 300 kDa (Fig. 6). Since the proteasome complex has a molecular size of approximately 670 kDa (13, 16, 21) it should be present only in the greater than 300-kDa cell fraction. The 0–300-kDa fraction was always prepared from radiolabeled cells whose proteins had been metabolically labeled by incorporation of [^{35}S]Met/Cys for either 2 h (short-lived proteins) or for 16 h plus a 2-h chase (long-lived proteins). These radiolabeled 0–300-kDa fractions were prepared from both untreated cells and from H_2O_2-treated cells, and were used as "substrate fractions" for proteolysis. The >300 kDa "proteasome fraction" was always prepared from non-labeled cells, either with no treatment, or following H_2O_2 exposure. When the proteasome fraction was prepared from control cells (Fig. 6*A*) it selectively catalyzed significant degradation of both short-lived and long-lived proteins in the 0–300-kDa substrate fractions from H_2O_2-treated cells. When the proteasome fraction was prepared from H_2O_2-treated cells (Fig. 6*B*) it again catalyzed the proteolysis of both long-lived and short-lived proteins in the 0–300-kDa substrate fractions from H_2O_2-treated cells, although the degradation of long-lived proteins was somewhat depressed in comparison with the results of Fig. 6*A*. The experiments of Fig. 6 are certainly permissive of a role for proteasome in the degradation of oxidatively modified cell proteins, and also indicate that stress-induced proteolysis is primarily the result of substrate modification or damage, rather than protease (proteasome) activation.

The fluoropeptide succinyl-leucine-leucine-valine-tyrosine-MCA (s-LLVY-MCA) has been extensively studied as a proteolytic substrate for proteasome (35, 36). To further test the possible importance of proteasome in degrading oxidatively modified Clone 9 liver cell proteins, we studied the effects of proteasome immunoprecipitation on the degradation of both s-LLVY-MCA and radiolabeled cell proteins, in lysates from control and H_2O_2-treated cells (Fig. 7). As shown in Fig. 7*A*,

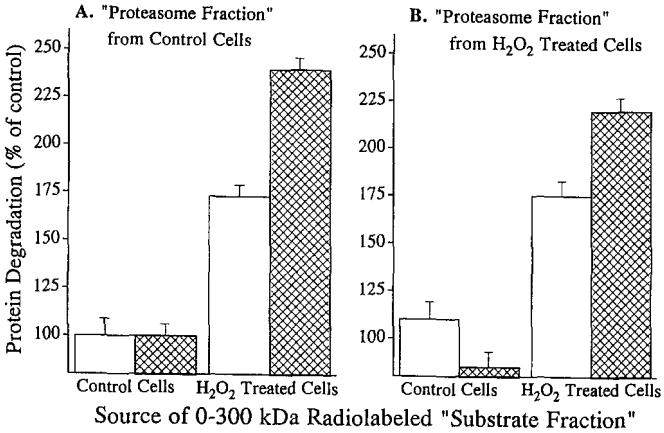

FIG. 6. **Proteolysis in recombined 0–300 kDa and >300 kDa Clone 9 liver cell fractions**. One set of cells was cultured (in the absence of radionucleotides), treated with 0.4 mM H_2O_2 or used as a control, lysed, centrifuged, and filtered through a 300-kDa cut-off membrane (Amicon™, Beverly, MA). The >300-kDa fraction retained by the membrane was re-solubilized and used as the (unlabeled) proteasome fraction for all experiments. Another set of cells was cultured identically, and then either short-lived or long-lived cellular proteins were metabolically labeled with [^{35}S]Met/Cys as per Figs. 1 or 4. After labeling the cells were either exposed to 0.4 mM H_2O_2, or used as controls. The labeled cells were then lysed, centrifuged, and filtered through the 300-kDa cut-off membrane. With these labeled cells, only the <300-kDa filtrate was retained for use as the substrate fraction for proteolysis. The unlabeled >300-kDa proteasome fraction and the <300-kDa radiolabeled substrate fraction were then recombined for proteolysis measurements, using the conditions of Fig. 5. *Panel A* shows experiments in which cells used for the proteasome fraction were untreated. *Panel B* shows experiments in which cells used for the proteasome fraction were pretreated by exposure to 0.4 mM H_2O_2 prior to lysis and filtration. Values reported are means ± S.E. of eight independent experiments.

proteasome immunoprecipitation depressed s-LLVY-MCA degradation by 75–85% in lysates of both control cells and H_2O_2-treated cells. The *inset* to Fig. 7*B* is a proteasome Western blot which shows major loss of several proteasome subunit bands following the immunoprecipitation procedure. The main panel of Fig. 7*B* reveals a 95% decrease in the H_2O_2 induced degradation of long-lived cellular proteins following proteasome immunoprecipitation.

We next repeated the immunoprecipitation studies of Fig. 7, but this time used H_2O_2 modified superoxide dismutase as the substrate for proteolysis in lysates of control and H_2O_2-treated cells. As shown in Fig. 8, proteasome immunoprecipitation decreased the degradation of H_2O_2-modified superoxide dismutase by approximately 55% in lysates of both control cells and H_2O_2-treated cells. When the immunoprecipitates were re-solubilized and incubated with H_2O_2-modified superoxide dismutase, more than 100% (actually 126–145%) of the initial activity was recovered (Fig. 8, *bars* labeled *Pcpt.*), suggesting a small activation of proteasome by the immunoprecipitation procedure.

A more rigorous test of proteasome involvement in the intracellular degradation of oxidatively modified proteins would be to use knock-out mutants in which one of the essential proteasome subunit genes was deleted. Unfortunately, for our purposes, proteasome appears to be essential for cell division and deletion mutations are typically lethal (42, 43). We, therefore, decided to use the more gentle approach of diminishing total cellular proteasome activity by prolonged exposure of cells to an antisense oligodeoxynucleotide for an essential proteasome subunit. For these studies we synthesized both sense and antisense oligodeoxynucleotides directed against the initiation codon region of the proteasome C2 subunit gene (44, 45).

After 7 days of daily exposure to the antisense message the cellular concentrations of several proteasome subunit polypeptides were significantly depressed, as shown by Western blot analysis (Fig. 9, *inset*). The degradation of labeled proteins in untreated cells was not affected by the sense oligodeoxynucleotide and the antisense message decreased basal proteolysis in control cells by only approximately 12% (Fig. 9, *main panel*). In contrast, the increased degradation of oxidized proteins normally seen in H_2O_2-treated cells was almost completely abolished in antisense oligodeoxynucleotide-treated cells. Interestingly, the sense oligodeoxynucleotide actually increased proteolysis in H_2O_2-treated cells, although only by a modest 16%, in comparison with cells that received no oligodeoxynucleotide treatment (Fig. 9, *main panel*).

It should be noted that cellular growth rates, rates of protein synthesis, and the incorporation of [^{35}S]Met/Cys into newly synthesized proteins (whose degradation was actually measured in Fig. 9), were all unaffected by either the sense or the antisense oligodeoxynucleotides (data not shown). The results of Fig. 9, thus, provide strong evidence for a major involvement of proteasome in the degradation of oxidatively modified Clone 9 liver cell proteins.

Most of the experimental data presented thus far would suggest that oxidative stress modifies cellular substrate proteins, making them more (or less) susceptible to proteolysis by proteasome. The results of Fig. 7*A* (*−IP*) and Fig. 8 (comparing total activity in re-solubilized immunoprecipitates), however, suggest that a small activation or induction of proteasome may actually occur with exposure of cells to H_2O_2 stress. To further investigate this possibility we compared the degradation of the proteasome substrate s-LLVY-MCA in control cells, and cells exposed to H_2O_2 or paraquat. For the most part both H_2O_2 and paraquat exposures caused no change in s-LLVY-MCA degrading activity, except for small increases at very high oxidant exposures which were actually very poor initiators of intracellular proteolysis (data provided to the reviewers). Thus neither activation nor induction of proteasome appears to explain our results.

DISCUSSION

To our knowledge, the present investigation is the first to conclusively demonstrate oxidative stress-induced intracellular proteolysis in a mammalian cell culture line. It would, thus, appear that the reasonable concern raised by Dean *et al.* (33), that such phenomena might be limited to bacteria and RBC, has been answered. Our studies reveal that mild forms of oxidative stress can increase the intracellular degradation of both short-lived and long-lived proteins in Clone 9 liver cells. In contrast, more severe levels of oxidative stress are less effective in initiating intracellular proteolysis and, at the extreme, can even depress protein degradation to below baseline values. Similar trends have now been observed in bacteria, isolated mitochondria, chloroplasts, erythrocytes, and reticulocytes, rat muscles, and primary hepatocytes (1–21, 25–32). It, thus appears clear that oxidative stress-induced intracellular proteolysis is a general property of both procaryotes and eucaryotes.

Our inhibitor profiles, proteasome immunoprecipitation experiments, and proteasome C2 subunit oligodeoxynucleotide studies all indicate that the multicatalytic proteinase complex, proteasome is largely responsible for the selective degradation of oxidatively modified proteins in oxidatively stressed Clone 9 liver cells. The same conclusion was previously reached with red blood cells (1–7, 9–11, 13–17, 19–21). It should also be noted that the multicatalytic proteinase complex, proteasome was the enzyme isolated by Rivett (31, 32) from primary hepatocyte cultures, based on its ability to selectively degrade oxidatively modified protein substrates.

The proteasome complex exists in both an ATP-independent 19–20 S (670–700 kDa) form, and an ATP-stimulated 26 S

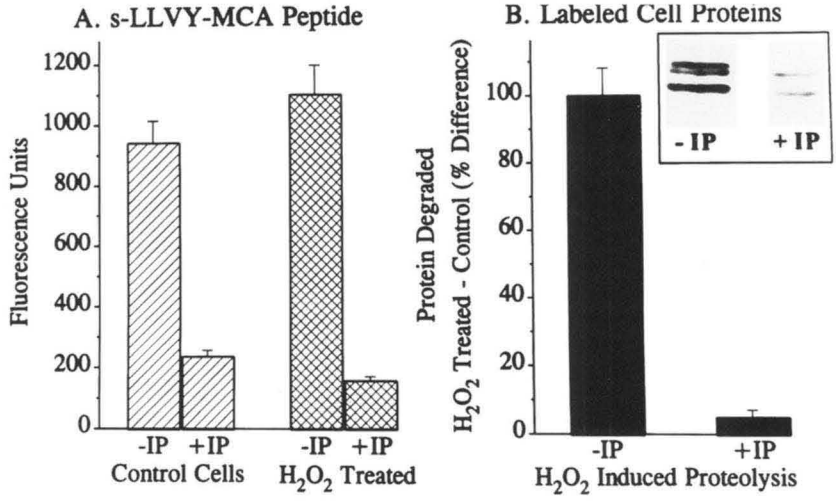

FIG. 7. **Proteolysis in lysates of H_2O_2-treated Clone 9 liver cells following proteasome immunoprecipitation**. Control and 0.4 mM H_2O_2-treated cells were lysed and centrifuged at 3 h after (30 min) H_2O_2 treatment or sham-treatment. Proteasome in the cell lysates was then either immunoprecipitated with a purified rabbit anti-rat IgG (39), directed against the 20 S proteasome, designated *+IP*, or sham-precipitated and designated *−IP*. Preliminary studies revealed that the optimal IgG/lysate protein ratio was 70 μg of IgG/mg of lysate protein, and this ratio was used. Cell lysates were incubated with IgG for 3 h at 4 °C, and then centrifuged at 34,000 × *g* for 30 min. Purified rabbit anti-rat IgG (38) was the kind gift of Dr. Keiji Tanaka (University of Tokushima, Tokushima, Japan) and immunoprecipitation was conducted according to Orino *et al.* (39). *Panel A* shows the effects of proteasome immunoprecipitation, in extracts from control and H_2O_2-treated cells, on degradation of the fluoropeptide s-LLVY-MCA. Degradation of s-LLVY-MCA was measured, as previously (14), in 34,000 × *g* supernatants by fluorescence at 365-nm excitation/460-nm emission (within the linear range for fluorescence response) in comparison with Pronase-digested standards. The fluoropeptide was dissolved in 10% Me_2SO and used at a final concentration of 50 μM in an assay buffer consisting of 50 mM Tris-HCl (pH 7.8), 20 mM KCl, 0.5 mM MgOAc, and 0.5 mg of cell lysate protein. After 1-h incubation at 37 °C proteolysis was terminated, prior to fluorescence measurements, by addition of 2.0 ml of a solution containing 0.1 M sodium borate (pH 9.0) and ethanol/water (144:16), to 200 μl of reaction mixture. The main portion of *panel B* shows the effects of proteasome immunoprecipitation on the H_2O_2 induced degradation of long-lived radiolabeled (16-h labeling plus a 2-h chase, as per Fig. 4) cell proteins, in 34,000 × *g* supernatants of proteasome immunoprecipitated (*+IP*) and sham-immunoprecipitated (*−IP*) cell lysates. It should be noted that the experiments of *panel B* involved a 30-min H_2O_2 treatment (or sham-treatment) of intact radiolabeled cells followed by cell lysis and centrifugation, immunoprecipitation (or sham-precipitation) for 3 h followed by centrifugation at 34,000 × *g* for 30 min, and a 37 °C proteolysis incubation for 6 h. The *inset* to *panel B* is a Western blot showing the effects of proteasome immunoprecipitation on proteasome subunit protein bands in the 34,000 × *g* cell supernatants. Western blots of control (*−IP*) and immunoprecipitated (*+IP*) cell lysates were performed according to Towbin *et al.* (40). To achieve best results a mixture of proteasome anitsera was used. The mixed proteasome antisera was 50% rabbit anti-chick (41) which was the kind gift of Dr. Chin Ha Chung (Seoul National University, Seoul, Korea), and 50% rabbit anti-rat (39) which was the kind gift of Dr. Keiji Tanaka (University of Tokushima, Tokushima, Japan). All values in both panels are means ± S.E. of at least six independent experiments.

(1,500 kDa) form in mammalian cells (35, 36, 46–59). Our previous work with RBC (1–7, 13–17, 19–21), and the work of Rivett (31, 32) with primary hepatocytes, provided experimental evidence that the ATP-independent 19–20 S (670–700 kDa) "core" proteasome complex is the form that recognizes and selectively degrades oxidatively damaged protein substrates. In this regard it is important to note that reticulocytes and terminally differentiated erythrocytes exhibit similar capacities to degrade oxidatively modified proteins, and similar inhibition profiles (1–8, 13–17, 19–21), despite the fact that the 26 S ATP-stimulated proteasome complex is lost during maturation, and erythrocytes retain only the 19–20 S ATP-independent core proteasome complex (13). The degradation of oxidized proteins in red cell lysates, and the degradation of oxidized protein substrates by the purified erythrocyte or reticulocyte 19–20 S proteasome complex, are inhibited by 15–20% upon addition of ATP (13–17, 19–21). In the present investigation ATP was also slightly inhibitory (by approximately 15%) to the selective degradation of oxidatively modified proteins in Clone 9 cell lysates, providing at least initial evidence that the 19–20 S core proteasome complex is also responsible for degrading oxidized proteins in Clone 9 liver cells.

The multicatalytic proteinase complex, proteasome, can bind (or release) both inhibitory and stimulatory polypeptide subunits (35, 36). Additionally, the entire proteasome complex is overexpressed under certain conditions, at discrete developmental stages, and in transformed cells (59). In initiating the present investigation we considered the hypothesis that oxidative stress might either activate proteasome (by releasing an inhibitory subunit or promoting expression of a stimulatory

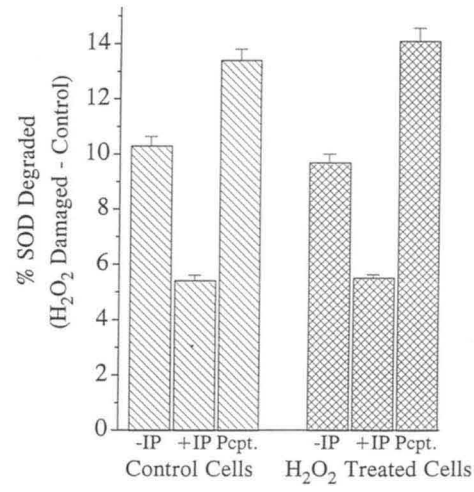

FIG. 8. **Degradation of H_2O_2-modified superoxide dismutase in lysates of Clone 9 liver cells following proteasome immunoprecipitation**. Centrifuged lysates of control or 0.4 mM H_2O_2-treated cells (with or without immunoprecipitation) were incubated with control and H_2O_2-modified [^{3}H]superoxide dismutase for 1 h, as per Fig. 5. The lysates were first either immunoprecipitated (*+IP*) with proteasome antibody as per Fig. 7, or were sham-precipitated (*−IP*), prior to centrifugation at 34,000 × *g*. Proteolysis was measured as described in the legend to Fig. 5 by release of acid-soluble counts, from [^{3}H]superoxide dismutase added to the 34,000 × *g* supernatants. Degradation of [^{3}H]superoxide dismutase was also measured by addition of this substrate to re-solubilized immunoprecipitates; designated *Pcpt*. Values for the difference between degradation of H_2O_2 modified and control [^{3}H]superoxide dismutase are means ± S.E. of at least three independent experiments.

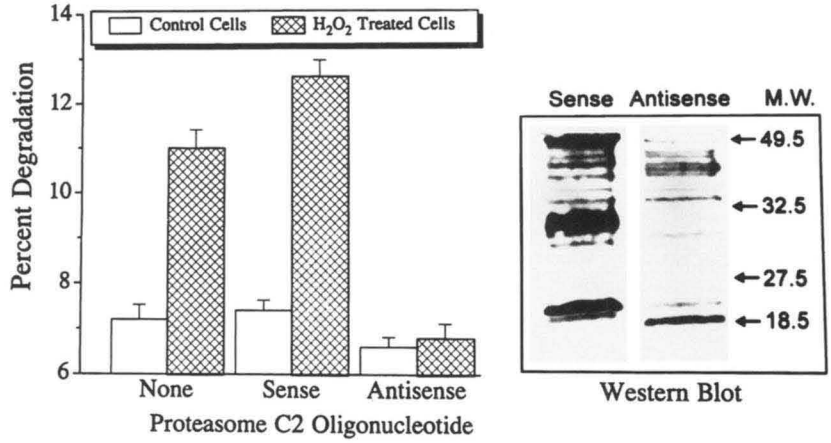

FIG. 9. **Degradation of long-lived proteins in Clone 9 liver cells treated with antisense oligodeoxynucleotide to the proteasome C2 subunit gene**. Cells were either untreated (*None*) or were exposed for 7 days to daily additions of 0.4 nmol/ml of either sense or antisense oligodeoxynucleotides to the initiation codon region of the proteasome C2 subunit gene (44). The sense oligonucleotide used was 5′-AGCTATGTT-TCGCAA-3′, and the antisense oligonucleotide was 5′-TTGCGAAACATAGCT-3′. Both oligonucleotides were synthesized on an ABI391 DNA synthesizer (Applied Biosystems), and extensively purified prior to use. Long-lived cell proteins were then metabolically radiolabeled with [^{35}S]Met/Cys for 16 h (plus a 2-h chase) as described in the legend to Fig. 4. The cells were next either exposed to 0.4 mM H_2O_2 (as per Fig. 1) or used as controls. Degradation of long-lived cellular proteins was measured during a 6-h incubation by production of acid-soluble counts, as per Fig. 1. Values in the main portion of the figure are means ± S.E. of 12 independent experiments. The *inset* is a Western blot showing the depletion of several proteasome subunit proteins in cells treated with antisense oligonucleotide for 7 days, in comparison with sense oligonucleotide-treated cells. Molecular weight standard markers are included in the *inset* for convenient identification of individual bands. The proteasome C2 subunit has an approximate molecular size of 30–32 kDa (44, 45).

subunit), or might induce proteasome synthesis. Our present studies, involving comparisons of control and oxidatively stressed cells, however, provide little or no evidence to support such an hypothesis. By Western blot analyses with polyclonal antiproteasome antibodies, Northern blot analyses with a cDNA probe directed against C2 subunit transcripts, and actual proteinase assays measuring total cellular capacity to degrade control and oxidatively modified protein substrates (superoxide dismutase and Hb added to cell lysates), no evidence for increased proteasome transcription, message stabilization, translation, or total activity was found. Small increases in peptidase activity with the proteasome fluoropeptide substrate s-LLVY-MCA were observed with severe oxidative stress, but such high stress levels actually depressed the degradation of oxidatively modified proteins. Thus, with the exception of a possible small activation or induction of peptidase activity, oxidative stress does not appear to either activate proteasome or induce proteasome synthesis in Clone 9 liver cells.

Since neither proteasome activation nor induction seem to explain the large increases in total intracellular protein degradation we observe following oxidative stress, and since oxidatively modified foreign proteins are selectively degraded in lysates of non-stressed Clone 9 liver cells, it seems clear that oxidative modification of substrate proteins must be the major cause of increased protein degradation following oxidative stress. This same conclusion was previously reported for erythrocytes and reticulocytes (1–7, 15–17, 19–21), isolated mitochondria (12), and bacteria (9, 10, 25–30), and now appears to be a fairly general biological phenomenon. Protein re-arrangement, with exposure of hydrophobic "patches" of amino acids, has been proposed as the substrate activation mechanism to explain increased degradation of oxidatively modified proteins by both RBC (13, 15–17, 19–21) and *E. coli* (9, 10, 30). The model foreign substrates (superoxide dismutase and Hb) used to link substrate hydrophobicity and proteolytic susceptibility in RBC (13, 15–17, 19–21) were the same substrates used in our present investigation, and proteasome appears responsible for the degradation of oxidized proteins in both cases. It, thus, seems reasonable to propose that increased intracellular proteolysis in oxidatively stressed Clone 9 liver cells may also be explained by modification of cell proteins to expose, proteasome sensitive, hydrophobic patches.

Although we were able to document oxidative stress-induced increases in intracellular proteolysis of 150% or more, the model oxidized substrates superoxide dismutase and Hb were degraded at severalfold higher rates (in comparison with the untreated proteins) in cell lysates. It is also abundantly clear from numerous studies that proteins vary widely in their susceptibility to various forms of oxidative stress, and to proteolysis (1–34). It, thus, seems probable that oxidative stress causes some generalized increase in the oxidation and degradation of cellular proteins, but that certain proteins are particularly susceptible. Further studies[2] have revealed the existence of at least five proteins in Clone 9 liver cells whose turnover (from radiolabeling experiments both before and after cellular oxidative stress) is increased severalfold following exposure to H_2O_2. The identities and mechanisms of oxidation/degradation of these, and other, Clone 9 liver cell proteins are under active investigation in this laboratory.

Acknowledgments—We thank Dr. Keiji Tanaka of the University of Tokushima in Tokushima, Japan, and Dr. Chin Ha Chung, of the Seoul National University in Seoul, Korea, for the kind gifts of proteasome antisera (KT and CHC) and purified anti-proteasome IgG (KT). Without the selfless generosity of such valued and respected colleagues much of the present work could not have been conducted. We also thank Mary Heiser for her excellent work in the preparation of the manuscript.

REFERENCES

1. Davies, K. J. A. (1986) *Free Radical Biol. Med.* **2,** 155–173
2. Davies, K. J. A. (1987) *J. Biol. Chem.* **262,** 9895–9901
3. Davies, K. J. A., Delsignore, M. E., and Lin, S. W. (1987) *J. Biol. Chem.* **262,** 9902–9907
4. Davies, K. J. A., and Delsignore, M. E. (1987) *J. Biol. Chem.* **262,** 9908–9913
5. Davies, K. J. A., Lin, S. W., and Pacifici, R. E. (1987) *J. Biol. Chem.* **262,** 9914–9920
6. Davies, K. J. A., and Goldberg, A. L. (1987) *J. Biol. Chem.* **262,** 8220–8226
7. Davies, K. J. A., and Goldberg, A. L. (1987) *J. Biol. Chem.* **262,** 8227–8234
8. Taylor, A., and Davies, K. J. A. (1987) *Free Radical Biol. Med.* **3,** 371–377
9. Davies, K. J. A., and Lin, S. W. (1988) *Free Radical Biol. Med.* **3,** 215–223
10. Davies, K. J. A., and Lin, S. W. (1988) *Free Radical Biol. Med.* **5,** 225–236

[2] T. Grune, T. Reinheckel, M. Talbot, and K. J. A. Davies, manuscript in preparation.

11. Salo, D. C., Lin, S. W., Pacifici, R. E., and Davies, K. J. A. (1988) *Free Radical Biol. Med.* **5,** 335–339
12. Marcillat, O., Zhang, Y., Lin, S. W., and Davies, K. J. A. (1988) *Biochem. J.* **254,** 677–683
13. Pacifici, R. E., Salo, D. C., Lin, S. W., and Davies, K. J. A. (1989) *Free Radical Biol. Med.* **7,** 521–536
14. Pacifici, R. E., and Davies, K. J. A. (1990) *Methods Enzymol.* **186,** 485–502
15. Salo, D. C., Pacifici, R. E., Lin, S. W., Giulivi, C., and Davies, K. J. A. (1990) *J. Biol. Chem.* **265,** 11919–11927
16. Pacifici, R. E., Kono, Y., and Davies, K. J. A. (1993) *J. Biol. Chem.* **268,** 15405–15411
17. Giulivi, C., and Davies, K. J. A. (1993) *J. Biol. Chem.* **268,** 8752–8759
18. Murakami, K., Lin, S. W., Davies, K. J. A., and Taylor, A. (1990) *Free Radical Biol. Med.* **8,** 217–222
19. Giulivi, C., and Davies, K. J. A. (1990) *J. Biol. Chem.* **265,** 19453–19460
20. Davies, K. J. A. (1993) *Biochem. Soc. Trans.* **21,** 346–353
21. Giulivi, C., Pacifici, R. E., and Davies, K. J. A. (1994) *Arch. Biochem. Biophys.* **311,** 329–341
22. Wolff, S. P., Garner, A., and Dean, R. T. (1986) *Trends Biochem. Sci.* **11,** 27–31
23. Dean, R. T., Thomas, S. M., and Garner, A. (1986) *Biochem. J.* **240,** 489–494
24. Wolff, S. P., and Dean, R. T. (1986) *Biochem. J.* **234,** 399–403
25. Levine, R. L., Oliver, C. N., Fulks, R. M., and Stadtman, E. R. (1981) *Proc. Natl. Acad. Sci. U. S. A.* **78,** 2120–2124
26. Fucci, L., Oliver, C. N., Coon, M. J., and Stadtman, E. R. (1983) *Proc. Natl. Acad. Sci. U. S. A.* **80,** 1521–1525
27. Levine, R. L. (1983) *J. Biol. Chem.* **258,** 11823–11827
28. Levine, R. L. (1983) *J. Biol. Chem.* **258,** 11828–11833
29. Nakamura, K., and Stadtman, E. R. (1984) *Proc. Nat. Acad. Sci. U. S. A.* **81,** 2011–2015
30. Cervera, J., and Levine, R. L. (1988) *FASEB J.* **2,** 2591–2595
31. Rivett, A. J. (1985) *J. Biol. Chem.* **260,** 300–305
32. Rivett, A. J. (1985) *J. Biol. Chem.* **260,** 12600–12606
33. Dean, R. T., Geiseg, S., and Davies, M. J. (1993) *Trends Biochem. Sci.* **18,** 437–441
34. Matthews, W., Tanaka, K., Driscoll, J., Ichihara, A., and Goldberg, A. L. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 2597–2601
35. Rivett, A. J. (1989) *Arch. Biochem. Biophys.* **268,** 1–8
36. Rivett, A. J. (1993) *Biochem. J.* **291,** 1–10
37. Michiels, C., and Remacle, J. (1991) *Toxicology* **66,** 225–234
38. Jentoft, N., and Dearborn, D. G. (1979) *J. Biol. Chem.* **254,** 4359–4365
39. Orino, E., Tanaka, K., Tamura, T., Sone, S., Ogura, T., and Ichihara, A. (1991) *FEBS Lett.* **284,** 206–210
40. Towbin, H. S., Staehelin, J., and Gordon, J. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76,** 4350–4354
41. Ahn, J. Y., Hong, S. O., Kwak, K. B., Kang, S. S., Tanaka, K., Ichihara, A., Ha, D. B., and Chung, C. H. (1991) *J. Biol. Chem.* **266,** 15746–15749
42. Fujiwara, T., Tanaka, K., Orino, E., Yoshimura, T., Kumatori, A., Tamura, T., Chung, C. H., Nakai, T., Yamaguchi, K., Shin, S., Kakizuka, A., Nakanishi, S., and Ichihara, A. (1990) *J. Biol. Chem.* **265,** 16604–16613
43. Ghislain, M., Udavardy, A., and Mann, C. (1993) *Nature* **366,** 358–360
44. Fujiwara, T., Tanaka, K., Kumatori, A., Shin, S., Yoshimura, T., Ichihara, A., Tokunaga, F., Aruga, R., Iwanaga, S., Kakizuka, A., and Nakanishi, S. (1989) *Biochemistry* **28,** 7332–7340
45. Tamura, T., Lee, D. H., Osaka, F., Fujiwara, T., Shin, S., Chung, C. H., Tanaka, K., and Ichihara, A. (1991) *Biochim. Biophys. Acta* **1089,** 95–102
46. Wilk, S., and Orlowski, M. (1980) *J. Neurochem.* **35,** 1172–1182
47. Dahlmann, B., Kuehn, L., and Reinauer, H. (1983) *FEBS Lett.* **160,** 243–247
48. Dahlmann, B., Rutschmann, M., Kuehn, L., and Reinauer, H. (1985) *Biochem. J.* **228,** 171–177
49. Hough, R., Pratt, G., and Rechsteiner, M. (1987) *J. Biol. Chem.* **262,** 8303–8313
50. DeMartino, G. N., and Goldberg, A. L. (1979) *J. Biol. Chem.* **254,** 3712–3715
51. Ray, K., and Harris, H. (1985) *Proc. Nat. Acad. Sci. U. S. A.* **82,** 7545–7549
52. Ishiura S., Sano, M., Kamakura, K., and Sagita, K. (1985) *FEBS Lett.* **189,** 119–123
53. McGuire, M. J., and DeMartino, G. N. (1986) *Biochim. Biophys. Acta* **873,** 279–289
54. Falkenburg, P. E., Haass, C., Kloetzel, P. M., Niedel, B., Kopp, F., Kuehn, L., and Dahlmann, B. (1988) *Nature* **331,** 190–192
55. Arrigo, A., Tanaka, K., Goldberg, A. L., and Welch, W. J. (1988) *Nature* **331,** 192–194
56. Baumeister, W., Dahlmann, B., Hegerl, R., Kopp, F., Kuehn, L., and Pfeifer, G. (1988) *FEBS Lett.* **241,** 239–245
57. McGuire, M. J., McCullough, M. L., Croall, D. E., and De Martino, G. N. (1989) *Biochim. Biophys. Acta* **995,** 181–186
58. Dahlmann, B., Kuehn, L., Ishiura, S., Tsukahara, T., Sugita, H., Tanaka, K., Rivett, A. J., Hough, R. F., Rechsteiner, M., Mykles, D. L., Fagan, J. M., Waxman, L., Ishi, S., Sasaki, M., Kloetzel, P.-M., Harris, H., Ray, K., Behal, F. J., Demartino, G. N., McGuire, M. J., Orlowski, M., and Wilk, S. (1988) *Biochem. J.* **255,** 750–751
59. Shimbara, N., Orino, E., Sone, S., Ogura, T., Takashina, M., Shono, M., Tamura, T., Yasuda, H., Tanaka, K., and Ichihara, A. (1992) *J. Biol. Chem.* **267,** 18100–18109