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Effect of heat shock on protein degradation in mammalian cells: involvement of the ubiquitin system

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Exposure of cultured rat hepatoma (HTC) cells to a 43°C heat shock transiently accelerates the degradation of the long-lived fraction of cellular proteins. The rapid phase of proteolysis which lasts ~2 h after temperature step-up is followed by a slower phase of proteolysis. During the first 2 h after temperature step-up there is a wave of ubiquitin conjugation to cellular proteins which is accompanied by a fall in ubiquitin and ubiquitinated histone 2A (uH2A) levels. Upon continued incubation at 43°C the levels of ubiquitin conjugates fall with a corresponding increase of ubiquitin and uH2A to initial levels. The burst of protein degradation and ubiquitin conjugation after temperature step-up is not affected by the inhibition of heat shock protein synthesis. Cells of the FM3A ts85 mutant, which have a thermolabile ubiquitin activating enzyme (E_1), do not accelerate protein degradation in response to a 43°C heat shock, whereas wild-type FM3A mouse cells do. This observation indicates that the ubiquitin system is involved in the degradation of heat-denatured proteins. Sequential temperature jump experiments show that the extent of proteolysis at temperatures up to 43°C is related to the final temperature and not to the number of steps taken to attain it. Temperature step-up to 45°C causes the inhibition of intracellular proteolysis. We propose the following explanation of the above observations. Heat shock causes the conformational change or denaturation of a subset of proteins stable at normal temperatures. These altered proteins are somehow recognized by the ubiquitin system and degraded by it. When the abnormal proteins formed shortly after temperature step-up have been broken down, intracellular proteolysis returns to a slower basal rate.

Key words: heat shock/protein degradation/ubiquitin

Introduction

Exposure of cells to slightly elevated temperatures elicits the heat shock response, the best known effect of which is the activation of a small set of heat shock protein genes (Bienz, 1985; Pelham, 1985; Lindquist, 1986; Schlesinger, 1986). Various types of stress other than heat shock also induce heat shock protein gene expression (Lindquist, 1986). It has been noted that many of the factors which elicit heat shock protein synthesis cause the formation of aberrant proteins (Finley *et al.*, 1984; Goff and Goldberg, 1985; Munro and Pelham, 1985). These include incubating cells with amino acid analogues (Kelley and Schlesinger, 1978; Hightower, 1980), certain mutations of major protein structural genes (Hiromi and Hotta, 1985), micro-injection of denatured proteins (Ananthan *et al.*, 1986) or, in bacteria, introduction of expression vectors producing large amounts of a foreign protein (Goff and Goldberg, 1985).

Aberrant proteins are selectively degraded within mammalian cells at elevated rates (Goldberg and St. John, 1976; Hershko and Ciechanover, 1982). For example the incorporation of some amino acid analogues into proteins (Knowles *et al.*, 1975; Neff *et al.*, 1979) or certain mutations (Capecchi *et al.*, 1974) lead to the accelerated degradation of the abnormal proteins produced. There is compelling evidence that in eukaryotic cells amino acid analogue substituted proteins are degraded via the ubiquitin system (Hershko *et al.*, 1982; Ciechanover *et al.*, 1984). It is inferred, although it has not been tested, that other aberrant proteins such as heat-denatured proteins, are also degraded by the ubiquitin system.

Recent observations establish a link between the ubiquitin system and the heat shock response. Ubiquitin itself is a heat shock protein in chicken fibroblasts and in yeast (Bond and Schlesinger, 1985; Finley and Varshavsky, 1985). Additional evidence supporting a functional relationship between ubiquitin and the heat shock response come from a mutant (ts85) of a mouse carcinoma cell line which has a temperature-sensitive ubiquitin-activating enzyme (E_1). In this mutant, heat shock proteins are induced at abnormally low temperatures (Ciechanover *et al.*, 1984). The above facts have led to the proposal of models in which the ubiquitin system couples heat shock protein gene activation to aberrant protein accumulation (Finley *et al.*, 1984; Munro and Pelham, 1985).

Although the observations outlined above imply that heat shock causes the denaturation and probably selective degradation of cellular proteins, this question has received very little attention. Munro and Pelham (1984) have found that heat shock inhibits the degradation of protein fragments produced by deletion mutants of the *hsp70* gene transfected into COS cells. However, to our knowledge the effects of heat shock on the degradation of normal cellular proteins has not been investigated. In the investigation described here we have examined the effects of heat shock on endogenous protein degradation in mammalian cells and the involvement of ubiquitin in this process. We find that moderate heat shock temperatures (43°C) cause a transient acceleration of protein degradation whereas higher temperatures inhibit proteolysis. The burst of proteolysis caused by heat shock appears to be mediated by the ubiquitin system.

Results

Heat shock protein synthesis

Figure 1 shows the pattern of general and heat shock protein synthesis of cells incubated for various times at 43°C. Equal numbers of cells pulse labelled for the same period of time were loaded onto each slot of the gel. Decreased overall protein synthesis is evident from 15-60 min after temperature step-up. A similar transient decrease in cellular protein synthesis has been observed in heat shocked HeLa cells (Hickey and Weber, 1982). Heat shock protein synthesis can be detected at 30 min and reaches maximum rates after 120 min. The major heat shock protein has an apparent M_r of about 70 kd and three other heat shock pro-

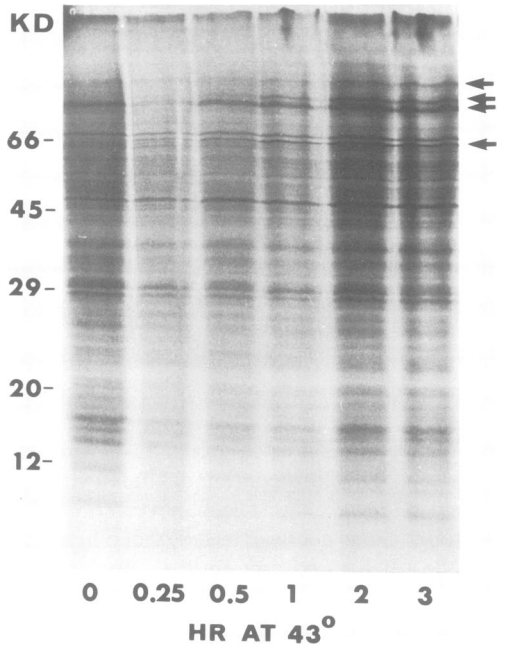

Fig. 1. General and heat-shock protein synthesis in HTC cells incubated at 43°C. HTC cells were incubated for the times indicated at 43°C and pulsed with [^{35}S]methionine for 15 min at 37°C, as described in Materials and methods. All slots of the gel were loaded with the same number of cells (7.5×10^5) in sample buffer.

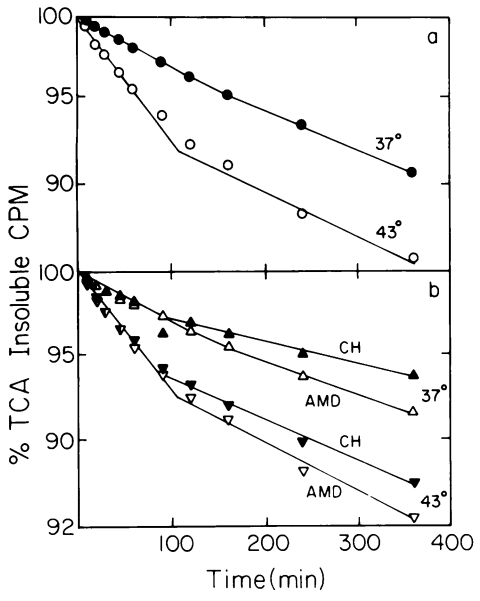

Fig. 2. Protein degradation in heat-shocked HTC cells: effect of cycloheximide and actinomycin D. HTC cells were incubated with [^{35}S]-methionine for 14.5 h at 37°C and chased for 2 h at 37°C with unlabelled methionine. Protein degradation was measured at 37°C or 43°C, as described in Materials and methods. (**a**) Protein degradation at 37°C (closed symbols) and at 43°C (open symbols). (**b**) Cycloheximide (2×10^{-4} M) (CH) or actinomycin D (2 μg/ml) (AMD) were added to the degradation system after the chase.

teins of higher mol. wt are also prominent (Figure 1, arrows). The pattern of heat shock proteins synthesized is similar to that described previously for rat cells by Welch *et al.* (1983).

Effect of heat shock on intracellular protein degradation

Figure 2a shows the effects of heat shock on the degradation of long-lived cellular proteins. These were labelled at 37°C for 15 h with [^{35}S]methionine followed by a 2-h chase to allow the breakdown of labile proteins. At 37°C these long-lived proteins break down at a slow exponential rate with a half-life of about 31 h. Following temperature step-up from 37 to 43°C protein degradation exhibits biphasic kinetics. Initially there is a burst of accelerated degradation (half-life of about 14 h) which decreases after about 2 h to a slower exponential rate (Figure 2a). Autoradiograms of SDS–polyacrylamide gels of cells labelled and chased as in Figure 2a did not reveal any striking changes in the rates of degradation of individual proteins after temperature step-up (not shown).

Effect of heat shock on intracellular levels of ubiquitin and ubiquitin conjugates

Figure 3A shows the effects of incubating cells at 43°C on the levels of free ubiquitin, uH2A (ubiquitinated histone 2A) and high mol. wt conjugates (M_r above 30 kd). Heat shock causes a transient fall in ubiquitin and uH2A levels which is accompanied by a corresponding increase in high mol. wt conjugate levels. Glover (1982) has previously reported a decrease of uH2A in heat-shocked *Drosophila* cells. Ubiquitin reaches a minimum level between 0.5 and 2 h after temperature step-up and returns to its original level between 2 and 4 h after step-up. uH2A levels decrease transiently similarly to ubiquitin levels (Figure 3A). Minimum levels of ubiquitin in cells incubated at 43°C ranged from 25 to 50% of its initial level in different experiments. The recent models which propose that heat shock protein synthesis is coupled to aberrant protein accumulation via the ubiquitin system (Finley *et al.*, 1984; Munro and Pelham, 1985) suggest that heat shock protein gene expression is activated by depletion of the free ubiquitin pool. The observed fall in ubiquitin levels seems to be rather small to act as a switch for heat shock protein synthesis, but more extensive measurements will be necessary to test the model. While this work was in progress Carlson and Rechsteiner (1985) reported that in HeLa cells a 45°C heat shock causes an increase of high mol. wt ubiquitin conjugates which is accompanied by a fall in uH2A and free ubiquitin.

Effect of inhibition of heat shock protein synthesis on protein degradation and ubiquitin conjugation

One possible explanation of the transient nature of accelerated protein degradation was that heat shock proteins protect cellular proteins against heat shock-induced degradation. We therefore examined the effects of inhibiting heat shock protein synthesis on intracellular proteolysis and ubiquitin conjugation. Actinomycin D (2 μg/ml) completely inhibits heat shock protein synthesis (not shown). Cycloheximide (2×10^{-4} M) inhibits cellular and heat shock protein synthesis (not shown).

Figure 2b shows that inhibition of heat shock protein synthesis with actinomycin D or cycloheximide does not markedly alter the biphasic kinetics of protein degradation after temperature step-up. Cycloheximide slightly inhibits the slow phase of degradation of long-lived proteins at 37 and 43°C but does not affect the rapid phase of degradation caused by heat shock.

In the presence of actinomycin D and cycloheximide, as in their absence, initial ubiquitin levels are restored after the burst of ubiquitin conjugation caused by temperature step-up (Figure 3B and C). We therefore conclude that the transient nature of heat shock-induced protein degradation and ubiquitin conjugation is not dependent on heat shock protein synthesis. An interesting observation is that actinomycin D prevents the return of uH2A to its initial level which is usually observed after prolonged incubation at 43°C (Figure 3C). In the presence of

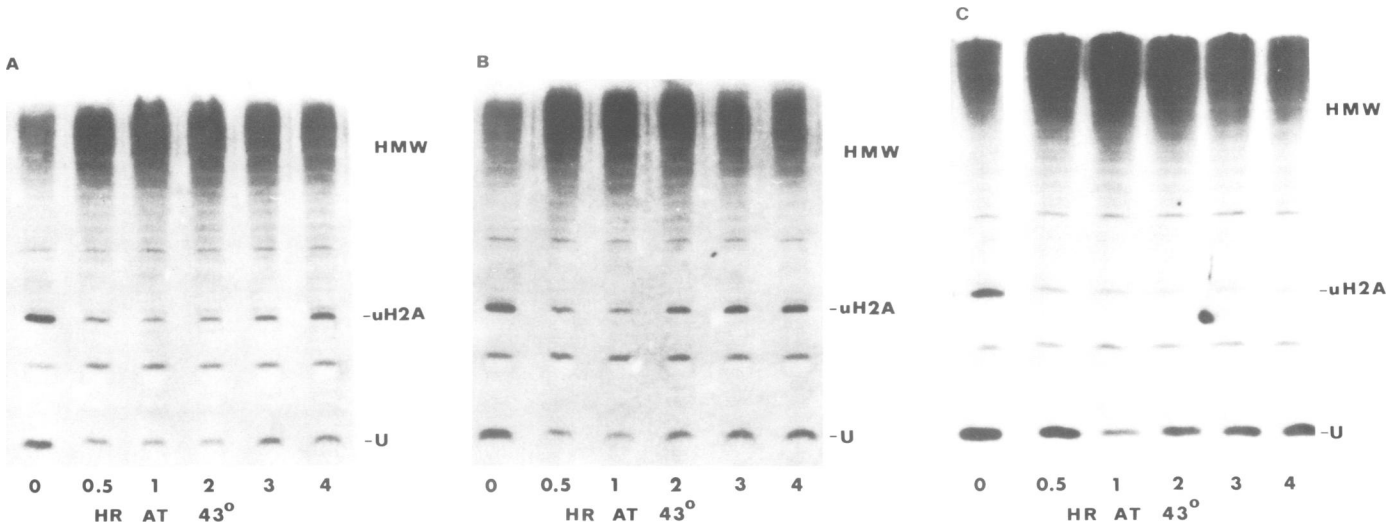

Fig. 3. Immunoblot of ubiquitin conjugates in heat-shocked cells: effect of cycloheximide and actinomycin D. HTC cells (10^6 cells/ml) were incubated at 37 or 43°C in growth medium (**A**); growth medium plus 2×10^{-4} M cycloheximide (**B**) or growth medium with 2 μg/ml actinomycin D (**C**). At the indicated times cells were removed, washed three times with tricine-buffered saline and resuspended in Laemmli (1970) sample buffer. After heating for 2 min in a boiling water bath the samples were subjected to electrophoresis. Conjugates in the gel were detected by immunoblotting with an anti-ubiquitin conjugate antibody as described in Materials and methods. (**A**) and (**B**) are from the same experiment and (**C**) is from a separate experiment.

cycloheximide (Figure 3B) the recovery of uH2A levels upon continued heat shock is the same as in the control (Figure 3A).

These experiments rule out a role of heat shock proteins in the biphasic response of the proteolytic system to temperature step-up. They do not, however, address the question whether the presence of heat shock proteins in cells at the time of temperature step-up prevents the denaturation and accelerated proteolysis of cellular proteins. When pre-heat shocked cells, which had accumulated heat shock proteins, were subjected to a second heat shock, protein degradation was accelerated to the same degree as during a first heat shock (not shown). Thus, the presence of heat shock proteins at the time of heat shock does not prevent the accelerated degradation of proteins.

Effect of a two-step temperature step-up on protein degradation

Another possible explanation of the biphasic protein degradation curve was that initially after temperature step-up the rate of proteolysis is elevated because of its temperature coefficient but eventually slows because of heat inactivation of the degradative system. We did the following 2-step temperature step-up experiments to test this possibility.

Figure 4a shows the effects on intracellular proteolysis of transferring cells from 37 to 41, 43 or 45°C. At 41 and 43°C similar biphasic degradation curves are obtained, except that a higher percentage of cellular proteins is degraded during the rapid phase at 43 than at 41°C. At both temperatures the rapid phase of degradation lasts about 2 h. The slow phase of degradation is similar at both temperatures. These kinetics are compatible with the hypothesis that the accelerated degradation is due to the selective breakdown of denatured proteins formed by temperature step-up and when this is complete degradation returns to a basal rate. After raising the temperature to 45°C, on the other hand, a short burst of rapid proteolysis lasting only about 30 min is followed by almost complete inhibition of intracellular proteolysis. These kinetics indicate that heat inactivation of the proteolytic system(s) occurs at 45°C.

In the experiment shown in Figure 4b cells were initially stepped up from 37 to 41°C. Two hours later, after completion of

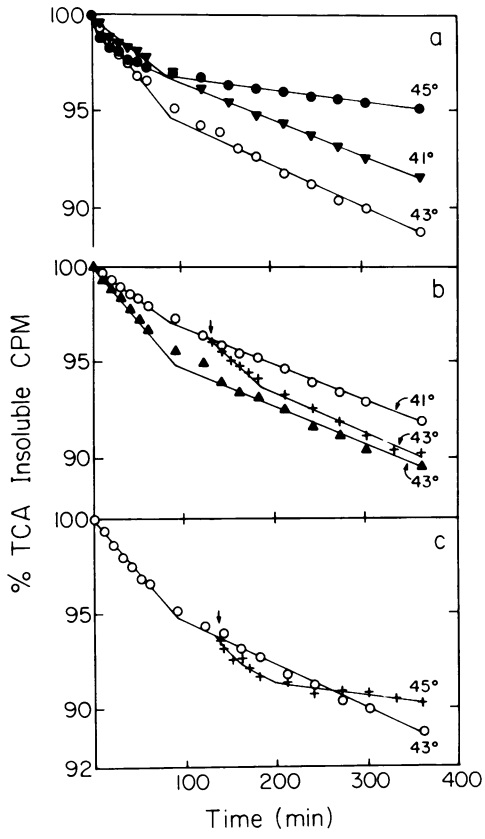

Fig. 4. Effect of two-step temperature jumps on protein degradation in HTC cells. HTC cells were incubated for 14 h with [^{35}S]methionine at 37°C and chased for 2 h at 37°C. After temperature step-up protein degradation was measured as described in Materials and methods. (**a**) Protein degradation at 41 (▼), 43 (○) and 45°C (●). (**b**) Protein degradation at 41 (○) and 43°C (▲). After 2 h some of the cells were transferred from 41 to 43°C (+). (**c**) Protein degradation at 43°C (○). After 2 h some of the cells were removed to 45°C (+). The results in (**a**) and (**c**) are from one experiment and those in (**b**) from another.

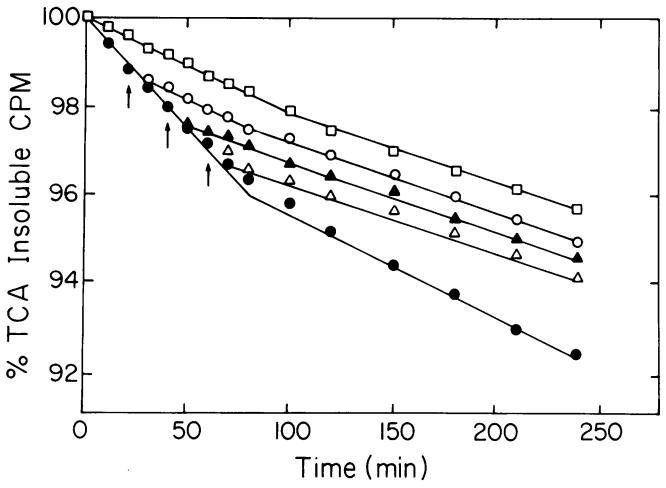

Fig. 5. Effect of pulses of heat shock on protein degradation in HTC cells. HTC cells were incubated for 13.5 h with [^{35}S]methionine at 37°C and chased for 2 h at 37°C with unlabelled methionine. Protein degradation was measured as described in Materials and methods at 37 (□) and at 43°C (●). Cells at 43°C were transferred to 37°C (arrows) after 20 (○), 40 (▲) or 60 min (△).

the rapid phase of degradation, they were exposed to a second temperature jump to 43°C. The second temperature jump causes an additional burst of protein degradation which is followed by a return to a slower rate of degradation. It is interesting that the same percentage of degradation of cellular proteins is obtained by a two-step jump to 43°C as by a single jump to 43°C (Figure 4b). This implies that the rapid phase of degradation is due to some intrinsic response of cellular proteins to a particular temperature. When cells are stepped up from 43 to 45°C (Figure 4c) there is a short burst of degradation which is followed by an inhibition of proteolysis, presumably due to the heat inactivation of the degradation system. However, both at 41 and 43°C the potential for additional accelerated protein degradation is retained after the initial burst of proteolysis is over. These experiments indicate that at temperatures of 43°C or less the biphasic nature of protein degradation after temperature step-up is not due to inactivation of the proteolytic system. At higher temperatures inactivation of proteolysis becomes significant. Inactivation of the proteolytic system can be detected already at 44°C (not shown).

Activation energies of protein degradation

Activation energies of the fast and slow phases of protein degradation were calculated from Arrhenius plots of data from the experiments in Figure 4 and similar experiments done at various temperatures ranging from 37 to 43°C. The activation energy of the rapid phase of degradation (up to 1.5 h after temperature step-up) is in the range of 26 ± 5 kcal/mol. The activation energy of the slow phase of degradation (between 3 and 6 h after temperature step-up) is much lower, in the range of 14 ± 5 kcal/mol. These data suggest that the fast and slow phases of degradation occur by different mechanisms. The activation energy of the fast phase of degradation is similar to that obtained by Hough and Rechsteiner (1984) for the degradation of ubiquitin conjugates by reticulocyte lysates (27 ± 5 kcal/mol).

Effect of pulses of heat shock on protein degradation

The finding that high mol. wt ubiquitin conjugates accumulate and ubiquitin levels fall shortly after temperature step-up (Figure 3) suggests that the proteolysis of conjugates is rate-limiting at

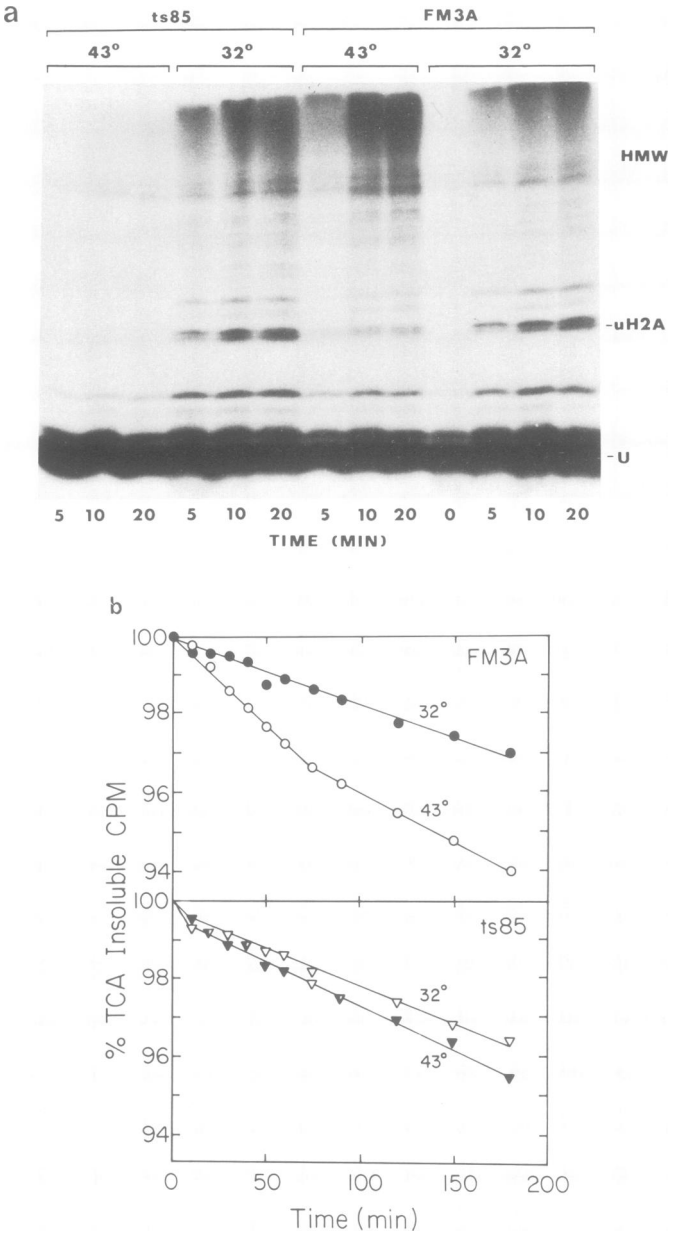

Fig. 6. Effect of heat shock on ubiquitin conjugation and protein degradation in FM3A wild-type and ts85 mutant cells. (**a**) The cells were permeabilized as described in Materials and methods and incubated at 32 or 43°C for 8 min. Then an ATP-generating system and [^{125}I]ubiquitin were added. U, ubiquitin; uH2A, ubiquitin conjugated to histone 2A; HMW, high mol. wt conjugates of ubiquitin with cellular proteins. (**b**) The cells were incubated for 20 h with [^{35}S]methionine at 32°C and chased for 3 h at 32°C. Protein degradation was measured at 32 or 43°C as described in Materials and methods.

this stage. The question arises whether denaturation of proteins occurs at the beginning of the phase of rapid proteolysis or whether denaturation occurs throughout this phase. In order to test this we exposed cells to a 43°C heat shock for 20, 40 and 60 min and examined the time course of proteolysis (Figure 5). In all cases rapid proteolysis continues for only 10 min after returning the cells to 37°C. This indicates either that denaturation of proteins is slow and continues through most of the phase of rapid proteolysis, or that returning the temperature to 37°C results in the rapid renaturation of most of the rapidly denatured proteins.

Is the ubiquitin system involved in heat shock-induced proteolysis?

To test if the ubiquitin system is involved in the degradation of heat-denatured proteins we used the ts85 mutant of the FM3A mouse mammary carcinoma cell line. This mutant has a temperature-sensitive ubiquitin-activating enzyme (E_1) and is defective in the degradation of amino acid analogue-substituted proteins at the non-permissive temperature (Finley *et al.*, 1984; Ciechanover *et al.*, 1984). Figure 6a shows that in permeabilized ts85 cells ubiquitin conjugation is abolished almost instantaneously at 43 °C whereas in wild-type FM3A mouse mammary carcinoma cells conjugation activity remains high at this temperature. Some of the effects of heat shock on whole cells (Figure 3) are reproduced in permeabilized cells. In permeabilized FM3A cells incubated at 43 °C the incorporation of ubiquitin into uH2A is inhibited and the incorporation of ubiquitin into high mol. wt conjugates is increased relative to control cells incubated at 32 °C (Figure 6a).

Figure 6b compares the effects of temperature step-up from 32 to 43 °C on intracellular proteolysis in wild-type FM3A cells and in ts85 cells. The wild-type cells, like HTC cells, respond to heat shock by a transient acceleration of protein degradation. In the ts85 mutant cells there is no burst of protein degradation upon temperature step-up, although the basal rate of proteolysis is preserved. These observations show that the ubiquitin system is involved in the accelerated proteolysis caused by heat shock. They also suggest that the basal degradation of stable proteins is not mediated by the ubiquitin system.

Discussion

The effect of heat shock on intracellular proteolysis has been the subject of much speculation (Finley *et al.*, 1984; Munro and Pelham, 1985; Ananthan *et al.*, 1986) but little experimental work has been done on this subject (Schlesinger, 1986). The data presented here show that exposure of mammalian cells to a moderate heat shock temperature (43 °C) transiently increases the degradation of endogenous proteins. A burst of ubiquitin conjugation with cellular proteins to form high mol. wt conjugates coincides with the burst of protein degradation following temperature step-up. This build-up of high mol. wt conjugates is accompanied by a transient fall in the levels of ubiquitin and uH2A. A possible explanation of the phemonema described in this paper is as follows. Heat shock causes the conformational change or denaturation of a subset of proteins stable at normal temperatures. These altered proteins are somehow recognized by the ubiquitin system and degraded by it. When the abnormal proteins formed shortly after temperature step-up have been broken down, intracellular proteolysis returns to a slower steady state rate. Points arising from this hypothesis will be discussed in more detail below.

Several possible reasons for the transient nature of heat shock-induced proteolysis were examined above. The involvement of heat shock proteins in the phenomenon seems to be ruled out since the biphasic kinetics of protein degradation persist when heat shock protein synthesis is inhibited (Figure 2b). Another possibility was that the acceleration of protein degradation after temperature step-up was the result of a high temperature coefficient of intracellular proteolysis (Hough and Rechsteiner, 1984) but was followed by heat inactivation of the proteolytic system. The latter explanation also seems unlikely since in sequential heat shock experiments (Figure 4) cells retain the potential for an additional burst of protein degradation when exposed to a second temperature step-up. Most likely the burst of protein degradation after temperature step-up stems from the intrinsic heat lability of some of the cellular proteins. With increasing temperature proteins undergo conformational transitions at specific transition temperatures which are sharp and characteristic of each protein domain (Privalov, 1982). Thus a jump from normal to heat shock temperature would be expected to cause some of the previously stable proteins to undergo conformational transitions. These altered proteins could then be selectively degraded by cellular scavenger systems.

Another feature of cellular protein denaturation implied by our results is that it occurs during a finite time period after temperature step-up, and not continuously as long as heat shock temperatures are maintained, as implied in the literature (Finley *et al.*, 1984; Munro and Pelham, 1985). The denaturation of pure proteins in solution is usually rapid (Cantor and Schimmel, 1980). However, since denaturation rates have usually been studied under non-physiological conditions (Cantor and Schimmel, 1980), it is difficult to extrapolate from these results to conditions prevailing in the cell. Since direct measurements of protein denaturation rates in whole cells were not feasible we tried to gain some insight into the time course of denaturation of cellular proteins from degradation measurements. The kinetics of protein degradation at 43 °C (Figures 2 and 4) imply that protein denaturation is complete by the end of the rapid phase of degradation, 2 h after temperature step-up. The failure of short pulses of heat shock to maintain accelerated protein degradation (Figure 5) rules out a rapid and irreversible denaturation of proteins, minutes after temperature step-up, followed by a slower degradation of the products. Rather, the experiment shows that the heat shock temperature must be maintained for continued accelerated protein degradation, indicating that either denaturation is slow in intact cells, or that renaturation upon restoring normal temperatures is rapid.

The finding that the ts85 mutant, which has a thermolabile ubiquitin-activating enzyme, does not respond to a 43 °C heat shock by transient acceleration of protein degradation, in contrast to wild-type FM3A cells (Figure 6), clearly implicates the ubiquitin system in the destruction of heat-denatured proteins. The burst of ubiquitin conjugation (Figure 3) coinciding with the burst of protein degradation (Figure 2) is consistent with such an hypothesis. It also implies that in this situation conjugate formation is more rapid than the proteolysis of conjugates. These findings supplement earlier evidence for the scavenger function of the ubiquitin system (Hershko *et al.*, 1982; Ciechanover *et al.*, 1984).

Other laboratories have reported that heat shock inhibits rather than accelerates the intracellular proteolysis of certain proteins. Munro and Pelham (1984) have found that heat shock inhibits the degradation of labile truncated proteins expressed from plasmids transfected into mammalian cells. These investigators have suggested that the inhibition may be due to the competition of heat-denatured cellular proteins with the single abnormal protein for free ubiquitin (Munro and Pelham, 1985). Thus, although the inhibition of degradation of individual proteins by heat shock apparently contradicts our findings, it could, in fact, be compatible with the view presented here that heat shock converts proteins which are not usually recognized by the ubiquitin system into good substrates of the system. It should, however, be kept in mind that the sharp temperature threshold of inactivation of the proteolytic system (Figure 4c) may play a role in heat shock inactivation of specific protein degradation.

An interesting implication of our findings is that the cell can somehow recognize and dispose of heat-denatured proteins. This

raises the question of the molecular basis for the selective degradation of some proteins in preference to others. Criteria for selective proteolysis by the ubiquitin system appear to include free *N*-terminal amino groups (Hershko *et al.*, 1984), the identity of the *N*-terminal amino acid residue (Bachmair *et al.*, 1986; Ferber and Ciechanover, 1986), oxidation of methionine residues (Hersko *et al.*, 1986), amino acid analogue substitution (Hershko *et al.*, 1982; Ciechanover *et al.*, 1984) and heat-denaturation (this paper). Earlier work in our laboratory showed that the degradation of some proteins micro-injected into HTC cells was accelerated if they were first denatured (Katznelson and Kulka, 1985). The denaturation of other proteins inhibited their degradation (Katznelson and Kulka, 1985). Since other evidence based on the lack of effect of methylation on the degradation of micro-injected proteins indicated that they were not degraded via the ubiquitin system (Katznelson and Kulka, 1983, 1985), the relationship of these findings to the present work is still not clear. Rogers *et al.* (1986) have recently presented evidence that proteins with regions rich in proline, glutamic acid, serine and threonine are particularly labile when micro-injected into mammalian cells. It is still uncertain whether or not these micro-injected proteins are degraded via the ubiquitin pathway. Much further work remains to be done to define the recognition codes for the selective degradation of intracellular proteins. An important consideration in determining such rules is the identification of the specific degradative pathway involved.

Materials and methods

Cells

Hepatoma tissue culture (HTC) cells clone GM22 were grown in suspension as described by Raboy *et al.* (1986).

The FM3A mouse mammary carcinoma cell line and its mutant ts85 (Mita *et al.*, 1980) were grown at 32°C in Dulbecco's modified Eagle medium supplemented with 10% newborn calf serum.

Heat shock protein synthesis

HTC cells were sedimented at 200 *g* for 5 min and resuspended at a density of 10^6 cells/ml in fresh growth medium. The cells were shaken gently in a rotatory water bath at 43°C. After various times samples of the cells were cooled rapidly to 4°C and washed twice in growth medium lacking methionine at a density of 5×10^5 cells/ml. The cells were suspended to 10^6 cells/ml in growth medium without methionine and [^{35}S]methionine (Amersham, 35 μCi/ml) was added. After 15 min at 37°C the cells were cooled to 4°C and washed twice at 5×10^5 cells/ml in tricine-buffered saline (160 mM NaCl and 20 mM tricine−NaOH, pH 7.4). The cells were suspended in Laemmli (1970) sample buffer, heated 2 min on a boiling water bath and subjected to electrophoresis in 18% SDS−polyacrylamide gels (Thomas and Kornberg, 1975). Radioactive bands were detected by autoradiography.

The incorporation of [^{35}S]methionine into total protein was measured by placing samples of cells onto Whatman 3MM paper discs, washing three times in 10% (w/v) trichloro-acetic acid, once in acetone, once in ether. The discs were counted in a scintillation counter with a mixture of 40% Lumax (Lumac) and 60% toluene.

Protein degradation

HTC cells. Cells were sedimented at 200 *g* for 5 min and resuspended in growth medium to a concentration of 4×10^5 cells/ml. Neomycin sulfate (50 μg/ml) and [^{35}S]methionine (6 μCi/ml) were added. The cells were incubated on a stirrer at 37°C for the time indicated (usually about 14 h). At the end of the incubation the cells were washed three times at 2.5×10^5 cells/ml at room temperature with growth medium supplemented with 1 mM methionine. The cells were resuspended in the same medium to 10^6 cells/ml and shaken gently in a rotatory water bath at 37°C for 2 h to allow the degradation of labile proteins. The cells were washed and suspended as before in growth medium containing 1 mM methionine and portions were transferred to baths at the appropriate temperatures. Measurements of protein degradation were started at this point, inhibitors being added as desired. Total protein c.p.m. at zero time were measured on samples removed to 3MM paper discs as described above. The cells were shaken in rotatory water baths at the indicated temperature. To measure the release of TCA-soluble c.p.m. samples were withdrawn to 10% (w/v) TCA, centrifuged and the radioactivity of the supernatant was measured. Degradation was calculated as the percentage of total zero time protein c.p.m. released as TCA-soluble c.p.m. Graphs show semi-logarithmic plots of percent TCA-insoluble counts remaining at each time point.

FM3A and ts85 cells. The cells were sedimented and resuspended to 5×10^5 cells/ml in growth medium. Neomycin sulfate (50 μg/ml) and [^{35}S]methionine (25 μCi/ml) were added and the cells were incubated for 20 h at 32°C in a CO_2 incubator. The cells were washed three times at 2.5×10^5 cells/ml and suspended to 10^6 cells/ml in growth medium supplemented with 1 mM methionine and 15 mM Hepes−NaOH (*N*-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer, pH 7.4. After a 3 h chase in a 32°C incubator, to allow the degradation of labile proteins, the cells were washed twice and suspended in growth medium supplemented with 1 mM methionine as before. Measurements of protein degradation were started at this point. Zero-time samples were withdrawn and the cells shaken gently at 32 or 43°C. Samples were removed and treated as described for HTC cells. Cell viability was monitored by mixing samples of cells with an equal volume of isotonic 0.4% (w/v) Trypan blue.

Immunoblotting

Ubiquitin and its conjugates were detected in whole cell extracts after SDS−polyacrylamide electrophoresis, by immunoblotting as described previously (Raboy *et al.*, 1986) with an antibody against ubiquitin conjugates generously provided by A.Ciechanover.

Cell permeabilization and incorporation of [^{125}I]ubiquitin

Cells were permeabilized by a modification (Raboy *et al.*, 1986) of the method of Schliwa *et al.* (1981) [^{125}I]Ubiquitin was prepared as described by Raboy *et al.* (1986) from ubiquitin generously given by A.Ciechanover and A.Hershko.

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