Microinjection of Ubiquitin: Changes in Protein Degradation in HeLa Cells Subjected to Heat-Shock

Noel Carlson,* Scott Rogers,* and Martin Rechsteiner*‡
Departments of *Biology and ‡Biochemistry, University of Utah, Salt Lake City, Utah 84112

Abstract. Ubiquitin was radiolabeled by reaction with 125I-Bolton-Hunter reagent and introduced into HeLa cells using erythrocyte-mediated microinjection. The injected cells were then incubated at 45°C for 5 min (reversible heat-shock) or for 30 min (lethal heat-shock). After either treatment, there were dramatic changes in the levels of ubiquitin conjugates. Under normal culture conditions, ~10% of the injected ubiquitin is linked to histones, 40% is found in conjugates with molecular weights greater than 25,000, and the rest is unconjugated. After heat-shock, the free ubiquitin pool and the level of histone-ubiquitin conjugates decreased rapidly, and high molecular weight conjugates predominated. Formation of large conjugates did not require protein synthesis; when analyzed by two-dimensional electrophoresis, the major conjugates did not co-migrate with heat-shock proteins before or after thermal stress.

Concomitant with the loss of free ubiquitin, the degradation of endogenous proteins, injected hemoglobin, BSA, and ubiquitin was reduced in heat-shocked HeLa cells. After reversible heat-shock, the decrease in proteolysis was small, and both the rate of proteolysis and the size of the free ubiquitin pool returned to control levels upon incubation at 37°C. In contrast, neither proteolysis nor free ubiquitin pools returned to control levels after lethal heat-shock. However, lethally heat-shocked cells degraded denatured hemoglobin more rapidly than native hemoglobin and ubiquitin-globin conjugates formed within them. Therefore, stabilization of proteins after heat-shock cannot be due to the loss of ubiquitin conjugation or inability to degrade proteins that form conjugates with ubiquitin.

AFTER heat treatment or exposure to various agents such as heavy metals, ethanol (2), or amino acid analogs (23), cells respond by increasing the synthesis of a small set of proteins called heat-shock proteins (HSPs) (for review see reference 13). This response is universal from bacteria to man (28, 39) and may confer upon the organism the ability to withstand a second exposure to heat (thermotolerance). Despite increasing interest in the heat-shock response, the functions of many HSPs are poorly understood.

Recently, ubiquitin was identified as an HSP in chicken embryo fibroblasts (6). Although ubiquitin is essential for ATP-dependent proteolysis in rabbit reticulocyte lysates (12, 21) and is a structural component of chromatin (7, 8), the relationship of these functions to the heat-shock response is not entirely clear. In the preceding paper (10), we studied the metabolism of injected ubiquitin in HeLa cells grown under normal culture conditions. Here, we describe changes that occur in the intracellular distribution, extent of incorporation into conjugates, and stability of injected ubiquitin in HeLa cells recovering from heat-shock. We also report on the rate of degradation of injected and endogenous proteins in heat-shocked cells.

Materials and Methods

Injection into Cultured Cells

Radioiodination of ubiquitin by the Bolton and Hunter procedure (5), cell culture, and red blood cell (RBC)-mediated microinjection were performed as described in the preceding paper (10).

Heat-Shock

8 or more hours after being injected with ubiquitin, HeLa cells were heat-shocked as follows. Cells plated at <50% confluency (1 × 10⁶ cells per 25-cm² culture flask) in 2.5 ml of Ham's F-12 medium were removed from 37°C incubators and immediately immersed in a 45°C water bath. The duration of heat-shock is considered to be the time between immersion in the water bath and return to 37°C.

Cell Viability after Heat-Shock

Viability was monitored by counting both surviving cells under phase-contrast and cell colonies present 1 wk after heat-shock. Cells shocked for 5 min resembled control cells in appearance, remained viable, and continued to grow. Cells that received a 30-min shock rounded up within 2 h, stopped growing, and eventually lysed; mortality was >50% by 48 h after

1. Abbreviations used in this paper: HMW, high molecular weight; HSP, heat-shock protein; RBC, red blood cell.

© The Rockefeller University Press, 0021-9525/87/03/547/9 $1.00
The Journal of Cell Biology, Volume 104, March 1987 547-555

547
Electrophoresis

Sample preparation and SDS PAGE analyses were performed as described in the previous paper (10). Two-dimensional PAGE was performed by the method of O'Farrell (34). Injected or pulse-labeled HeLa cells were removed from the monolayer with 0.1% trypsin in Ca**+/Mg**+-free saline, rinsed twice in PBS, dissolved in O'Farrell's lysis buffer, and immediately frozen at −80°C. After thawing, a 50-μl sample (~1 × 10⁶ cells) was focused for 7,200 volt-hours; the final pH gradient ranged from 4.6 to 7.3. First-dimension gels were expelled from the glass tubes, equilibrated in SDS sample buffer, and frozen at −80°C before analysis in the second dimension on 8.7% acrylamide gels. Molecular weights were calibrated as described (10).

Labeling of Endogenous Proteins with [³⁵S]Methionine

Fused or unfused HeLa cells were incubated for 20 min with methionine-free F-12 medium supplemented with 50 μCi/ml [³⁵S]methionine to label proteins for two-dimensional electrophoretic analysis. Cells that had been exposed to 45°C for 5 min (5-min heat-shock) were incubated at 37°C for 2 h before labeling. For degradation experiments, HeLa cell proteins were labeled before heat-shock by incubating cells for 18 h in F-12 medium supplemented with 1 μCi/ml [³⁵S]methionine (long pulse), or by incubating cells for 20 min in methionine-free F-12 medium supplemented to 50 μCi/ml of [³⁵S]methionine (short pulse). After the pulse, cells, were either heat-shocked as described or grown continuously at 37°C, then chased at 37°C in F-12 medium containing 20-fold the usual level of methionine. [³⁵S]Methionine was obtained from Amersham Corp. (Arlington Heights, IL).

Autoradiography

Autoradiography of thin sections was performed as described previously (30). The intracellular location of injected ubiquitin was determined by quantitation of the grain distribution over the nucleus and cytoplasm (see Table I). However, varying amounts of [³⁵S] were lost from the cells during fixation (40% loss from control cells, 20% from 5-min heat-shock cells, and 5% from 30-min heat-shocked cells). Free ubiquitin may be preferentially extracted since the amounts of radiolabel lost during fixation were proportional to the levels of free ubiquitin in the cell (see Fig. 1). Additionally, >90% of the radiolabel extracted from cells during fixation chromatographed on Sephadex G-100 with an apparent molecular weight of less than 20,000 (data not shown). Therefore, it is likely that autoradiography detects mainly ubiquitin conjugates.

Results

Ubiquitin Pools after Heat-Shock

In the previous paper (10), we arbitrarily assigned intracellular ubiquitin to one of three metabolic compartments: the free pool, histone conjugates, and high molecular weight (HMW) conjugates. As shown in Fig. 1 A, the partitioning of ubiquitin among these metabolic compartments changed dramatically in cells recovering from heat-shock. The proportion of ubiquitin in the free pool and in histone conjugates decreased, and nearly 90% of the labeled protein was converted to HMW conjugates. Similar results were obtained when ubiquitin was injected into HeLa cells that had been heat-shocked before fusion.

Since the synthesis of HSPs is induced after heat-shock (see reference 13), redistribution of ubiquitin might depend upon synthesis of one or more of the HSPs. To test this possibility, HeLa cells that had received an injection of ubiquitin were heat-shocked, and protein synthesis was immediately blocked with 100 μM cycloheximide. Although incorporation of [³⁵S]methionine was reduced by >98%, the redistribution of ubiquitin to HMW conjugates was unaffected (Fig. 1 B). Hence, the increase in HMW conjugates does not require the synthesis of new HSPs.

Relationship between Ubiquitin Conjugates and Heat-Shock Proteins

To assure that HSPs were actually synthesized after thermal stress and that RBC-mediated microinjection did not in some way affect the heat-shock response, HeLa cells were fused with RBCs, heat-shocked, and labeled with [³⁵S]methionine 2 h later. Analysis of newly synthesized proteins from control and 5-min heat-shocked cells by two-dimensional gel electrophoresis revealed several prominent proteins which were present only in the heated cells (Fig. 2). Their molecular weights and isoelectric points presented in the legend to Fig. 2 are similar to those reported for HSPs in HeLa cells (15, 41, 44). Identical results were obtained with unfused cells, indicating that fusion of RBCs to HeLa cells does not induce or inhibit the production of HSPs.

After heat-shock, some newly synthesized HSPs might accumulate as ubiquitin conjugates. To test this possibility, HeLa cells were injected with ubiquitin, and conjugates from control and heat-shocked cells were analyzed by two-dimensional gel electrophoresis. As shown in Fig. 3, prominent ubiquitin conjugates did not co-migrate with HSPs, nor did the pattern of discrete ubiquitin conjugates change after heat-shock.
distribution of ubiquitin after heat-shock. HeLa cells which had been injected with ubiquitin were prepared as in A except that just before heat-shock, cycloheximide was added to a final concentration of 100 μM. There were 20,000 cpm of ubiquitin in each lane; autoradiographic exposure was for 2 d.

Figure 2. Appearance of HSPs in HeLa cells after heat-shock. HeLa cells were fused with RBCs, then given a 20-min pulse of [35S]methionine (see Materials and Methods). Proteins extracted from these cells were then analyzed by two-dimensional PAGE and regions of the gel where HSPs appear are outlined. The proteins analyzed in Fig. 3 A were synthesized in cells grown continuously at 37°C. The proteins analyzed in Fig. 3 b were synthesized in cells subjected for 5 min to 45°C 2 h before addition of [35S]methionine. Each gel contained 10⁶ cpm of [35S]methionine; autoradiographic exposure was 7 d. The molecular weights and isoelectric points of the prominent HSPs are as follows: 200,000, pI 5.8; 107,000, pI 5.9; 76,000, pI 6.3; 73,000, pI 6.2; 41,000, pI 5.6; and 24,000, pI 6.4. On the gel shown above, actin migrated with an apparent molecular weight of 46,000 and an isoelectric point of 5.8; the corresponding published values are 43,000 and 5.4, respectively (17).
Rather, the additional HMW conjugates that form after heat treatment are predominantly found as “smears” in the molecular weight region above 150,000 consistent with the hypothesis that ubiquitin is forming conjugates with a variety of denatured proteins (see Fig. 3 B).

Since the HSPs listed in the legend to Fig. 2 are not ubiquitin conjugates, we can eliminate the possibility that prominent HSPs arise by conjugation of ubiquitin to pre-existing proteins. This does not imply that HSPs are incapable of forming conjugates with ubiquitin, merely that the major HSPs are not ubiquitinated. If ubiquitin–HSP conjugates are present, then they should appear exclusively in heat-shocked cells, and some conjugates enriched in stressed cells do correspond in size to mono-ubiquitinated HSPs. They are observed as minor species at 86,000 (pI 5.0), 47,000 (pI 6.4), 46,000 (pI 5.7), and 32,000 (pI 5.8–6.1). Of course, these ubiquitin conjugates may simply be intermediates in the degradation of HeLa proteins denatured by heat treatment.

Localization of Ubiquitin after Heat-Shock

Some HSPs accumulate in the nucleus (43, 45), and others associate with the cytoskeleton (29) after heat-shock. To determine whether the location of ubiquitin changed after heat-shock, the intracellular distribution of injected ubiquitin in control and heat-shocked cells was determined by two methods, autoradiography of sectioned cells and extraction of cells with buffers containing 0.5% Triton X-100. It is apparent from the results presented in Table I that ubiquitin redistributes to the Triton X-100-insoluble fraction in cells that have been heat-shocked. However, autoradiography of thin sections showed no major shift of ubiquitin to either the nucleus or cytosol after heat-shock.

Ubiquitin Stability in Heat-Shocked Cells

Under normal culture conditions, ubiquitin is degraded in HeLa cells with a half-life <20 h (10, 48); after heat-shock, its half-life increases substantially (Table II). In HeLa cells heat-shocked for 5 min and in those receiving a 30-min heat-shock, ubiquitin redistributes from the free pool and histone conjugates into HMW conjugates (Fig. 1). However, the fate of cells differs significantly after the two treatments. Cells heat-shocked for 5 min do not die, the degradation of ubiquitin is only transiently decreased, and the levels of free ubiquitin, histone conjugates, and HMW conjugates eventually return to control levels (Fig. 4). The recovery of a steady-state pool of free ubiquitin molecules is accompanied by reformation of histone conjugates, but the degradation of ubiquitin lags behind both events (see Fig. 4). Thus, degradation of ubiquitin does not appear to depend solely on the availability of free ubiquitin molecules, but may require reactivation of the system that degrades it. In contrast, after a 30-min heat-shock, HeLa cells never recover, and as late as 50 h after heat-shock, ubiquitin remains in HMW forms (Fig. 5). Ubiquitin is also greatly stabilized in these cells (see Table II).

Proteolysis after Heat-Shock

Under normal culture conditions an increase in proteolysis of abnormal substrates is accompanied by an increase in intracellular ubiquitin conjugates (11, 22). The levels of HMW ubiquitin conjugates are also increased after heat-shock; so one might expect an increase in proteolysis. However, pulse-chase experiments using [35S]methionine revealed a decrease in the rate of proteolysis of both short-lived and long-lived endogenous proteins after heat-shock (Fig. 6). Degradation of injected BSA, hemoglobin, and denatured hemoglobin was also reduced in heat-shocked cells (Table II and Fig. 7). However, proteolytic selectivity, per se, was not affected by heat treatment because stressed cells degraded phenylhydrazine-denatured hemoglobin faster than native hemoglobin (Fig. 7). Moreover, the data in Fig. 8 illustrate that labeled hemoglobin and labeled ubiquitin both form conjugates in...
control and heat-shocked cells. Since heat-shocked cells contain little free ubiquitin, formation of substantial amounts of ubiquitin–globe conjugates must have required mobilization of ubiquitin from pre-existing HMW forms.

Discussion

Ubiquitin Conjugates and Proteolysis after Heat-Shock

We have presented data on ubiquitin metabolism in HeLa cells recovering from heat-shock. The most apparent change after heat-shock is the reduction of free and histone-conjugated ubiquitin and conversion of almost all cellular ubiquitin to HMW conjugates (Fig. 1). These characteristic responses, combined with the ability of heat-shocked cells to form ubiquitin–globe conjugates (Fig. 8), indicate that the ubiquitin-conjugating pathway remains intact even after a lethal heat-shock. In addition to its altered distribution, ubiquitin was degraded at a markedly reduced rate in heat-shocked cells. Resistance to proteolysis is not unique to ubiquitin since endogenous proteins (Fig. 6), injected proteins (Table II), and other polypeptides (31, 46) are also stabilized in heat-shocked HeLa cells. Because reduced proteolysis cannot be attributed to impairment of the ubiquitin conjugation pathway, the activity of proteases that degrade conjugated proteins (26, 27) might be labile to heat treatment. However, denatured globe, a likely substrate for this sort of protease (11), is still degraded more rapidly than native hemoglobin in heat-shocked cells. Thus, it would seem that ubiquitin-dependent proteases retain some activity.

Heat-shock-induced redistribution of ubiquitin to the Triton X-100–insoluble fraction may also contribute to reduced rates of degradation. As shown previously, injected proteins that partition to the Triton-insoluble fraction of cells are, on the average, more stable than Triton-soluble proteins (38).
Figure 5. Ubiquitin pools after a 30-min heat-shock treatment. HeLa cells were injected with $^{25}$I-ubiquitin, heat-shocked for 30 min, and returned to 37°C. Proteins in cells harvested at 24 and 54 h after heat-shock were analyzed by SDS PAGE. Each lane contained 6,000 cpm of $^{25}$I-ubiquitin, and autoradiographic exposure was for 11 d.

Hence, it is noteworthy that the shift of ubiquitin conjugates to the Triton-insoluble fraction (Table I) coincides with the decrease in overall proteolysis.

Two other models, one based on competition of substrates for ubiquitination, the other based on competition for proteases, could account for the stabilization of proteins after heat-shock. Competition for limiting amounts of ubiquitin could reduce proteolysis if attachment of multiple ubiquitins were required to signal peptide bond cleavage (21). Even though there is an increase in the overall level of ubiquitin

Table II. Half-lives of Microinjected Proteins*

| Protein                      | Method of radio-iodination | Pretreatment of injected cells | Half-life of injected protein | Increase in half-life |
|------------------------------|-----------------------------|--------------------------------|------------------------------|-----------------------|
| Hemoglobin (oxy) BH/CT        | None                        | 5-min hs                       | 174.5                        | 1                     |
| Hemoglobin (met) BH/CT        | None                        | 5-min hs                       | 175.4                        | 1                     |
| Hemoglobin (apo) CT           | None                        | 5-min hs                       | 407.6                        | 2.3                   |
| Hemoglobin (apo) BH           | None                        | 30-min hs                      | 71.9                         | 2.3                   |
| Hemoglobin (apo) CT           | None                        | 5-min hs                       | 85.4                         | 1.2                   |
| Hemoglobin (apo) CT           | None                        | 30-min hs                      | 151.0                        | 2.1                   |
| Hemoglobin (apo) CT           | None                        | 5-min hs                       | 30.4                         | 1.1                   |
| Hemoglobin (apo) CT           | None                        | 30-min hs                      | 36.1                         | 1.3                   |
| Hemoglobin (apo) BH           | None                        | 5-min hs                       | 28.9                         | 1.2                   |
| Hemoglobin (apo) BH           | None                        | 30-min hs                      | 30.8                         | 1.3                   |
| BSA CT                       | None                        | 5-min hs                       | 26.5                         | 1.5                   |
| BSA CT                       | None                        | 30-min hs                      | 145.2                        | 8.4                   |

* The proteins listed were microinjected into HeLa cells, and their degradation was determined by the release of acid-soluble radiolabel into the medium. Half-lives were then calculated, and the stabilization in heat-shocked cells is expressed as the relative increase in half-life compared to control cells.

† BH, Bolton-Hunter; CT, chloramine-T.

§ hs, heat-shock.

Figure 6. Effect of heat-shock on proteolysis of endogenous HeLa proteins. HeLa cells were pulsed at 37°C with [35S]-methionine for 20 min or for 18 h. Proteolysis of endogenous proteins was then measured in cells which had not been heat-shocked (solid circle), or from cells which had been shocked for 5 (open triangle) or 30 (solid triangles) minutes and returned to 37°C. The graph shows the acid-soluble radioactivity released into the media from each set of cells (33).
conjugates after heat-shock, individual proteins might be insufficiently ubiquitinated to elicit proteolysis. In the second model, the accumulation of ubiquitin conjugates could saturate the protease and, by competition, stabilize proteins such as denatured globin. This proposal is consistent with both the accumulation of ubiquitin conjugates and the stabilization of some substrates. Still, if the ubiquitin pathway is responsible for most proteolysis before heat treatment, the hypothesis cannot account for the overall decrease in degradation of endogenous proteins (Fig. 6). To do so, one has to invoke the possibility of another proteolytic pathway(s) which is labile to heat. The existence of alternate pathways could explain why denatured hemoglobin is rapidly degraded after heat treatment, whereas other proteins, such as BSA and ubiquitin, are markedly stabilized, and the overall rate of proteolysis is reduced. Finally, heat treatment may affect proteolysis through changes in pH or the concentration of various ions. Reduced proteolysis or loss of μH2A (3) resulting from a decrease in nucleotide triphosphate pools is unlikely since ATP levels in Chinese hamster ovary cells are unaltered unless exposure to 45°C exceeds 45 min (9).

The Stress Response and Proteolysis

In 1980, Hightower suggested that HSPs are synthesized in response to the generation of abnormal proteins and that HSPs may be involved in the degradation of these newly formed substrates (23). This hypothesis has received in-

Figure 7. Effect of heat-shock on proteolysis of injected hemoglobin. HeLa cells were injected with 125I-hemoglobin, and the release of acid-soluble radiolabel into the media was measured for control cells (open squares), cells heat-shocked for 5 min (open circles), and cells heat-shocked for 30 min (solid squares). Some cells were also treated with phenylhydrazine to follow the degradation of denatured globin: control cells (solid circles), cells heat-shocked for 5 min (open triangles), and cells heat-shocked for 30 min (solid triangles).

Figure 8. Formation of ubiquitin–globin conjugates after heat-shock. HeLa cells were injected with 125I-hemoglobin or 125I-ubiquitin. Injected cells were either grown continuously at 37°C (control) or were heat-shocked for 5 or 30 min and returned to 37°C. After heat-shock, one flask from each group was treated with phenylhydrazine to denature the injected hemoglobin, and the increase in ubiquitin–globin conjugates was measured by SDS PAGE. Arrows mark bands that correspond in molecular weight to the addition of one, two, or four ubiquitin molecules to globin. There were ∼8,000 cpm of 125I-hemoglobin per lane in the gel on the left; autoradiographic exposure was for 14 d. Lanes in the gel on the right contained about 10,000 cpm of 125I-ubiquitin; exposure was for 3 d.
increasing support over the past 6 years. HSPs are synthesized in *Drosophila* cells producing mutant actins (25); HSP synthesis can be induced in oocytes after injection of denatured proteins (1); agents that stabilize proteins can inhibit certain inducers of stress response (24); and two known HSPs, *Escherichia coli* protease La and ubiquitin, are components of proteolytic pathways (6, 35). It remains to be determined whether other proteolytic pathway components are also HSPs. The small HSPs (20,000–30,000) are similar in size to E2's, the ubiquitin carrier proteins (36), and to the subunits of a large, neutral protease present in a number of tissues (14, 37, 47).

**Proteolysis and Heat-Shock Transcription Factors**

In *E. coli*, the synthesis of protease La (the lon gene product) (4, 20) and various other HSPs (49) are under the control of the heat–shock transcription factor, htpR. Goff and Goldberg (19) proposed that the usually short-lived htpR could be stabilized after heat–shock if heat-denatured proteins competed for the protease. Stabilization of htpR would then allow synthesis of protease La, as well as other HSPs. In eukaryotic cells similar schemes involving ubiquitination of an as-yet-undefined, heat–shock transcriptional activator have been proposed (16, 32). The hypothetical heat–shock transcription factor would be ubiquitinated under normal conditions, thereby either making it a substrate for ubiquitin-dependent proteolysis as suggested by Finley et al. (16) or inactivating it as suggested by Munro and Pelham (32). After heat–shock, this transcription factor would be stabilized by competition with other substrates for ubiquitin-dependent proteolysis (16) or activated by conversion to its non-ubiquitinated form (32). In either case, the extent of ubiquitination of a heat–shock transcription factor would determine whether HSP synthesis occurred. In fact, one could propose that ubiquitinated histones alone are sufficient to prevent transcription of heat–shock genes. Regulation of the stress response would then be sensitive to levels of abnormal proteins as suggested by Hightower (23) and could be reversed by increasing ubiquitin levels or by degrading the abnormal proteins, thereby restoring the free ubiquitin pool. Such models are consistent with the data presented in this study and can explain why ts85 cells after a shift to the nonpermissive temperature may result in decreased protein synthesis initiating factors in crude HeLa cell lysates by two-dimensional polyacrylamide gel electrophoresis. Hightower, L. E. 1980. Cultured animal cells exposed to amino acid ana-

We would like to thank LeRoy Kuehl, Kevin Rote, and Kent Redman for their helpful suggestions on the manuscript. The typing of Anne Kidd and the expert word processing of Maurine Vaughan are also much appreciated. This study was supported by grants GM27159 and GM30445 from the National Institutes of Health.

Received for publication 26 August 1986, and in revised form 6 November 1986.

**References**

1. Anaithan, J., A. L. Goldberg, and R. Voelmin. 1986. Abnormal proteins serve as eukaryotic stress signals and trigger the activation of heat shock genes. Science (Wash. DC). 232:522–524.

2. Ashburner, M., and J. J. Bonner. 1979. Induction of gene activity in *Drosophila* by heat shock (review). Cell. 17:241–254.

3. Atida, J., and R. G. Kulk. 1982. Formation of conjugates by 3H-labeled ubiquitin microinjection into cultured hepatoma cells. FEBS (Fed. Eur. Biochem. Soc.) Lett. 142:72–76.

4. Baker, T. A., A. D. Grossman, and C. A. Gross. 1984. A gene regulating the heat shock response in *Escherichia coli* also affects proteolysis. Proc. Natl. Acad. Sci. USA. 81:6779–6783.

5. Bolton, A. E., and W. M. Hunter. 1973. The labeling of proteins to high specific radioactivities by conjugation to a 3H-containing acylating agent. Biochem. J. 133:529–539.

6. Bond, U., and M. J. Schlesinger. 1985. Ubiquitin is a heat shock protein in chicken embryo fibroblasts. Mol. Cell. Biol. 5:949–956.

7. Busch, H. 1984. Ubiquitination of proteins. Methods Enzymol. 106:238–262.

8. Busch, H., and I. L. Goldknopf. 1981. Ubiquitin-protein conjugates. Mol. Cell. Biochem. 40:173–187.

9. Calderwood, S. K., E. A. Bump, M. A. Stevenson, J. V. Kersen, and G. M. Hahn. 1985. Investigation of adenylate energy charge, phosphorylation potential, and ATP concentration in cells stressed with starvation and heat. J. Cell. Physiol. 124:261–268.

10. Carlson, N., and M. Rechsteiner. 1987. Microinjection of ubiquitin: intracellular distribution and metabolism in HeLa cells maintained under normal physiological conditions. J. Cell Biol. 104:537–546.

11. Chin, D. T., L. Kuehl, and M. Rechsteiner. 1982. Conjugation of ubiquitin to denatured hemoglobin is proportional to the rate of hemoglobin degradation in HeLa cells. Proc. Natl. Acad. Sci. USA. 79:5897–5901.

12. Ciechanover, A., D. Finley, and A. Varshavsky. 1984. The ubiquitin-mediated proteolytic pathway and mechanisms of energy-dependent intracellular protein degradation. J. Cell. Biochem. 24:27–58.

13. Craig, E. A. 1985. The heat shock response. CRC Crit. Rev. Biochem. 18:239–280.

14. Dahlmann, B., L. Kuehn, M. Rutschmann, and H. Reinauer. 1985. Purification and characterization of a multicatalytic high-molecular-mass protease from rat skeletal muscle. Biochem. J. 228:161–170.

15. Duncan, R., and J. W. B. Hershey. 1983. Identification and quantitation of levels of protein synthesis initiating factors in crude HeLa cell lysates by two-dimensional polyacrylamide gel electrophoresis. J. Biol. Chem. 258:7228–7238.

16. Finley, D., A. Ciechanover, and A. Varshavsky. 1984. Thermolability of ubiquitin-activating enzyme from the mammalian cell cycle mutant ts85. Cell. 37:43–55.

17. Garrels, J., and W. Gibson. 1976. Identification and characterization of multiple forms of actin. Cell. 9:793–805.

18. Gerner, E. W., R. Boone, W. G. Conner, J. A. Hicks, and M. L. M. Boone. 1976. A transient thermotolerant survival response produced by single thermal doses in *HeLa* cells. Cancer Res. 36:1035–1040.

19. Goff, S. A., and A. L. Goldberg. 1985. Production of abnormal proteins in *E. coli* stimulates transcription of *lon* and other heat shock proteins. Cell. 37:587–595.

20. Goff, S. A., L. P. Casson, and A. L. Goldberg. 1984. Heat shock regulatory gene *Hpr* influences rates of protein degradation and expression of the ion gene in *Escherichia coli* Proc. Natl. Acad. Sci. USA. 81:6647–6651.

21. Herskso, A., and A. Ciechanover. 1982. Mechanisms of intracellular protein breakdown. Annu. Rev. Biochem. 51:335–364.

22. Herskso, A., E. Eynan, A. Ciechanover, and A. L. Haas. 1982. Immunochemical analysis of the turnover of ubiquitin-protein conjugates in intact cells. J. Biol. Chem. 257:13964–13970.

23. Hightower, L. E. 1980. Cultured animal cells exposed to amino acid analogs or puromycin rapidly synthesize several polypeptides. J. Cell Physiol. 102:407–427.

24. Hightower, L. E., P. T. Guidon, Jr., S. A. Whelan, and C. N. White. 1985. Changes in Eukaryotic Gene Expression in Response to Environmental Stress. G. B. Atkinson and D. B. Walden, editors. Academic Press, Inc., New York. 197–210.

25. Hiromi, Y., and Y. Hotta. 1985. Actin gene mutations in *Drosophila*; heat shock activation in the indirect flight muscles. EMBO (Eur. Mol. Biol. Organ.) J. 4:1681–1687.
26. Hough, R., and M. Rechsteiner. 1986. Ubiquitin-lysozyme conjugates: purification and susceptibility to proteolysis. *J. Biol. Chem.* 261:2391–2399.
27. Hough, R., G. Pratt, and M. Rechsteiner. 1986. Ubiquitin-lysozyme conjugates: identification of an ATP-dependent protease from reticulocytes. *J. Biol. Chem.* 261:2400–2408.
28. Kurtz, S., J. Rossi, L. Perko, and S. Lindquist. 1986. An ancient developmental induction: heat-shock proteins induced in sporulation and oogenesis. *Science (Wash. DC).* 231:1154–1157.
29. Leicht, B. B., H. Biessmann, K. B. Palter, and J. J. Bonner. 1986. Small heat shock proteins of *Drosophila* associate with the cytoskeleton. *Proc. Natl. Acad. Sci. USA.* 83:90–94.
30. McGarry, T., R. Hough, S. Rogers, and M. Rechsteiner. 1983. Intracellular distribution and degradation of immunoglobulin G and immunoglobulin G fragments injected into HeLa cells. *J. Cell Biol.* 96:338–346.
31. Munro, S., and H. Pelham. 1984. Use of peptide tagging to detect proteins expressed from cloned genes: deletion mapping functional domains from *Drosophila* hsp 70. *EMBO (Eur. Mol. Biol. Organ.) J.* 3:3087–3993.
32. Munro, S., and H. Pelham. 1985. What turns on heat shock genes? *Nature (Lond.)* 317:477–478.
33. Neff, N. T., L. Bourret, P. Miao, and J. F. Dice. 1981. Degradation of proteins microinjected into IMR-90 human diploid fibroblasts. *J. Cell Biol.* 91:184–194.
34. O'Farrell, P. H. 1975. High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* 250:4007–4021.
35. Phillips, T. T., R. VanBogelen, and F. Neidhardt. 1984. Lon gene product of *Escherichia coli* is a heat-shock protein. *J. Bacteriol.* 159:283–287.
36. Pickart, C. M., and I. A. Rose. 1985. Functional heterogeneity of ubiquitin carrier proteins. *J. Biol. Chem.* 260:1573–1581.
37. Ray, K., and H. Harris. 1985. Purification of neutral lens endopeptidase: close similarity to a neutral protease from pituitary. *Proc. Natl. Acad. Sci. USA.* 82:7545–7549.
38. Rogers, S., and M. C. Rechsteiner. 1985. Degradation rates and intracellular distributions of structurally characterized proteins injected into HeLa cells. *In Intracellular Protein Catabolism. E. A. Khairallah, J. S. Bond, and J. W. C. Bird, editors. Alan R. Liss, Inc., New York.* 405–416.
39. Schlessinger, M. J., M. Ashburner, and A. Tissieres, editors. 1982. Heat Shock: From Bacteria to Man. Cold Spring Harbor Laboratory, New York. 440 pp.
40. Siegelman, M., M. W. Bond, W. M. Gallatin, T. St. John, H. T. Smith, V. A. Fried, and I. L. Weissman. 1986. Cell surface molecule associated with lymphocyte homing is a ubiquitinated branched-chain glycoprotein. *Science (Wash. DC).* 231:823–829.
41. Slutter, A., A. C. B. Cato, G. M. Sillar, J. Kioussis, and R. H. Burdon. 1981. The pattern of protein synthesis induced by heat shock of HeLa cells. *Eur. J. Biochem.* 117:341–346.
42. St. John, T., W. M. Gallatin, M. Siegelman, H. T. Smith, V. A. Fried, and I. L. Weissman. 1986. Expression cloning of a lymphocyte homing receptor cDNA: ubiquitin is a reactive species. *Science (Wash. DC).* 231:845–850.
43. Tanguay, R. M. 1985. Intracellular localization and possible functions of heat shock proteins. *In Changes in Eukaryotic Gene Expression in Response to Environmental Stress. B. G. Atkinson and S. B. Walden, editors. Academic Press, Inc., New York.* 91–113.
44. Thomas, G. P., W. J. Welch, M. P. Mathews, and J. R. Feramisco. 1982. Molecular and cellular effects of heat-shock and related treatments of mammalian tissue-culture cells. *Cold Spring Harbor Symp. Quant. Biol.* 46:985–996.
45. Velazquez, J. M., and S. Lindquist. 1984. Hsp 70: nuclear concentration during environmental stress and cytoplasmic storage during recovery. *Cell.* 36:655–662.
46. Wheatly, D. N. 1985. Investigation of the mechanisms of protein turnover in HeLa S-3 cells by incubation at elevated temperatures. *Exp. Cell Res.* 157:157–171.
47. Wilk, S., and M. Orłowski. 1983. Evidence that pituitary cation-sensitive neutral endopeptidase is a multicatalytic protease complex. *J. Neurochem.* 40:842–849.
48. Wu, R. S., K. W. Kohn, and W. M. Bonner. 1981. Metabolism of ubiquitinated histones. *J. Biol. Chem.* 256:5916–5920.
49. Yamamuro, T., and T. Yura. 1982. Genetic control of heat-shock protein synthesis and its bearing on growth and thermal resistance in *Escherichia coli* K-12. *Proc. Natl. Acad. Sci. USA.* 79:860–864.

Carlson et al. *Ubiquitin Metabolism after Heat-Shock* 555