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Ibrahim PİRİM

Characterisation of a Polyclonal Antiserum Against Ubiquitin and its use in the Investigation of Ubiquitin Conjugate Distribution in Normal and Stressed 3T3-L1 Cells

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Abstract: Antiserum against ubiquitin was used to identify ubiquitin conjugates in 3T3-L1 cells, and to investigate changes in the amount and distribution of these conjugates in cells subjected to stress. Stress was induced by incubation with canavanine, an amino acid analogue, and by exposure of cells to temperatures of 43°C (heat shock).

SDS-PAGE, followed by immunoblotting, produced no observable change in ubiquitin conjugation with the canavanine as used in this protocol. Heat shock, in a variety of different exposure protocols, appeared to produce an increase in a wide spectrum of ubiquitin conjugates of molecular weight >70kd.

The conjugates were present in the Triton-extracted cell residues, which are nuclear-cytoskeletal-enriched preparations and this fact is discussed in the light of evidence for a site for protein sequestration prior to degradation which localises to a nuclear-intermediate filament-enriched cell fraction.

Indirect immunofluorescence was employed in an attempt to visualize changes in the distribution of ubiquitin in whole cells which had been exposed to stress. No changes were observed, and this is discussed in terms of the specificity of the antiserum for different forms of ubiquitin, and in the light of other work which was successfully used the antiserum to visualize ubiquitin associated with intracellular inclusions.

Experiments are proposed to investigate more fully the changes in ubiquitin conjugation and their likely meaning with respect to protein degradation. Modification of procedures for microscopy are proposed, in order to visualize changes in ubiquitin distribution in whole cells, and to assess whether any such changes have relevance to pathological processes involving ubiquitin.

Key Words: Ubiquitin, Heat Shock, Stress.

Ataturk University, Department of Biochemistry,
Faculty of Medicine, Erzurum-Turkey

Introduction

The exposure of all organisms, from bacteria to humans, to heat or stress, results in a rapid increase in the rate of synthesis of a group of proteins known as heat shock proteins (hsp), also called stress proteins (1, 2). Apart from heat (e.g. a rise in temperature to 44°C for a few minutes for mammalian cells), stressors include cold, heavy metals, amino acid analogues, anoxia, reactive oxygen species, ethanol, sulphhydryl reagents, virus infections, etc. This wide variety of stressors that stimulate the response are believed to share the common characteristics of either denaturing or damaging cellular proteins or causing cells to synthesize aberrant proteins. The role of the stress proteins is not clearly understood but it is thought that in some way they can protect cells against harmful traumas and preserve cell viability, enabling a rapid return to normal cellular activities once

the stress has ceased. Certainly a number of the hsp can act as molecular chaperons assisting in the folding or refolding of partially denatured proteins (3, 4). The major hsp's are classified into families based on molecular weight and sequence homology. They include the 8 kDa ubiquitin, 60-65 kDa, 70-78 kDa, 90 kDa and 100 kDa proteins (4-6).

The discovery that ubiquitin, 76 aa with 8.5 kDa MW polypeptide, is a heat shock protein suggests an important role for protein breakdown in the protection of cells from high temperatures and other types of stress (7, 8). In fact, after a thermal shift, ubiquitination increases markedly and the content of the free protein falls (9). Under these conditions, there is also an increased rate of degradation of normally stable cell proteins (10). A change in protein ubiquitination has recently been described in chronic neurodegenerative diseases, such as

Alzheimer's disease in which the neurofibrillary tangles have been found to be excessively ubiquitinated. Ubiquitin immunocytochemistry has also revealed unsuspected filamentous inclusions in another neurological disease. Spinal cord anterior horn neurons and motor neurons in the brain cortex can be seen to contain ubiquitinated filamentous inclusions in amyotrophic lateral sclerosis (motor neurone disease). These filamentous inclusions had not been detected before the advent of ubiquitin immunocytochemistry (15). In addition to these, several studies have revealed that ubiquitin concentrations in body fluids are increased in patients with various diseases: serum ubiquitin in chronic renal failure, in parasitic and allergic diseases. The origin and metabolism of ubiquitin in body fluid have not been clarified (16).

The aim of this study was to use a polyclonal antiserum against ubiquitin to visualize changes in ubiquitin distribution in 3T3-L1 cells which had been heat shocked or incubated with an amino acid analogue. In vitro cultured 3T3-L1 cells produced a heat shock response and also showed immunoreaction to ubiquitin which is the key protein in inclusions of many neurodegenerative diseases.

Materials and Methods

Ubiquitin antibody in all cases was bovine red blood cell ub (Sigma U-6253). 3T3-L1 cells (mouse preadipocytes) were grown in 90 mm culture dishes (Nunc) in Dulbecco's modified eagle's medium supplemented with 10% newborn calf serum at 37°C in a humidified 10% CO₂ atmosphere. Cells were fed approximately every 4 days, and passaged when confluent. The medium was aspirated from confluent plates, and the cells treated with trypsin/EDTA to remove them. The resulting suspension was added to 10 mls culture medium, and centrifuged for ten minutes at 1200 rpm. The supernatant was aspirated and the pellet resuspended in culture medium prior to plating out.

When treating cells with heat shock, the culture dishes were removed from the 37°C incubator and placed in an incubator at 43°C; 5 min was allowed for the temperatures to equilibrate. At the end of the period of heat shock, the culture dishes were returned to incubation at 37°C.

When treating cells with canavanine, the culture medium was aspirated, and replaced with culture medium which contained 0.4 mM canavanine, pre-warmed to 37°C.

Gel Electrophoresis and Western Blotting

The medium was aspirated and the cells washed twice

in PBS (phosphate buffered saline, 137 mM NaCl, 8 mM Na₂HPO₄, pH 7.4). The cell suspension was collected into Eppendorf tubes, spun for 1 minute at low speed (approx. 6000xg) in a microcentrifuge and the supernatant aspirated. The pellet was dissolved in 300 µl sample buffer (1.25M Tris-HCl pH6.8, 2-Mercaptoethanol, SDS, Bromophenol blue) and boiled for 3 minutes prior to loading onto the gel which was set up as gradients from 5-15% acrylamide, with a 2.5% stacking gel. Materials run on the gel was transferred onto a nitrocellulose membrane. The membrane was treated with the ubiquitin antibody to show the staining on the nitrocellulose.

Immunofluorescence and Microscopy

Both heat shock and canavanine treated 3T3-L1 cells were fixed on coverslips and blocked with blocking solution (3% bovine serum albumin in PBS) to hamper nonspecific binding sites for 1hr at room temperature (RT). 50µl anti-ubiquitin antibody (1:100 dilution in blocking solution) was pipetted onto each coverslip for 1hr at RT. The other coverslips where cells were not treated with heat shock or canavanine were left in the blocking solution as the control group. Cells were then washed with PBS and 50 µl fluoresceinated anti-rabbit globulin (FARG; 1:100 dilution in blocking solution) was pipetted as in the ubiquitin antibody procedure. The coverslips were then washed, dried in darkness and mounted. The cells were examined with a Zeiss microscope with a UV light attachment (Zeiss DRC). Phase contrast and UV light (with a fluorescence filter) were used in the imaging of cells.

Results

Immunoblots

Figure 1. shows a western blotting stained with anti-ubiquitin antibody. Lanes;

- 1: High molecular weight standards
- 2: Control cells
- 3-6: Canavanine treated cells
- 7-12: Heat shocked cells

The bands in lanes 7-12 (i.e heat shocked cells) appear denser in the region of 120-140kDa (apparent MW) than in either the control (lane 2) or canavanine treated cells (lanes 3-6). The smears in lanes 3-6 are denser in the region of 50-60kDa (apparent MW) than lanes 7-12. Lanes 7-12 exhibited strongly stained bands at MW~30kDa. All gels were 5-15% gradient gels and therefore the 8.5kDa ubiquitin is not at the region of

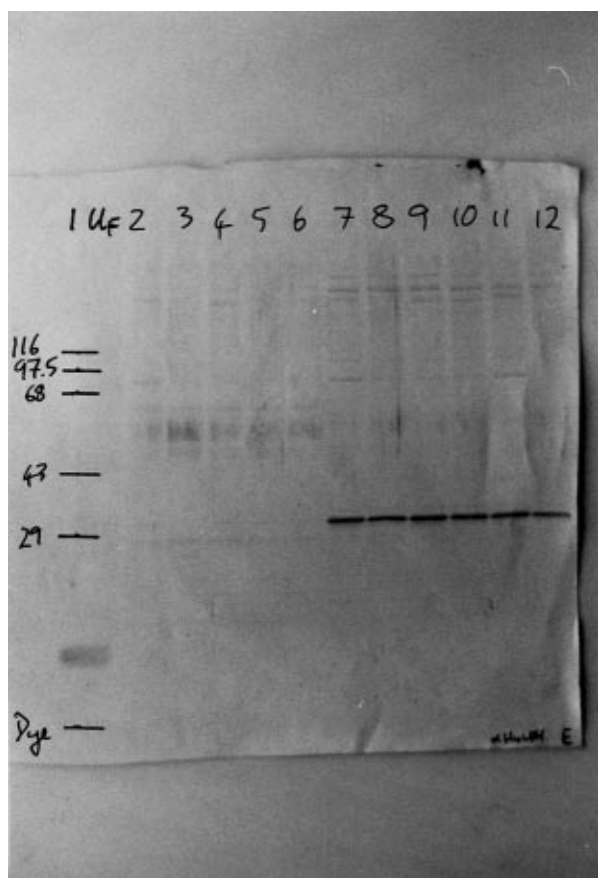


Figure 1. Blot analysis for ubiquitin content in the fraction of 3T3-L1 cells treated with heat shock at 43°C and canavanine. Lanes loaded with control cells (lane 2), canavanine treated cells (lanes 3-6) and heat shocked cells (lanes 7-12). Detailed information is given in the results section of the text.

8.5mw. Thus, due to problems of exact extrapolation, ubiquitin has an apparent mw of 30 kDa in the blot.

Microscopy

In all experiments, no first antibody (anti-Ub) controls exhibited a negligible signal under fluorescent light (results not shown), implying that results were due to binding of the first antibody. In all fluorescence photographs, the field of view was the same as the corresponding phase picture, allowing comparison with respect to cell outlines and nuclear position.

Figure 2. shows control and heat shocked cells stained with anti-ubiquitin antibody. A and C show phase views of control and heat shocked cells respectively; the cell outlines are marked by arrows. B and D show fluorescent views of these cells. In the control cells, filaments can be seen extending well into the periphery of the cytoplasm. The very strong fluorescent signal can be

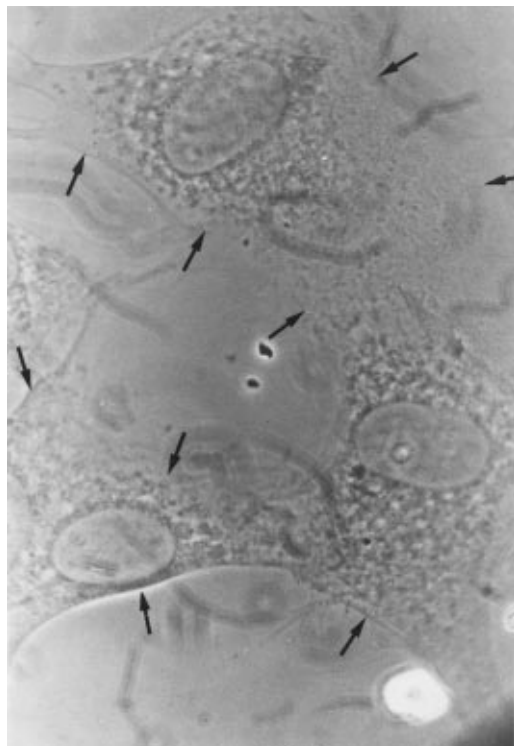
seen in the perinuclear region of the cell, often extending only part way around the nucleus. In the heat shocked cells, the filaments adopted a more perinuclear location, with only a few extending toward the periphery. In the phase photograph, the grainy cytoplasm appears more perinuclear than in the control cells. This is especially noticeable in the cell marked "A". This could be due to a redistribution of organelles on heat shock.

Figure 3. shows control and canavanine treated cells stained with anti-ubiquitin antibody. A and C show phase views of control and experimental cells respectively. B and D show the corresponding fluorescent views. The fluorescent view showed that unstressed cells display bright signals close to the nucleus and to the periphery of the cytoplasm. In the canavanine treated cells, the cytoplasm under the phase appears to display a contracted appearance with increasing phase density in the perinuclear region (marked D).

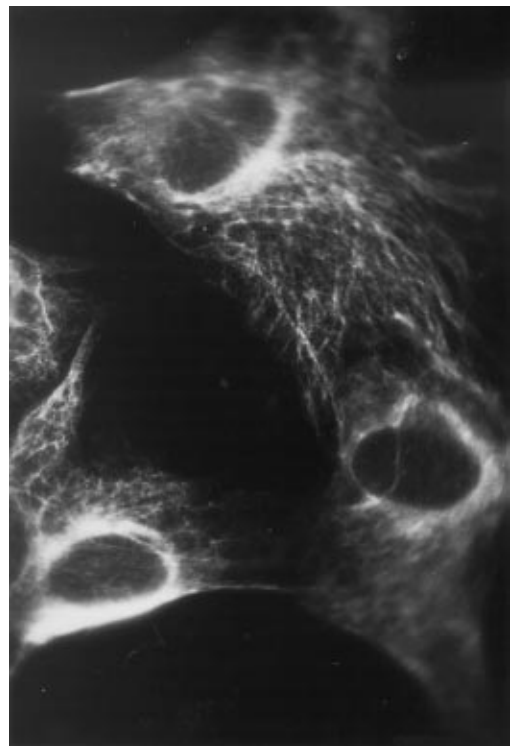
Discussion

Several papers have reported a characteristic pattern in SDS-PAGE experiments in which ubiquitin and its conjugates were visualised by either autoradiography using ^{125}I (11), or immunoblotting (12, 13). This pattern consists of smear staining in the high molecular weight region coupled with the variable presence of distinct bands in the low molecular weight region, some of which are usually attributed to histon H2A, a putative dimer of ubiquitin and free ubiquitin (14). The smear extends no further than 30 kDa, but the extent of the smear appears to vary according to the cell type studied. The smear appearance in high molecular weight staining is not due to a poor resolution of the gel, as other bands are resolved sharply and the pattern is reproducible under different PAGE conditions. It may, rather, imply heterogeneity in the conjugates of ubiquitin to high molecular weight proteins. Studies in which cell extracts are treated with isopeptidase to remove ubiquitin from conjugates would be useful as a comparison, as it would be expected that such treatment would markedly decrease any staining due to the presence of C-terminal ubiquitin conjugates. In figure 1, it appears that heat shock causes an increase in the number of high molecular weight conjugates to ubiquitin.

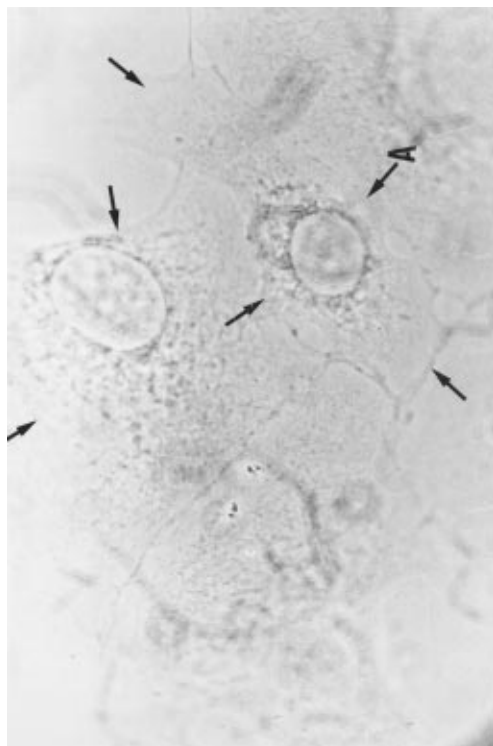
In canavanine treated cells, the staining of 50-60 kDa and in heat shock treated cells, the staining of 120-140 kDa proteins might show that the effect of the heat is different from the effect of the amino acid analog. Moreover, the appearance of probably free ubiquitin in only heat shocked cells is not clear. It is, however, known



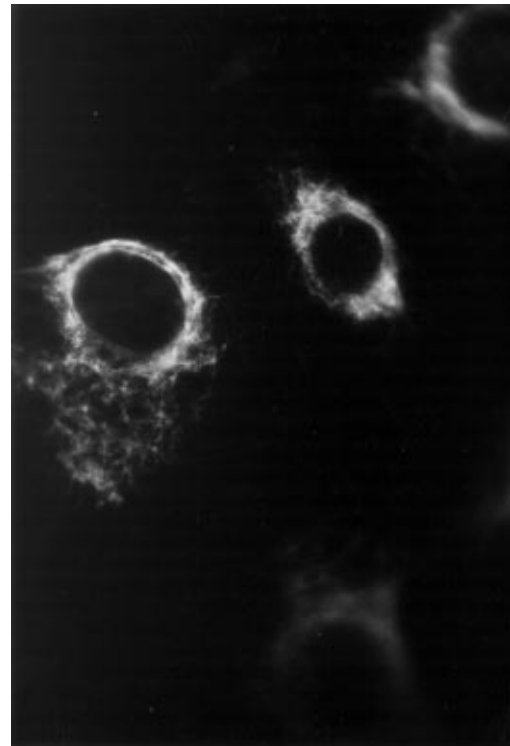
2A



2B

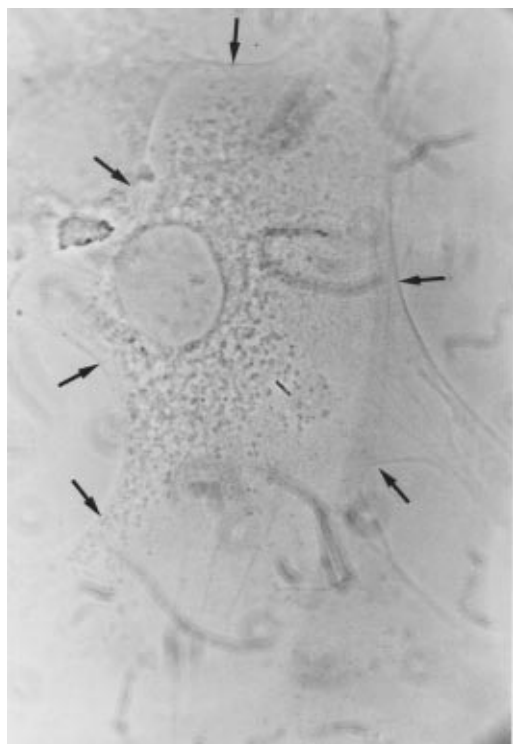


2C

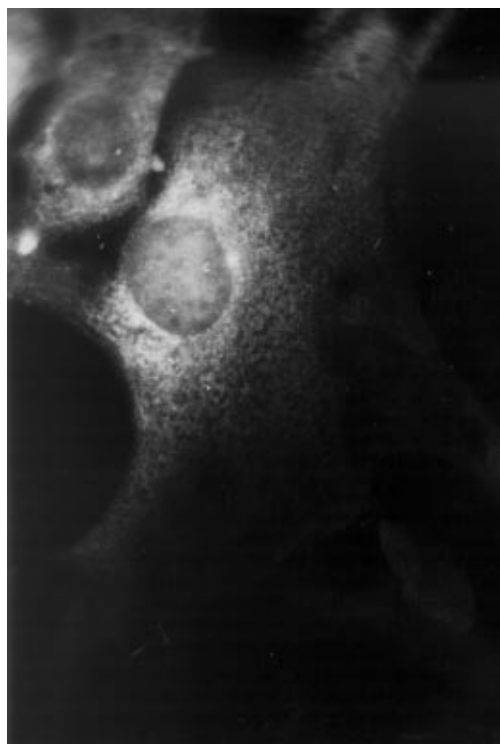


2D

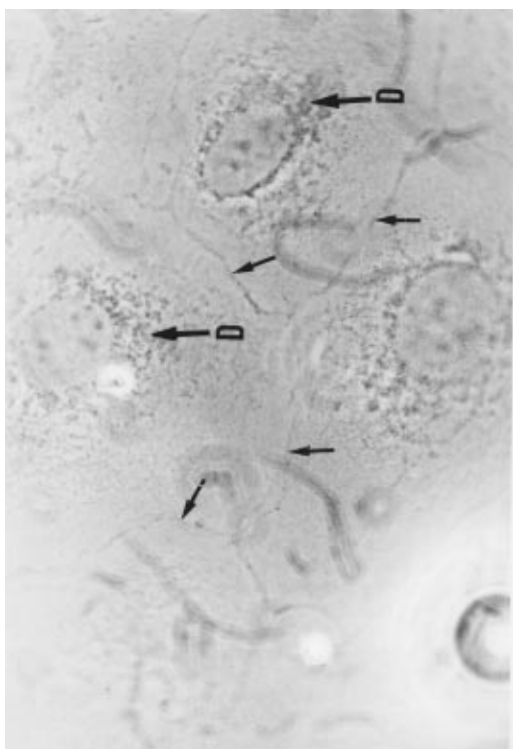
Figure 2. The effect of heat shocked on 3T3-L1 cells. All cells were stained with anti-ubiquitin antibody. A and C, phase views of control and heat shocked cells respectively. B and D, fluorescent views of these cells.



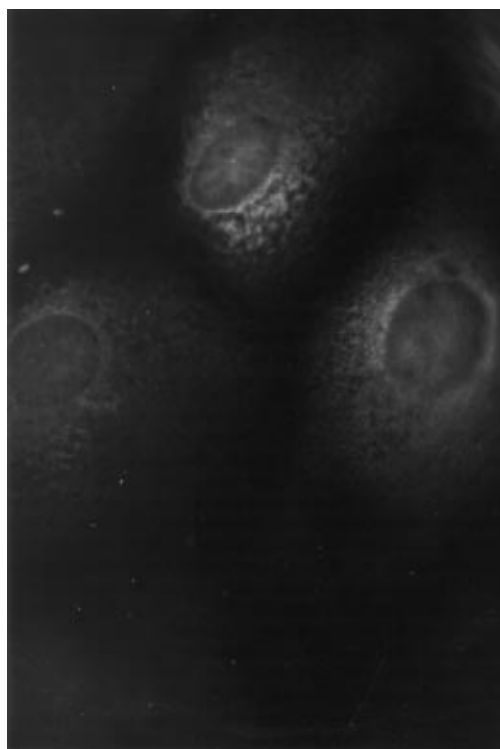
3A



3B



3C



3D

Figure 3. The effect of amino acid analogue (canavanine) on 3T3-L1 cells. All cells were stained with anti-ubiquitin antibody. A and C, phase views of control and canavanine treated cells respectively. B and D, fluorescent views of these cells.

that any stress to the cells causes induction of ubiquitin production (9, 10). Increased staining in heat shock treatment could therefore be due to either conjugates entering this fraction for the first time, or equally may be due to an increase in ubiquitination of proteins already present in this fraction. Besides heat shock, it has been shown that sendai virus HN and F proteins are translocated from the plasma membrane to the perinuclear region concurrently with intermediate filament collapse in 3T3-L1 cells by showing similar effects of heat shock with which there is intermediate collapse (12).

There is no apparent difference in the canavanine treated cells (in fig. 1), despite incubation with amino acid analogues being one stimulus for the stress response and despite ubiquitin mediated degradation being cited as important in the removal of the abnormal proteins thus produced (10).

A variety of explanations may be proposed to account for this: eg 3T3-L1 cells may react differently to other cells with respect to their stress response to amino acid analogues; the ubiquitination of proteins in heat shock has a different function from that in the removal of 'mutant' proteins. However, it is also possible that the conditions of the incubation did not allow time for a sufficiently large amount of abnormal protein to build up, especially as canavanine was only present in equimolar concentration with arginine, and so further experiments are proposed to investigate different conditions of analogue-incubation and their effect on ubiquitin conjugation, before other inferences are made.

Microscopy Results

The picture of intermediate filaments (IF) collapse seen in figure 2 is a typical finding in heat shock. No pictures were alike for cells treated with canavanine, which would have provided information on the homology between these two methods of inducing stress in cells.

The phase pictures reveal a cytoplasmic contraction on

heat shock which is possibly caused by the IF collapse. The contraction does not appear to result in shrinkage of the cell outline, but seems to result in phase dense elements of the cytoplasm (probably organelles) moving into the perinuclear region.

Figures 2 and 3 imply that only solid structures i.e. organelles relocate in heat shock. Ubiquitin distribution on heat shock may therefore be attributable to it being associated with solid structures within the cells. The ubiquitin antibody we used recognizes both free and conjugated ubiquitins. Any ubiquitin will therefore be shown, if it is present in a high enough concentration to overcome this low reactivity and produce a sufficiently strong signal. The least reactive species will be free, native ubiquitin and in a large proportion of the cell, ubiquitin will probably be in this form, hence staining may be poor. Any increase in conjugated ubiquitin on heat shock might be detected, as reactivity of the antisera should be higher against conjugates, but the results obtained have not clearly shown this. It may well be that the lack of denaturation reduces the signal so much as to make the method insensitive to the levels of change in the conjugates caused by the treatments employed here. Carlson et al. also failed to detect redistribution of ubiquitin on heat shock, using autoradiograms of cells loaded with radio-labeled ubiquitin (9, 11).

Anti-ubiquitin antibody has been used to identify ubiquitin in inclusions in cells both in human pathology specimens (13, 14) and in 3T3-L1 cells (12). In both cases, it is likely that the ubiquitin is present in sufficiently high amounts in the inclusions to be shown by the antiserum.

Acknowledgments

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