FORMATION OF CONJUGATES BY ^{125}I-LABELLED UBIQUITIN MICROINJECTED INTO CULTURED HEPATOMA CELLS

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1. Introduction

Ubiquitin is a polypeptide of 8500 M_r, with a sequence that has been highly conserved during evolution [1–3]. It is widely distributed in animals, plants, yeasts and bacteria [1,2]. In animal cells ubiquitin has been found both in free form [1,4] or covalently bound by its carboxyl terminal to other proteins [5–8]. In the chromatin of interphase cells, ~10% of histone 2A and ~1% of histone 2B are covalently linked to ubiquitin [8]. Since the H2A-ubiquitin conjugate, protein A24, is absent from metaphase chromosomes, it has been suggested that ubiquitin plays a role in the cell cycle [9,10]. Amounts of ubiquitin linked to H2A decrease in nucleoli undergoing hypertrophy [11], in 'active' chromatin [12] and during chicken erythrocyte maturation [13]. Ubiquitin may be involved in intracellular protein degradation in reticulocytes [7,14–16]. Conjugates of ubiquitin with other proteins could act as substrates of intracellular proteolytic systems [6,7].

The wide distribution of ubiquitin, its highly conserved sequence and the variety of conjugates which it forms with other proteins indicate its importance. However, its roles in the cell are still not clear. One way of gaining more information about its functions is to introduce labeled ubiquitin into whole cells and to observe its behavior. Here, we have examined the fate of ^{125}I-labelled ubiquitin introduced into cultured hepatoma cells by erythrocyte ghost-mediated microinjection [17]. Autoradiograms show that ubiquitin is rapidly conjugated to other cellular proteins.

Abbreviations: HTC cells, hepatoma tissue culture cells; PMSF, phenylmethyl-sulfonyl fluoride; SDS, sodium dodecyl sulfate; TLCK, *Nα-p*-tosyl-L-lysine-chloromethyl ketone; TPCK, L-1-tosylamide-2-phenylethyl-chloromethyl ketone

2. Materials and methods

2.1. *Cells and media*

HTC cells clone GM22 were grown in suspension in modified S-77 medium plus 10% newborn calf serum as in [18]. 'Solution Na^+' contained 160 mM NaCl and 20 mM Tricine–NaOH (pH 7.4). PBS was phosphate-buffered saline [19] without Ca^{2+}.

2.2. *Iodination*

This was done essentially as in [6]. To 500 μg pure ubiquitin dissolved in 20 μl 0.5 M phosphate buffer (pH 7.5), were added 10 μl of a solution containing 1 mCi carrier-free Na^{125}I. Chloramine T (10 μl, 2 mg/ml) were added, and the sample was mixed vigorously at room temperature for 60 s. The reaction was terminated by adding 10 μl sodium metabisulfite (2 mg/ml). Free Na^{125}I was removed by centrifugation through a column of Sephadex G-25 pre-treated with 100 μg bovine serum albumin [20]. The preparations contained ~20% of trichloroacetic acid-soluble ^{125}I.

2.3. *Loading of erythrocyte ghosts*

Human erythrocyte ghosts were loaded with ubiquitin essentially as in [21]. Briefly, washed erythrocytes, from blood collected <1 week before use, were swollen by suspending in PBS diluted with 0.7 vol. water. To 75 μl of swollen erythrocytes were added 100 μg bovine serum albumin and ~250 μg ^{125}I-labelled ubiquitin in 100 μl water. After mixing in a vortex mixer for 15 s, the lysate was kept for 5 min at 0°C. Then, 6.5 μl solution containing 2.2 M KCl and 10 mM $MgCl_2$ was added, and the mixture was incubated at 37°C for 30 min to reseal the ghosts. At this point the resealed ghosts could be stored overnight at 4°C. The ghosts were washed 3 times with 'solution Na^+' and suspended in the same solution.

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2.4. *Fusion of loaded ghosts with HTC cells and incubation*

The procedure was essentially as in [17]. The fusion system contained 1×10^6 HTC cells/ml, 2×10^7 ghosts/ml, 0.15 mM La^{3+} and Sendai virus (10–40 μg protein/ml). After addition of the virus, the mixture was kept for 5 min at 0°C and then incubated for 30 min at 37°C with gentle shaking. The cells were washed twice at $180 \times g$ for 5 min with S-77 growth medium to remove ghosts, and resuspended in the same medium. The cells were incubated either in suspension with shaking or in monolayer in 60 mm Petri dishes. At the end of the incubation, samples containing ~10^6 cells were washed with 2.0 ml 'solution Na^+' and resuspended in 2.0 ml of 'solution Na^+' containing 0.25 mM *N*-ethylmaleimide, 0.1 mM PMSF, 0.1 mM TLCK, 0.1 mM TPCK, and 0.25 mM EDTA. Trichloroacetic acid was added to a final concentration of 10% (w/v). After 30 min at 0°C, the trichloroacetic-insoluble material was collected by centrifugation and its radioactivity was measured in a γ counter.

2.5 *Electrophoresis and autoradiography*

SDS–Polyacrylamide gel electrophoresis was done as in [22]. Slab gels of 10×14 cm were used, and the concentrations of acrylamide are noted in the legends. To prepare the samples, the trichloroacetic acid-soluble material was dissolved in sample buffer [22] and neutralized with NaOH. The gels were stained with Coomassie blue for 40 min and destained with methanol/acetic acid/water (250:75:675, by vol.) for 60 min, dried and autoradiographed with Agfa-Gevaert Curex RP2 X-ray film, using Agfa-Gevaert MR 400 intensifying screens.

2.6. *Preparation of nuclear and cytoplasmic fractions*

Cells (5×10^6) were washed with 10 ml 'solution Na^+' and suspended in 2 ml homogenizing buffer, consisting of 20 mM Tricine–NaOH buffer (pH 7.4), 2 mM $CaCl_2$, 1 mM $MgCl_2$, and containing 1.25 mM *N*-ethylmaleimide, 0.5 mM PMSF, 0.5 mM TLCK and 0.5 mM TPCK. The swollen cells were homogenized in a Dounce homogenizer. All operations were performed at 0–4°C. Nuclei were sedimented by centrifuging for 5 min at $1200 \times g$. The supernatant containing the cytoplasmic fraction was centrifuged again at $1200 \times g$. The nuclear pellet was washed with 2.0 ml homogenizing buffer.

2.7. *Materials*

Pure ubiquitin was the generous gift of Professor A. Hershko (Faculty of Medicine, Technion-Israel Institute of Technology). $Na^{125}I$ was from The Nuclear Research Center (Negev). Other fine chemicals were from Sigma (St Louis).

3. Results

^{125}I-Labelled ubiquitin microinjected into HTC cells forms high-M_r conjugates with cellular proteins. Fig.1 shows the electrophoretic patterns of radioactive cellular proteins from HTC cells microinjected with ^{125}I-labelled ubiquitin by Sendai virus-induced fusion with loaded erythrocyte ghosts. The following pattern

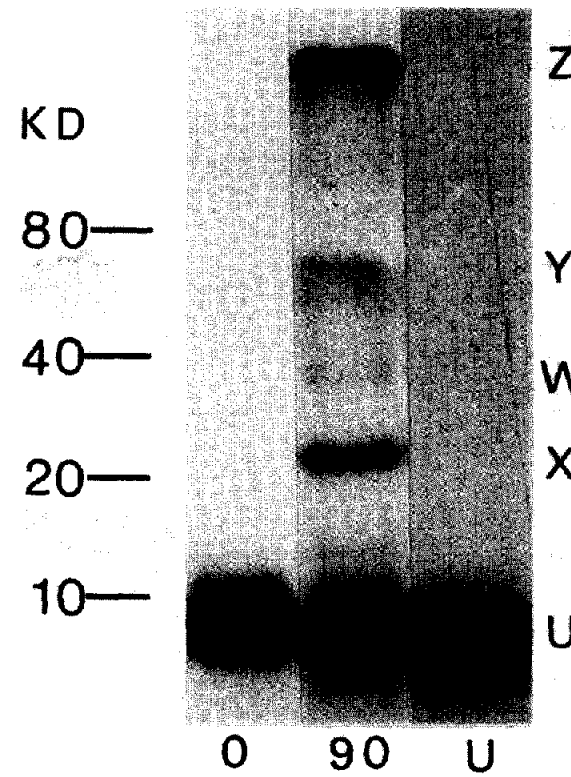

Fig.1. Electrophoretic pattern of radioactive bands from HTC cells microinjected with ^{125}I-labelled ubiquitin. Cellular proteins were subjected to electrophoresis on 12.5% (w/v) polyacrylamide gels. The M_r markers were bovine serum albumin (67 000), ovalbumin (44 000), β-lactoglobulin (17 500), lysozyme (14 000); lane 0, zero time sample taken immediately after termination of fusion; 90, cells incubated for 90 min after termination of fusion; U, ^{125}I-labelled ubiquitin.

of radioactive bands, listed in order of increasing M_r, is observed with a high degree of reproducibility: free ubiquitin (8500 M_r), a strong band, X (21 000–26 000 M_r), one or more weak bands, W (31 000–35 000 M_r), a group of bands Y, with a major one at 60 000–67 000 M_r, and a second group of bands Z, near the origin of the gel. With the exception of free ubiquitin, these bands were absent from controls (not shown). The controls included ^{125}I-labelled ubiquitin-loaded erythrocyte ghosts incubated with HTC cells without virus and unloaded erythrocyte ghosts fused with HTC cells in the presence of external ^{125}I-labelled ubiquitin.

The formation of ubiquitin conjugates is extremely rapid. The intensity of the new bands detected on autoradiograms usually reached a maximum within 30 min incubation after the termination of the Sendai virus-induced fusion (fig.2). In many experiments the bands were formed already during the fusion procedure itself.

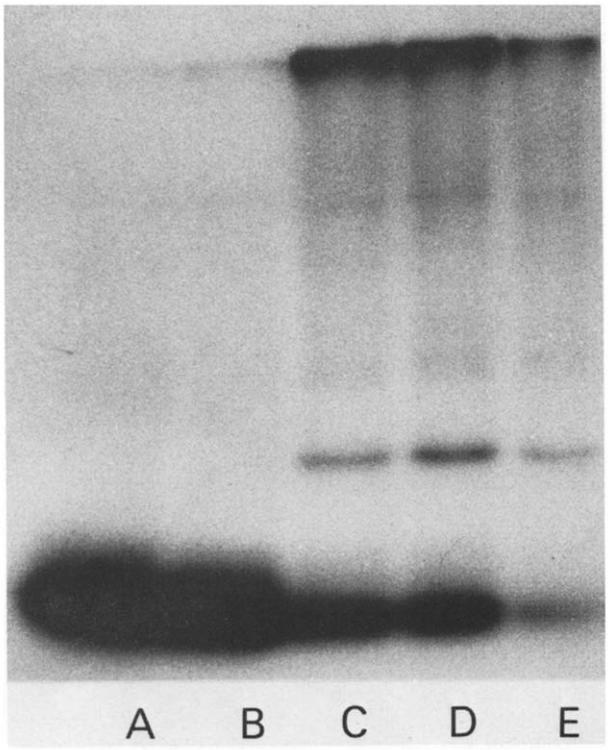

Fig.2. Kinetics of formation of conjugates by microinjected ^{125}I-labelled ubiquitin: (A) Zero time fusion; (B) end of fusion, zero time incubation; (C) 30 min incubation; (D) 60 min incubation; (E) 90 min incubation; (C,D) incubated in suspension; (E) incubated in monolayer. Gels contained 11.5% (w/v) polyacrylamide.

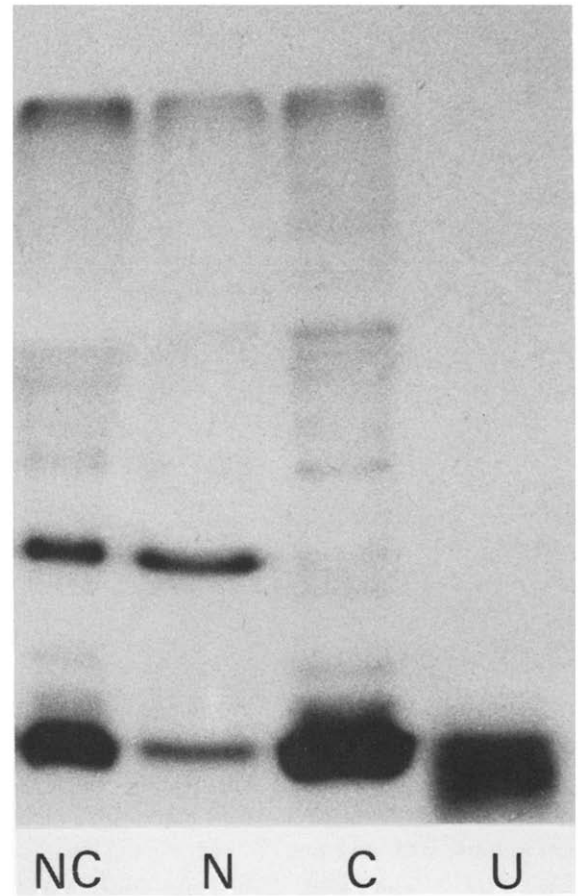

Fig.3. Pattern of bands of ^{125}I-labelled ubiquitin conjugates in nuclear and cytoplasmic fractions. Gels contained 12.5% (w/v) polyacrylamide; (NC) whole cell extracts; (N) nuclear fraction; (C) cytoplasmic fraction; (U) ubiquitin.

Fig.3 compares the autoradiographic pattern of whole cell extract with those of nuclear and cytoplasmic fractions. Band X was predominantly, if not exclusively, present in the nuclear fraction. All the other bands were found predominantly in the cytoplasmic fraction, although not entirely absent from the nuclear fraction. Since the nuclear fraction was to some extent contaminated with unbroken cells and cytoplasmic tabs, it is still uncertain whether the faint bands represent nuclear material or are cytoplasmic contaminants.

Fig.4 shows the effects of ATP-depletion on the formation of ubiquitin conjugates. In ATP-depleted cells (fig.4B,B′,D,D′) the intensity of band X was greatly reduced relative to the controls (fig.4A,A′). The intensity of several bands in the region W was

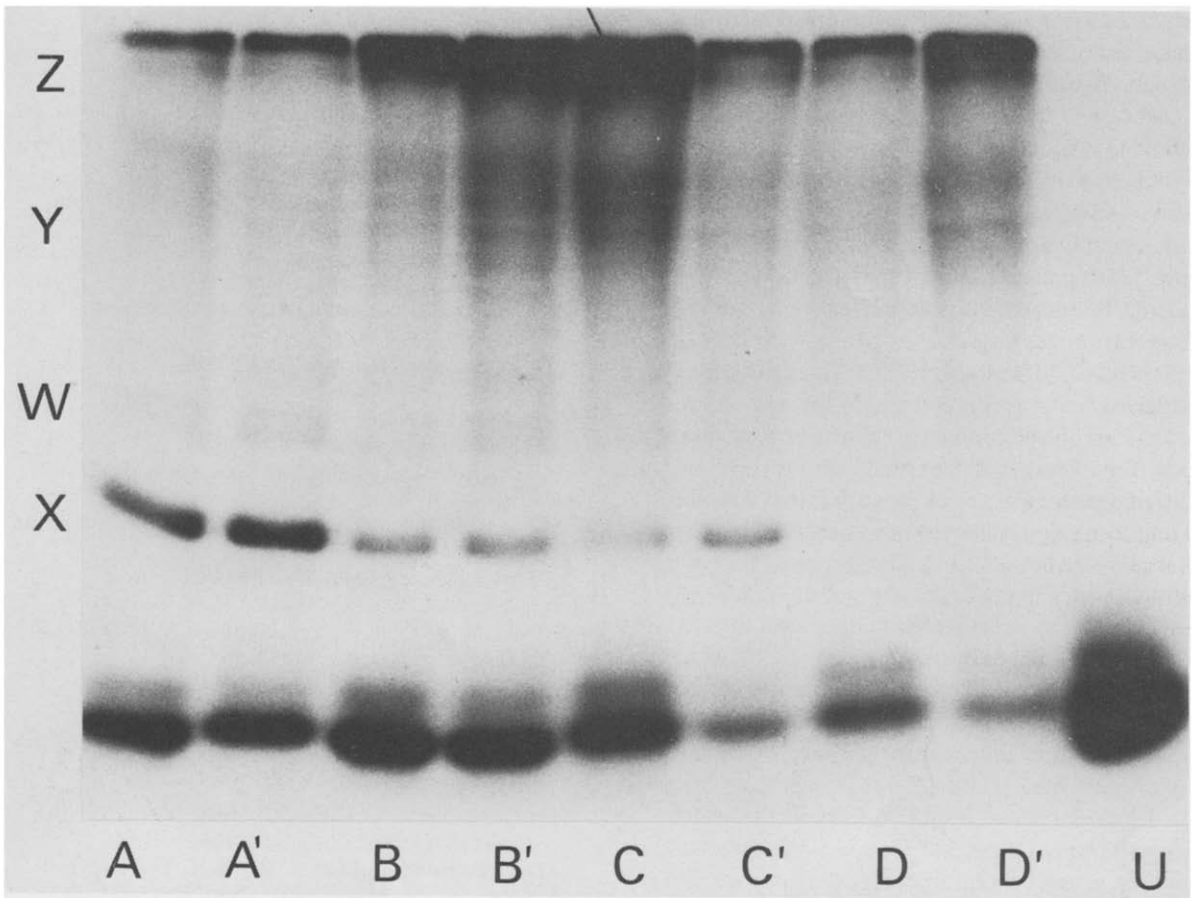

Fig.4. Effects of ATP-depletion on the formation of conjugates by microinjected ^{125}I-labelled ubiquitin. ATP-depleted cells were incubated for 60 min in medium without glucose, containing 10 mM 2-deoxy-D-glucose and 1 mM 2,4-dinitrophenol (ATP-depletion medium). The ATP levels in nmol/10^6 cells were in control cells before fusion 10.0, after fusion 2.7, and in ATP-depleted cells before fusion 2.6 and after fusion 0.49. After fusion with ^{125}I-labelled ubiquitin-loaded erythrocyte ghosts, control cells were divided into 2 batches incubated in: complete medium plus 0.2 mM adenine for (A) 60 min or (A') 180 min; ATP-depletion medium for (B) 60 min or (B') 180 min. Fused ATP-depleted cells were divided into two batches incubated in: complete medium plus 0.2 mM adenine for (C) 60 min or (C') 180 min; ATP-depletion medium for (D) 60 min or (D') 180 min. Lane (U) ^{125}I-labelled ubiquitin. The ATP levels at the time of sampling were in nmol/10^6 cells: (A) 2.8; (A') 3.0; (B) 0.89; (B') 0.39; (C) 1.5; (C') 1.5; (D) 0.03; (D') 0. Gels contained 11.5% (w/v) polyacrylamide.

also markedly decreased under these conditions. However, ATP-depletion caused a marked increase in the radioactivity in the upper third of the gel (regions Y and Z, fig.4). The effects of ATP-depletion were at least partially reversible. When cells depleted before fusion with erythrocyte ghosts were transferred to conditions of ATP-regeneration, bands appeared in the X and W regions (fig.4C,C'). Conversely, if, after bands in the X and W regions had formed, cells were transferred to conditions of ATP depletion, the bands disappeared (not shown). Thus, bands X and W are labile in the absence of ATP.

4. Discussion

The above results show that ^{125}I-labelled ubiquitin microinjected into HTC cells is rapidly incorporated into complexes which appear on polyacrylamide gel electrophoresis as bands of higher M_r than ubiquitin itself. Since electrophoresis was conducted under conditions which disrupt non-covalent bonds and –S–S bridges between polypeptide chains, these bands presumably represent covalent conjugates of ubiquitin with cellular proteins.

One of the most prominent bands, labeled X on our

autoradiograms, has a mobility indicating ~23 000 M_r. This corresponds with the M_r of nuclear protein A24 (22 500 M_r) which consists of histone 2A (14 000 M_r) bound to ubiquitin (8500 M_r) [5]. The probability that band X in our cells is protein A24 is strengthened by the observation that it is confined to the nucleus. If band X is, indeed, protein A24, then the rapidity with which ubiquitin is incorporated into it is consistent with indications that ubiquitin bound to histone 2A in the nucleus is rapidly exchanged with free ubiquitin [10,23].

It is more difficult to interpret the significance of the bands found predominantly in the cytoplasm. Conjugates of ubiquitin with cellular proteins have been formed in extracts of reticulocytes [6,7], by the ATP-dependent linkage of the carboxyl terminal of ubiquitin to the lysine residues of other proteins via an isopeptide bond [7,16]. These conjugates were rapidly broken down in the absence of ATP [7]. In our experiments ATP-depletion decreased the amount of band X and bands in the W region (fig.4), which is consistent with the interpretation that these conjugates consist of ubiquitin linked by its carboxyl-terminal to lysine residues of other proteins. However, the intensity of other bands, in the Z and Y regions of the gel (fig.4), was increased by ATP-depletion. A possible interpretation of these findings is that the latter bands are conjugates of ubiquitin with cellular proteins formed by an ATP-independent reaction. Such conjugates could, for example, be formed by transglutaminases [24]. Since ATP-depletion was incomplete, another possibility is that bands in the Y and Z regions of the gel (fig.4) accumulate because their formation requires low levels of ATP, whereas their disappearance requires high levels of ATP.

It has been proposed that conjugates of ubiquitin with cellular proteins are intermediates of intracellular protein degradation [7]. We have obtained no evidence, so far, that any of the conjugates seen in our experiments play a role in protein degradation.

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