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Conjugation of [^{125}I]ubiquitin to cellular proteins in permeabilized mammalian cells: comparison of mitotic and interphase cells

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[^{125}I]Ubiquitin introduced into permeabilized hepatoma tissue culture (HTC) cells rapidly forms conjugates with endogenous proteins. A characteristic pattern of low mol. wt conjugates is obtained which includes the ubiquitinated histone, uH2A, and unknown molecular species with M_rs of 14, 23, 26 (two bands) and 29 kd. A broad spectrum of higher mol. wt conjugates is also produced. The formation of all conjugates is absolutely dependent on ATP, and upon depletion of ATP they are rapidly broken down. The 14, 23 and 29 kd species are found in all subcellular fractions examined. uH2A is located exclusively in the nuclear fraction. The pair of 26 kd bands is specifically associated with the ribosome fraction. A considerable percentage of the higher mol. wt conjugates sediments with the small particle (100 000 *g*) fraction in the ultracentrifuge but is solubilized with deoxycholate, indicating that there are many membrane-associated conjugates. The pattern of ubiquitin conjugation in interphase and metaphase cells was compared. The incorporation of ubiquitin into uH2A was markedly reduced in metaphase cells whereas its incorporation into other low mol. wt conjugates and into high mol. wt conjugates was affected slightly, if at all. This shows that the known decrease of uH2A levels in metaphase is due to a specific effect on histone ubiquitination and not to a general decrease in ubiquitination activity or increase of isopeptidase activity. Changes in the levels of uH2A during mitosis measured by immunoblotting were similar to those estimated in permeabilized cells. These experiments indicate that permeabilized cells provide a useful approach to the study of rapidly turning over ubiquitin conjugates in mammalian cells.

Key words: ubiquitin/permeabilized cells/cell cycle/histones

Introduction

Ubiquitin, a 76 amino acid residue protein, is present in all eukaryotic cells and its sequence is conserved to an unprecedented degree. Ubiquitin is involved in a number of basic cellular functions which include intracellular protein degradation, cell cycle control and heat shock response. The properties and activities of ubiquitin have been discussed in detail in several recent reviews (Hershko and Ciechanover, 1982; Hershko, 1983; Ciechanover *et al.*, 1984; Finley and Varshavsky, 1985).

A common denominator of all the known functions of ubiquitin is its covalent linkage to other proteins. It is surprising, therefore, that little is known about the naturally occurring conjugates of ubiquitin with cellular proteins. Ubiquitin forms conjugates with a broad spectrum of endogenous proteins (Hershko *et al.*, 1980; Atidia and Kulka, 1982; Finley *et al.*, 1984; Haas and Bright, 1985). However, the only well-characterized conjugates are the ubiquitinated histones uH2A and uH2B. In uH2A ubiquitin is linked by its terminal carboxyl via an isopeptide bond to lysine 119 of H2A (Goldknopf and Busch, 1977, 1980), while uH2B has a similar, but not precisely known, structure (West and Bonner, 1980).

Earlier work in this and other laboratories has shown that iodinated ubiquitin microinjected into mammalian cells rapidly forms a wide variety of conjugates (Atidia and Kulka, 1982; Chin *et al.*, 1982). However, the radioactivity of the ubiquitinated proteins obtained by this method was too low to allow the identification of intracellular conjugates other than uH2A. In the work reported here we show that iodinated ubiquitin introduced into permeabilized cells in the presence of an ATP-generating system rapidly forms highly radioactive conjugates with cellular proteins. This has revealed a characteristic and reproducible pattern of ubiquitin conjugation to endogenous proteins. The pattern is changed in metaphase cells, the labeling of uH2A being specifically reduced.

Results

ATP-dependent conjugation of [^{125}I]ubiquitin to endogenous proteins

Figure 1 shows the conjugates formed when [^{125}I]ubiquitin is incubated with permeabilized cells in the presence of an ATP-generating system. A number of characteristic low mol. wt bands are formed. One of these is uH2A, as deduced from its comigration with a uH2A marker. The other bands, which are as yet unidentified conjugates, have been labeled up13, up14, up23, up26a, up26b and up29 according to their apparent mol. wts. In addition, a broad spectrum of high mol. wt conjugates (M_r > 30 kd) is formed, which is referred to as HMW. Because of the closeness of the two bands up26a and up26b, and their similar behaviour, they have been treated as a single entity in the rest of the paper. The order of intensity of the low mol. wt bands is usually up14 > uH2A ≥ up29 > up23 ≥ up26a and b. Up13 is a minor species not always clearly seen.

The formation of conjugates is absolutely dependent on ATP (Figure 1); no conjugates are formed without added ATP in permeabilized cells depleted of ATP before the addition of [^{125}I]ubiquitin (Figure 1). When permeabilized cells not previously depleted of ATP are incubated with [^{125}I]ubiquitin in the absence of added ATP, some conjugates may be formed as a result of endogenous ATP (not shown).

Time course of [^{125}I]ubiquitin incorporation into conjugates

[^{125}I]Ubiquitin is rapidly incorporated into conjugates during the first 20–25 min of incubation but the label in conjugates falls off after longer incubation (Figure 2). This fall in radioactivity is probably due to the breakdown of conjugates by isopeptidases (Andersen *et al.*, 1981a,b; Matsui *et al.*, 1982) following the exhaustion of ATP. Figure 2 shows that when ATP is exhausted prematurely by the addition of an ATP-depletion system, there is a rapid exponential decay of radioactivity in the conjugates.

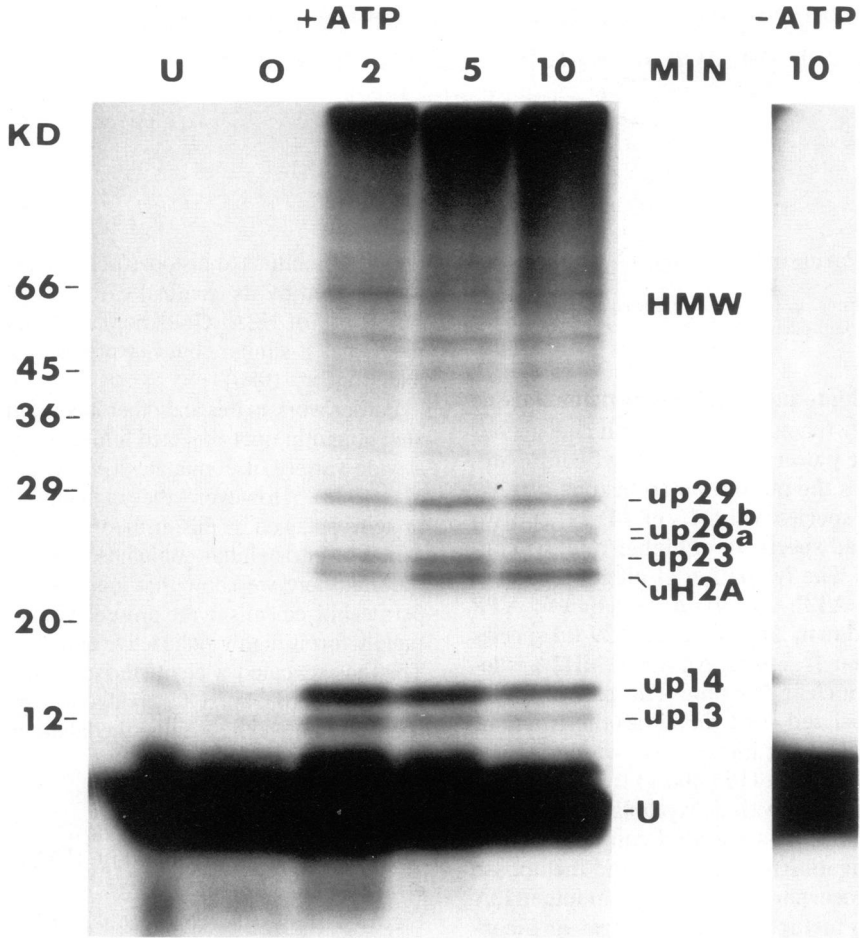

Fig. 1. Formation of conjugates by [^{125}I]ubiquitin in permeabilized cells: dependence on ATP. Cells were permeabilized and incubated with [^{125}I]ubiquitin for the times shown with an ATP-generating system (+ATP), as described in Materials and methods. Other permeabilized cells (−ATP) were depleted of ATP by preincubation with 1 mM 2,4-dinitrophenol and 10 mM 2-deoxy-D-glucose before the addition of the [^{125}I]ubiquitin. U, ubiquitin; HMW, high mol. wt conjugates; up13−up29, low mol. wt conjugates.

Only uH2A and high mol. wt conjugates are shown in Figure 2 since the kinetic behaviour of the other low mol. wt conjugates is similar to that of uH2A. The half-lives of conjugates in the absence of ATP are somewhat variable from experiment to experiment, but are usually in the order of 10 min.

Intracellular distribution of ubiquitin conjugates

Figure 3 shows the distribution of ubiquitin conjugates in subcellular fractions of permeabilized cells. As expected from previous work (Atidia and Kulka, 1982), uH2A is located exclusively in the nuclear fraction (lanes 5 and 6) and is extracted by acid (lane 8) but not by 0.3 M NaCl (lane 7). Conjugates up14, up23 and up29 are, like ubiquitin, distributed among all the cellular fractions. The up26 doublet, on the other hand, is found in the post-nuclear supernatant (lane 3) and sediments in the ultracentrifuge at 100 000 *g* (lane 1), suggesting that it is associated with small particles.

To test if the up26 pair is associated with ribosomes, the 100 000 *g* pellet was treated with 1% deoxycholate and sedimented through a sucrose cushion. The up26 doublet is associated with the ribosomal pellet (lane 9) and absent from the supernatant (lane 10). This fractionation also reveals several other ribosome-associated conjugates (arrows, lane 9) not detected in other fractions or in whole-cell extracts.

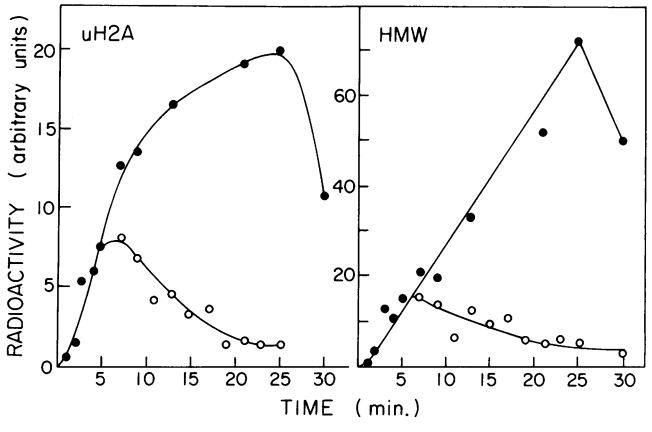

Fig. 2. Time course of incorporation of [^{125}I]ubiquitin into uH2A and high mol. wt conjugates: effect of ATP-depletion. Permeabilized cells were incubated with [^{125}I]ubiquitin with an ATP-generating system as described in Materials and methods for the times shown (closed circles). After 5 min an ATP-depletion system consisting of 1 mM 2,4-dinitrophenol, 20 mM 2-deoxyglucose and 30 units/ml of hexokinase was added to a portion of the cells (open circles). Arbitrary radioactivity units were derived from densitometric scans of autoradiograms of gels. The region scanned for high mol. wt conjugates is marked in Figure 4A.

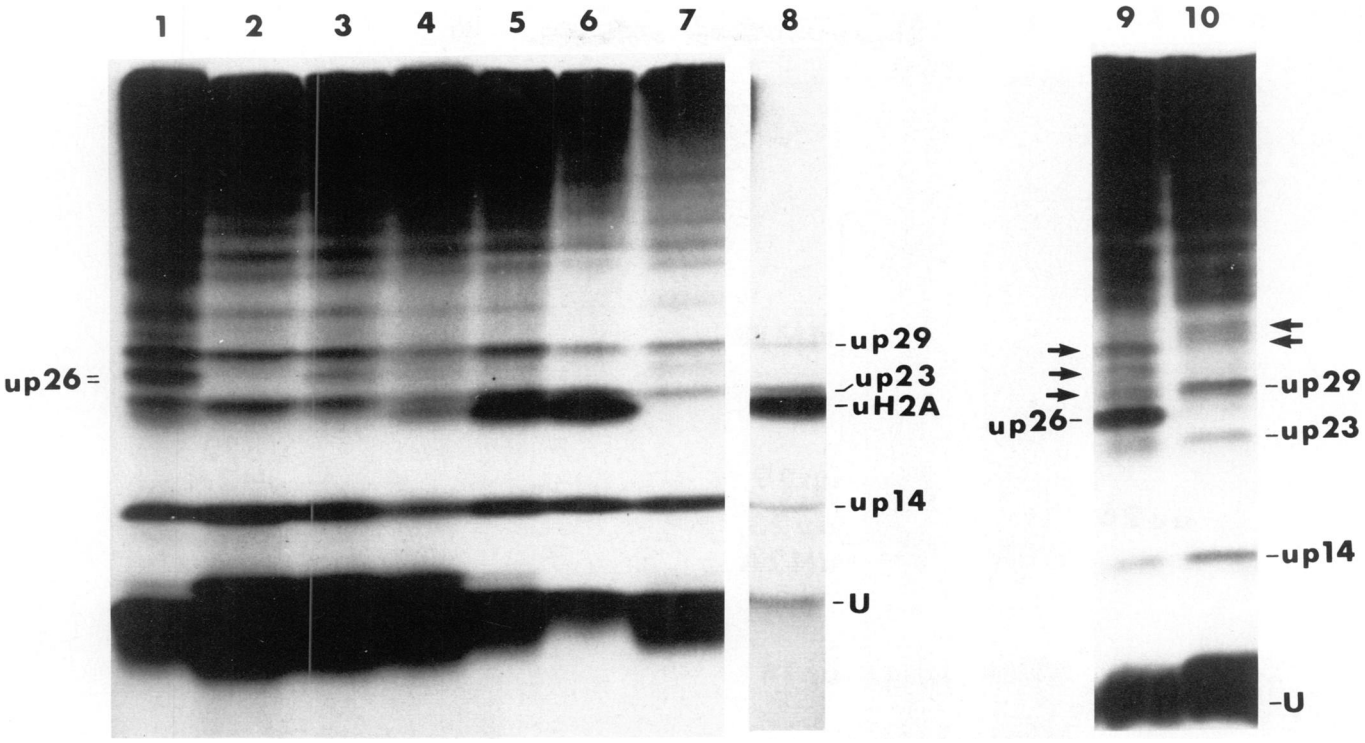

Fig. 3. Distribution of ubiquitin conjugates in subcellular fractions of permeabilized cells. Permeabilized cells were incubated with [^{125}I]ubiquitin and an ATP-generating system for 15 min and subcellular fractions were prepared as described in Materials and methods. **Lane 1,** 100 000 *g* pellet of post-nuclear supernatant; **lane 2,** 100 000 *g* supernatant; **lane 3,** post-nuclear supernatant; **lane 4,** whole-cell extract; **lane 5,** nuclei; **lane 6,** salt-washed nuclei; **lane 7,** salt extract of nuclei; **lane 8**, acid extract of nuclei; **lane 9**, deoxycholate pellet; **lane 10**, deoxycholate supernatant. **Lanes 1–8** are from a different gel than **lanes 9** and **10**.

A large portion of the high mol. wt conjugates is bound to small particles sedimenting at 100 000 *g* in the ultracentrifuge (lane 1) but a considerable percentage of these becomes soluble upon treatment with deoxycholate (lane 10). This suggests that many ubiquitin conjugates are membrane-associated. The deoxycholate supernatant contains previously unresolved conjugate bands (arrows, lane 10) which may be membrane-associated.

Conjugation of ubiquitin in metaphase cells

Previous work in other laboratories has shown that levels of the ubiquitinated histones uH2A and uH2B are greatly reduced in metaphase cells (Matsui *et al.*, 1979; Wu *et al.*, 1981; Mueller *et al.*, 1985). We therefore examined whether this change in ubiquitination pattern is reflected in the incorporation of [^{125}I]-ubiquitin into permeabilized metaphase cells.

Figure 4 shows that the labeling of uH2A is specifically and strikingly decreased in metaphase cells, whereas the incorporation of ubiquitin into other conjugates is not markedly affected. Colcemid-treated interphase cells, taken from the same flasks as the mitotic cells (Figure 4B, lane C) incorporated ubiquitin into uH2A similarly to untreated control cells (Figure 4A, lane I). Thus the reduced incorporation of ubiquitin into uH2A is not the result of drug treatment.

Figure 5 compares the time course of incorporation of [^{125}I]-ubiquitin into conjugates of mitotic and control cells. The incorporation of label into uH2A in metaphase cells rapidly reaches a plateau much lower than that in control cells. Incorporation of ubiquitin into high mol. wt conjugates is not detectably changed and incorporation into low mol. wt conjugates other than uH2A is slightly reduced. Thus the change in label of uH2A at metaphase is due to specific effect and not to a general decrease in ubiquitination activity.

Detection of ubiquitin conjugates by immunoblotting in metaphase and interphase cells

Figure 6 shows the results of an immunoblot of whole cell extracts of metaphase and unsynchronized cells with an antibody raised against a synthetic peptide containing amino acid residues 6–19 of ubiquitin (Swerdlow *et al.*, 1986). This antibody reacts strongly with ubiquitin–protein conjugates. The most prominent band is uH2A which is greatly reduced in intensity in metaphase cells. Below uH2A is a 21 kd band which is also much reduced in metaphase (up21, Figure 6). Preliminary experiments show that up21 is a nuclear protein. In some experiments a decrease in intensity of a 29 kd band (arrow, Figure 6) could be detected in metaphase cells. This band coincides with a 29 kd ubiquitin-containing band (Figure 6, arrow) previously found to be present in core histones and suspected to be a ubiquitinated H2A derivative, possibly diubiquitinated H2A (Kulka, Finley and Varshavsky, unpublished). Since the autoradiogram in Figure 6 was exposed for sufficient time to clearly show the 29 kd band, contrast was lost between the uH2A bands of mitotic and unsynchronized cells.

Table I compares densitometric measurements of the uH2A band in immunoblots and in autoradiograms of permeabilized cells incubated with [^{125}I]ubiquitin. In immunoblots the estimated level of uH2A in metaphase cells is 1–9% of its level in interphase cells. In permeabilized cells the labeling of uH2A is 9–18% of that of metaphase cells.

Discussion

Permeabilized mammalian cells have previously been used to investigate various aspects of the cell cycle and for other purposes (Miller *et al.*, 1978; Schliwa *et al.*, 1981; Cande, 1982). Here

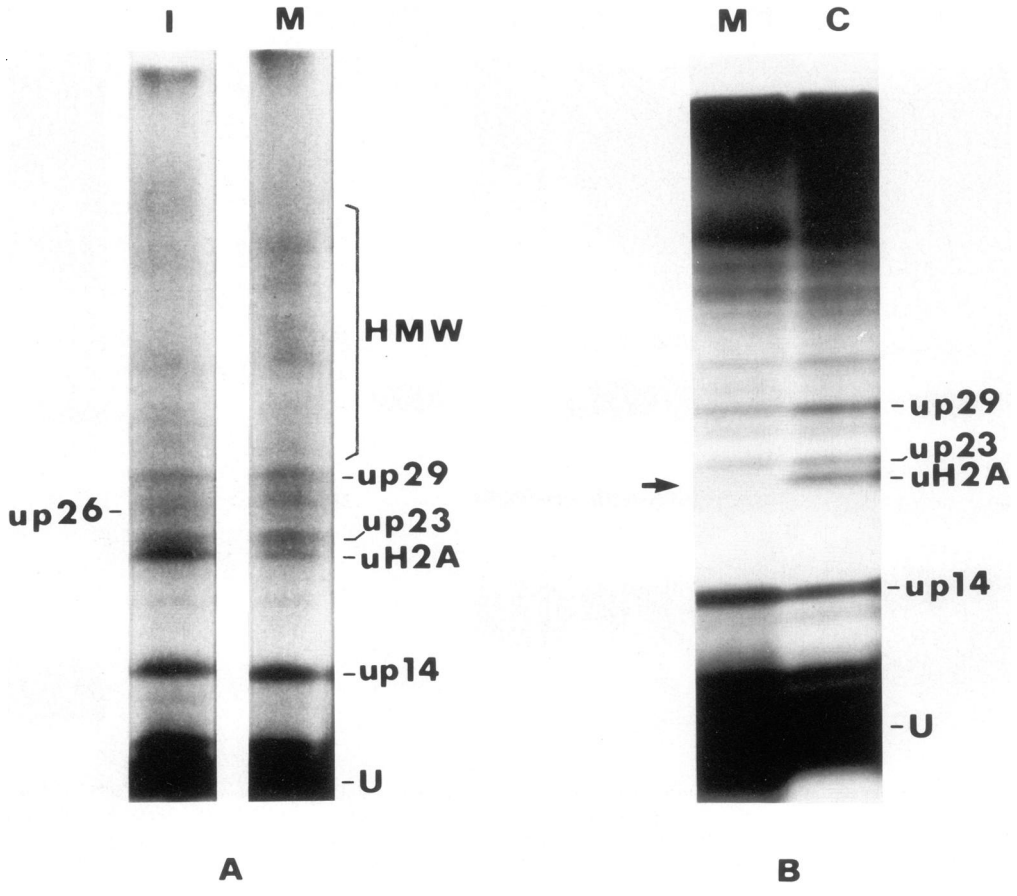

Fig. 4. Conjugation of ubiquitin in permeabilized interphase and metaphase cells. Metaphase cells were prepared as described in Materials and methods. **A.** M, metaphase cells, I, unsynchronized cells from spinner culture. Permeabilized cells were incubated for 10 min with [^{125}I]ubiquitin. The data in Figure 5 are from the same experiment. **B.** M, metaphase cells; C, control colcemid-treated interphase cells remaining in flask as M after shake-off of the metaphase cells.

we have shown that permeabilized cells provide a useful tool for studying the conjugation of ubiquitin to the endogenous proteins of mammalian cells. Immunoblotting has also been used recently for the characterization and quantitation of naturally occurring ubiquitin conjugates (Haas and Bright, 1985). The use of permeabilized cells complements immunoblotting in that it allows kinetic measurements of ubiquitin conjugation to be made on cells in a variety of metabolic states. The experiments summarized in Figures 4–6 indicate that permeabilized metaphase cells retain to a large measure the ubiquitination pattern of the intact cells. The molecular species labeled in permeabilized cells are presumably the major rapidly turning over conjugates in the cell, while those detected by immunoblotting are the quantitatively important ubiquitin-containing molecules.

In permeabilized mammalian cells ubiquitin forms a number of clearly recognizable conjugates with endogenous proteins. One of these is the well-known ubiquitinated histone uH2A (Goldknopf and Busch, 1980) while the others have not yet been characterized. Some of the conjugates described here resemble ubiquitinated species observed previously. Up14 is probably identical to a 14–15 kd molecule found in other laboratories to be formed from iodinated ubiquitin in extracts of *ts*85 cells and reticulocytes, and thought to be a dimer of ubiquitin (Finley *et al.*, 1984; Haas and Bright, 1985). The rapid labeling of up14 in the present work indicates that this putative dimer of ubiquitin has an important cellular function. Ubiquitinated species with similar mobilities to up29 have also been reported elsewhere. Immunoblotting experiments have revealed a 29 kd molecule in core histones and in mammalian cells (Kulka, Finley and Varshavsky, unpublished). Preliminary experiments suggest that this is a ubiquitinated derivative of H2A, perhaps diubiquitinated H2A. Recently Haas and Bright (1985) have found that one of the most prominent ubiquitinated molecules in reticulocyte extracts is a 29 kd species.

Additional insight into the properties and possible functions of conjugates formed by permeabilized cells comes from subcellular fractionation studies (Figure 3). All fractions contain a wide but different spectrum of conjugates. Some of the major low mol. wt conjugates are associated with specific cellular organelles. As expected from previous work (Atidia and Kulka, 1982), uH2A is exclusively found in the histone fraction of the nucleus. The up26 pair and several other conjugates are specifically associated with the ribosomal fraction. An intriguing possibility is that ubiquitination of ribosomal or ribosome-associated proteins is involved in the regulation of translation. A large percentage of the conjugates in the post-nuclear supernatant sediment in the ultracentrifuge, but are solubilized by sodium deoxycholate, suggesting that they are membrane-associated. A question which still remains to be answered is whether all the observed conjugates are intermediates of proteolysis or whether ubiquitin conjugation has some other independent function. It is possible that one of the activities of ubiquitin is the modification of protein function (Munro and Pelham, 1985).

Both the synthesis of conjugates and the maintenance of conjugate pools is dependent on ATP. It has previously been noted

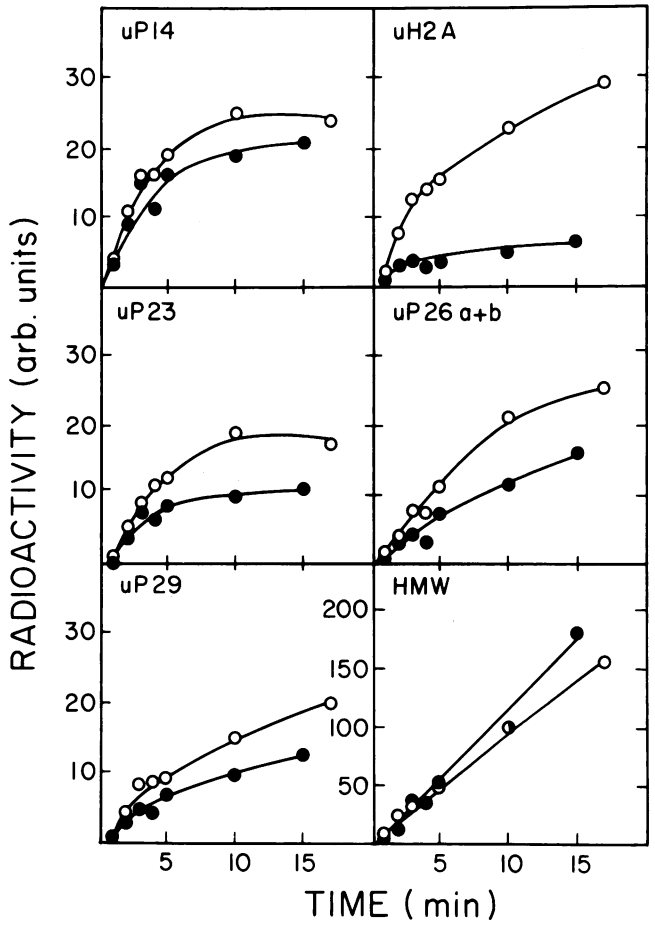

Fig. 5. Time course of conjugation of [^{125}I]ubiquitin in metaphase and interphase permeabilized cells. Metaphase cells (closed circles) and unsynchronized cells from spinner culture (open circles) were permeabilized and incubated with an ATP-generating system as described in Materials and methods. The metaphase cell system contained 85% metaphase cells. Samples were withdrawn at intervals. After electrophoresis and autoradiography, the relative radioactivity of conjugates was determined by densitometry. The region scanned in the high mol. wt (HMW) region by the densitometer is indicated by the line in Figure 4A.

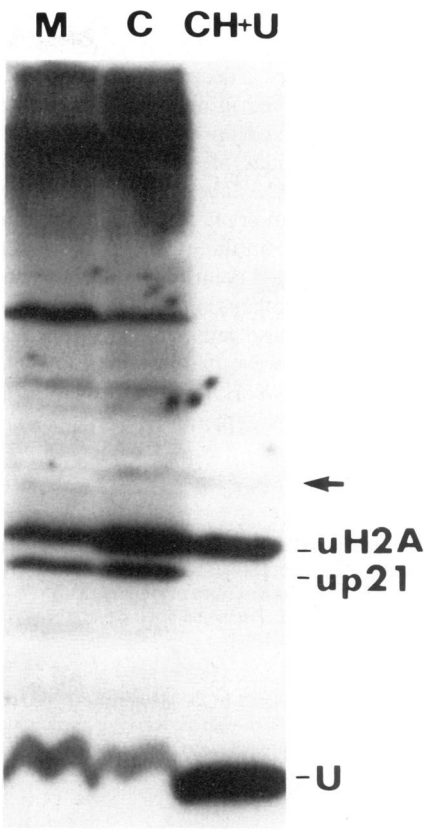

Fig. 6. Immunoblot of ubiquitin conjugates in mitotic and unsynchronized cells. Metaphase (M) and unsynchronized cells (C) and a mixture of calf thymus core histones with ubiquitin (CH + U) were taken up in Laemmli (1970) sample buffer. After heating for 2 min in a boiling water bath, the samples were subjected to electrophoresis. Conjugates in the gel were revealed by immunoblotting with an anti N-terminal peptide antibody, as described in Materials and methods.

Table I. uH2A in metaphase and unsynchronized cells: a comparison of permeabilization and immunoblotting experiments

Assay	Number of experiments	Ratio of intensities of uH2A bands metaphase	
		mean	range
Permeabilized cells	3	0.13	0.09–0.18
Immunoblot	4	0.04	0.01–0.09

Autoradiograms were scanned with a densitometer as described in Materials and methods. Permeabilized cells were incubated with [^{125}I]ubiquitin and an ATP-generating system for 15 min. The ratios were calculated after correcting values for colcemid-arrested cells for contamination with non-mitotic cells and values for unsynchronized cells for contamination with mitotic cells. Immunoblotting experiments were performed with anti-ubiquitin conjugate antibody (three experiments) and anti-N-terminal peptide antibody (one experiment).

that the ubiquitin moiety of uH2A is constantly being exchanged with free ubiquitin by an ATP-dependent process (Seale, 1981; Wu *et al.*, 1981; Atidia and Kulka, 1982; Matsumoto *et al.*, 1983). Many of the ubiquitin conjugates described here appear to behave similarly. This conclusion is based on the observation that radioactive ubiquitin rapidly enters the conjugates with an absolute dependence on ATP (Figure 1) and that upon depletion of ATP there is a rapid decay of label in the conjugates (Figure 2). Similar observations on conjugate stability have been made with reticulocyte extracts (Hershko *et al.*, 1980; Haas and Rose, 1981). The most plausible explanation of these results is that the steady state levels of ubiquitin conjugates in the cell are the result of the opposing effects of the ubiquitin conjugation system on the one hand, and isopeptidases (Andersen *et al.*, 1981a,b; Matsui *et al.*, 1982) and/or ubiquitin-dependent proteolysis (Hershko, 1983) on the other hand. The reason for the existence of such an energy-wasteful process in the cell is puzzling. It has been suggested that isopeptidases play a role in the selection of cellular proteins for proteolysis (Hershko and Ciechanover, 1982).

The present work confirms and extends previous observations on the decrease in uH2A during metaphase (Matsui *et al.*, 1979; Wu *et al.*, 1981; Mueller *et al.*, 1985). We find that the incorporation of iodinated ubiquitin into uH2A in permeabilized metaphase cells is strongly decreased, whereas its incorporation into other conjugates is affected little, if at all (Figures 4 and 5). These experiments show that the fall in uH2A is due to a specific effect and not to a general decrease of ubiquitination activity or increase of isopeptidase activity during metaphase. The level of uH2A decreases >10-fold in metaphase (Table I). Estimates of initial rates of uH2A synthesis in the experiment in Figure 5 and in similar experiments indicate that there is a decrease of ≤3-fold

in metaphase relative to interphase cells (not shown). Although initial rate measurements are inevitably inaccurate because of the limitations of densitometry, a decrease in the rate of uH2A synthesis is consistently observed in metaphase cells, and apparently contributes to the observed fall in uH2A levels. However, the estimated decrease in initial rate seems to be too low to account for the 8-fold fall in uH2A labeling of permeabilized cells observed in Table I. Matsui *et al.* (1982) have reported a translocation of isopeptidase from the cytoplasm to the chromosomes in metaphase which may contribute to the disappearance of uH2A. An alternative explanation is that the condensation of the chromosomes at metaphase causes the H2A ubiquitination sites to be masked in some way, making them unavailable for the attachment of ubiquitin. Work is in progress to further elucidate the mechanism of uH2A depletion at metaphase.

Materials and methods

Cells and media

Hepatoma tissue culture (HTC) cells, clone GM22, were grown in suspension in modified Swim's 77 medium plus 10% newborn calf serum. The composition of the modified medium was as described previously (Kulka *et al.*, 1972) except that 75 mM Tricine was added. Tricine-buffered saline contained 160 mM NaCl and 20 mM Tricine-NaOH, pH 7.4.

Iodination

Ubiquitin was iodinated with $Na^{125}I$ by the chloramine-T method, as previously described (Atidia and Kulka, 1982), with the following modifications: the reaction mixture contained 20 μg of pure ubiquitin (the generous gift of A.Hershko and A.Ciechanover) and, after termination of the iodination, 10 μl of KI (30 mg/ml) were added as carrier.

Preparation of mitotic cells

HTC cells (1.5×10^7 total) were plated onto 175-cm^2 T-flasks. $CaCl_2$ (2 mM) was added to the growth medium to promote cell adherence. After 5–7 h non-adhering cells were removed by vigorous shaking. Fresh growth medium containing 2 mM $CaCl_2$ and 0.1 μg/ml colcemid was added to the remaining cells. After 12 h of colcemid block, mitotic cells were collected by gentle shake-off. The mitotic index obtained was 85–95%.

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

Cells were permeabilized by the following modification of the method of Schliwa *et al.* (1981). HTC cells were washed three times at 4°C at a concentration of 5×10^5 cells/ml with Tricine-buffered saline at 200 *g* for 5 min and once at a concentration of 5×10^6 cells/ml with stabilizing buffer containing 60 mM Pipes [piperazine-N,N'-bis(2-ethane sulfonic acid)], 25 mM Hepes (N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid), 10 mM EGTA [ethylene glycol-bis(β-aminoethylether)-N,N,N,N'-tetraacetic acid] and 2 mM $MgCl_2$, final pH 7.0. Cells at a concentration of 5×10^7 cells/ml in stabilizing buffer were permeabilized by the addition of Brij 58 to a final concentration of 0.2% (w/v). After 10 min at 4°C the percentage of permeabilized cells was checked by mixing a sample of the cells with an equal volume of isotonic 4% (w/v) Trypan blue.

The incorporation system (150 μl) contained 100 μl of permeabilized cells in stabilizing buffer, 3×10^6 c.p.m. of [^{125}I]ubiquitin, 1.5 mM dithiothreitol and an ATP-generating system containing 1.5 mM ATP, 25 mM creatine phosphate and 16 units/ml of creatine kinase. After incubation at 37°C, samples were removed at intervals into Laemmli (1970) sample buffer, heated for 2 min in a boiling water bath, and subjected to electrophoresis. Radioactive bands were detected by autoradiography.

Preparation of subcellular fractions

After incorporation of [^{125}I]ubiquitin into 1.5×10^8 permeabilized cells in 1.5 ml for 10–15 min, they were cooled to 4°C and lysed by repeated pipetting with 5 ml of a solution containing 10 mM Tris-HCl buffer pH 7.5, 2 mM $MgCl_2$ and 0.25% (v/v) Triton X-100. This and all other solutions used for fractionation were supplemented with 10 mM N-ethylmaleimide and 1 mM phenylmethylsulfonyl fluoride (PMSF) to inhibit isopeptidases and serine proteases. All operations were carried out at 4°C.

The lysate was centrifuged at 2500 *g* for 10 min to sediment the nuclei. The pellet was washed once with 5 ml of lysis buffer. Salt-washed nuclei were prepared by suspending the nuclear pellet in 1.0 ml of 10 mM Tris-HCl, pH 7.5, containing 0.3 M NaCl and 1 mM EDTA, and centrifuging at 2500 *g* for 10 min. This operation was repeated once. Acid extracts of salt-washed nuclei were prepared by vortexing 7.5×10^7 nuclei in 0.5 ml of 0.5 M HCl for 60 min at 4°C.

The cytoplasmic fraction obtained after sedimenting the nuclei from the original lysate was further fractionated as follows. First mitochondria and other large particles were removed by centrifuging at 12 000 *g* for 20 min. The resultant clear supernatant was centrifuged for 60 min at 100 000 *g* and the pellet obtained was suspended in 1 ml of TKM buffer (50 mM Tris-HCl, pH 7.5, 25 mM KCl and 10 mM $MgCl_2$) containing 0.25 M sucrose. Sodium deoxycholate was added to a final concentration of 1%. The sample was layered on a 1.45 M sucrose cushion in TKM buffer and centrifuged for 2.5 h at 100 000 *g* (De Groot *et al.*, 1976). The ribosomal pellet obtained was resuspended in TKM buffer and the ratio of absorbances at 260 nm and 280 nm was determined. All fractions were mixed with Laemmli (1970) sample buffer and heated for 2 min in a boiling water bath prior to electrophoresis.

Gel electrophoresis

SDS-polyacrylamide gels containing 18% (w/v) acrylamide, as described by Thomas and Kornberg (1975), were used.

Immunoblotting

A slight modification of the method of Swerdlow *et al.* (1986) was used. Briefly, proteins were transferred by electrophoresis to 0.45 μm nitrocellulose filters (Schleicher and Schuell, BA85). The transfer buffer contained 25 mM Tris, 190 mM glycine, 0.01% (w/v) SDS and 20% (v/v) methanol. The filters were washed with ANT [50 mM Tris-HCl, pH 8.0, 150 mM NaCl and 0.02% (w/v) NaN_3]. After heating in boiling water for 20 min the filters were blocked with 10% fetal calf serum and 5% bovine serum albumin in ANT for 60 min. They were then treated with antibody in the blocking solution for 120 min. After washing four times for 10 min in ANT, [^{125}I]protein A (Amersham) in blocking solution (0.1 μCi/ml) was applied for 60 min. After four 10-min washes in ANT the filters were autoradiographed. Two antibody preparations reacting with ubiquitin conjugates were used. The first of these, which was raised against a synthetic N-terminal peptide of ubiquitin (residues 6–19) and which was affinity purified on a ubiquitin–Sepharose column, was the generous gift of A.Varshavsky and D.Finley ('anti-N-terminal peptide antibody'). The other antibody, which was raised against ubiquitin conjugates, was the generous gift of A.Ciechanover ('anti-conjugate antibody').

Densitometry of autoradiograms

Autoradiograms were scanned with a Biomed Instruments Inc. soft laser scanning densitometer. Intensities of bands were measured in a density range previously shown to be linear with radioactivity. The radioactivity of high mol. wt conjugates (HMW) was measured by scanning the region marked in Figure 4A. The radioactivity of ubiquitin in each lane was assayed by densitometry of autoradiograms, exposed for appropriately short periods, and was used to correct for sampling errors.

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