Nuclear localization of the ubiquitin-activating enzyme, E1, is cell-cycle-dependent

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The mechanisms that regulate ubiquitin-mediated degradation of proteins such as the mitotic cyclins at defined stages of the cell cycle are poorly understood. The initial step in the conjugation of ubiquitin to substrate proteins involves the activation of ubiquitin by the ubiquitin-activating enzyme, E1. Previously we have described the subcellular localization of this enzyme to both nuclear and cytoplasmic compartments. In the present study, we have used the 1C5 anti-E1 monoclonal antibody in immunofluorescent-microscopy and subcellular-fractionation techniques to examine the distribution of E1 during the HeLa cell cycle. E1 is both cytoskeletal and nuclear during the G1-phase. As the cells progress into S-phase, E1 is exclusively

cytoskeletal and has a perinuclear distribution. During G2-phase, E1 reappears in the nucleus before breakdown of the nuclear envelope. In mitotic cells, E1 localizes to both the mitotic spindle and the cytosol, but is absent from the chromosomes. Immunoblot analysis reveals multiple forms of E1 in HeLa whole cell extract. This heterogeneity is not a result of polyubiquitination and may represent inactive pools of E1. Only the characteristic E1 doublet is able to activate ubiquitin. Cell-fractionation studies reveal a differential distribution of specific E1 isoforms throughout the cell cycle. Therefore we propose that the subcellular localization of E1 may play a role in regulating cell-cycle-dependent conjugation of ubiquitin to target proteins.

INTRODUCTION

Ubiquitination is a covalent modification of various cellular proteins. This post-translational modification is involved in various physiological processes, the best understood of which is the ubiquitin-dependent degradative pathway (Finley and Chau, 1991). In this case, multiply ubiquitinated proteins are targeted for degradation. In addition, stable monoubiquitin adducts are found intracellularly, such as those involving nuclear histones and cytoplasmic arthrin and the cytoplasmic domain of receptors for platelet-derived growth factor and growth hormone (Yarden et al., 1986; Ball et al., 1987; Leung et al., 1987; Bonner et al., 1988).

The first step in the ubiquitin-conjugation system is the activation of ubiquitin to a high-energy intermediate. This reaction is catalysed by E1, the ubiquitin-activating enzyme (Ciechanover et al., 1982). The two-step reaction involves the formation of a ubiquitin adenylate intermediate and the transfer of ubiquitin to a thiol site (Haas and Rose, 1982). The ubiquitin is then transferred to one of the ubiquitin-carrier proteins (E2s) to generate a similar thiol ester linkage (Pickart and Rose, 1985). At this point, ubiquitin can either be linked directly to a target protein or conjugated to proteins destined for degradation via the ubiquitin-protein ligases (E3s) (Ciechanover and Schwartz, 1989; Hershko, 1992). Thus E1 provides the initial activated form of ubiquitin necessary for any conjugation reaction to occur. In this capacity, E1 plays a key regulatory role in any process affected by ubiquitin modification.

We (Handley et al., 1991) and others (Hatfield et al., 1990; Zachsenhaus and Scheinen, 1990; McGrath et al., 1991) have cloned and sequenced the cDNA for E1 from human, yeast and wheat. A single ubiquitous E1 mRNA of 3.5 kb predicts a

mature protein of ~117 kDa. A new testis-specific gene, *Sby*, has also been cloned which shows extensive similarity to human E1 (Kay et al., 1991; Mitchell et al., 1991). *Sby* has been proposed as a candidate for the spermatogenesis gene, *Spv*, which is required for the survival and proliferation of A spermatogonia during spermatogenesis (Burgoyne et al., 1986). Indeed, ubiquitination of proteins and histones is thought to be involved in the marked changes in protein turnover, chromatin structure and cell-cell interactions that occur during spermatogenesis (Agell and Mezquita, 1988).

The ubiquitin-mediated proteolytic system is known to play a key role in cell-cycle progression. Three mammalian cell lines have recently been described that appear to have a single temperature-sensitive defect in E1. At the non-permissive temperature, A1S9, ts85 and ts20 cells are arrested at the S/G2-phase transition (Sheinin, 1976; Ciechanover et al., 1984; Finley et al., 1984; Kulka et al., 1988; Mayer et al., 1989). As the removal of ubiquitin from histone 2A is a prerequisite for chromosome condensation, the role of E1 in these cell lines could be related to its ability to activate ubiquitin and thus permit its covalent attachment to histones 2A and 2B during interphase (Bonner et al., 1988). Indeed, A1S9 cells lose the capacity to convert newly replicated small DNA strands into chromosomal DNA at the non-permissive temperature (Sheinin, 1976).

Ubiquitin-mediated degradation is also essential for cell-cycle progression from metaphase. In this case, degradation of both cyclin A and cyclin B appear to be mediated by the ubiquitin system (Glutzer et al., 1991; Hershko et al., 1991). The mechanisms whereby the mitotic cyclins are specifically degraded at metaphase are poorly understood. Both cyclin A and B are known to form complexes with the highly conserved phosphoserine/threonine kinase, p34cdc2. The cyclin B-cdc2

complex is thought to activate the cyclin destruction pathway presumably by phosphorylation/dephosphorylation of an unknown substrate, driving the cells into interphase of the next cell cycle (Luca et al., 1991). Given that the timing of cyclin destruction is determined by the cell-cycle stage of the cytoplasm rather than the cell-cycle stage of the cyclins themselves (Luca et al., 1991), possibly one or more of the components of the ubiquitin system are modified in such a way that degradation of a specific protein is initiated.

We have previously described the subcellular localization of ubiquitin-activating enzyme, E1 (Schwartz et al., 1992; Trausch et al., 1993), and found its distribution within exponentially growing populations of cells to be variable, with some cells having positive nuclei and others negative nuclei (Trausch et al., 1993). We consider it possible that the ubiquitin system may perform distinct essential roles by changing subcellular localization of E1 during the cell cycle. Indeed, in support of the notion of a proteolytic system within the nucleus, Tsuneoka and Mekada (1992) have demonstrated the rapid intranuclear degradation of an abnormal malformed protein expressed transiently in COS cells. In this study we have used the IC5 anti-E1 monoclonal antibody in immunofluorescence and cell-fractionation techniques to address the possibility that the subcellular localization of E1 varies with the cell cycle. We demonstrate here that the appearance and localization of E1 isoforms is cell-cycle-dependent, and may be regulated by post-translational modifications.

MATERIALS AND METHODS

Antibodies/labelling reagents

IC5 is a mouse IgM monoclonal antibody raised against human E1 (Schwartz et al., 1992). Affinity-purified goat anti-mouse IgM-rhodamine was from Chemicon and goat anti-mouse IgG-fluorescein was obtained from Boehringer. Rabbit polyclonal ubiquitin-conjugated antibodies have been described previously (Schwartz et al., 1987). Rabbit anti-lamin (A,B,C) was a gift from L. Gerace (Scripps Clinic, La Jolla, CA, U.S.A.).

Synchronization procedures

Exponentially growing cells were arrested at various stages of the cell cycle as described by Pines and Hunter (1989) and Tobey et al. (1990). To accumulate HeLa cells in G₀-phase. Confluent monolayers were maintained for 36 h in serum-deficient medium (0.02 %). Isoleucine-deficient F-10 medium supplemented with 5 % dialysed calf serum was used to synchronize exponentially growing HeLa cells in G₁-phase. G₁/S-phase cells were obtained by culturing exponentially growing cells for 24 h in Dulbecco's modified Eagle's medium containing 5 µg/ml aphidicolin. A 24 h incubation in 1 mM hydroxyurea or 3.3 µM nocodazole synchronized the cells in S- and M-phase respectively, and a 15 h treatment with Hoechst 33342 (0.1 µg/ml) arrested the cells in G₂-phase.

Flow-cytometric analysis of DNA

Cells were prepared for fluorescence-activated cell sorting (FACS) by resuspending 5×10^6 cells in 1 ml of 0.1 % sodium citrate containing propidium iodide (0.05 mg/ml), 0.3 % Nonidet P40 and 0.02 mg/ml ribonuclease A. The cells were then analysed on a Coulter Elite FAC scan. For example, in asynchronous

populations of cells, approximately 80 % of cells had a DNA content of 2N (diploid) and 8 % 4N (tetraploid); in cells arrested in M-phase approximately 98 % had 4N (tetraploid), whereas in S-phase over 90 % of cells were in a peak between 2N and 4N.

Immunofluorescence

Immunofluorescence experiments were conducted as described previously (Trausch et al., 1983). Briefly, HeLa cells grown in glass coverslips were fixed in 4 % paraformaldehyde in PBS (2.7 mM KCl, 2.9 mM KH₂PO₄, 137 nM NaCl, 14.1 mM Na₂HPO₄, 1 mM CaCl₂, 0.5 mM MgCl₂, pH 7.4) for 20 min. The cells were washed twice, and the paraformaldehyde quenched with 0.1 M ethanolamine (pH 8). After two rinses, the cells were permeabilized by a 10 min incubation in 1 % Triton X-100. Any non-specific antibody binding was subsequently blocked using 1 % BSA/0.01 % Tween 80/PBS, and the cells were incubated for 45 min at 37 °C with IC5 monoclonal antibody tissue culture supernatant. Thereafter, the cells were rinsed twice, and incubated for 30 min in goat anti-mouse IgM-rhodamine (diluted 1:100 in blocking buffer). The cells were washed three times, and mounted on glass slides and sealed. All dilutions and rinses were performed in PBS/1 % BSA/0.01 % Tween 80, and all steps were performed at 25 °C except where noted. Double-label immunofluorescence was achieved by using IC5 monoclonal antibody tissue culture supernatant containing rabbit anti-lamin antibodies at a dilution of 1:100. Goat anti-mouse IgM-rhodamine (diluted 1:100 in blocking buffer) and fluorescein isothiocyanate-conjugated goat anti-rabbit IgG were used as secondary antibodies to detect the IC5 and anti-lamin antibodies respectively. In order to prevent cross-reactivity with IgM, the anti-mouse IgG-fluorescein was preincubated with 170 µg/ml mouse IgM before addition to the cells. Immunofluorescence was detected in a Zeiss Axioskop fluorescence microscope.

Whole cell extracts

Whole cell extracts from HeLa cells were prepared in ice-cold extraction buffer [5 mM Na₂HPO₄, pH 7.4, 0.01 % digitonin, 0.5 % Nonidet P40, 50 mM NaCl, 150 mM sucrose, 5 mM KCl, 2 mM dithiothreitol (DTT), 1 mM MgCl₂, 0.1 mM phenylmethanesulphonyl fluoride (PMSF), 5 µg/ml aprotinin, 2.5 µg/ml leupeptin, 1 mM sodium orthovanadate, 0.1 mM sodium molybdate]. Generally 2×10^8 cells were lysed in 100 µl of buffer which was then clarified by centrifugation (14000 g for 10 min). The resultant supernatant was used for all experiments on whole cell extracts. No E1 was detected in the pellet by immunoblot analysis.

Cell fractionation

Cell fractionation was carried out by a modified procedure of Boyle et al. (1984, 1985). Briefly, cells were lysed with ice-cold lysis buffer [5 mM sodium phosphate (pH 7.4), 50 mM NaCl, 150 mM sucrose, 5 mM KCl, 2 mM DTT, 1 mM MgCl₂, 0.5 mM CaCl₂, 0.1 mM PMSF and 0.01 % digitonin]. Nuclei were then collected from lysed cells on a cushion of a mixture of 30 % sucrose, 2.5 mM Tris/HCl, pH 7.4, and 10 mM NaCl, after centrifugation at 1000 g for 10 min at 4 °C. Nuclei were subsequently extracted twice in Nonidet P40 buffer (0.5 % Nonidet P40, 10 mM sodium phosphate buffer, pH 7.4, 120 mM NaCl, 0.1 mM PMSF) for 30 min on ice. The nuclei were then collected by centrifugation (1000 g for 10 min at 4 °C) and boiled for 10 min at 110 °C in Laemmli reducing sample buffer

(Laemmli, 1970). Characterization of the cytoplasmic and nucleoplasmic fractions was performed in two ways. Quantitative immunoblots were evaluated for β -tubulin, which was present at more than 95% in the cytoplasmic fraction. In addition, lactate dehydrogenase activity was assayed in all fractions; less than 1.6% was detected in the nuclear fraction.

Immunoblot

Samples were diluted in Laemmli sample buffer, boiled for 10 min at 110 °C, and fractionated by SDS/PAGE. The separated proteins were electrophoretically transferred to nitrocellulose paper at 10 °C. 1C5 tissue culture supernatant was diluted to 1:10 in Tris-buffered saline (20 mM Tris/HCl, pH 7.5, 137 mM NaCl, 0.1% Tween 20) and incubated with the blot at room temperature for 1 h. Bound antibody was subsequently detected with either 125 I-labelled goat anti-mouse IgM and autoradiography as described previously (Schwartz et al., 1986) or colorimetrically using diaminobenzidine tetrahydrochloride (Aldrich) and hydrogen peroxide.

Isolation of pH 9 eluate

A crude preparation of E1, E2s and E3s (i.e. pH 9 eluate) was prepared from rabbit reticulocytes by the affinity-chromatography procedure described by Ciechanover et al. (1982).

Purification of active E1 from HeLa whole cell extract

Active E1 was prepared from HeLa whole cell extract by the affinity-chromatography procedure described by Ciechanover et al. (1982), with the following modification; fraction II was concentrated using an Amicon 30 kDa centriprep concentrator in place of $(\text{NH}_4)_2\text{SO}_4$ precipitation.

Ubiquitin-conjugation assays

Ubiquitin-conjugation assays were performed essentially as described (Mayer et al., 1989; Schwartz et al., 1992). Briefly, the reaction mixture contained, in a final volume of 50 μ l, 20 mM Tris/HCl, pH 7.6, 2 mM DTT, 5 mM MgCl_2 , 20 μ g of ox RNAase, 1 μ M 125 I-ubiquitin and pH 9 eluate/crude cell extract/fraction II. In addition, the reaction mixtures were supplemented with either an ATP-regenerating system (+ATP lanes) (i.e. 500 μ M ATP, 10 μ g of creatine kinase, 10 mM phosphocreatine) or an ATP-depleting system (–ATP lanes) (i.e. 50 mM 2-deoxyglucose, 2.5 μ g of hexokinase). After incubation at 37 °C for 30 min, the reaction was terminated by the addition of Laemmli sample buffer. Conjugates were resolved by SDS/PAGE (12.5% acrylamide), and detected by autoradiography.

RESULTS

We have previously used immunofluorescent microscopy to determine the subcellular distribution of E1 in exponentially growing cells (Trausch et al., 1993). Using monoclonal antibody 1C5, we observed that the subcellular distribution of E1 varied within a given population of cells. In order to establish whether this was due to differential localization of E1 with the cell cycle, we used immunofluorescence with 1C5 to assess the subcellular localization of E1 in HeLa cells arrested at various stages of the cell cycle. Figures 1(a)–1(f) are arranged according to the well-

defined stages of the cell cycle. Cells arrested in G0-phase display exclusively cytoplasmic staining of E1, which appears to be cytoskeletal (Figure 1a). In contrast, G1-phase cells show both nuclear and cytosolic E1 (Figure 1b). The nuclear staining is diffuse and extranucleolar. The cytoplasmic staining, although still cytoskeletal, differs from that of resting cells and appears filamentous. The relative staining of E1 changes in cells arrested at the G1/S boundary by aphidicolin (Figure 1c); the nuclear staining becoming less prominent. The cytoplasmic distribution of E1 also alters, with E1 found in a more perinuclear distribution. Negative nuclei and perinuclear staining are also characteristics of S-phase cells (Figure 1d). During G2-phase, E1 was detected throughout the nucleus, except in the nucleoli (Figure 1e), and the cytoplasmic staining was similar to that of late S-phase. Thus the subcellular distribution of E1 changes as a cell progresses through the cell cycle. Examination of exponentially growing HeLa cells revealed the presence of all the cellular staining described above (Figure 1f), thus eliminating the possibility that the subcellular distribution of E1 described in Figures 1(a)–1(e) is an artifact of the treatments used to arrest the cells.

We next determined the distribution of E1 during M-phase. As a portion of E1 colocalizes with microtubules (Trausch et al., 1993), we scanned exponentially growing populations of HeLa cells for mitotic cells rather than arresting the cells with microtubule-disrupting agents such as nocodazole or vinblastine. Figure 2 shows a composite of mitotic HeLa cells as observed by immunofluorescence using 1C5 antibody. During prophase (Figure 2a), E1 is diffusely distributed throughout the cell. During metaphase (Figures 2b and 2c), E1 is localized on the mitotic spindle, but is excluded from the chromosomes. E1 is also uniformly distributed throughout the cytoplasm of the cell. As the cell progresses into anaphase (Figure 2d), the E1 remains

Figure 1 Immunolocalization of E1 throughout the cell cycle of HeLa cells

HeLa cells growing on coverslips were synchronized as described in the Materials and methods section. Cells were subsequently fixed and processed for immunofluorescence using mouse monoclonal antibody 1C5. (a) G0-phase cells; (b) G1-phase cells; (c) G1/S-phase boundary cells; (d) S-phase cells; (e) G2-phase cells; (f) exponential-phase cells.

Figure 2 Immunolocalization of E1 in mitotic cells

Exponentially growing HeLa cells on coverslips were fixed, stained for E1 as described for Figure 1 and scanned for mitotic cells. (a) Prophase; (b) and (c) metaphase; (d) anaphase; (e) and (f) telophase.

associated with the mitotic spindles. The two sets of daughter chromosomes are visible as dark plates against the fluorescent structures. Cells in telophase (Figures 2e and 2f), which show the characteristic cleavage furrow, demonstrate E1 associated with the intracellular bridge. E1 remains associated with the intracellular bridge long after the two daughter cells have become nucleated (Figures 2e and 2f). This pattern of immunofluorescent staining suggests that E1 is not associated with the bulk of chromatin, but is associated with the mitotic spindle throughout mitosis.

It has recently been shown that cyclin A is degraded before breakdown of the nuclear envelope (Minshull et al., 1990). In order for E1 and the ubiquitin system to be instrumental in cyclin A degradation, E1 would have to enter the nucleus while the nuclear lamin is still intact. Alternatively, E1 could activate ubiquitin in the cytoplasm, and the activated ubiquitin bound to E2 could be transported into the nucleus. In order to distinguish between these two possibilities, we used double-label immunofluorescence to detect both E1 and nuclear lamin in cells arrested in the G₂-phase. Figures 3(a) and 3(b) show that in G₂-phase, E1 is localized in the nucleus of cells that have a bright nuclear lamin ring. This suggests that E1 is detectable in the nucleus before the breakdown of the nuclear envelope.

Thus these studies demonstrate the changes in the subcellular compartmentalization of E1 at various stages of the cell cycle. We next sought to determine whether post-translational modifications of E1 within a single compartment regulate the activity of this enzyme. Monoclonal antibody 1C5 recognizes

Figure 3 Immunolocalization of E1 and nuclear lamin during G₂-phase

HeLa cells growing on coverslips were synchronized in G₂-phase, fixed, and double-stained for (a) E1 and (b) nuclear lamin as described in the Materials and methods section.

Figure 4 Analysis of E1 throughout the cell cycle

Whole cell extract was prepared from synchronized cells. The proteins were separated by SDS/PAGE, transferred to nitrocellulose, and probed with 1C5, as described in the Materials and methods section. Lane A, exponential-phase cells; lane B, G₀-phase cells; lane C, G₁-phase cells; lane D, G₁/S-phase cells; lane E, S-phase cells; lane F, G₂-phase cells; lane G, M-phase cells. The arrows denote species of E1. Molecular-mass markers are on the left in kDa.

multiple forms of E1 by immunoblot analysis in a variety of cell lines (Tausch et al., 1993). The cellular distribution of these isoforms, however, is unknown (Figures 1–3) (Schwartz et al., 1992; Tausch et al., 1993). We therefore used immunoblot techniques to identify the E1 isoforms present in whole cell extracts from arrested cells. Proteins from whole cell extracts were separated by SDS/PAGE and immunoblotted with 1C5 antibody. There are at least eight forms of E1 identified in exponentially growing HeLa cells (Figures 4 and 5; results not shown). These forms of E1 are referred to here according to their migration on reducing SDS/polyacrylamide gels, and include E1(150), E1(146), E1(133), E1(126), E1(119), E1(112), E1(97) and E1(73), where E1(112/119) represents the well-documented 'doublet' (Finley et al., 1984; Hatfield and Vierstra, 1989; Handley et al., 1991). Figure 4 shows that some isoforms, for example E1(112/119), are present at all stages of the cell cycle. In contrast, the appearance of forms such as E1(133) are cell-cycle-dependent (compare lanes f and g). This suggests that the appearance of E1 isoforms may be important for the function(s) of this enzyme during the cell cycle.

It is well established that the function of the E1 doublet [E1(112/119)] is to activate ubiquitin. Moreover, these two forms of E1 are easily purified from cell lysates via ion-exchange chromatography (yielding 'fraction II') followed by ubiquitin-affinity chromatography (Ciechanover et al., 1982; Hatfield and

Figure 5 Purification of active E1 from HeLa whole cell extract

Active E1 was purified from whole cell extract by the procedure outlined in the Materials and methods section: lane 1, whole cell extract; lane 2, flowthrough from DEAE-cellulose DE-52 column; lane 3, DE-52 low-salt wash; lane 4, DE-52 eluate (fraction II); lane 5, flowthrough from ubiquitin-Sepharose column; lane 6, ubiquitin-Sepharose salt wash; lane 7, AMP/PP_i-containing eluent; lane 8, DTT-containing eluent. Molecular-mass markers are on the left in kDa.

Vierstra, 1989; Mayer et al., 1989). In order to assess a possible contribution of the other E1 isoforms in ubiquitin activation, fraction II was prepared from HeLa whole cell extracts, and E1 purified by ubiquitin-affinity chromatography. Figure 5 clearly shows that whole cell extracts contain multiple species of E1 (lane 1), and that these forms of E1 bound to the ion-exchange column and were eluted as fraction II (lane 4). The ability of these forms to activate ubiquitin was subsequently tested by ubiquitin-affinity chromatography. When HeLa-cell fraction II was applied to the ubiquitin-Sepharose column [although more E1(112) than E1(119)], a proportion of E1(112/119) bound to the ubiquitin-Sepharose column, consistent with previous studies (Ciechanover et al., 1984), and was eluted by AMP/PP_i (lane 7), and DTT-containing buffer (lane 8). In contrast, E1(126)–E1(150) proteins failed to bind to the affinity column, and were detected in the column flowthrough and wash fractions (lanes 5 and 6). This suggests that only E1 isoforms E1(112) and E1(119) are capable of activating ubiquitin. The nature and function of E1(126)–E1(150) are currently unknown. Although the 1C5 monoclonal antibody recognizes multiple species of E1, two of which are capable of activating ubiquitin, it is not known at

present which of these species are identified in the formaldehyde-fixed cells. Future studies will be directed toward characterization of the biochemical differences in the individual species of E1.

As E1 is autoubiquitinated (Arnold and Gevers, 1990), we considered it possible that some of the E1 isoforms may represent part of an E1–ubiquitin ‘ladder’. To test this hypothesis, we sought to establish whether E1–ubiquitin conjugates could be generated by a system reconstituted *in vitro*. Both rabbit reticulocyte fraction II and a crude preparation of E1, E2s and E3s (pH 9 eluate) supported ATP-dependent ubiquitin-conjugation (results not shown). The predominant high-molecular-mass reaction product in pH 9 eluate was a single ubiquitinated protein, which appears to correspond to E1(119) (Figure 6b, lane 5). Addition of purified E1 resulted in an increase in this monoubiquitinated species of E1 (Figure 6b, lane 7). This did not represent the previously characterized E1–s-ubiquitin, as all samples were subjected to boiling for 10 min at 110 °C in Laemmli sample buffer containing 710 mM 2-mercaptoethanol, a treatment known to cleave the thiol–ester bond (Ciechanover et al., 1982). Similar results were obtained when the experiment was conducted in the presence of rabbit reticulocyte fraction II (Figure 6a). High-molecular-mass conjugates of E1 were not observed in either case (Figures 6a and 6b). These results demonstrate that E1 is monoubiquitinated, but not poly-ubiquitinated.

We next determined the subcellular localization of all of the E1 isoforms at various stages of the cell cycle. Crude cytoplasmic and nuclear fractions were prepared from synchronized HeLa cells. The proteins contained within each fraction were separated by SDS/PAGE, transferred to nitrocellulose, and immunoblotted with 1C5 antibodies. Figure 7 clearly shows that E1 from HeLa cells can be found in the digitonin-soluble cytosol (lane 1), the Nonidet P40-extracted nucleoplasm (lane 2) and the insoluble nuclear skeleton (lane 3). In all cases, the predominant forms of E1 in the cytosol were the E1 doublet [E1(112/119)] and E1(126). Furthermore, these cytosolic forms remained constant throughout the cell cycle (Figures 7a–7f, lanes 1). During M-phase, most forms of E1 were located in the cytosol fraction (Figure 7f, lane 1). The E1 isoforms detected within the nucleoplasm varied considerably according to the cell-cycle stage. In resting cells and during G1-phase, the predominant forms in the nucleoplasm were E1(119) and E1(133) (Figures 7a and 7b, lane 2). As the cells progressed from the G1/S boundary to G2, the E1 pattern

Figure 6 Formation of monoubiquitinated E1

Rabbit reticulocyte fraction II (a) or rabbit pH 9 eluate (b) was incubated with ¹²⁵I-ubiquitin in the presence of an ATP-regenerating (+ATP) or depleting (–ATP) system as described in the Materials and methods section. The reaction mixtures were incubated for 15 min at 37 °C and terminated by boiling in Laemmli sample buffer. Samples (lanes 1–4) were fractionated by SDS/PAGE (7.5% gels), transferred to nitrocellulose, and blotted with 1C5, and bound antibody was detected colorimetrically. Duplicate samples were fractionated by SDS/PAGE, and the ¹²⁵I-ubiquitin conjugates subsequently detected by autoradiography (lanes 5–8). Molecular-mass markers are on the left in kDa. Arrow denotes the ubiquitinated E1 species.

Figure 7 Subcellular distribution of E1 isoforms throughout the cell cycle

Synchronized HeLa cells were fractionated as described in the Materials and methods section. Fractions containing equal amounts of protein were separated by SDS/PAGE, transferred to nitrocellulose, and immunoblotted with 1C5. (a) G0-phase cells; (b) G1-phase cells; (c) G1/S-phase cells; (d) S-phase cells; (e) G2-phase cells; (f) M-phase cells. Lanes 1, cytosolic E1; lanes 2, nucleoplasmic E1; lanes 3, nuclear skeleton-associated E1. Molecular-mass markers are on the left in kDa.

became more complex (Figures 7c–7e, lane 2). There were no forms of E1 present in the nucleoplasm fraction of M-phase cells as predicted (Figure 7f, lane 2). E1 isoforms were also found associated with the nuclear skeleton. E1(133) was identifiable at both the G1/S transition and during G2–M (Figure 7c, lane 3), whereas E1(126) was only present during G1/S (Figures 7e and 7f, lane 3). There were no detectable forms of E1 associated with the nuclear skeleton during G0-, G1- or S-phase (Figures 7a, 7b and 7d, lanes 3). However, immunoblot analysis of cellular fractions suggests that E1 is predominantly cytoplasmic in both G0- and G1-phase, whereas immunofluorescence suggests that E1 is mainly cytoplasmic in G0, but nuclear in G1. It is possible that the epitope recognized by monoclonal antibody 1C5 changes in accessibility during the cell cycle or under different assay conditions. Future studies with epitope-tagged constructs should clarify this issue in the future. Nonetheless, taken together we conclude that both the number and subcellular localization of E1 isoforms are cell-cycle-dependent.

DISCUSSION

We have previously used immunoelectron and immunofluorescent microscopy to describe the subcellular localization of ubiquitin-activating enzyme, E1 (Schwartz et al., 1992; Trausch et al., 1993). We identified both a nuclear pool of E1, associated with the heterochromatin, and a cytoplasmic pool of E1, associated with the endosomal/lysosomal membranes, rough endoplasmic reticulum and cytoskeleton. In the course of these studies, we noted that the subcellular localization of E1 within an exponentially growing population of cells varied. Given that ubiquitination of substrates such as histones and cyclins is cell-cycle-dependent (Bonner et al., 1988; Glotzer et al., 1991), we considered it possible that E1 and/or other components of the ubiquitin system may perform distinct essential roles by localizing to different subcellular compartments within the cell. Two methods have been used in this study to establish the subcellular localization of E1 in HeLa cells during the cell cycle: (i) immunofluorescence microscopy using monoclonal antibody 1C5

and (ii) subcellular fractionation followed by immunoblot analysis with 1C5. Using these approaches, we have identified distinct cell-cycle-dependent changes both in the forms of E1 and their subcellular localization. Although the present studies have utilized a variety of pharmacological agents to arrest cells at various stages of the cell cycle, we recognize that not all aspects of the cell-cycle progression are tightly coupled under these conditions. Future studies will extend the present observations by taking advantage of non-perturbing means of cell synchronization.

We have confirmed and extended previous observations regarding the heterogeneity of E1 (Finley et al., 1984; Mayer et al., 1989; Hatfield and Vierstra 1989, 1993; Trausch et al., 1993). We have identified about eight forms of E1 from HeLa cells, only two of which are able to activate ubiquitin as judged by the ability to bind to ubiquitin–Sepharose in an ATP-dependent manner. The nature and function of E1 heterogeneity are at present unknown. Multiple functions and/or forms have also been demonstrated for other components of the ubiquitin system, e.g. the ubiquitin-carrier proteins (E2s) as a result of multiple genes, each encoding an E2 with a distinct substrate specificity (Jentsch et al., 1990). As E1 mutations are lethal in both yeast and mammalian cells (Finley et al., 1984; Kulkla et al., 1988; Zachsenhaus and Scheinen, 1990; McGrath et al., 1991), it is presumed that E1 is the product of a single gene. Indeed, a single gene encoding E1(112) has been isolated from yeast and human cells (Zachsenhaus and Scheinen, 1990; Handley et al., 1991; McGrath et al., 1991), and maps to the X chromosome (Zachsenhaus and Scheinen, 1990). However, the isolation of multiple E1 genes from wheat germ (Hatfield et al., 1990; Hatfield and Vierstra, 1993), together with our observations that E1 isoforms are not freely interchangeable, raises the possibility that heterogeneity of mammalian E1s may be the result of multiple genes. Indeed, a gene encoding an E1 homologue has recently been identified on the Y chromosome of mice (Kay et al., 1991; Mitchell et al., 1991).

Alternatively, E1 heterogeneity may be a consequence of post-translational modification(s) of a single gene product. The

possibility that the higher-molecular-mass forms of E1 represent polyubiquitinated forms of E1(112) may be excluded. However, our data do support the hypothesis (Finley et al., 1984; Arnold and Gevers, 1990) that E1(119) is a monoubiquitinated form of E1(112). Other monoubiquitin adducts are found intracellularly [e.g. nuclear histones (Bonner et al., 1988)]. The physiological significance of monoubiquitinated adducts in general is not clear, although it is suggested that these species are not rapidly degraded (Finley and Chau, 1991). Our finding that ubiquitinated E1(119) is found predominantly in the nucleoplasm fraction of HeLa cells suggests that such a modification may be necessary for the functioning and/or retention of E1 within the nucleus and/or cytoskeleton.

Our observation that only the E1(112/119) 'doublet' is able to support ubiquitin-conjugating activity raises important questions with regard to the function of the other E1 isoforms. As both the appearance and subcellular localization of the inactive E1 isoforms change as a cell progresses through the cell cycle, it is tempting to speculate that E1 plays a role in cell-cycle control which is unrelated to ubiquitin-activation. E1 is essential for cell-cycle progression, at both the S/G2 transition and M-phase. However, as transfection of the cDNA for a truncated form of E1(112) rescues the E1 mutation in A1S9 cells, and M-phase appears to be dependent on a ubiquitin-activating form of E1 (Zachsenhaus and Scheinen, 1990; Glotzer et al., 1991; Hershko et al., 1991), the role that a non-ubiquitin-activating form of E1 may play is unclear at present. An alternative interpretation is that the high-molecular-mass species of E1 represent inactive forms of E1(112/119). Post-translational modification(s), oligomer formation and/or interaction with inhibitor protein(s) are all possible mechanisms whereby E1(112/119) could be rendered inactive. The conversion of an inactive form of E1 to a ubiquitin-conjugating form could represent an important mechanism whereby ubiquitin-mediated proteolysis is regulated.

Quantitative immunoblot analysis reveals that the cellular abundance of free ubiquitin and E1(112) are approximately equal (Schwartz et al., 1987, 1992). As microinjection studies have revealed that free ubiquitin is equally distributed between the cytosol and the nucleus (Carlson and Rechsteiner, 1987; Carlson et al., 1987), movement of E1 from one subcellular compartment to another may represent a mechanism whereby ubiquitin-conjugating activity is regulated. Indeed, ubiquitin-mediated degradation of the plant photoreceptor, phytochrome, correlates with changes in subcellular localization of the substrate (McCurdy and Pratt, 1986; Jabben et al., 1989). Thus subcellular localization of the components of the ubiquitin system may represent a common mechanism for regulating ubiquitin-mediated proteolysis. The immunofluorescence and immunoblot evidence presented in this study suggests that both the subcellular localization and form of E1 are cell-cycle-dependent. Alternatively, the possibility exists that availability of the IC5 epitope varies with the stage of the cell cycle, as has been observed in other systems (e.g. Turner et al., 1985). However, as discussed above, different assay systems (immunofluorescence, immunoblot of cellular fractions) with non-identical limitations were used in the present study. Future studies using epitope-tagged E1 constructs will clarify these issues. As E1 is known to play a key role in cell-cycle regulation, it is possible that changes in its subcellular localization contribute towards the specific ubiquitination and/or degradation of substrates at precise phases of the cell cycle.

Studies on protein import into the nucleus have revealed that the information required for targeting is encoded in a short polybasic amino acid sequence (Dingwall and Laskey, 1991). The best-studied nuclear-localization sequence is that of the

Simian Virus (SV40) T-antigen. The seven-amino acid sequence -PKKKRKV- is both necessary and sufficient for its nuclear localization. Many nuclear targeting sequences are now thought to be more complex than that of the SV40 T-antigen, and a functional bipartite signal motif has been proposed as another consensus for nuclear targeting (Dingwall and Laskey, 1991). Human E1 contains three polybasic -KKRR-, -SKKRRV- and -SKRKLGR- sequences which resemble the nuclear targeting sequence of the SV40 T-antigen, but lack a bipartite signal motif (Zachsenhaus and Scheinen, 1990; Handley et al., 1991). However, as the nature of E1 heterogeneity is uncertain, and as it is not known which form(s) of E1 are translocated to the nucleus, the mechanism(s) responsible for nuclear import of this enzyme have yet to be elucidated. Mutation analysis and transfection studies are currently underway in our laboratory to assess the contribution of the polybasic sequences in human E1 to nuclear translocation of this enzyme.

The present study provides a framework for further elucidation of the role of the ubiquitin system in the degradation of nuclear proteins during the cell cycle. The mechanisms for intranuclear protein degradation are poorly understood. Several nuclear proteins are rapidly degraded *in vivo* and also serve as substrates for the ubiquitin-mediated system *in vitro* (Ciechanover et al., 1991). Furthermore, Ellison and Hochstrasser (1991) have suggested that the ubiquitin system is responsible for the degradation of the yeast nuclear α_2 repressor. Most recently, Tsuneoka and Mekada (1992) have demonstrated the rapid intranuclear degradation of an abnormally folded nuclear-localized protein in transfected COS cells, presumably mediated by the ubiquitin system. Taken together these studies suggest a potential role for the ubiquitin system in the turnover of nuclear proteins.

We thank Susan Starbuck for preparation of the manuscript, Sharon Bader and Cathy Allen for help with FACS analysis, Tom Steinberg for the use of the microscope, Larry Gerace for anti-lamin and Jonathan Gitlin for constructive criticism. This study was supported by the NIH, Monsanto, and the National Foundation. A.C. is supported by the U.S.-Israel Binational Science Foundation and Israel Cancer Association.

REFERENCES

- Agell, N. and Mezquita, C. (1988) *Biochem. J.* **250**, 883-889
- Arnold, J. E. and Gevers, W. (1990) *Biochem. J.* **267**, 751-757
- Ball, E., Karlik, C. C., Beall, C. J., Saville, D. L., Sparrow, J. C., Bullard, B. and Fyrberg, E. A. (1987) *Cell* **51**, 221-228
- Bonner, W. M., Hatch, C. L. and Wu, R. S. (1988) in *Ubiquitin* (Rechsteiner, M., ed.), pp. 157-172, Plenum Publishing Corporation, New York
- Boyle, W. J., Lampert, M. A., Lipsick, J. S. and Baluda, M. A. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 4265-4269
- Boyle, W. J., Lampert, M. A., Li, A. C. and Baluda, M. A. (1985) *Mol. Cell. Biol.* **5**, 3017-3023
- Burgoyne, P. S., Levy, E. R. and McLaren, A. (1986) (London) **320**, 170-176
- Carlson, N. and Rechsteiner, M. (1987). *J. Cell Biol.* **104**, 537-546
- Carlson, N., Roger, S. and Rechsteiner, M. (1987) *J. Cell Biol.* **104**, 547-555
- Ciechanover, A. and Schwartz, A. L. (1989) *Trends Biochem. Sci.* **14**, 483-488
- Ciechanover, A., Elias, S., Heller, H. and Hershko, A. (1982) *J. Biol. Chem.* **257**, 2537-2542
- Ciechanover, A., Finley, D. and Varshavsky, A. (1984) *Cell* **37**, 57-66
- Ciechanover, A., Digioseppe, J. A., Bereovich, B., Orain, A., Richter, J. D., Schwartz, A. L. and Brodeur, G. M. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 139-143
- Dingwall, V. and Laskey, R. A. (1991) *Trends Biochem. Sci.* **16**, 478-481
- Ellison, M. J. and Hochstrasser, M. (1991) *J. Biol. Chem.* **266**, 21150-21157
- Finley, D. and Chau, V. (1991) *Annu. Rev. Cell Biol.* **7**, 25-69
- Finley, D., Ciechanover, A. and Varshavsky, A. (1984) *Cell* **37**, 43-56
- Glotzer, M., Murray, A. W. and Kirschner, M. W. (1991) *Nature (London)* **349**, 132-138
- Haas, A. L. and Rose, I. A. (1982) *J. Biol. Chem.* **257**, 10329-10337
- Handley, P. M., Mueckler, M., Siegal, N. R., Ciechanover, A. and Schwartz, A. L. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 258-262
- Hatfield, P. M. and Vierstra, R. D. (1989) *Biochemistry* **28**, 735-742
- Hatfield, P. M. and Vierstra, R. D. (1993) *J. Biol. Chem.* **267**, 14799-14803
- Hatfield, P., Callis, J. and Vierstra, R. (1990) *J. Biol. Chem.* **265**, 15813-15817

- Hershko, A. (1992) *Trends Biochem. Sci.* **16**, 265–268
- Hershko, A., Ganoth, D., Pehrson, J., Palazzo, R. and Cohen, L. (1991) *J. Biol. Chem.* **266**, 16376–16379
- Jabben, M., Shanklin, J. and Vierstra, R. D. (1989) *J. Biol. Chem.* **264**, 4998–5005
- Jentsch, S., Seufert, W., Sommer, T. and Reins, H. (1990) *Trends Biochem. Sci.* **15**, 195–198
- Kay, G. F., Ashworth, A., Penny, G. D., Dunlop, M., Swift, S., Brockdorff, N. and Rastan, S. (1991) *Nature (London)* **354**, 486–489
- Kulka, R. G., Raboy, B., Schuster, R., Parag, H. A., Diamond, G., Ciechanover, A. and Marcus, M. (1988) *J. Biol. Chem.* **263**, 15726–15731
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Leung, D., Spencer, S. A., Sachianes, G., Hammonds, G., Collins, G., Henzel, W. J., Barnard, R., Waters, M. J. and Woods, W. I. (1987) *Nature (London)* **330**, 537–543
- Luca, F. C., Shibuya, E. K., Dohrmann, C. E. and Ruderman, J. V. (1991) *EMBO J.* **10**, 4311–4320
- Mayer, A., Gropper, R., Schwartz, A. L. and Ciechanover, A. (1989) *J. Biol. Chem.* **264**, 2060–2068
- McCurdy, D. W. and Pratt, L. H. (1986) *J. Cell Biol.* **103**, 2541–2550
- McGrath, J. P., Jentsch, S. and Varshavsky, A. (1991) *EMBO J.* **10**, 227–236
- Minshull, J., Golsteyn, R., Hill, C. S. and Hunt, T. (1990) *EMBO J.* **9**, 2865–2875
- Mitchell, M. J., Woods, D. R., Tucker, P. K., Opp, J. S. and Bishop, C. E. (1991) *Nature (London)* **354**, 483–486
- Pickart, C. and Rose, I. A. (1985) *J. Biol. Chem.* **260**, 1573–1581
- Pines, J. and Hunter, T. (1989) *Cell* **58**, 833–846
- Schwartz, A. L., Ciechanover, A., Merritt, S. and Turekewitz, A. (1986) *J. Biol. Chem.* **261**, 15225–15231
- Schwartz, A. L., Ciechanover, A., Brandt, R. A. and Geuze, H. J. (1987) *EMBO J.* **7**, 2961–2966
- Schwartz, A. L., Trausch, J. S., Ciechanover, A., Slot, J. W. and Geuze, H. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 5542–5546
- Sheinin, R. (1976) *Cell* **7**, 49–57
- Tobey, R. A., Oishi, N. and Crissman, H. A. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 5104–5108
- Trausch, J. S., Grenfell, S. J., Handley-Gearhart, P. M., Ciechanover, A. and Schwartz, A. L. (1993) *Am. J. Physiol.* **264**, C93–C102
- Tsuneoka, M. and Mekada, E. (1992) *J. Biol. Chem.* **267**, 9107–9111
- Turner, B. M., Davies, S. and Whittfield, W. G. F. (1985) *Eur. J. Cell Biol.* **38**, 344–352
- Yarden, Y., Escobedo, J. A., Kuang, W. J., Yang-Fent, T., Daniel, T. O., Tremble, P., Ehen, E., Ando, M., Harkens, R., Franke, U., Fried, V. and Williams, L. T. (1986) *Nature (London)* **323**, 226–232
- Zachsenhaus, E. and Scheinen, R. (1990) *EMBO J.* **9**, 2923–2929