The ubiquitin-activating enzyme E1 is phosphorylated and localized to the nucleus in a cell cycle-dependent manner*

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The ubiquitin-activating enzyme E1 exists as two isoforms, E1a (117 kDa) and E1b (110 kDa). E1a is phosphorylated, whereas E1b is not. In the present study we have demonstrated the cell cycle dependence of E1a phosphorylation: a 2-fold increase in the specific phosphorylation of E1a in G2 compared with the basal level of phosphorylation in the other stages of the cell cycle. Two-dimensional gel electrophoresis resolved E1 into the two isoforms E1a and E1b; E1a resolved further as three phosphorylated forms and one nonphosphorylated form, while E1b resolved as one nonphosphorylated form. However, the distribution of E1a among these different phosphorylated forms was not cell cycle-dependent. We next evaluated the enzymatic activity of E1 as well as its subcellular localization throughout the cell cycle. 32P-Pyrophosphate exchange activity of E1 did not vary along the cell cycle; however, the amount of ubiquitin-protein conjugates decreased by 50% in G2. Nuclear and cytosolic fractionation of cells revealed the nuclear to cytosolic ratio of phosphorylated E1a was 3-fold greater in G2 compared with the other stages of the cell cycle. Finally, purified nuclear extracts supported E1-dependent ubiquitin conjugation of exogenous substrates as did purified cytosol. However, in nuclear extracts but not in cytosol the amount of E1 activity was rate-limiting. Thus we establish nuclear E1-dependent protein ubiquitination and propose that an increase in phosphorylation of E1a in G2 functions to increase the import and/or retention of E1a in the nucleus and may modulate nuclear protein ubiquitination.

The ubiquitin-proteasome pathway is responsible for the degradation of abnormal, short lived, and regulatory proteins within the cell (reviewed by Ciechanover (1994) and Hochstrasser (1995)). Proteins are selectively targeted for degradation by the covalent attachment of ubiquitin (Ub)1 to an accessible lysine residue on the protein substrate by a multi-enzyme cascade. The initial step requires the activation of ubiquitin, catalyzed by the ubiquitin-activating enzyme (E1). A high energy thiol-ester linkage is formed between E1 and the carboxy-terminal glycine residue of ubiquitin with the production of AMP and PPi. Activated ubiquitin is then transferred to one of a group of enzymes known as ubiquitin-conjugating enzymes or ubiquitin-carrier enzymes (E2s). E2s can then conjugate ubiquitin directly onto the target protein or transfer ubiquitin to one of a group of enzymes, ubiquitin protein ligases (E3), which conjugate ubiquitin to the target substrate. Multiple rounds of ubiquitin conjugation result in the formation of a polyubiquitin chain that is recognized by the 26 S proteasome (Deveraux et al., 1994). This large proteolytic particle (whose catalytic core is the 20 S proteasome) completely degrades the substrate and results in the release of reusable ubiquitin (reviewed by Rivett (1994)).

Several cell lines with temperature-sensitive mutations in enzymes of the ubiquitin pathway result in cell cycle arrest (Finley et al., 1984; Kulka et al., 1988; Goebi et al., 1988), indicating an involvement of this pathway in cell cycle progression. Recent studies have determined specific events that require the participation of the ubiquitin system. Ubiquitin-mediated degradation of mitotic cyclins is required for the exit from mitosis (Glotzer et al., 1991, Hershko et al., 1991, Holloway et al., 1993). Cyclin-ubiquitin conjugates were only observed in mitotic but not interphase extracts (Glotzer et al., 1991). This process requires a novel E2 active throughout the cell cycle and another activity that was found only in mitotic extracts (Hershko et al., 1994). Combined biochemical and genetic approaches have revealed that this mitotic activity is a large 20 S complex that contains Cdc16, Cdc27, and Cdc23 (King et al., 1995; Imrieger et al., 1995). This complex functions as an E3 and is regulated by Cdc2 (Sudakin et al., 1995). In addition to mitotic cyclins, G1 cyclins are degraded by the ubiquitin-proteasome pathway in a p34cdc28-dependent manner (Yaglom et al., 1995; Deshaies et al., 1995). Evidence that the cyclin-dependent kinase inhibitors p27 (Pagano et al., 1995) and p40src1 (Schwob et al., 1994) are degraded by the ubiquitin-proteasome system confirms the importance of this system in cell cycle control.

Since ubiquitin must be activated before it can participate in any downstream reaction, E1 plays a key role in the ubiquitin-proteasome system. Purified human E1 exists as two isoforms; E1a (117 kDa) and E1b (110 kDa) (Cook and Chock 1992; Handley-Gearhart et al., 1994a). Of these isoforms E1a is phosphorylated in vivo (Handley-Gearhart et al., 1994a) on a serine residue (Cook and Chock, 1995); however, E1b is not phosphorylated. The physiological significance of this modification is not known. Analysis using epitope-tagged cDNA constructs of E1 suggests that these isoforms may result from alternative translational start sites in the E1 mRNA (Handley-Gearhart et al., 1994a). Immunofluorescence (Trausch et al., 1993) and immunoelectron microscopy (Schwartz et al., 1992) revealed

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1 The abbreviations used are: Ub, ubiquitin; E1, ubiquitin-activating enzyme; E2, ubiquitin carrier protein; E3, ubiquitin-protein isopeptide ligase; Fr II, rabbit reticulocyte fraction II; IEF, isoelectric focusing; FAC5, fluorescence-activated cell sorting; DTT, dithiothreitol; ATPγS, adenosine 5’-O-(thiotriphosphate).
that E1 is localized to both the nucleus and the cytoplasm. Furthermore, the observation that cell lines mutant for E1 display cell cycle arrest at S/G2 and G2 (Finley et al., 1984; Kulka, et al., 1988) suggests a potential role for nuclear E1 in cell cycle progression. In the present study we examined the phosphorylation of E1 during the cell cycle and the resultant effect on E1 activity and nuclear localization. Our data demonstrate nuclear E1-dependent ubiquitin-protein conjugate formation as well as increased E1 phosphorylation and nuclear localization in G2.

MATERIALS AND METHODS

Reagents—Ub-Sehparose was prepared according to the method of Ciechanover et al., (1982). The anti-E1 rabbit polyclonal antibody was described previously (Handley-Gearhart et al., 1994a). 125I was from DuPont NEN, and [32P]orthophosphoric acid and [35S]methionine-cysteine Translabel were from ICN. [32P]Sodium pyrophosphate was from DuPont NEN. Rabbit reticulocyte lysate fraction II (FrII) was prepared as described earlier (Ciechanover et al., 1978).

Cell Culture and Synchronization Procedure—ts20 cells were cultured in minimal essential medium-a (Life Technologies, Inc.) supplemented with 4.5 g/liter glucose and 10% fetal calf serum and maintained at 30°C and 5% CO2 in a humidified chamber as described previously (Handley-Gearhart et al., 1994b). HeLa cells were cultured in Dulbecco's modified Eagles medium and 10% fetal calf serum and maintained at 37°C and 5% CO2 in a humidified chamber as described previously (Trausch et al., 1993). Exponentially growing cells were arrested at various stages of the cell cycle as summarized in Table 1.

Flow Cytometric Analysis of Cellular DNA Content—Cells (1 × 10⁶) were prepared for fluorescent activated cell sorting (FACS) by washing twice with phosphate-buffered saline and then resuspending in 5 ml of 0.1% sodium citrate containing 0.05 mg/ml propidium iodide, 0.3% Nonidet P-40, and 0.02 mg/ml ribonuclease A (buffer A). The cells were incubated on ice in the dark for 30 min and then resuspended in 1 ml of buffer A and analyzed on a Coulter Elite FACS.

Metabolic Labeling, Immunoprecipitation, and Immunoblot Analysis of E1—Cells were metabolically labeled with [32P]orthophosphoric acid as described previously (Handley-Gearhart et al., 1994a). Cells were lysed in 20 mM Tris, pH 7.6, 0.25% Triton X-100, 0.02% DTT containing 0.2 mM phenylmethylsulfonyl fluoride, 2.5 mM leupeptin, 1 mM pepstatin, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate, and 5 mM sodium fluoride. The lysates were incubated on ice for 20 min and then centrifuged at 14,000 rpm for 15 min. Protein concentrations of cleared lysates were determined in triplicate using the Bio-Rad protein assay (Bio-Rad) with bovine serum albumin as standard. E1 was immunoprecipitated from radiolabeled extracts (500 μg of protein) as described previously (Handley-Gearhart et al., 1994a) but with the following modification. The immunoprecipitation buffer contained the following phosphate inhibitors: 1 mM β-glycerophosphate, 1 mM sodium orthovanadate, and 5 mM sodium fluoride. Samples were resolved on a 7.5% reducing gel and then transferred to nitrocellulose. E1 was visualized using a rabbit polyclonal antibody raised against E1, followed by a peroxidase-conjugated goat anti-rabbit IgG (Handley-Gearhart et al., 1994a). Immunoprecipitated proteins were detected in cys-todinated nitrocellulose using Enhanced chemiluminescence (Amersham Corp.) and quantified using a Molecular Dynamics densitometer. The immunoblot was then exposed in a Molecular Dynamics Phosphorimager cassette, and the radiolabeled proteins were quantified using a Molecular Dynamics densitometer.

Two-dimensional Gel Electrophoresis—Isoelectric focusing (IEF) was performed using a Hoefer GT2 system. IEF tubes (130 × 15 mm) contained 3% acrylamide, 9% urea, 2% Nonidet P40, and 1% am-photates (pH 5–8). Gels were prerun for 30 min at 200 V and then for 30 min at 300 V using 10 mM phosphoric acid as the anolyte and 10 mM sodium hydroxide as the catholyte. ts20 cells were metabolically labeled with [35S]methionine/cysteine Translabel and [32P]orthophosphoric acid as described previously (Handley-Gearhart et al., 1994a). Immunoprecipitated E1 was eluted from protein A beads with IEF sample buffer (9 M urea, 4% Nonidet P-40, 2% amphotates, 1% DTT), loaded onto the IEF gel, and focused for 18 h at 200 V. After the gel was equilibrated in SDS buffer (0.125 M Tris, 2% SDS, 10% glycerol, 4.9 mM DTT, pH 6.8) for 20 min, the IEF gels were placed on a reducing 7.5% SDS-PAGE gel (18 × 16 cm) and electrophoresed for 7 h at 40 mA as the second dimension. The gel was fixed for 30 min and fluorohanced in Amplify (Amersham). The dried gel was then exposed to film for autoradiography at −80°C.

E1 Purification and Pyrophosphate Exchange Assay—E1 was purified from arrested cells with Ub-Sepharose. Arrested extracts (3000 μg of protein) in a final concentration of 57 mM Tris, pH 7.2, 11 mM MgCl₂, 5.7 mM ATP, 0.2 mM DTT were added to 100 μl of Ub-Sepharose beads previously equilibrated in 50 mM Tris, pH 7.2, 1 mM ATP, 0.2 mM DTT, 5 mM MgCl₂ and incubated at ambient temperature for 30 min. The Ub-Sepharose beads were then washed with 1 mM KCl, 50 mM Tris, pH 7.2, and then equilibrated in 50 mM Tris, pH 7.2. Bound proteins were eluted with 50 mM Tris, pH 9, 20 mM DTT, 2 mM AMP, 0.04 mM PPi in a final volume of 100 μl. The eluted proteins were then dialyzed overnight against 50 mM Tris, pH 7.6. Pyrophosphate exchange assays were performed as described before (Ciechanover et al., 1981). The assay was linear to 0.1 μg of purified human E1. All assays were performed in duplicate and were within this linear range.

Ubiquitin Conjugation Activity—Ubiquitin conjugation was measured according to the method of Hershko et al. (1983) with the following modifications. The reaction mixture (i.e. “plus ATP”) contained 100 μg of cell lysate in 50 mM Tris, pH 7.6, 5 mM MgCl₂, 2 mM DTT, 50 pmol of [16C]Ub, 1 mM ATP, 10 μg of creatine phosphokinase, 10 mM creatine phosphate in a final volume of 50 μl. For the “minus ATP” condition, parallel incubations included 2.5 μg of hexokinase and 10 mM 2-deoxyglucose in place of the creatine phosphokinase and creatine phosphate, and the exogenous ATP was omitted. The “plus ATP” reactions were all conducted in triplicate. The reactions were incubated at 37°C for 30 min. The assays were linear for 1 hr. The resulting radiolabeled Ub conjugates were resolved by reducing SDS-PAGE and autoradiography. The dried gel was exposed to film at 80°C, and 125I-labeled ubiquitinated proteins were quantified using a Molecular Dynamics computing densitometer.

Cell Fractionation—Cells were fractionated according to the method of Dignam et al. (1983) with the following modifications. HeLa or ts20 cells were washed twice with phosphate-buffered saline and then resuspended in 5 cell pellet volumes of buffer B (10 mM Hepes, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 1 mM DTT, 0.2 mM phenylmethylsulfonyl fluoride, 2.5 mM leupeptin, 1 mM pepstatin, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate, and 5 mM sodium fluoride). Cells were allowed to swell on ice for 10 min and then briefly pelleted at 14,000 rpm in a refrigerated centrifuge for 5 s. Cells were resuspended in 2 cell pellet volumes of buffer B and dounced with 15 strokes of a “B-type” pestle (Kontes Glassware Corp.). The disrupted cells were briefly pelleted at 14,000 rpm for 5 s, and the supernatant was saved. Cytosol was obtained by centrifuging the supernatant for 30 min at 100,000 × g at 4°C. The pellet from the disrupted cells was washed once in buffer B and then resuspended in 1 cell pellet volumes of buffer B. After centrifugation for 20 min at 14,000 rpm and 4°C the supernatant was discarded. The pellet was resuspended in 2 volumes of 20 mM Hepes, pH 7.9, 25% (v/v) glycerol, 1.5 mM MgCl₂, 0.2 mM EDTA, 420 mM NaCl, 1 mM DTT, 0.2 mM phenylmethylsulfonyl fluoride, 2.5 mM leupeptin, 1 mM pepstatin, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate, and 5 mM sodium fluoride. Contaminating cytosol was determined by centrifugation at 14,000 rpm for 30 min at 4°C. Contamination of cytosolic extract by nuclear proteins was determined by quantification of nuclear histones via immunoblot using a histone pan antibody (Boehringer Mannheim). Routinely no histones were immu-noprecipitated from extracts (i.e. less than 2% contamination of cytosol). Cytosolic protein contamination of nuclear extracts was assessed by determination of lactate dehydrogenase activity. Nuclear extracts contained less than 6% and at times as little as 1% of total lactate dehydrogenase activity.

Purification of Human E1—Baculovirus containing the human E1 cDNA (Handley et al., 1991) was a gift from Dr. Yuval Reiss. Sf9 cells were infected with this virus for 72 h and lysed, and enzymatically active human E1 was purified to homogeneity on a Ub-Sepharose column (Ciechanover et al., 1982).

TABLE 1

| Cell cycle arrest conditions for ts20 cells |
|-------------------------------------------|
| G₁   | 400 μM mimosine, 36 h*  |
| Gₙ/S| 5 μg/ml aphidicolin, 36 h*  |
| S   | 1 mM hydroxyurea, 36 h**  |
| G₂   | 5 μg/ml aphidicolin, 36 h* release into 2 μg/ml bisbenzimide H33342, 36 h*  |
| M   | 3.3 μM nocodazole, 24 h  |

* Treatments were the same for HeLa cells, except incubation times were decreased to either 24 h (* or 36 h (**).
RESULTS

Phosphorylation of E1—While the involvement of the ubiquitin-proteasome pathway in progression through the cell cycle is well documented (Deshaies, 1995), no studies to date have focused on the role of the ubiquitin-activating enzyme in the cell cycle. To address this question we first determined the conditions necessary to arrest ts20 and HeLa cells at specific stages of the cell cycle (Fig. 1). As only E1a is phosphorylated, we evaluated the specific phosphorylation level of E1 through the cell cycle. ts20 cells were arrested and metabolically labeled with [32P]orthophosphoric acid, and E1 was immunoprecipitated from cell lysates with polyclonal anti-E1 antibody. To control for any variations in the amount of E1 during the cell cycle, the immunoprecipitated proteins were resolved by SDS-PAGE and transferred to nitrocellulose, and the total amount of immunoreactive E1 was determined via immunoblot analysis with the polyclonal antibody (Fig. 2A). The nitrocellulose was subjected to autoradiography to determine [32P]incorporation into E1 (Fig. 2B). The amount of E1 is constant throughout the cell cycle and consists of an equal amount of both isoforms, E1a and E1b (Fig. 2A). However, there is an increase in the phosphorylation state of E1a in G2 compared with the basal amount of phosphorylation in the other stages of the cell cycle (Fig. 2B). To determine the specific phosphorylation of E1, the total amount of E1 was quantified using densitometry, and [32P]incorporation was determined by PhosphorImager analysis at each stage of the cell cycle. As seen in Fig. 2C, there was a 2-fold increase in the specific phosphorylation of E1a in G2. The phosphorylation levels of E1 during the cell cycle varied from experiment to experiment; however, the increase at G2 was always between 1.5 and 3-fold greater than the basal level. Similar results have been obtained in HeLa cells (data not shown).

Analysis of E1 by Two-dimensional Gel Electrophoresis—To determine the extent of E1 modification by phosphorylation, the isoforms were resolved by two-dimensional gel electrophoresis. Asynchronously growing ts20 cells were metabolically labeled with either [32P]phosphoric acid or [35S]methionine/cysteine, E1 was immunoprecipitated from cell lysates, and the resultant proteins were separated by isoelectric focusing in the first dimension and by SDS-PAGE in the second dimension (Fig. 3). [35S]-Labeled E1 was resolved into a single spot corresponding to E1b and four spots corresponding to E1a. In the IEF dimension the E1a spots migrated toward the cathode, consistent with a modification by phosphorylation. [32P]-Labeled E1 separated into three discrete spots (Fig. 3, spots 1, 2, and 3), migrating in the same position as [35S]-labeled E1a (Fig. 3, spots 0, 1, 2, and 3). These results suggest that E1a exists in three phosphorylated forms and one nonphosphorylated form and further confirms the lack of modification of E1b by phosphorylation. However, the number and ratio of these [32P]-labeled spots did not vary throughout the cell cycle (data not shown).

Pyrophosphate Exchange Activity of E1 throughout the Cell Cycle—Previous analysis has demonstrated that phosphorylation of E1 in vitro resulted in a small (approximately 2-fold) stimulation in activity (Kong and Chock, 1992). To determine what effect phosphorylation of E1 has on the activity of E1 in vivo, pyrophosphate exchange activity was determined throughout the cell cycle. E1 was purified from arrested ts20 cell extracts using Ub-Sepharose beads. The amount of E1 protein eluting from the Ub-Sepharose beads was quantified by Western blot and densitometry. The purified E1 was assayed for pyrophosphate exchange activity, and the activity was nor-

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**Fig. 1. FACS analysis of arrested ts20 cells.** Asynchronously growing ts20 or HeLa cells were arrested in G1, G1/S, S, G2, and M phase of the cell cycle according to Table I. DNA stained with propidium iodide was sorted using a Coulter Elite FACScan. The FACS profiles of ts20 and HeLa cells were identical.

**Fig. 2. Phosphorylation of E1 throughout the cell cycle.** Arrested ts20 cells were metabolically labeled with [32P]orthophosphoric acid for 5 h. E1 was immunoprecipitated from 500 μg of lysate protein, resolved by SDS-PAGE, and transferred to nitrocellulose. E1 was visualized using a polyclonal antibody against E1 and chemiluminescence (A). The nitrocellulose was then exposed to film to detect [32P]-labeled proteins (B). Total E1 protein and [32P]-labeled E1 was quantified by densitometry and PhosphorImager analysis, and the specific phosphorylation of E1 was determined at each stage of the cell cycle (C).

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**Fig. 3.** Two-dimensional gel electrophoresis of E1. Asynchronously growing ts20 cells were metabolically labeled with [32P]phosphoric acid or [35S]methionine/cysteine, E1 was immunoprecipitated from cell lysates, and the resultant proteins were separated by isoelectric focusing in the first dimension and by SDS-PAGE in the second dimension (Fig. 3). [35S]-Labeled E1 was resolved into a single spot corresponding to E1b and four spots corresponding to E1a. In the IEF dimension the E1a spots migrated toward the cathode, consistent with a modification by phosphorylation. [32P]-Labeled E1 separated into three discrete spots (Fig. 3, spots 1, 2, and 3), migrating in the same position as [35S]-labeled E1a (Fig. 3, spots 0, 1, 2, and 3). These results suggest that E1a exists in three phosphorylated forms and one nonphosphorylated form and further confirms the lack of modification of E1b by phosphorylation. However, the number and ratio of these [32P]-labeled spots did not vary throughout the cell cycle (data not shown).
phosphate exchange activity would have been expected in G2. How-
stimulated the activity of E1, then an increase in pyrophos-
malized for E1 protein (Table II). If phosphorylation in vivo
stabilized the activity of E1, then an increase in pyrophos-
phorImager analysis (Fig. 4).

Cohen, 1991) had no effect on the amount of ubiquitin conju-
gation, one would predict that in G2 where there is maximal
phosphorylation a different distribution of E1a would be ob-
lished for HeLa cells. Nuclear protein contamination of the

Conjugation Activity through the Cell Cycle—The effect of E1
phosphorylation on ubiquitin-protein conjugate formation was
determined at each stage of the cell cycle. Arrested extracts of
ts20 cells were assayed for their ability to conjugate 125I-Ub to
dogenous proteins in the presence of either an ATP-regener-
ating or -depleting system. 125I-Ub conjugates were only seen
in the presence of ATP (Fig. 4A) and were quantified by Phos-
phorImager analysis (Fig. 4B). The amount of Ub conjugates
was relatively similar throughout the cell cycle apart from G2,
in which there was a decrease of approximately 50% (Fig. 4B).
The exact amount of conjugates varied among different experi-
ments. However, in G2 they were always at a minimum. The
levels of ubiquitin conjugates are dependent on their rate of
formation (requiring E1, E2, and E3) and disassembly (requir-
ing the 26 S proteasome and/or isopeptidases). The addition of
ATP-ßS (which inhibits 26 S proteasome activity) (Johnson and
Cohen, 1991) had no effect on the amount of ubiquitin conju-
gates formed (data not shown).

Distribution of Phosphorylated E1a in Nuclear and Cytosolic
Extracts—Previous analyses have demonstrated that phos-
phorylation of residues adjacent to a nuclear localization sequence
can affect the localization of a protein (Tagawa et al., 1995; Rihs
et al., 1991). If the phosphorylation of E1a effects its localiza-
tion, one would predict that in G2, where there is maximal
phosphorylation a different distribution of E1a would be ob-
served compared with other stages of the cell cycle. In order to
address this question a cell fractionation protocol was estab-
lished for HeLa cells. Nuclear protein contamination of the
cytosolic extract was assessed via Western blot analysis for
histones. Cytosolic protein contamination of nuclear extract
was assessed by lactate dehydrogenase activity. HeLa cells
arrested in G1, S, and G2 phase were metabolically labeled with
32P j phosphoric acid and fractionated as described under “Ma-
terials and Methods.” M phase cells could not be fractionated,
as nocodazole arrests the cells after nuclear membrane break-
down. To compare the amount of E1 in nuclear and cytosolic
extracts, equal proportions of these extracts were used. E1 was
immunoprecipitated, resolved by SDS-PAGE, and transferred
to nitrocellulose. The total amount of E1 was determined by
Western blot analysis (Fig. 5A). The nitrocellulose was then
exposed to film to determine the 32P incorporation (Fig. 5B).

The ratio of E1a to E1b is equal and constant in G1, S, and G2
cytosolic extracts. However, in the nuclear extracts there is
10-fold less E1b than E1a (Fig. 5A); thus, the nuclear extract is
deficient in E1b. The amount of total E1 (expressed as µg/mg of
protein) in cytosolic extracts is approximately 4-fold more than
in nuclear extracts in all of the stages of the cell cycle (when
corrected for cytosolic contamination of the nuclear extract (see
Fig. 5 legend)). When the specific phosphorylation of E1a is
quantified in both nuclear and cytosolic extracts and the ratio
is determined (Fig. 5C), there is approximately 3-fold more
phosphorylated E1a in nuclear than in cytosolic extracts in the
G2 phase compared with G1 or S.

Ubiquitin Conjugation Activity in Nuclear and Cytosolic Ex-
tracts—The amount of E1 (on a protein basis) is 4-fold less in
nuclear extracts than in cytosolic extracts (Fig. 5A); this im-
plies that the ubiquitin activation potential in the nuclear
extracts may be decreased if E1 is limiting. To determine if
nuclear extracts have a decreased ubiquitin activation poten-
tial, we compared the ubiquitin conjugation activity in reticu-
locyte lysate fraction II (Fr II) (Ciechanover et al., 1981) and

![Fig. 3. Two-dimensional gel electrophoresis of E1.](image)

![Fig. 4. Ubiquitin conjugation activity throughout the cell cycle.](image)

| Specific pyrophosphate exchange activity | units |
|---|---|
| G1 | 0.148 ± 0.001 |
| S | 0.094 ± 0.023 |
| G2 | 0.135 ± 0.049 |
| M | 0.115 ± 0.021 |

Table II

Pyrophosphate exchange activity of E1

E1 was purified on a Ub-Sepharose column from arrested ts20 ex-
tracts (3000 µg of protein) and was subsequently assayed for
[32P] pyrophosphate exchange activity as described under “Materials and

Methods.” The amount of E1 in each purification was quantified by
immunoblot analysis and densitometry and then used to determine
the specific pyrophosphate exchange activity (units are expressed as
percentage of [32P] pyrophosphate exchange/densitometrically quantified
E1 protein). Results are presented as mean ± S.D. (n = 3).

Endogenous proteins in the presence of either an ATP-regener-
ating or -depleting system. 125I-Ub conjugates were only seen
in the presence of ATP (Fig. 4A) and were quantified by Phos-
phorImager analysis (Fig. 4B). The amount of Ub conjugates
was relatively similar throughout the cell cycle apart from G2,
in which there was a decrease of approximately 50% (Fig. 4B).
The exact amount of conjugates varied among different experi-
ments. However, in G2 they were always at a minimum. The
levels of ubiquitin conjugates are dependent on their rate of
formation (requiring E1, E2, and E3) and disassembly (requir-
ing the 26 S proteasome and/or isopeptidases). The addition of
ATP-ßS (which inhibits 26 S proteasome activity) (Johnson and
Cohen, 1991) had no effect on the amount of ubiquitin conju-
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Cohen, 1991) had no effect on the amount of ubiquitin conju-
gates formed (data not shown).

The amount of ubiquitin conjugation activity in reticu-
locyte lysate fraction II (Fr II) (Ciechanover et al., 1981) and
FIG. 5. Nuclear and cytosolic fractionation of 32P-labeled E1. HeLa cells arrested in G1, S, and G2 phase were metabolically labeled with [32P]orthophosphoric acid for 7 h. Cells were then fractionated as described under "Materials and Methods." The cytosolic contamination of G1, S, and G2 nuclear extracts, as determined by lactate dehydrogenase activity, was 6, 3, and 4%, respectively. No nuclear contamination was observed in the cytosolic extracts. E1 was immunoprecipitated from an equal portion of the nuclear and cytosolic extracts, resolved by SDS-PAGE, and transferred to nitrocellulose. The total amount of E1 was quantified by immunoblot analysis (A). The nitrocellulose was exposed to film to detect 32P-labeled E1 (B). Immunoreactive and 32P-labeled E1 were quantified by densitometry, and the specific phosphorylation of nuclear and cytosolic E1 in G1, S, and G2 was determined. The partition of phosphorylated E1 in nuclear and cytosolic extracts was then expressed as a ratio (C).

nuclear and cytosolic extracts. Equal amounts of Fr II, nuclear, or cytosolic extract (150 μg of protein) were assayed for conjugation activity as described under "Materials and Methods." 125I-Ub protein conjugates were resolved by SDS-PAGE and quantified by densitometry. The upper panel in Fig. 6A shows 125I-Ub conjugates generated from Fr II, nuclear, and cytosolic extracts; 30-fold more of the nuclear conjugation reaction was loaded compared with that of cytosolic extract in order to visualize the nuclear Ub conjugates. Thus, the ubiquitin activation potential in nuclear extract was substantially lower than Fr II and cytosolic extract. To address whether the low ubiquitin activation potential in nuclear extract was because E1 was limiting we added purified human E1 (0.1 or 1 μg) to each of the conjugation reactions (Fig. 6A, middle and lower panels). The addition of 1 μg of E1 represents a 138 or 46% increase in the total E1 in either cytosolic or nuclear extracts, respectively. The relative amounts of 125I-Ub conjugates in Fr II, cytosolic, and nuclear extracts are shown in Fig. 6B. There is an 11-fold increase in the amount of 125I-Ub conjugates generated in the nuclear extract compared with a 3-fold increase in cytosolic extract with the addition of 1 μg of purified human E1. Fr II showed essentially no increase in 125I-Ub conjugates with the addition of exogenous E1. This increase in 125I-Ub conjugates from the nuclear extract was consistently between 9 and 11-fold, and that of the cytosolic extract was between 1 and 3-fold. Leakage of E1 from nuclear extract does not account for these observations because immunoblot analysis could not detect any E1 in the various washes during the cell fractionation procedure. Control assays using added polyclonal rabbit-anti E1 antibody confirmed the E1 dependence of the generated 125I-Ub conjugates (data not shown). Thus, these observations demonstrated that the decreased ubiquitin activation potential in nuclear extract is because E1 is limiting.

DISCUSSION

In the present study we have analyzed the phosphorylation state, activity, and subcellular localization of the ubiquitin-activating enzyme, E1, during progression through the cell cycle. The total amount of E1 is constant in the different phases of the cell cycle. This observation is indeed expected for a protein with a half-life of approximately 20 h.2 In addition, the levels of the two E1 isoforms (E1a and E1b) do not vary during the cell cycle (Fig. 2A). However, there is a 2-fold increase in the specific phosphorylation of E1a in G2 (Fig. 2C). No phosphorylation of E1b was observed, consistent with previous observations (Handley-Gearhart et al., 1994a; Cook and Chock 1995).

Analysis of E1 from asynchronously growing cells by twodimensional gel electrophoresis separated E1b as a distinct species that was not phosphorylated (Fig. 3). E1a separated into one nonphosphorylated form and three phosphorylated forms (Fig. 3, spots 0, 1, 2, and 3, respectively). In asynchronously growing cells more 35S-labeled E1a migrates as spots 2 and 3, indicating that it is in the more highly phosphorylated forms. The increase in phosphorylation of E1a in G2 could result from either a general increase in phosphorylation at all sites or from a cell cycle-specific phosphorylation event. If the

2 A. L. Schwartz, unpublished results.
latter were the case one would expect to observe an increase in the amount of E1a migrating as one of the phosphorylated forms. However, no difference was observed throughout the cell cycle in the pattern of the phosphorylated forms of E1a. Thus, the increase in E1a phosphorylation at G2 most probably is a result of a general increase in phosphorylation at all available sites.

The consequence of increased phosphorylation of E1 could be a change in the activity of E1, a change in the specificity of E1 for certain E2s, or a change in the subcellular localization of E1. Pyrophosphate exchange assays performed on E1 purified from cell extracts at different stages of the cell cycle did not reveal any correlation between phosphorylation of E1 and activity (Table II). To determine the role of E1 phosphorylation on ubiquitin conjugate formation, arrested cell extracts were assayed for $^{125}$I-ubiquitin conjugation activity (Fig. 4). A decrease in Ub conjugate levels to 50% was observed in G2 (where E1 is maximally phosphorylated). This was unexpected, as no change in E1 activity determined by pyrophosphate exchange was observed. However, levels of Ub conjugates are dependent on both their formation (requiring E1, E2, and E3) and disassembly (either by 26 S proteasome and/or isopeptidases). The 26 S proteasome did not appear to be responsible for the decrease in Ub conjugates in G2 as inclusion of ATP-$\gamma$S in the assay did not alter the amount of Ub conjugates detected (data not shown). Since a multienzyme process is required for generating the steady state levels of Ub conjugates it is not possible to determine with assurance which component is responsible for the 50% decrease in Ub conjugates in G2. Mahaffey et al. (1993) did not detect any differences in Ub conjugation, 26 S proteasome, or isopeptidase activity in Xenopus egg extracts throughout the cell cycle. However, these different observations in Ub conjugate formation may reflect differences in the somatic and early embryonic cell cycle. In addition, whereas the early embryonic cycle consists of alternate DNA synthesis and mitosis, the interphase of somatic cells is divided into discrete phases and represents a more tightly controlled system.

To delineate changes in subcellular localization in phosphorylated E1, HeLa cells were fractionated into cytosolic and nuclear extracts (Fig. 5). The ratio of specific phosphorylated E1 in nuclear to cytosolic extracts in G2 was 3-fold that of G0 or S (Fig. 5C). These observations suggest that phosphorylation of E1a may stimulate its transport to and/or retention within the nucleus. The rate of nuclear protein accumulation may be enhanced via phosphorylation of a casein kinase II site proximal to the nuclear localization sequence in E1. Thus, it is tempting to speculate that phosphorylation at these residues is responsible for the increase in phosphorylated E1a in nuclear extracts. Studies addressing this possibility are currently under way.

The concentration of E1 in nuclear and cytosolic extracts of HeLa cells is 4.8 $\mu$g/mg of total protein and 14.5 $\mu$g/mg total protein, respectively, as determined by our cell fractionation studies. Thus, approximately 20% of total cellular E1 is localized in the nucleus, and 80% is localized in the cytosol. This is similar to the distribution found using immunoelectron microscopy in HepG2 cells, which demonstrated that the nuclear and cytoplasmic distribution was 37 and 56%, respectively (Schwartz et al., 1992). This difference probably reflects differences in the cell types as well as limitations in quantification following cell fractionation or immunoelectron microscopy. Such a difference in the amount of E1 in nuclear and cytosolic extracts suggests that these intracellular pools may have different Ub conjugation activity. Nuclear extracts displayed a much lower capacity for generating $^{125}$I-ubiquitin-protein conjugates than cytosolic extracts (Fig. 6). This relative deficiency was abrogated by the addition of exogenous human E1. Thus, there is a decreased ubiquitin activation potential in the nuclear extract in which E1 is rate-limiting.

The increase in E1 phosphorylation in G2 may function to increase the ubiquitin activation potential within the nucleus. Currently no G2-specific events that require ubiquitination have been described, although removal of the DNA synthesis machinery, which is localized to the nucleus, may be required for progression to mitosis. Additionally, events such as centrosome migration and chromosome condensation or nuclear membrane disassembly prior to mitosis may require protein ubiquitination.

Alternatively, E1a and E1b may have different specificities for distinct E2s. For example phosphorylated E1a may be required to interact with an exclusively nuclear E2 linked to specific mitotic events (Seufert et al., 1995). If phosphorylated E1 interacts only with a specific subset of nuclear E2s this may explain the decreased conjugation activity in the G2 phase of the cell cycle. In order to address these hypotheses directly, additional studies are required. For example, identification of the sites of phosphorylation on E1a and their subsequent mutation and analysis will aid in defining the precise function of E1 phosphorylation within the nucleus.

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E1 Is Phosphorylated in a Cell Cycle-dependent Manner

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