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Ubiquitin-activating enzyme, E1, is phosphorylated in mammalian cells by the protein kinase Cdc2

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SUMMARY

The ubiquitin-activating enzyme (E1) is the first enzyme in the pathway leading to formation of ubiquitin-protein conjugates. E1 was found to be phosphorylated in cells of a mouse mammary carcinoma cell line, FM3A. Peptide mapping of trypsin digests of labeled E1 indicated that two oligopeptides were mainly phosphorylated in vivo. The same oligopeptides were also labeled in vitro on Cdc2 kinase-mediated phosphorylation of E1, affinity-purified from the same cell line. The Cdc2 kinase is a key enzyme playing a pivotal role in G_2/M transition in the cell cycle. The phosphorylation of one of the two oligopeptides was prominent at the G_2/M phase of the cell cycle, and dependent upon the Cdc2 kinase activity in vivo since it was significantly reduced in tsFT210, a mutant cell line deficient in Cdc2 kinase. Mutation analysis indicated that the serine residue at the fourth position of the E1 enzyme was a phosphorylation site of Cdc2 kinase. These findings suggest that E1 is a target of Cdc2 kinase in the cell, implying that the ubiquitin system may be dynamically involved in cell cycle control through phosphorylation of this key enzyme.

Key words: ubiquitin-activating enzyme, Cdc2 kinase, cell cycle control

INTRODUCTION

Ubiquitin, a highly conserved protein with 76 amino acid residues, is present in all eukaryotic cells and is covalently ligated to various target proteins (Hershko, 1991; Hershko and Ciechnover, 1992; Jentsch, 1992a,b; Rechsteiner, 1991). The conjugation of ubiquitin to proteins involves multiple enzymatic reactions. First, the C-terminal glycine of ubiquitin is adenylated by the ubiquitin activating enzyme (E1) in the presence of ATP, and forms a thiolester bond with a Cys residue of E1. The activated ubiquitin is then transferred to one of a family of ubiquitin-conjugating enzymes (E2s), which recognize specific substrates, and finally donates the ubiquitin to a lysine residue(s) of the substrates. Another enzyme, one of the ubiquitin ligases (E3s) is involved in a subset of the final conjugation step. Ubiquitin conjugation is thought to be an essential step in a major cellular proteolytic pathway because proteins tagged with ubiquitin are rapidly degraded ATP-dependently by the proteasome, a huge complex of proteases (Matthews et al., 1989; Tanaka et al., 1988, 1992). In addition to its function in selective removal of abnormal or denatured proteins, the ubiquitin-conjugating system is also involved in other diverse cellular functions, including the stress response, DNA repair, chromosome condensation and decondensation, and cell cycle control (Hershko and Ciechnover, 1992; Jentsch, 1992b), presumably via proteolytic breakdown of essential proteins associated with these processes.

A series of key proteins regulating cell proliferation or cell cycle progression have been shown to be ubiquitinated. Oncoproteins such as N-Myc, c-Myc, c-Fos and p53 were reported to be degraded by a ubiquitin-mediated pathway(s) at least in a reticulocyte lysate (Ciechanover et al., 1991). An increased level of mitotic promoting factor (MPF) at the onset of mitosis requires the synthesis of cyclin B, and the subsequent degradation of cyclin B is apparently responsible for the inactivation of MPF at the metaphase-anaphase transition (Murray et al., 1989; Nurse, 1990). Glotzer et al. (1991) showed that the programmed and rapid degradation of cyclin B was mediated by a ubiquitin-dependent proteolytic pathway in *Xenopus* oocyte extracts. Mos, the c-*mos* proto-oncogene product, has been shown to be degraded by the proteasome in a ubiquitin-dependent fashion during its rapid turnover in an early stage of meiotic maturation of *Xenopus* oocytes (Ishida et al., 1993; Nishizawa et al., 1993). Thus, the conjugation of ubiquitin is associated with various steps that are essential for cell growth or cell-cycle progression. However, the molecular basis of the specificity and timing during the cell cycle of ubiquitin conjugation to target proteins are largely unknown.

Mutations of E1 that block steps of ubiquitin-conjugation are lethal to cells, in some cases arresting them at specific

stages of the mitotic cell cycle. A temperature-sensitive (ts) mutant of mouse mammary carcinoma cell line FM3A, named ts85, has been shown to be defective in E1 and to be arrested in the late S to G_2 period of the cell cycle at the restrictive temperature (Finley et al., 1984; Mita et al., 1980). Recently, other types of E1 mutants of mammalian cell lines have also been reported. Although they belong to the same complementation group, some of these mutants are arrested predominantly at the S phase (Ayusawa et al., 1992; Mori et al., 1993; Zacksenhaus and Sheinin, 1990), while others are arrested at the G_1, S and G_2/M phases under restrictive conditions (Yamao et al., 1993), indicating that multiple steps of the cell cycle are tightly regulated by ubiquitination of specific proteins involved in cell-cycle progression. No mammalian cell lines with mutations of E2 are yet available, but more than 10 genes for a variety of E2s have been identified in yeast (Jentsch, 1992b). Two genes among them were found to be associated with specific cell cycle control. *UBC3*/*CDC34*, a gene in *Saccharomyces cerevisiae* conferring cell cycle progression at the G_1/S boundary was proved to encode one of the family of E2s (Goebl et al., 1988). On the contrary, a disruption of another cell cycle E2 gene, *UBC9*, was reported to result in arrest at the G_2 phase (Jentsch, 1992a,b; Seufert et al., 1995). Thus, independent ubiquitin pathways via distinct E2s play diverse roles in ubiquitin conjugation in cell-cycle control. This raises the issue of how E1 recognizes the specific E2 at a distinctive point of the cell cycle to activate the particular ubiquitin pathway.

A variety of mammalian cyclins and associated kinases have been identified to form a family of cyclin-dependent protein kinases (Cdks) (Pines, 1993). The transitions of the critical points in the cell cycle are controlled by the Cdks. Cdc2 is required for the G_2/M transition, and its kinase activity peaks during mitosis (Lee and Nurse, 1987; Riabowol et al., 1989). The G_1/S transition requires another Cdk, Cdk2, the kinase activity of which rises in the late G_1 or early S phase and declines during mitosis (Pagano et al., 1993; Tsai et al., 1993). Recently, more Cdks have been isolated, including Cdk3, Cdk4 and Cdk5, the functions of which are not known but are widely thought to be regulating progression through particular parts of the cell cycle. It is of great interest to find out not only which cyclin associates with which kinases and its timing, but also the in vivo substrates of the kinases. To investigate the coupling of the ubiquitin system with cell cycle control by cyclin-dependent kinases, in this study we examined the Cdc2 kinase-mediated phosphorylation of the ubiquitin-activating enzyme, E1.

MATERIALS AND METHODS

Cell lines, culture and labeling of cells

FM3A, a mouse mammary carcinoma cell line, and its derivative, tsFT210 (Th'ng et al., 1990; Yasuda et al., 1991), a ts mutant of Cdc2 kinase, were used. Cells were cultured in suspension at 33.5°C in growth medium (α minimum essential medium (αMEM; Gibco) supplemented with 7.5% (v/v) fetal bovine serum (FBS; Hazelton Biologics Inc., Lenexa, KS) and streptomycin at 200 μg/ml) in a humidified atmosphere containing 5% CO_2. For labeling with ^{32}P, 1.5×10^7 exponentially growing cells were incubated at 33.5°C for 6 hours in 40 ml of labeling medium (phosphate-free αMEM with 8% dialyzed FBS and 0.1 mCi/ml [^{32}P]orthophosphate (9,000 Ci/mmol, DuPont-NEN)). For arrest of wild type cells for labeling at the G_1/S, or at the M phase, they were cultured at 33.5°C in growth medium containing 1 μg/ml aphidicolin (Sigma), and 0.5 μg/ml nocodazole (Sigma), respectively, for 10 hours. They were washed by centrifugation and suspended at a cell density of 3×10^5 cells/ml in 20 ml of labeling medium with the respective drug and incubated at 33.5°C for 5 hours. For labeling M phase-arrested tsFT210 cells, 6×10^6 cells grown at 33.5°C for 16 hours in 20 ml of growth medium containing 1 μg/ml aphidicolin were released from G_1/S arrest by washing out the drug, and incubated at 33.5°C for 3.5 hours. After changing to labeling medium supplemented with 0.2 μM monobasic sodium phosphate and 0.06 μg/ml colcemid, half the cells were incubated at 33.5°C and half at 39°C for 8 hours.

Purification of E1 from mouse cells

E1 was purified from FM3A cells essentially as described by Haas and Bright (1988). Cells (5×10^9) were disrupted by homogenization in buffer consisting of 20 mM *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid] (HEPES), pH 7.5, and 0.25 mM dithiothreitol (DTT), and the lysate was centrifuged for 1 hour at 100,000 ***g*** at 4°C. The resulting S100 extract was applied to a DEAE-cellulose column, and materials in the 0.5 M KCl eluate was precipitated with 25%-85% ammonium sulfate. The precipitated proteins were suspended in 20 mM Tris-HCl, pH 7.5, containing 1 mM DTT, dialyzed against the same buffer, and then applied to a ubiquitin-Sepharose column (18 mg ubiquitin/ml resin). E1 was eluted with a solution of 50 mM Tris-HCl, pH 7.2, containing 2 mM AMP and 0.04 mM sodium pyrophosphate. The fractions containing activity for ubiquitin-dependent ATP-PP_i exchange were pooled and used as purified E1.

Immunoprecipitation of E1

Rabbit antiserum against human E1 was a gift from M. Rechsteiner, University of Utah School of Medicine (Deveraux et al., 1990). Cells ($1\text{-}2\times10^7$) were lysed in RIPA buffer composed of 10 mM Tris-HCl, pH 8.0, 0.14 M NaCl, 0.1% sodium dodecyl sulfate (SDS), 1% Triton X-100, 1% sodium deoxycholate, 20 mM sodium fluoride and 1 mM phenylmethylsulphonyl fluoride (PMSF), and centrifuged at 15,000 ***g*** at 4°C for 30 minutes. The resulting cell extract was pre-adsorbed with Protein A-Sepharose CL-4B (Pharmacia), then incubated with antiserum at 4°C for 1.5 hours. The immunocomplex was precipitated with Protein A-Sepharose CL-4B, and washed twice with RIPA buffer.

Expression of E1 proteins in *E. coli*

A mouse cDNA for ubiquitin-activating enzyme was described previously (accession no. D10576 for DDBJ, GenBank and EMBL data base) (Imai et al., 1992). Its complete coding sequence was ligated to the initiation codon for gene 10 in an expression vector, pET3b, to obtain pET-MuE1. This plasmid expressed the complete sequence of the E1 protein in *E. coli* BL21pLys cells in the presence of 2 mM isopropylthio-β-D-galactoside (IPTG) with the solubilized form in the cytoplasm. This protein was partially purified from an *E. coli* extract by stepwise elution with 0.2 M KCl from a DEAE-cellulose column, followed by separation with protein-pak G-DEAE (Waters). Two types of mutations, serine to alanine at the 4th position (Ala4) and serine to glycine at the 835th position (Gly835), were generated by primer-directed mutagenesis by polymerase chain reaction (Ito et al., 1991). Two deletions, *del*1 (lacking Leu194-Gly654 of the E1 sequence) and *del*2 (Arg119-Gly654), were made using the restriction sites for *Aat*I and *Eco*NI. pET-MuE1 carrying *del1* or *del2* produced recombinant proteins, which were purified by continuous and preparative polyacrylamide gel electrophoresis (PrepCell, Bio-Rad).

Preparation of Cdc2 kinase and in vitro phosphorylation

Anti-Cdc2 antibody raised against the C-terminal peptide of human $p34^{cdc2}$ was purchased from Gibco-BRL. Cells of FM3A or its deriv-

ative, tsFT210, were grown in growth medium containing 0.1 μg/ml nocodazole at 33.5°C for 16 hours, and were arrested in the mitotic phase. They were suspended at a cell density of 2×10^7 cells/ml in hypotonic buffer composed of 10 mM Tris-HCl, pH 7.5, 3 mM $MgCl_2$ and 1 mM PMSF, and stood on ice for 10 minutes. Nonidet P-40 (NP40) was added to 0.1%, and the mixture was centrifuged at 400 ***g*** for 10 minutes (Yasuda et al., 1990). The precipitate containing crude nuclei was suspended in 0.5 ml of hypotonic buffer with 0.4 M NaCl and 0.1% Brij-35, incubated at 4°C for 30 minutes, and centrifuged at 10,500 ***g*** for 10 minutes. The supernatant nuclear extract was diluted threefold with hypotonic buffer, resulting in a concentration of 0.13 M NaCl. The solution was pre-adsorbed with Protein A-Sepharose CL-4B, and incubated with anti-human Cdc2 antibody or rabbit IgG at 4°C for 1.5 hours. The immunocomplex was precipitated with Protein A-Sepharose CL-4B, and washed twice with the hypotonic buffer. The phosphorylation was carried out at 30°C for 30 minutes in 20 μl of kinase buffer (20 mM Tris-HCl, pH 7.4, 4.5 mM β-mercaptoethanol, 1 mM ethyleneglycol bis[2-aminoethyl ethel] tetraacetic acid (EGTA), 10 mM $MgCl_2$, 50 μM ATP) containing 10 μCi of [γ-^{32}P]ATP (3,000 Ci/mmol; Dupont-NEN), protein substrate (1 μg) and the immunopurified Cdc2 kinase. The reaction was stopped with 2× Laemmli sample buffer, and the mixture was subjected to 7.5% or 12% SDS-polyacrylamide gel electrophoresis (PAGE) (Laemmli, 1970), followed by autoradiography with image analyzer, Fujix Bas2000.

Peptide mapping

Tryptic phosphopeptide mapping was carried out by the method of Moll et al. (1991) with a slight modification. The region containing the E1 protein was cut out from the dried gel after autoradiography, rehydrated and washed with 10% methanol. Protein in the lyophilized gel slice was oxidized in performic acid for 1 hour at 4°C, and lyophilized. The protein was digested with 20 μg of TPCK(L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone)-treated trypsin (Worthington) in 0.2 ml of 50 mM ammonium bicarbonate at 25°C for 24 hours. The lyophilized digests were resolved in H_2O and separated on a thin-layer cellulose plate (Merk) by electrophoresis at 50 V/cm for 28 minutes in 1% ammonium carbonate in the first dimension, and chromatography with the solvent *n*-butanol/pyridine/acetic acid/H_2O (75:50:15:60) in the second dimension.

Phosphoamino acid analysis

^{32}P-Labeled E1 proteins were eluted from dried SDS-gel and treated with 6 M HCl at 110°C for 2 hours. The hydrolyzed samples were then separated by electrophoresis as described by Jonathan et al. (1983).

Flow cytometry

Cells (2×10^6) were fixed with 85% (v/v) ethanol, treated with DNase-free RNase A (2 mg/ml), and stained with 0.05% propidium iodide. Samples of 20,000 cells were analyzed with a FACScan (Becton Dickinson).

RESULTS

E1 is phosphorylated in cells

To determine if the ubiquitin-activating enzyme E1 is phosphorylated in vivo, we immunoprecipitated E1 with the rabbit antiserum raised against human E1 from a whole extract of FM3A cells that had been labeled with ^{32}P-orthophosphate. The antiserum against human E1 reacted with the mouse enzyme, which is understandable since the amino acid sequences of the two E1's are highly homologous, showing more than 95% identity as deduced from their cDNA sequences (Imai et al., 1992). The antiserum mainly recognized and precipitated two of the total cellular proteins (data not shown), the sizes of which corresponded to those of the two E1 isoforms (105 kDa and 113 kDa forms; see next section). Only one of them, however, was found to be phosphorylated in the cells (Fig. 1A, lane 1), which comigrated in the SDS gel with the phosphorylated 113 kDa form of E1 (data not shown). Addition of mouse E1 that was purified from FM3A cells (see next section) to the labeled extract before immunoprecipitation competitively abolished the precipitation of the labeled 113 kDa protein (Fig. 1A, lane 2). These results indicated that one (the 113 kDa form) of the two isoforms of E1 is exclusively phosphorylated in the cells. E1 was phosphorylated predominantly at serine residues. The phosphorylation also occurred at threonine residues but to a lesser extent when compared with that at serine residues. Phosphotyrosine was undetectable (Fig. 1B).

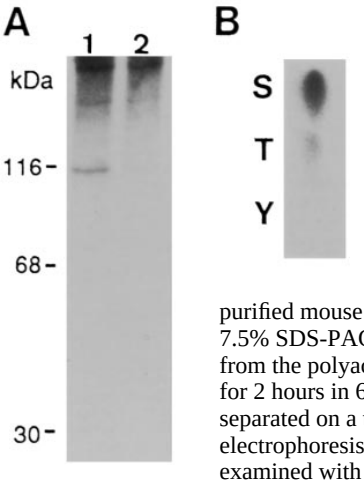

Fig. 1. Phosphorylation of E1 protein in vivo. (A) FM3A cells (1.5×10^7) were labeled for 6 hours with [^{32}P]orthophosphate as described in Materials and Methods. The immunoprecipitates with anti-E1 serum from the extract in the absence (lane 1) or presence (lane 2) of purified mouse E1 (3 μg) were separated by 7.5% SDS-PAGE. (B) ^{32}P-Labeled E1 eluted from the polyacrylamide gel, was hydrolyzed for 2 hours in 6 M HCl. The hydrolysate was separated on a thin-layer plate by electrophoresis from bottom to top, and examined with an image analyzer. S, T and Y indicate the positions of phosphoserine, phosphothreonine and phosphotyrosine, respectively.

In vitro phosphorylation of E1 by Cdc2 kinase

E1 from FM3A cells was prepared by affinity column chromatography with ubiquitin for use as substrate in an in vitro phosphorylation assay. The affinity-purified E1 was composed of two isoforms as determined by SDS-PAGE with apparent M_r values of 113,000 and 105,000 in a molar ratio of 1:4 (data not shown). The intracellular levels of the isoforms and their ratio during the cell cycle did not vary (data not shown). Both isoforms could form a thiolester bond with ubiquitin in the presence of ATP, and reacted with rabbit antiserum raised against human E1 (data not shown).

A mouse Cdc2 kinase was prepared by immunoprecipitation from the nuclear extract of mitotic FM3A cells with the antibody raised against the human p34^{cdc2} (Yasuda et al., 1990). The immunopurified Cdc2 kinase phosphorylated histone H1 very efficiently (Fig. 2A, lane 5), while the immunoprecipitate with control rabbit IgG had no significant H1 kinase activity (Fig. 2A, lane 6).

As shown in Fig. 2A (lane 1), both isoforms acted as substrate for the Cdc2 kinase in vitro. The 105 kDa form of the E1 was, however, phosphorylated less than the 113 kDa form: 65% of the phosphate incorporated into the E1 proteins was associated with the 113 kDa form, although the amount of this form was only one fourth of that of the 105 kDa form in

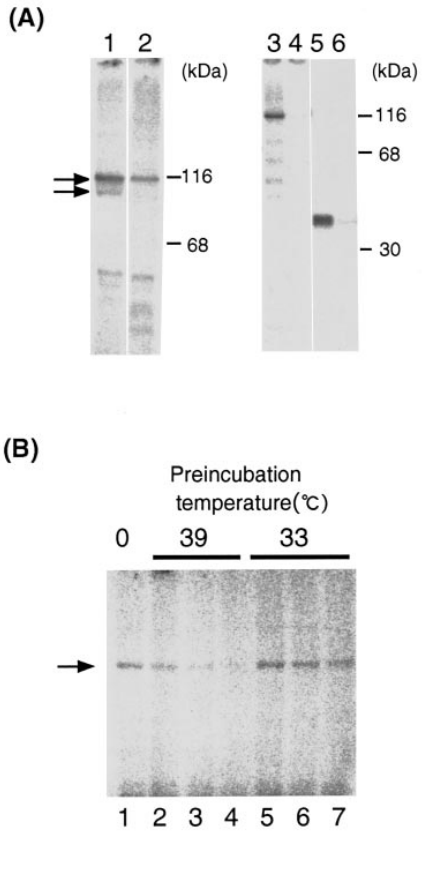

Fig. 2. In vitro phosphorylation of E1 by Cdc2 kinase. (A) 1 μg of affinity purified E1 from mouse FM3A cells (lane 1), recombinant mouse E1 protein expressed in *E. coli* (lanes 2, 3 and 4) and bovine H1 histone (lanes 5 and 6) were incubated with anti-Cdc2 immunoprecipitate (lanes 1, 2, 3 and 5) or control IgG-immunoprecipitate (lanes 4 and 6) in the presence of [γ-^{32}P]ATP and the labeled proteins were separated by 7.5% (lanes 1 and 2) or 12% (lanes 3-6) SDS-PAGE as described in Materials and Methods. Though panels for lanes 3-4 and lanes 5-6 are separated, they derived from the same gel electrophoresis and exposure. Arrows indicate the positions of E1. (B) Cdc2 kinase prepared by immunoprecipitation from tsFT210 cell extract was used as a kinase source for phosphorylation of recombinant E1 protein with [γ-^{32}P]ATP, without preincubation (lane 1) or after preincubation at 39°C (lanes 2 to 4) or 33.5°C (lanes 5 to 7) for 2 minutes (lanes 2, 5), 5 minutes (lanes 3, 6) and 10 minutes (lanes 4, 7). The labeled products were analyzed as for (A). The arrow indicates the position of E1 protein. (C) Cdc2 kinase prepared from FM3A (open circles) or tsFT210 (filled circles) was preincubated at 39°C for the indicated times, and the activity for phosphorylation of recombinant E1 protein was assayed as for (B). The residual activity of E1 phosphorylation after heat inactivation was quantified by measuring the radioactivity in the E1 protein separated by electrophoresis, and expressed as a percentage of the activity of each Cdc2 preparation without preincubation.

the E1 preparation. That is, the 113 kDa form of the E1 was more than 10 times more efficient as a substrate of the Cdc2 kinase in vitro. The phosphorylation of the E1 from FM3A cells by cdc2 kinase was comparable with that of bacterially-expressed E1 protein (Fig. 2A: lane 1 and 2). The efficiency for the latter one was about one fifth of that for H1 histone so far judged from the autoradiogram (Fig. 2A: lane 3 and 5). Cdc2 kinase phosphorylates H1 histone at 5 or 6 sites in vitro (Langan et al., 1981) while phosphorylating 2 sites in E1 (see later section). These suggest that the E1 could be a possible substrate of cdc2 kinase in vitro.

To confirm that the Cdc2 kinase phosphorylated E1, we prepared an immunoprecipitate from a mitotic cell extract of tsFT210, a derivative of FM3A with a ts mutation in Cdc2 kinase (Yasuda et al., 1991). As shown in Fig. 2B,C, the activity of the immunoprecipitate from tsFT210 cells to phosphorylate E1 was heat-sensitive while that from wild-type cells was not: the immunoprecipitate from wild-type cells did not show loss of phosphorylation activity after 5 minutes incubation at 39°C, whereas the kinase activity from tsFT210 cells decreased to 25% of the initial level on heat treatment (Fig. 2C). Furthermore, scarcely any activity for E1 phosphorylation was observed in the immunoprecipitate from an extract of tsFT210 cells that had been incubated at restrictive temperature before harvesting, whereas that from cells that had not been exposed to the restrictive temperature showed normal activity (data not shown). Thus, we concluded that the kinase responsible for E1 phosphorylation in the immunoprecipitate was Cdc2 kinase.

Peptide mapping of labeled E1

E1 labeled in vitro or in vivo, was purified by SDS-PAGE, and subjected to peptide mapping analysis. Trypsin digests of the labeled E1 were separated two-dimensionally by electrophoresis and thin-layer chromatography. As shown in Fig. 3a, two oligopeptides were found to be phosphorylated in vivo (spots 1 and 2 in the Figure). Some minor spots other than spots 1 and 2 were found in some cases, but were not reproducibly detected. Two major labeled oligopeptides (spots 1 and 2) and a minor one (spot 3) were detected in the peptide map of the in vitro labeled 113 kDa form of E1 (Fig. 3b). The peptide map of the 108 kDa isoform was essentially the same as that of the 113 kDa form (data not shown). The oligopeptides of spots 1 and 2 labeled in vitro were identical to those of in vivo labeled E1, since they comigrated, when mixed together, under the conditions for peptide mapping (Fig. 3c). The oligopeptide in spot 3 that was weakly labeled in vitro, showed scarcely any phosphorylation in vivo under the conditions of our labeling experiments.

Cdc2-mediated phosphorylation of recombinant E1 proteins

We determined the primary structures of human and mouse cDNAs for E1 (Ayusawa et al., 1992; Imai et al., 1992), and searched for possible target sequences of Cdc2 kinase, (S or T)-P or the more restrictive (S or T)-P-X-K (Nigg, 1993), which are conserved in both species along with the amino acid sequences deduced from the cDNA. As shown in Fig. 4, there were five matches to the target motif, three near the N-terminal

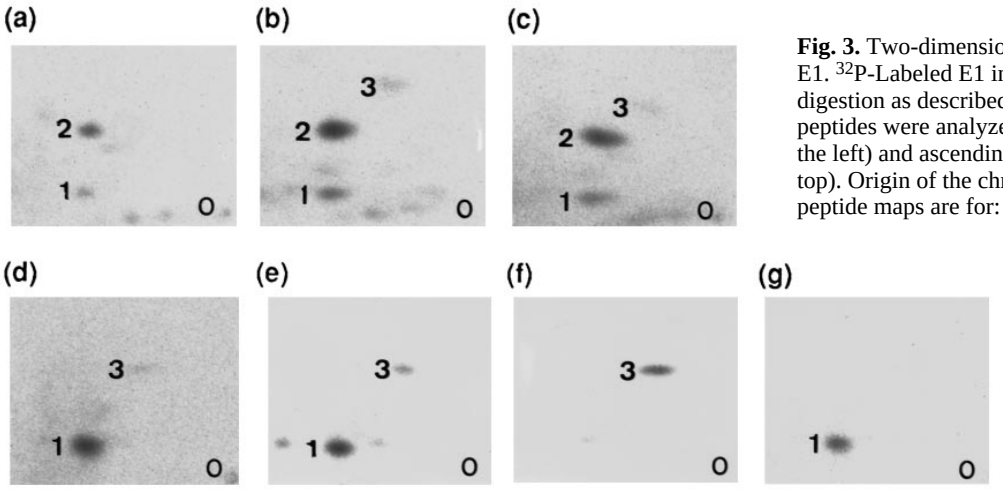

Fig. 3. Two-dimensional tryptic phosphopeptide mapping of E1. ^{32}P-Labeled E1 in pieces of gel was subjected to trypsin digestion as described in Materials and Methods. The tryptic peptides were analyzed by electrophoresis (pH 8.9, anode to the left) and ascending chromatography (from bottom to top). Origin of the chromatography is shown by O. The peptide maps are for: (a) E1 immunoprecipitated from ^{32}P-labeled growing cells of FM3A; (b) affinity purified mouse E1 labeled in vitro with anti-Cdc2 immunoprecipitants; and (c) a mixture of half the amount of each of the digests used in (a) and (b); and in vitro phosphorylated recombinant E1 of (d) wild type; (e) *del*1; (f) *del*1Ala4; and (g) *del*1Gly835.

and two in the C-terminal half. For determination of whether any of these sites were targets of Cdc2 kinase, the site-directed mutations Ala4 and Gly835, shown in Fig. 4, were introduced into cDNA for the mouse E1, which were integrated into the pET3b vector, producing mutant E1 proteins in *E. coli* cells.

The E1 protein synthesized in *E. coli* from a full length E1 cDNA with the wild type sequence was found to be phosphorylated in vitro by the immunopurified Cdc2 kinase (Fig. 2A, lanes 2 and 3). A peptide map of trypsin digests of this protein showed two labeled oligopeptides (spots 1 and 3 in Fig. 3d). Judging from their comigration in the peptide map, spots 1 and 3 were identical to spots 1 and 3, respectively, of the in vitro labeled E1 enzyme (data not shown). Spot 3, however, was somehow less efficiently labeled in E1 protein from *E. coli*, than in the in vitro labeled E1 enzyme. Phosphorylation of the oligopeptide corresponding to spot 2, observed on in vitro phosphorylation of the E1 enzyme did not occur in the E1 protein synthesized in *E. coli*. To improve induction of recombinant E1 protein in *E. coli*, we used the deletion derivatives of E1 cDNA *del1* and *del2*, producing small proteins lacking amino acid residues Leu194 to Gly654 and Arg119 to Gly654, respectively. These small proteins gave the same peptide maps as that derived from full length cDNA, except for enhanced labeling of spot 3 (Fig. 3e), indicating that the middle region including Ser167, one of the possible target sites of Cdc2 kinase (Fig. 4), was not responsible for the in vitro phosphorylation of the oligopeptides in spots 1 and 3. Fig. 3f showed that spot 1 disappeared when Ser4 was mutated to Ala, strongly suggesting that the N-terminal fragment of E1 protein is a target of Cdc2 kinase and the serine at the fourth position is the phosphorylated residue. On the other hand, spot 3 was missing in the map of the labeled E1 protein with a mutation of Gly835 (Fig. 3g). Furthermore, a synthetic oligopeptide corresponding to the E1 sequence between Glu828 and Lys843 (ELKATLPSPDKLPGFK) was phosphorylated by Cdc2 kinase and its trypsin digests showed spot 3 on peptide mapping (data not shown). Thus, the serine at position 835 is a phosphorylation site corresponding to spot 3.

Cell cycle-dependent phosphorylation of E1

Exponentially growing FM3A cells were treated with aphidicolin at 33.5°C, resulting in a mixture of cells arrested at the G_1 and S phases (Fig. 5 (G_1/S)). Cells arrested in the M phase (mitotic index >80%) were prepared by treating them with nocodazole (Fig. 5(M)). The arrested cells were labeled with [^{32}P]orthophosphate for 5 hours in the presence of the drug, and then E1 was precipitated with antiserum. Total incorporation of phosphate into E1 did not vary significantly between the G_1/S and M phases of the cell cycle (data not shown). The molar ratios, however, of radioactivity in spots 1 and 2 in the peptide maps of E1 labeled at the G_1/S, and M phases were 35:65 and 65:35, respectively, indicating that phosphorylation of the oligopeptide in spot 1 mainly occurred in the M phase when the Cdc2 kinase was mostly activated. Labeling of the oligopeptide in spot 1 was significantly less, but still detectable at the G_1/S boundary. This residual activity could be explained

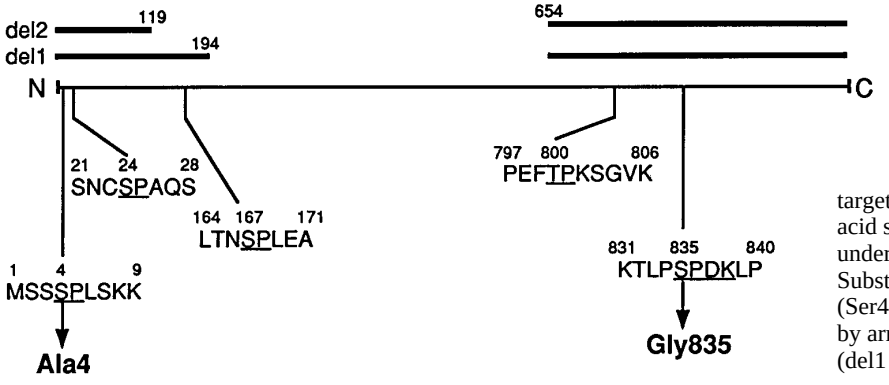

Fig. 4. Schematic diagram of possible target sites for cyclin-dependent kinase in E1. The mouse E1 protein with 1058 amino acid residues, is shown as a continuous line. Numbers indicate the positions from the N terminus of the sequence. The five possible target sites for Cdc2 kinase are shown with their amino acid sequences in a one-letter code. The residues underlined are consensus sequences for the kinase. Substitutions of amino acid residues in the two mutants (Ser4 and Gly835) used in the experiments are shown by arrows. The structures of two deletion derivatives (del1 and del2) are shown at the top.

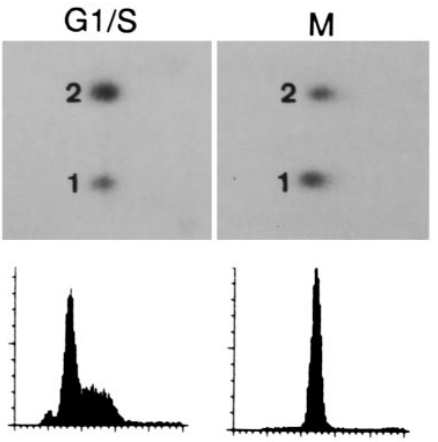

Fig. 5. Phosphopeptide map of E1 in vivo during the cell cycle. FM3A cells (6×10^6) were synchronized in the G_1/S phase with aphidicolin or in the M phase with nocodazole and labeled for 5 hours with [^{32}P]orthophosphate. Phosphorylated E1 was immunoprecipitated from the cell extracts with anti-E1 antiserum as for Fig. 1. Tryptic phosphopeptides of ^{32}P-labeled E1 were mapped and numbered as for Fig. 3. Flow cytometric patterns of synchronized cells are shown below.

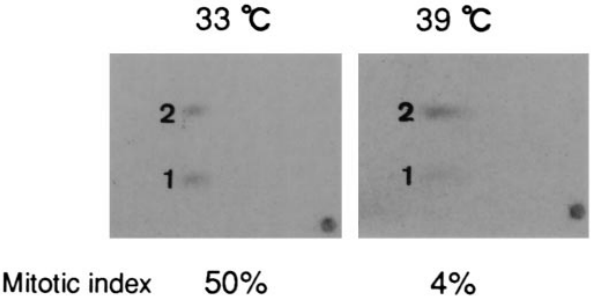

Fig. 6. Phosphorylation pattern of E1 in cdc2^{ts} mutant cells. An equal number of FT210 cells, synchronized in the S phase with aphidicolin were cultured in drug-free medium at 33.5°C or 39°C for 4 hours, and then labeled with [^{32}P]orthophosphate in the presence of colcemid. Incorporation of phosphate into E1 at 39°C was around 60% of that at 33°C when normalized with labeled cell number. An equal count of tryptic phosphopeptides from ^{32}P-labeled E1 at each temperature were mapped and numbered as for Fig. 3. The mitotic indices of the cells at the time of harvest are shown.

by supposing that the cells were not all arrested at the G_1/S boundary, and that some were in the late S phase when Cdc2 kinase was partially activated (Fig. 5(G_1/S)). Alternatively, some kinases other than Cdc2 might phosphorylate this oligopeptide at the G_1/S boundary before Cdc2 kinase was fully activated. Unlike spot 1, spot 2 was rather more phosphorylated in cells arrested in the G_1 and S phases than in those in the M phase. This suggested that some kinase other than Cdc2 is involved in phosphorylation of this oligopeptide in the cells, although Cdc2 kinase could phosphorylate this oligopeptide in vitro.

Involvement of Cdc2 kinase in the phosphorylation of E1 in the cells

tsFT210 cells synchronized around the G_2/M phase were prepared by releasing aphidicolin-arrested cells by incubation in the presence of colcemid for 4 hours at either 33.5°C or 39.5°C, and were then labeled with [^{32}P]orthophosphate at the respective temperatures. Incorporation of phosphate into E1 at the restrictive temperature tended to be around 60% of that at the permissive temperature, suggesting that Cdc2 kinase was responsible, at least in part, for the in vivo phosphorylation of E1. Fig. 6 shows a comparison of the peptide maps of tryptic digests from an equal count of E1 labeled at the restrictive and permissive temperatures.

The phosphorylation of the oligopeptide in spot 1 was not completely abolished, but significantly reduced when compared with its phosphorylation at the permissive temperature. When normalized with the labeled cell number, the count of spot 1 from cells labeled at high temperature was reduced to around 15% of that labeled at low temperature whereas counts of spot 2 were comparable each other. This estimation was based on three independent experiments. The mitotic index of cells labeled at the higher temperature was 4%, indicating that the activity of Cdc2 kinase during the labeling of E1 was effectively repressed though most cells were arrested at the G_2 phase. Although spot 1 was phosphorylated at the permissive temperature, it was labeled less efficiently than in the same type of labeling experiments with wild type cells (compare Fig. 6 (33°C) and Fig. 5(M)). This finding was consistent with the fact that at the permissive temperature the mitotic index of tsFT210 cells was about 50% in the presence of colcemid, whereas that of wild type cells was usually about 80%, indicating mutational damage of Cdc2 kinase activity even at the permissive temperature. Two possibilities on the phosphorylation of E1 in the cells would be suggested. The Cdc2 kinase itself was responsible for the phosphorylation of the materials in spot 1. Alternatively, kinases other than Cdc2, which were active during only M, not the late S to G_2 phases, or were activated by Cdc2 kinase, were involved in its phosphorylation.

Unlike the materials in spot 1, the oligopeptide of spot 2 was not significantly affected by temperature shift, indicating that it was labeled by a kinase other than Cdc2.

DISCUSSION

Kong and Chock (1992) showed that E1 purified from rabbit reticulocytes could be phosphorylated by protein kinase C in vitro. We showed here that the E1 of the mouse cell line was a substrate of the Cdc2 kinase in vivo as well as in vitro. This is the first report of in vivo phosphorylation of E1.

Two major oligopeptides of E1 phosphorylated in vivo were detected. Phosphorylation of one of these two oligopeptides, which was derived from the N terminus of E1, occurred predominantly in the M phase of the cell cycle when Cdc2 kinase was fully activated in the cells. Its phosphorylation was dependent on Cdc2 kinase activity in the cells, since it was not fully phosphorylated in tsFT210 cells at their restrictive temperature. Hence, this oligopeptide was phosphorylated in vivo either by Cdc2 kinase or some other kinase, whose activity was dependent on the activation of the Cdc2 kinase. The fact, however, that Cdc2 kinase phosphorylated the same oligopeptide in vitro strongly suggests that Cdc2 kinase itself was responsible for phosphorylation of this particular oligopeptide

in vivo. Labeling this oligopeptide in asynchronously cultured cells (Fig. 3a) was rather inefficient when compared with that in in vitro phosphorylation (Fig. 3b). This could be due to the possibility that a limited level of phosphate in the labeling medium interfered with the ability of the cells to proceed into S phase and hence to enter G_2 phase in which cdc2 kinase is fully activated.

The second oligopeptide phosphorylated in vivo did not seem to be targeted by Cdc2 kinase since its phosphorylation was not affected by the absence of Cdc2 kinase activity in tsFT210 cells. Furthermore, this phosphorylation tended to occur in the G_1 and S phases rather than in the M phase. These findings indicate that some other kinase distinct from Cdc2 is also involved in the phosphorylation of E1 in the cells. This oligopeptide was phosphorylated in vitro by immunopurified Cdc2 kinase though we did not identify the site of this phosphorylation. Therefore, provided that it was phosphorylated at the same site in vitro as in vivo, a Cdc2-related kinase that was mainly activated during the G_1/S period and that had a similar target sequence to that of Cdc2, might be involved in the phosphorylation of this peptide in vivo. Contrary to the in vitro phosphorylation of this peptide in the cellular E1 protein, it was not detected in the bacterially-expressed protein. A possible explanation for this is that the phosphorylation occurs dependently on yet unknown post-translational modification of the E1 protein in the mammalian cells.

The third oligopeptide that was substantially phosphorylated in vitro, had the most tightly fitting target sequence of Cdc2 kinase, SPDK, and the serine residue, located at position 835 of the E1 sequence, was a phosphorylation site of the fragment. We could not detect the phosphorylation of this residue in vivo. This does not, however, necessarily mean that it was not phosphorylated in vivo since the sensitivity of our in vivo labeling experiments was not high enough to detect minor phosphorylations. Thus, Cdc2 kinase or some kind of CDK may phosphorylate this site in the cells to some extent.

The existence of two isoforms of E1 has been reported independently by two groups. Kudo et al. (1991) showed that two electrophoretically different sizes of mouse E1, of 105 kDa and 113 kDa, and also of human E1, of 108 kDa and 116 kDa, were copurified by affinity chromatography, by a procedure in which the enzyme was bound to ubiquitin-Sepharose in the presence of ATP, and then specifically eluted with AMP and pyrophosphate. Recently, Cook and Chock (1992) reported that two electrophoretically different bands of E1 protein from HeLa cells were isoforms of the enzyme. This conclusion was based on the observations that the two forms were immunologically very similar and gave an almost identical peptide map, and that both of them had the enzymatic activity characteristic of E1. E1 has been suggested to be ubiquitinated because the size difference between the isoforms coincides with the molecular size of monomer ubiquitin. However, western blot analysis has shown no immunoreactivity of the isoforms with antibodies against ubiquitin. We found here that only the minor species of E1 protein from the mouse cell line FM3A, migrating slowly on SDS-PAGE, was phosphorylated in the cells. This finding coincides with the fact that the 113 kDa isoform was a much more efficient substrate of Cdc2 kinase than the other form in an in vitro phosphorylation system. Thus, the susceptibilities of the two isoforms to Cdc2 kinase are different. It remains, however, to be determined whether there is a functional difference between the isoforms, and, if so, whether it is associated with their phosphorylation.

We have not yet examined the possibility of whether the phosphorylation of E1 is essential for its functions. Since Cdc2 kinase is closely associated with cell cycle control, phosphorylation of E1 mediated by Cdc2 kinase may reflect dynamic involvement of the ubiquitin system in cell cycle regulation. The extent of cellular functions regulated by ubiquitin conjugation seems to depend on the molecular and functional variety of the ubiquitin conjugating enzymes, E2s, each of which seems to have a specific or preferred target protein(s). Various E2s are probably specifically associated with progression of the various phases of the cell cycle. This is the case in the yeast *S. cerevisiae*, with regard to the *UBC3*/*CDC34* and *UBC9* gene products for progression of the G_1 and G_2 phase, respectively. The specific ubiquitin pathway via distinct E2s associated with cell cycle regulation could be activated at particular points of the cell cycle. The most feasible way for this kind of regulation would be cell cycle-dependent expression of the genes for particular E2s. More dynamically, however, it could be accomplished by the recognition of particular E2s by E1, which would not be possible until E1 was phosphorylated by the key kinase for cell cycle control. Cyclin B proteolysis has been suggested to be induced by Cdc2 kinase itself, providing a negative feedback to terminate mitosis (Felix et al., 1990; Luca et al., 1991). Felix et al. (1990) showed that the destruction of cyclin B was triggered by adding Cdc2 kinase to a cell-free extract of interphase *Xenopus* eggs. In their model, mitotic Cdc2 kinase increased the phosphorylation of a protein, X, which in turn activated cyclin B degradation. Taking these findings into consideration, we consider that cyclin B might be one of the substrates targeted by the specific ubiquitin conjugation pathway activated by the phosphorylation of E1 with Cdc2 kinase.

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