Identification of a Consensus Cyclin-dependent Kinase Phosphorylation Site Unique to the Nuclear Form of Human Deoxyuridined Triphosphate Nucleotidohydrolase

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In the preceding report (Ladner, R. D., McNulty, D. E., Carr, S. A., Roberts, G. D., and Caradonna, S. J. (1996) J. Biol. Chem. 271, 7745–7751), we identified two distinct isoforms of dUTPase in human cells. These isoforms are individually targeted to the nucleus (DUT-N) and mitochondria (DUT-M). The proteins are nearly identical, differing only in a short region of their amino termini. Despite the structural differences between these proteins, they retain identical affinities for dUTP (preceding article). In previous work, this laboratory demonstrated that dUTPase is posttranslationally phosphorylated on serine residue(s) (Lirette, R., and Caradonna, S. (1990) J. Cell. Biochem. 43, 339–353). To extend this work and determine if both isoforms of dUTPase are phosphorylated, a more in-depth analysis of dUTPase phosphorylation was undertaken. [32P]Orthophosphate-labeled dUTPase was purified from HeLa cells, revealing that only the nuclear form of dUTPase is phosphorylated. Electrospray tandem mass spectrometry was used to identify the phosphorylation site as Ser-11 in the amino-terminal tryptic peptide PCSEETPAIpSPSKR (the NH2-terminal Met is removed in the mature protein). Mutation of Ser-11 by replacement with Ala blocks phosphorylation of dUTPase in vivo. Analysis of the wild type and Ser-11 → Ala mutant indicates that phosphorylation does not regulate the enzymatic activity of the DUT-N protein in vitro. Additionally, experiments with the Ser-11 → Ala mutant indicate that phosphorylation does not appear to play a role in subunit association of the nuclear form of dUTPase. The amino acid context of this phosphorylation site corresponds to the consensus target sequence for the cyclin-dependent protein kinase p34cdc2. Recombinant DUT-N was specifically phosphorylated on Ser-11 in vitro with immunoprecipitated p34cdc2. Together, these data suggest that the nuclear form of dUTPase may be a target for cyclin-dependent kinase phosphorylation in vivo.

Human dUTPase1 was first purified from HeLa cells by Caradonna and Adamkiewicz (2). The enzyme was characterized as a homodimer with a monomeric molecular weight of 22,500 and a Km for dUTP of 2.5 μM. The dUTPase monomers associate in the presence of divalent cations such as magnesium or manganese to form the active enzyme. In later work, we identified the human enzyme as a serine phosphoprotein (1). Studies on herpesvirus infection of HeLa cells have shown that cellular dUTPase activity decreases postinfection, while the virus-encoded dUTPase activity increases. It was postulated that the associated decrease of cellular dUTPase activity was not due to rapid degradation but rather correlated with dephosphorylation of the host dUTPase protein. These data suggest that phosphorylation may play a role in regulating the enzymatic activity of the human dUTPase protein. More recently, Strahler and co-workers demonstrated that, upon peripheral blood lymphocyte stimulation, there is a large induction of the phosphorylated form of dUTPase. This induction of dUTPase protein coincided with the onset of DNA replication, suggesting a link between dUTPase phosphorylation and the proliferation status of the cell (4).

In this report we extend previous work involving dUTPase phosphorylation. A single site of phosphorylation correlating to Ser-11 of the nuclear isoform of dUTPase was identified. Although both the DUT-N and DUT-M contain the identical site, phosphorylation of Ser-11 is unique to the nuclear isoform. This site correlates with the consensus sequence for cyclin-dependent kinase phosphorylation and is specifically phosphorylated by p34cdc2 in vitro, suggesting a link to the cyclin signaling pathway. Studies with a Ser-11 → Ala mutant of DUT-N suggest that this modification is unrelated to both enzymatic activity and subunit association.

EXPERIMENTAL PROCEDURES

Cell Culture and Labeling—HeLa S3 cells (CCL 2.2) were purchased from the American Type Culture Collection and maintained in Dulbecco’s modified Eagle’s medium supplemented with 5% calf serum (Life Technologies, Inc.). COS 7 cells (CRL 1651) were purchased from the American Type Culture Collection and maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (Life Technologies, Inc.). 32P-Labeling of dUTPase was accomplished by incubating HeLa cells for 24 h in phosphate-deficient Dulbecco’s modified Eagle’s medium supplemented with 5% fetal calf serum and [32P]orthophosphate.

Purification of dUTPase—Purification of dUTPase from HeLa, Cos-7, and Sf21 cells was performed using a modification of the method developed by guest on July 22, 2018http://www.jbc.org/Downloaded from
Phosphorylation of the Nuclear Isoform of Human dUTPase

1

2

-29 kD

-18

FIG. 1. The nuclear form of dUTPase is phosphorylated. HeLa S3 cells were labeled with [32P]orthophosphate and dUTPase was immunoprecipitated, utilizing dUTPase-specific monoclonal antibodies, from total cell extracts. The immunoprecipitates were fractionated by SDS-PAGE and transferred to nitrocellulose. dUTPase protein was detected by immunoblot analysis utilizing affinity-purified, dUTPase-specific polyclonal antibody. The protein bands were visualized using a chemiluminescent detection system. Lane 1, immunoblot analysis demonstrates the presence of the major nuclear form of dUTPase (DUT-N) and the larger, less abundant mitochondrial isoform (DUT-M). The chemiluminescent detection reaction was quenched by exposing to visible light, and the membrane was subjected to autoradiography to detect the 32P labeling. Lane 2, autoradiograph demonstrating the phosphorylation of DUT-N. There is no detectable phosphorylation associated with the DUT-M isoform.

centrifuge (Savant Instruments) and reconstituted in 50 μl of 1:1 methanol: H2O (v/v), 0.2% in formic acid. The sample was examined by electrospray mass spectrometry on a PE-Sciex API-III triple quadrupole mass spectrometer fitted with a fully articulated ionspray probe and an atmospheric pressure ionization source. Approximately 5 μl of the sample (at 0.2–0.5 pmol/μl) was introduced into the mass spectrometer by infusion with an infusion pump (Harvard Apparatus) at a flow rate of 1 μl/min. Electrospray mass spectra were acquired by scanning quadrupole 1 from m/2 10 to 2400 with a mass step of 0.2 Da and 10-ms dwell/step. Dually charged parent ions, (M + 2H)2+, were selected for fragmentation and structural analysis with quadrupole 1. The mass-selected parent ion was subjected to collision-induced decomposition in quadrupole 2 of the triple quadrupole. Quadrupole 3 was scanned from m/2 10 to 2400 with a mass step of 1.0 Da and 10-ms dwell/step. Argona was used as the collision gas with a calculated collision energy of approximately 28 eV. Parent ion transmission was maximized by reducing the resolution of quadrupole 1 to transmit approximately a 2–3 m/2 window about the selected parent ion.

Detection of the Stoichiometry of dUTPase Protein Phosphorylation—Procedures for determining the extent of dUTPase phosphorylation followed those of Sefton (3). Briefly, HeLa cells were labeled for 24 h with [32P]orthophosphate. dUTPase protein was recovered by immunoprecipitation and fractionated by SDS-PAGE. dUTPase was quantitated by Coomassie staining of the gel and comparison to recombinant dUTPase protein standards run on the same gel. The 32P-labeled dUTPase protein band was excised, and the amount of associated radioactivity was determined by scintillation counting. The specific activity of 32P was determined by counting an aliquot of the media and dividing by the mol of inorganic phosphate in the media (based on fetal calf serum containing 3.8 ± 0.7 m mol inorganic phosphate; Life Technologies, Inc.). The mol of phosphate/mol of dUTPase protein was then calculated.

RESULTS

The Nuclear Form of dUTPase Is Phosphorylated—We have previously demonstrated that human dUTPase is a serine phosphoprotein (1). In order to determine if one or both of the dUTPase isoforms are phosphorylated in vivo, HeLa cells were labeled with [32P]orthophosphate, and dUTPase was immunoprecipitated from total cell extracts. The immunoprecipitates were fractionated by 15% SDS-PAGE and selected for Western blot analysis. The immunoblot shown in Fig. 1, lane 1, demonstrates the presence of both DUT-N and DUT-M isoforms. After quenching the chemiluminescent immunoblot reaction, the nitrocellulose membrane was exposed to x-ray film to detect [32P]orthophosphate labeling. As seen in Fig. 1, lane 2, all of the radioactivity is associated with the lower molecular weight nuclear form of dUTPase (DUT-N) and not the mitochondrial isoform (DUT-M).

Identification of Ser-11 as the Vivo Phosphorylation Site of dUTPase—HeLa cells were radiolabeled with [32P]orthophos-
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To further confirm that Ser-11 is the site of DUT-N phosphorylation, transfected cells were labeled with $^{32}$P orthophosphate for 10 h at 50–60 h posttransfection. At 60 h, cells were harvested and extracts were prepared. Each sample was subjected to immunoprecipitation analysis using a human dUTPase-specific monoclonal antibody. The immunoprecipitates were resolved by 15% SDS-PAGE and visualized by autoradiography. The wild type DUT-N protein is readily phosphorylated in COS-7 cells (Fig. 3B, lane 2). Phosphorylation of the mutant DUT-N however, is blocked by the Ser-11 → Ala mutation (Fig. 3B, lane 3). The monoclonal antibody used in this experiment was determined to quantitatively immunoprecipitate both the wild type and mutant dUTPase. COS-7 were transfected as above, and cells were labeled with $^{32}$P orthophosphate between 50 and 60 h posttransfection. At 60 h cells were harvested, and dUTPase was immunoprecipitated from total cell extracts. Immunoprecipitates were washed and fractionated by SDS-PAGE. The dried gel was exposed to x-ray film for 12 h at –80°C. Lane 1, pEUK-C1, no insert; lane 2, pEUK-C1/Ser-11 to Ala mutant dUTPase; lane 3, pEUK-C1-native dUTPase; lane 3, pEUK-C1/Ser-11 to Ala mutant dUTPase. 

Estimation of Stoichiometry of dUTPase Phosphorylation by Biosynthetic Labeling—The stoichiometry of dUTPase phosphorylation was determined by the procedure described by

Fig. 2. Mass spectrometry analysis of the DUT-N phosphopeptide. Electrospray tandem mass spectrometry of the (M + 2H)$^{2+}$ (m/z 820) of approximately 1 pmoI of the phosphopeptide. Fragment nomenclature is according to Biemann and Roepstorff (20, 21). The numbering above the single-letter code sequence refers to $y_n$ ions formed by cleavage of the peptide bond of the nth amino acid from the COOH terminus with H-rearrangement to form a charged, COOH-terminal peptide fragment (NH$_2$-CHR$^1$-CO...NHCHR$^1$-CO$^\cdot$H + H)$^+$. The numbering below the single-letter code sequence refers to $b_n$ ions formed by charge retention on the NH$_2$-terminal acylium fragments (NH$_2$-CHR$^1$-CO...NHCHR$^1$-CO$^\cdot$); loss of CO from the $b_n$ ions yields the $a_n$ ion series. The mass increment between the $y_n$ and $y_{n+1}$ ions (167 Da), and the presence of satellite peaks formed by the loss of either H$_3$PO$_4$ (98 Da) on all of the $y_n$ ions that are formed by cleavage COOH-terminal to the Pro-11 indicate that the phosphate is located on Ser-10.

Fig. 3. In vivo verification of phosphorylation on Ser-11 by site-directed mutagenesis. A, Ser-11 was changed to Ala by site-directed mutagenesis. The dUTPase native and mutant open reading frames were cloned into the eukaryotic expression vector pEUK-C1. COS-7 cells were transfected with these constructs and control vector (with no insert) using Lipofectin. Cells were harvested after 60 h. Immunoblot analysis utilizing affinity-purified polyclonal antibodies demonstrates the transient expression of native and mutant dUTPases. Lane 1, pEUK-C1 alone; lane 2, pEUK-C1/native dUTPase; lane 3, pEUK-C1/Ser-11 to Ala mutant dUTPase. B, COS-7 were transfected as above, and cells were labeled with $^{32}$P orthophosphate between 50 and 60 h posttransfection. At 60 h cells were harvested, and dUTPase was immunoprecipitated from total cell extracts. Immunoprecipitates were washed and fractionated by SDS-PAGE. The dried gel was exposed to x-ray film for 12 h at –80°C. Lane 1, pEUK-C1, no insert; lane 2, pEUK-C1/native dUTPase; lane 3, pEUK-C1/Ser-11 to Ala mutant dUTPase. Both the monoclonal and polyclonal antibodies to human dUTPase do not cross-react with COS-7 derived dUTPase.
Fig. 5. Phosphorylation of dUTPase by p34cdcd in vitro. Recombinant DUT-N protein was phosphorylated in vitro with immunoprecipitated p34cdcd from HeLa cells as described under “Experimental Procedures.” DUT-N was subsequently immunoprecipitated with a monoclonal antibody and fractionated by SDS-PAGE. The dried gel was exposed to x-ray film for 12 h. Lane 1, recombinant DUT-N protein; lane 2, DUT-N protein, competition experiment with p34cdcd peptide; lane 3, Ser-11 → Ala mutant. All reactions were carried out under identical conditions.

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envelope is shown to be fully functional, possessing identical $K_m$ values of 2.5 \( \mu M \) (data not shown). These values are in close agreement with previous determinations of the $K_m$ for the native DUT-N and DUT-M enzymes derived from HeLa cells (22). In addition, apparent velocities at saturating dUTP concentrations were similar for both the wild type and Ser-11 \rightarrow Ala mutant. Specific activity in each case is about 15 nmol of dUMP formed per min per \( \mu g \) of purified protein. These experiments suggest that phosphorylation of Ser-11 does not significantly alter the enzymatic activity of the DUT-N protein in vitro.

We previously hypothesized that phosphorylation may regulate subunit association (1). Both the wild type and Ser-11 \rightarrow Ala mutant were assayed for the ability to undergo magnesium-dependent multimerization as described previously (2). Both the wild type and mutant forms of the DUT-N protein formed higher molecular weight complexes, demonstrating that monomer association is independent of the phosphorylation state of the protein (data not shown).

**DISCUSSION**

**Nuclear and Mitochondrial dUTPase Structural Differences**—Data presented in the previous paper (22) indicate DUT-N and DUT-M differ only in a short region of their amino termini (see Fig. 4 of this report for comparison). Specifically, DUT-M contains an additional 19 amino acids. This presumably accounts for the higher molecular weight compared with DUT-N. In addition, the next 5 amino acids of DUT-N (Pro-20 through Pro-24) are different from DUT-M. The remaining amino acid sequence appears to be identical between DUT-N and DUT-M. In this study we further distinguish between these two isoforms on the basis of posttranslational phosphorylation. A single phosphorylation site correlating to Ser-11 of the nuclear isoform was identified. Stoichiometry analysis of phosphorylation indicates that approximately 81% of the DUT-N protein is phosphorylated in HeLa cells. Although both DUT-N and DUT-M contain the identical consensus phosphorylation site, only the nuclear form of dUTPase is phosphorylated in HeLa cells. A possible explanation for the exclusive phosphorylation of the DUT-N isoform is that the relevant protein kinase is sequestered away from the mitochondrial form. It is conceivable that the kinases acting on DUT-N are protein kinase is sequestered away from the mitochondrial phosphorylation of the DUT-N isoform is that the relevant phosphorylation indicates that approximately 81% of the these two isoforms on the basis of posttranslational phosphorylation (18).

**The Nonconserved dUTPase Phosphorylation Site Is a Consensus p34<sup>cdc2</sup> Sequence**—There are five highly conserved motifs common to dUTPases (8) that are implicated in active site function. Two of these characteristic motifs (II and V) contain highly conserved serine residues. However, the DUT-N phosphorylation site lies in the nonconserved amino-terminal region of the protein. It is possible that the human dUTPase phosphorylation site is a relatively recent adaptation of this enzyme, performing a role that is unique to higher eukaryotes. It is presently unknown whether dUTPases from other mammalian or higher eukaryotes also possess phosphorylated residues.

The amino acid context of the DUT-N phosphorylation site resembles the consensus target sequence of the cyclin-dependent kinase p34<sup>cdc2</sup> (9). In order to determine that the Ser-11 phosphorylation site was authentic, we performed in vitro experiments demonstrating that dUTPase is specifically phosphorylated on Ser-11 by immunoprecipitated p34<sup>cdc2</sup> from HeLa S3 cells (Fig. 5). However, these experiments do not confirm that p34<sup>cdc2</sup> directly phosphorylates dUTPase in vivo. There is a family of related kinases that share extensive homology with p34<sup>cdc2</sup> (e.g. CDK2 through CDK5) (12). It is possible that DUT-N is a target for one or several of these p34<sup>cdc2</sup>-related kinases in vivo.

The Functional Role of dUTPase Phosphorylation—Protein phosphorylation/dephosphorylation controls a wide range of cellular functions including aspects of cell cycle control and signal transduction (13, 14). Although relatively few CDK substrates have been identified, the functional significance of phosphorylation by CDKs appears to be diverse, including promoting protein complex formation and modulating enzymatic activity. For example, CDK phosphorylation of pp60<sup>src</sup> is correlated with a 3–7-fold increase in pp60<sup>src</sup> tyrosine kinase activity (15). Phosphorylation of lamins by p34<sup>cdc2</sup> plays a major role in lamina disassembly (16). In addition, it has been suggested that the phosphorylation of lamins, which complex with CDKs such as p34<sup>cdc2</sup>, affects the regulation of kinase activity (17).

The role of dUTPase phosphorylation remains to be elucidated. In a previous report, we postulated that dUTPase phosphorylation may regulate enzymatic activity (1). Several lines of evidence, presented in this work, suggest that phosphorylation does not regulate the enzymatic activity of DUT-N. Mutagenesis of Ser-11 \rightarrow Ala prevents phosphorylation; however, experiments with the mutant protein demonstrate that enzymatic activity is not significantly altered in vitro. Second, a recombinant human dUTPase protein has been expressed that lacks the first 22 amino acids present in the nuclear form of dUTPase. This recombinant protein does not contain the phosphorylation site yet still retains full enzymatic activity (18). A third line of evidence arguing against the regulation of dUTPase activity by phosphorylation comes from a comparison of the enzymatic activities of the nuclear and mitochondrial isoforms. DUT-M contains the identical site for phosphorylation as DUT-N but is not phosphorylated in vivo. Although DUT-M is not phosphorylated, it exhibits identical kinetic characteristics (\( K_m = 2.5 \mu M \)) to the phosphorylated nuclear form. Taken together, these observations suggest that the phosphorylation of dUTPase does not significantly govern enzymatic activity under the assay conditions utilized in vitro.

Another possible role of phosphorylation is the formation of the dUTPase multimer. Caradonna and Adamkiewicz (2) first described human dUTPase as a homodimer, although molecular modeling of human dUTPase based on the E. coli dUTPase crystal structure (19) suggests that the human protein is a homotrimer.\(^2\) Experiments utilizing both the DUT-N recombinant and the Ser-11 \rightarrow Ala mutant demonstrate that multimerization is independent of Ser-11 phosphorylation. Additional evidence supporting this again comes from a truncated recombinant form of dUTPase lacking 22 amino-terminal residues. This protein was shown to trimerize independent of the Ser-11 phosphorylation site (18).

DUT-N phosphorylation may also regulate its intracellular localization. The DUT-N and DUT-M isoforms differ exclusively in their aminoterminal. This distinction appears to confer the ability of DUT-M to localize in the mitochondria. It is conceivable that the exclusive phosphorylation of DUT-N may play a role in nuclear targeting of this protein. Taken a step further, Ser-11 may confer the ability of DUT-N to localize in specific regions of the nucleus where the dUTPase function is

\(^2\) C. Tucker and S. Ealick, personal communication.
required. The Ser-11 → Ala mutant should aid in the testing of these hypotheses.

In summary, we have continued our investigation of the detailed biochemistry of dUTPase in human cells, uncovering an additional layer of detail. Elucidation of two distinct isoforms localized to the mitochondria and nucleus, respectively, as well as identification of a CDK phosphorylation site specific to the nuclear isoform, all suggest that this enzyme function is highly regulated within the cell.

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