Identification of a Region within the Ubiquitin-activating Enzyme Required for Nuclear Targeting and Phosphorylation*

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The ubiquitin-activating enzyme exists as two isoforms: E1a, localized predominantly in the nucleus, and E1b, localized in the cytoplasm. Previously we generated hemagglutinin (HA) epitope-tagged cDNA constructs, HA1-E1 (epitope tag placed after the first methionine) and HA2-E1 (epitope tag placed after the second methionine) (Handley-Gearhart, P. M., Stephen, A. G., Trausch-Azar, J. S., Ciechanover, A., and Schwartz, A. L. (1994) J. Biol. Chem. 269, 33171–33178), which represent the native isoforms. HA1-E1 is exclusively nuclear, whereas HA2-E1 is found predominantly in the cytoplasm. Using high resolution isoelectric focusing and SDS-polyacrylamide gel electrophoresis, we confirm that these epitope-tagged constructs HA1-E1 and HA2-E1 represent the two isoforms E1a and E1b. HA1-E1/E1a exists as one non-phosphorylated and four phosphorylated forms, and HA2-E1/E1b exists as one predominant non-phosphorylated form and two minor phosphorylated forms. We demonstrate that the first 11 amino acids are essential for phosphorylation and exclusive nuclear localization of HA1-E1. Within this region are four serine residues and a putative nuclear localization sequence (NLS; PLSKKRR). Removal of these four serine residues reduced phosphorylation levels by 60% but had no effect on nuclear localization of HA1-E1. Each serine residue was independently mutated to an alanine and analyzed by two-dimensional electrophoresis; only serine 4 was phosphorylated. Disruption of the basic amino acids within the NLS resulted in loss of exclusive nuclear localization and a 90–95% decrease in the phosphorylation of HA1-E1. This putative NLS was able to confer nuclear import on a non-nuclear protein in digitonin-permeabilized cells in a temperature- and ATP-dependent manner. Thus the predominant requirement for efficient phosphorylation of HA1-E1/E1a is a functional NLS, suggesting that E1a may be phosphorylated within the nucleus.

The ubiquitin-activating enzyme (E1) catalyzes the first reaction in the ubiquitin (Ub) conjugation pathway. Activation of Ub occurs by the formation of a high energy thiol-ester bond between E1 and the C-terminal glycine of Ub and the production of AMP and PPi. Activated Ub is then transferred to a ubiquitin-conjugating enzyme (ubiquitin-carrier enzyme, E2). E2 proteins conjugate Ub directly to a target substrate or, alternatively, transfer Ub to a ubiquitin-protein ligase (E3), which then conjugates Ub to a target protein (reviewed in Ref. 1 and 2). Multiple rounds of Ub conjugation result in the rapid degradation of the target protein by the 26 S proteasome (3). Recent published results, however, suggest ubiquitination plays an indirect role in protein degradation as well (reviewed in Ref. 4). Ubiquitination on cell surface receptors such as Ste2 (5), yeast α mating receptor (6), and growth hormone receptor (7) triggers their endocytosis and degradation within the lysosome.

Because Ub requires activation prior to participation in any subsequent reactions, E1 plays a key role in the Ub-conjugating pathway. E1 is localized in both the nucleus and the cytoplasm (8, 9) and exists as two isoforms E1a (117 kDa) and E1b (110 kDa) (10, 11). To investigate the nature of these isoforms, epitope-tagged cDNA constructs of E1 were generated where the hemagglutinin (HA) epitope tag was placed after the first methionine (amino acid 1; HA1-E1) or after the second methionine (amino acid 40; HA2-E1) (11). HA1-E1 localized exclusively to the nucleus and displayed the same molecular weight as E1a, whereas HA2-E1 localized predominantly in the cytoplasm and displayed the same molecular weight as E1b (11). These observations are consistent with the hypothesis that the E1 isoforms are a result of alternate translational start sites. Of these isoforms, E1a/HA1-E1 is phosphorylated in vivo (11) on a serine residue (12), whereas E1b/HA2-E1 is not phosphorylated. Phosphorylation of E1a occurs in a cell cycle-dependent manner (maximal in G2) and the resultant phosphorylated E1a was concentrated within nuclear extracts (13). On the basis of these observations, we proposed that an increase in phosphorylation of E1a functions to increase the import and/or retention of E1a in the nucleus (13).

The present study uses HA epitope-tagged cDNA constructs of E1 to identify amino acids that are important for nuclear localization and phosphorylation and whether phosphorylation of E1 is a prerequisite for its nuclear localization. We identify a specific serine residue that is phosphorylated in addition to a region of basic amino acids that is required for both nuclear localization and phosphorylation. Our data suggest that phosphorylation is not required for nuclear import, but that E1 may

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The abbreviations used are: E1, ubiquitin-activating enzyme; E2, ubiquitin-carrier enzyme; E3, ubiquitin-protein ligase; BSA, bovine serum albumin; Cy3, indocarbocyanine; HA, influenza hemagglutinin monoclonal antibody epitope; IEF, isoelectric focusing; NLS, nuclear localization sequence; Ub, ubiquitin; PCR, polymerase chain reaction; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; sulfos-MCC, sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate.
require a functional nuclear localization sequence for efficient phosphorylation.

MATERIALS AND METHODS

Cell Culture and Transfection—HeLa cells were cultured in Dulbecco’s modified Eagle’s medium and 10% fetal calf serum and maintained at 37 °C and 5% CO2 in a humidified chamber as described previously (9). Transient transfections were performed using a calcium phosphate precipitation method as described by Chen and Okayama (14); cells were processed 48–48 h following transfection.

Construction of HA-E1 Mutants—HA1-E1, HA2-E1, and HA1-E1-11 del-11 constructs were described previously (11). The N-terminal deletion constructs, HA1-E1-del-4, HA1-E1-del-22, and HA1-E1-del-30, were constructed by annealing a primer encoding the HA epitope tag (YPYDVPDYAS) and a 30-base overlap with the E1 sequence, beginning at amino acid 5, 23, or 31 (nucleotides 142, 196, or 220). The first point mutations (HA1-E1-12A, HA1-E1-33A, HA1-E1-8A, and HA1-E1-7A) were constructed using an HA-containing primer where serine 2, 3, 4, or 7 encoded within the 30-base overlap was replaced with an alanine (nucleotide 133, 136, 139, and 148). Using the same protocol, HA1-E1-R10A, R11A had lysines 10 and 11 replaced with alanines. HA1-E1-del-4,57A, was constructed using an HA encoding primer with a 30-base pair overlap (which included a point mutation to alanines. HA1-E1-del-4,57A was constructed using an HA-containing primer whose sequence included a unique I site within the E1 sequence. PCR was performed using the Ericomp Twinblock thermocycler, and T3 DNA polymerase (Promega) with an annealing temperature of 55 °C. The human E1 cDNA in pGem7zf-E1(Gem) was used as the template. PCR products were subcloned into the pCRII vector (TA cloning kit, Promega) and sequenced. Full-length E1 was constructed by replacement of a BamHI and NcoI fragment of ElpGem with the HA-E1 fragment generated by PCR. Full-length HA-E1 constructs were then subcloned into the mammalian expression vector pcDNA3 (In Vitrogen). HA1-E1-del-8-11 was generated where the amino acids 8–11 were removed using the Sculptor Mutagenesis kit (Amersham). All restriction enzymes were from Promega.

Metabolic Labeling, Immunoprecipitation, and Immunoblot Analysis of HA-E1 Constructs—HeLa cells were metabolically labeled with [32P]orthophosphoric acid (ICN) as described previously (13). Cells were lysed in 20 mM Tris, pH 7.6, 0.25% Triton X-100, 0.2% DTT containing 0.2 mM phenylmethylsulfonyl fluoride, 2.5 μg/ml leupeptin, 1 mM pepstatin, 100 μg/ml benzamidine, 1 mM sodium orthovanadate, 1 mM sodium fluoride. The lysates were incubated on ice for 30 min, then centrifuged at 14,000 rpm for 15 min. Protein concentrations of cleared lysate were determined using the Bio-Rad protein assay with bovine serum albumin as standard. HA-tagged E1 was immunoprecipitated from radiolabeled extracts (200 μg of protein) using the 12CA5 monoclonal antibody directed against the HA-E1 epitope tag, as described previously (11). The immunoprecipitation buffer contained the following phophatase 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. HA-tagged E1 was visualized using the 12CA5 antibody, followed by a peroxidase-conjugated goat anti-mouse IgG (11). Immunoreactive proteins were detected using enhanced chemiluminescence (Amersham) and quantified using a Molecular Dynamics densitometer. The immunoblot was visualized using the 12CA5 antibody, followed by a peroxidase-conjugated goat anti-mouse IgG (11). Immunoreactive proteins were detected using enhanced chemiluminescence (Amersham) and quantified using a Molecular Dynamics Storm Optical Scanner.

Immunofluorescence—Immunofluorescence on transiently transfected HeLa cells was performed as described previously (11). HA-tagged E1 was detected using the 12CA5 monoclonal antibody followed by rhodamine-conjugated goat anti-mouse IgG (Bios). Immunoreactive proteins were detected using enhanced chemiluminescence (Amersham) and quantified using a Molecular Dynamics Storm Optical Scanner.

Conjugation of Peptides to BSA and Labeling with Cy3—Peptides were synthesized (Washington University) and cross-linked to the non-nuclear protein bovine serum albumin (BSA) (Calbiochem) using sulfo-SMCC. The key modification was the addition of in phosphatase buffer (Promega). The lysates were incubated on ice for 20 min. The dry strip was placed on a reducing 7.5% SDS-PAGE gel (18 × 16 cm) and electrophoresed for 5 h at 40 mA as the second dimension. The gel was fixed for 30 min and then washed with 50% ethanol in Acrylamide. The dried gel was then exposed to film for autoradiography at –80 °C. Two-dimensional gels were analyzed using a Molecular Dynamics Storm Optical Scanner.

Dephosphorylation of E1—HeLa cells metabolically labeled with either [32P]orthophosphoric acid or Tran35S-label were lysed as described above but without the addition of phosphatase inhibitors. Lysate was then dialyzed overnight into 100 mM Tris/HC1, pH 7.0, 150 mM NaCl, 1 mM dithiothreitol. Dialyzed [32P]- or Tran35S-labeled lysate (300 μg of protein) was incubated with 1 unit of potato acid phosphatase (Sigma) in 100 mM sodium citrate, pH 5.8, 10 mM MgCl2 overnight at 4 °C (final volume of 80 μl). The following day another 1 unit of phosphatase was added and incubated for 2 h at ambient temperature. Parallel incubations were included without the addition of phosphatase. E1 was immunoprecipitated from the lysate as described previously (13). Tran35S-Labeled E1 was resolved by SDS-PAGE and transferred to nitrocellulose, and E1 was visualized by an anti-E1 polyclonal antibody. Tran35S-Labeled E1 was immunoprecipitated and resolved by two-dimensional gel electrophoresis (as described previously).

RESULTS

Two-dimensional Electrophoresis of HA1-E1, HA2-E1, and Human E1—E1 exists as two isoforms E1 (117 kDa) and E1b (110 kDa). Previously we prepared E1 cDNA constructs in which an HA-epitope tag was placed after the first methionine (HA1-E1) or the second methionine (HA2-E1) (Fig. 1A; Ref. 11). HA1-E1 and HA2-E1 were similar in their molecular weight and phosphorylation state to E1a and E1b, respectively (11). Two-dimensional gel electrophoresis analysis of E1 from Chinese hamster (ta20) cells resolved E1 into the two isoforms E1a and E1b; E1a resolved further as three phosphorylated and one non-phosphorylated forms, whereas E1b resolved as one non-phosphorylated form (13). To further determine the relationship between human E1, HA1-E1, and HA2-E1, we analyzed...
these species by two-dimensional gel electrophoresis. HeLa cells were metabolically labeled with Tran$^{35}$S-label or [$^{32}$P]orthophosphoric acid, and human E1 was immunoprecipitated with a polyclonal antibody raised against human E1 (11). HeLa cells transiently transfected with HA1-E1 or HA2-E1 were metabolically labeled with either Tran$^{35}$S-label or [$^{32}$P]orthophosphoric acid, and immunoprecipitated proteins were resolved by IEF in the first dimension, followed by SDS-PAGE in the second dimension (B). HeLa cells transiently transfected with HA1-E1 or HA2-E1 were metabolically labeled with either Tran$^{35}$S-label or [$^{32}$P]orthophosphoric acid, and immunoprecipitated proteins were resolved by IEF and SDS-PAGE (C).

![Fig. 1](http://www.jbc.org/) Two-dimensional gel electrophoresis of HA epitope-tagged and wild type E1. HA epitope tags were placed after the first (HA1-E1) or second (HA2-E1) methionine in the E1 sequence as described under “Materials and Methods” (A). HeLa cells were metabolically labeled with either Tran$^{35}$S-label or [$^{32}$P]orthophosphoric acid for 5 h. Immunoprecipitated proteins were resolved by IEF in the first dimension, followed by SDS-PAGE in the second dimension (B). HeLa cells transiently transfected with HA1-E1 or HA2-E1 were metabolically labeled with either Tran$^{35}$S-label or [$^{32}$P]orthophosphoric acid, and immunoprecipitated proteins were resolved by IEF and SDS-PAGE (C).
FIG. 2. Phosphorylation of HA-tagged E1 N-terminal truncation mutants. A, a series of HA-tagged E1 species were generated which had successive 10 or 11 amino acids segments removed form their N terminus (A). HA1-E1 and these constructs were transiently transfected into HeLa cells and metabolically labeled with [32P]orthophosphoric acid for 5 h. Immunoprecipitated proteins were resolved by SDS-PAGE and transferred to nitrocellulose. The HA-encoding constructs were visualized using the 12CA5 antibody and chemiluminescence (B). The nitrocellulose was then exposed to film to detect 32P-labeled proteins (B).

Identical to E1a and E1b and is consistent with our hypothesis that the two E1 isoforms result from alternate translational start sites at the first and second in-frame methionines in the E1 sequence. These results also demonstrate that addition of the HA epitope tag to E1 did not alter its phosphorylation state.

To further confirm our observations, we dephosphorylated human E1 by treatment with potato acid phosphatase. Dephosphorylated 35S-labeled E1a resolved as a pattern of spots similar to E1b; it was found predominantly as spot 0 with minor species migrating as spot -1, 1, and 2 (data not shown). There was very little change in the pattern of 35S-labeled E1b spots after dephosphorylation; only a slight decrease in spot -1 was observed. Taken together, these data suggest that HA1-E1/E1a exists predominantly as four phosphorylated and a non-phosphorylated form; HA2-E1/E1b exists as two minor phosphorylated forms and as a predominant non-phosphorylated form. Furthermore, in addition to the phosphorylated forms, both HA1-E1/E1a and HA2-E1/E1b can be resolved into other non-phosphorylated charged species. A relatively abundant species (spot 1) is detected in 35S-labeled HA2-E1/E1b preparations, although the precise nature of these charged variants is currently not known.

Construction of HA Epitope-tagged E1 N-terminal Truncation Mutants—We and others have previously determined that E1a/HA1-E1 are phosphorylated, whereas no detectable phosphorylation has been demonstrated for E1b/HA2-E1 (11–13). However, in our current study it appears that HA2-E1/E1b is phosphorylated but at levels approximately 100-fold less than HA1-E1/E1a (Fig. 1). Others have recently determined that E1 is only phosphorylated on serine residues (12). As HA1-E1 is 40 amino acids longer than HA2-E1, it is tempting to speculate that this region at the N terminus contains the phosphorylation residues. Within this 40 amino acid region, there 10 serine residues. Thus, to identify which serines are involved in phosphorylation of HA1-E1, a series of N-terminal truncation mutants were generated (Fig. 2A). Constructs were made where the HA epitope was attached to the N-terminal region of E1 to create proteins that were truncated in segments of about 10 amino acids (Fig. 2A). These HA1-tagged E1 truncation mutants were transiently expressed in HeLa cells and metabolically labeled with [32P]orthophosphoric acid. HA-tagged constructs were then immunoprecipitated using the 12CA5 monoclonal antibody and resolved by SDS-PAGE and transferred to nitrocellulose. The total amount of phosphorylated E1a/HA1-E1 and E1b/HA2-E1 at the N terminus also resulted in no detectable phosphorylation of the HA-tagged constructs. These data thus suggest the first 11 amino acids contain residues that are essential for efficient phosphorylation of HA1-E1/E1a.

Immunolocalization of HA-tagged E1 N-terminal Truncation Mutants—Phosphorylation of certain proteins correlates with their retention in either the nucleus or cytoplasm (reviewed in Ref. 16). For example SWI5 is excluded from the nucleus when phosphorylated (17), whereas STAT-3 is translocated to the nucleus after phosphorylation (18). Indeed this is the case with E1; HA1-E1 is localized exclusively in the nucleus and phosphorylated, whereas HA2-E1 is found almost exclusively in the cytoplasm and is phosphorylated approximately 100-fold less than HA1-E1 (11). Previously we have determined the subcellular localization of HA1-E1del-11 by immunofluorescence using the 12CA5 monoclonal antibody (17). Removal of the first 11 amino acids of the E1 sequence (HA1-E1del-11) resulted in no detectable phosphorylation. Removal of 22 (HA1-E1del-22), 30 (HA1-E1del-30), and 40 amino acids (HA2-E1) at the N terminus also resulted in no detectable phosphorylation of the HA-tagged constructs. These data thus suggest the first 11 amino acids contain residues that are essential for efficient phosphorylation of HA1-E1/E1a.
Identification of Amino Acids Which Are Required for Phosphorylation and Nuclear Localization

The first 11 amino acids of the E1 sequence contains, in addition to serines 2, 3, 4, and 7, a putative nuclear localization sequence (NLS), PL-skRrR11. To determine which residues are responsible for phosphorylation or for nuclear localization, two additional HA-tagged E1 constructs were prepared: HA1-E1-del-7, where the first 7 amino acids (including serines 2, 3, 4, and 7) were removed, and HA1-E1-del-8–11, where the basic region of the putative NLS (8KKRR11) was deleted (Fig. 3A). These constructs along with HA1-E1 were transiently transfected into HeLa cells and metabolically labeled with [32P]orthophosphoric acid as described previously. Immunoprecipitated proteins were resolved by SDS-PAGE and transferred to nitrocellulose. The total amount of HA-encoding proteins was determined by immunoblot analysis (B). The nitrocellulose was then exposed to film to detect 32P-labeled proteins (B). The total HA-encoding E1 and 32P-labeled HA-E1 was quantified by densitometry and phosphorimaging analysis, and the specific phosphorylation of each construct was determined. Each construct was compared with the wild type HA1-E1, which was normalized to 100% (B). HeLa cells were transfected with each construct and immunofluorescence using the 12CA5 antibody performed (C).

E1 Requires an Intact NLS for Efficient Phosphorylation

Identification of Amino Acids Which Are Required for Phosphorylation and Nuclear Localization—The first 11 amino acids of the E1 sequence contains, in addition to serines 2, 3, 4, and 7, a putative nuclear localization sequence (NLS), PL-skKRR11. To determine which residues are responsible for phosphorylation or for nuclear localization, two additional HA-tagged E1 constructs were prepared: HA1-E1-del-7, where the first 7 amino acids (including serines 2, 3, 4, and 7) were removed, and HA1-E1-del-8–11, where the basic region of the putative NLS (8KKRR11) was deleted (Fig. 3A). These constructs along with HA1-E1 were transiently transfected into HeLa cells, labeled with [32P]orthophosphoric acid, resolved by SDS-PAGE, and transferred to nitrocellulose. The total amount of HA-tagged construct was determined by immunoblot analysis, and 32P incorporation was determined by autoradiography (Fig. 3B). To determine the specific phosphorylation of the HA-tagged constructs, the total amount of HA-tagged E1 constructs was quantified by densitometry, and 32P incorporation was determined by PhosphorImager analysis (Fig. 3B). Removal of the basic amino acids (8KKRR11) in the putative NLS from HA1-E1 drastically reduced the level of specific phosphorylation to 5% of HA1-E1 (Fig. 3B). Additionally HA1-E1-del-8–11 was no longer localized exclusively in the nucleus but found predominantly in the cytoplasm (approximately 90% of the cells displayed completely negatively stained nuclei) (Fig. 3C). This distribution is identical to that observed with HA1-E1-del-11. This implies that the basic region (8KKRR11) of the putative nuclear localization sequence at the N terminus of E1 is required for the exclusive nuclear localization of HA1-E1. The dramatic decrease in phosphorylation of HA1-E1-del-8–11 and the predominant cytoplasmic distribution strongly suggest a correlation between nuclear localization and phosphorylation.

The specific phosphorylation of HA1-E1-del-7 (deletion including serines 2, 3, 4, and 7) was approximately 50% that of HA1-E1 (Fig. 3B). Additionally HA1-E1-del-8–11 was no longer localized exclusively in the nucleus but found predominantly in the cytoplasm (approximately 90% of the cells displayed completely negatively stained nuclei) (Fig. 3C). This distribution is identical to that observed with HA1-E1-del-11. This implies that the basic region (8KKRR11) of the putative nuclear localization sequence at the N terminus of E1 is required for the exclusive nuclear localization of HA1-E1. The dramatic decrease in phosphorylation of HA1-E1-del-8–11 and the predominant cytoplasmic distribution strongly suggest a correlation between nuclear localization and phosphorylation.
plasmic localization was observed (Fig. 4A). This observation implies that phosphorylation of any of the serine residues within the first 7 amino acids (including serines 2, 3, 4, and 7) is not required for exclusive nuclear localization of HA1-E1. The dramatic reduction in specific phosphorylation and predominant cytoplasmic localization of HA1-E1-del-8–11 suggests that phosphorylation occurs within the nucleus. An alternative explanation may be that the kinase which phosphorylates HA1-E1 on one or more of the serine 2, 3, 4, and 7 residues binds to the basic region of the NLS (8KKRR11). Thus removal of this region would disrupt both nuclear targeting and phosphorylation. In an attempt to reconcile these two possibilities a further HA-tagged construct was generated where arginine residues 10 and 11 were mutated to alanines (HA1-E1-R10A,R11A; Fig. 4A). Removal of these two arginine residues also disrupts nuclear targeting (Fig. 4C) and substantially reduces the specific phosphorylation to levels comparable with HA1-E1-del-8–11 (Fig. 4B; 9% of HA1-E1). Although these results are not conclusive, they do suggest that phosphorylation of HA1-E1 may occur within the nucleus.

**The E1 NLS Efficiently Imports a Non-nuclear Protein into the Nucleus**—The archetypal NLS is that of SV40 large T antigen, which consists of a basic patch of amino acids on an \(\alpha\)-helix (19, 20). The putative E1 NLS (\(^{5}\)PLSKKRR\(^{11}\)) shows considerable sequence similarity to the SV40 NLS (71% homology over 7 amino acids) (Fig. 5A). Digitonin-permeabilized cells have been used to assay putative NLS motifs for their ability to import non-nuclear proteins to the nucleus (15). This assay system was employed to determine if the putative E1 NLS could import BSA into the nucleus of digitonin-permeabilized HeLa cells. Peptides were synthesized and cross-linked to BSA using sulfoSMCC via a terminal cysteine residue. The BSA peptide was then labeled with the fluorescent compound Cy3. Using reticulocyte lysate as a cytosol source, Cy3-BSA peptides were then assayed for their ability to be imported into the nucleus in digitonin-permeabilized HeLa cells (Fig. 5B). Assays were carried out in the presence of ATP at 30 °C, the absence of ATP at 30 °C, or the presence of ATP at 4 °C. Using this system nuclear import of BSA-NLS\(^{SV40}\) was dependent upon both ATP and temperature (data not shown), consistent with previously published data (15). The E1 NLS imported BSA to the nucleus in the presence of ATP and at 30 °C, but not in the absence of ATP nor at 4 °C (Fig. 5B). When KKRR were replaced with alanines (BSA-NLS\(^{E1mutant}\)), BSA was no longer imported to the nucleus (data not shown).

Others have demonstrated that phosphorylation at or near a
NLS may increase the rate of nuclear import or nuclear retention (16, 21). Within the putative E1 NLS (\textit{\textsuperscript{5PLSKKRRV12}}, there is one serine residue. We did not determine whether this serine residue was phosphorylated when this peptide was conjugated to BSA. However, a third BSA peptide was constructed where serine 7 was changed to an alanine (BSA-NLSE1-S7A; \textit{\textsuperscript{5PLAKKRRV12}}) to determine if this serine played a role in nuclear import. There was no substantial difference in nuclear import between BSA-NLSE1-S7A and BSA-NLS E1 (Fig. 5 B).

Thus, we conclude that the E1 sequence \textit{\textsuperscript{5PLSKKRRV12}} is able to confer nuclear import on a non-nuclear protein, and within this region KKRR is essential, whereas serine 7 is not.

Identification of Individual Serine Residues Which are Phosphorylated—To address which serine residues are phosphorylated within the first 7 amino acids of HA1-E1, a series of serine point mutants were generated where serine residues 2, 3, 4, or 7 were individually changed to an alanine (Fig. 6 A). When the specific phosphorylation of each of these constructs was compared with HA1-E1 (Fig. 6 B), HA1-E1-S4A was consistently the lowest (approximately 60%), HA1-E1-S2A, HA1-E1-S3A, and HA1-E1-S7A generally had levels of specific phosphorylation similar to the wild type HA1-E1. However, between experiments the level of specific phosphorylation of each construct compared with HA1-E1 varied somewhat. Therefore, to determine definitively which residues are phosphorylated, each construct was analyzed by IEF followed by SDS-PAGE. Each construct was transiently transfected into HeLa cells and metabolically labeled with \textit{\textsuperscript{32P}}orthophosphoric acid; HA-containing proteins were immunoprecipitated and subjected to two-dimensional electrophoresis (Fig. 6 C). HA1-E1 migrates as four phosphorylated species when analyzed by two-dimensional electrophoresis. Serine point mutants HA1-E1-S2A, HA1-E1-S3A, and HA1-E1-S7A all migrated as four phosphorylated species (Fig. 6 C). However, HA1-E1-S4A migrated as only three phosphorylated species indicating that changing serine 4 to an alanine removed one phosphorylation site from HA1-E1. Thus serine 4 is phosphorylated in HA1-E1/E1a.

E1b/HA2-E1 exists as two minor phosphorylated forms, a predominant non-phosphorylated form, and several forms which migrated toward the anode (Fig. 1). Densitometric quantification of \textit{\textsuperscript{35S}}-labeled E1b/HA2-E1 revealed that only 2.5–4% is found as the phosphorylated forms. Thus only a very small fraction of E1b is phosphorylated and may explain why previous studies by ourselves (11) and others (12, 22) were unable to detect it. E1a/HA1-E1 resolves as four phosphorylated and one non-phosphorylated forms (Fig. 1). Densitometric quantification of the \textit{\textsuperscript{35S}}-labeled E1a/HA1-E1 spots revealed that 92–97% is found in the phosphorylated forms, respectively. Several minor positively charged species were also observed (as described previously for E1b/HA2-E1). Dephosphorylation of E1a changed the electrophoretic migration to one very similar to E1b. This suggests that in the basal non-phosphorylated state E1 exists as a heterogeneous mixture of charged forms. This pattern of charged forms was extremely reproducible and was also observed with hamster E1 (data not shown). This heterogeneous mixture of charged forms may arise from modifications such as deamination; however, further work will be required to resolve this.

In an attempt to identify which serine residues are phosphorylated in HA1-E1, N-terminal truncation mutants were generated (Fig. 2 A). Removal of only the first 11 amino acids completely abolished phosphorylation of the HA-tagged E1 (Fig. 2B). Removal of further segments of the N terminus also...
resulted in no detectable phosphorylation (Fig. 2B). Thus the first 11 amino acids contain the necessary information for complete phosphorylation of HA1-E1/E1a. HA1-E1 is localized exclusively in the nucleus; however, removal of the first 11 amino acids changes the subcellular localization to predominantly cytoplasmic (11). Consistent with this, HA1-E1-del-22, HA1-E1-del-30, and HA2-E1 are all found predominantly in the cytoplasm (data not shown and Ref. 11). Hence, the first 11 amino acids in the E1 sequence contain the necessary information for exclusive nuclear localization and complete phosphorylation.

For efficient nuclear transport, proteins larger than approximately 50 kDa require a NLS (23). The putative NLS (5PL-SKKRR) at the extreme N terminus of E1 shares 71% homology with the SV40 NLS, which is regarded as the archetype. Deletion of the four basic residues from this putative NLS (HA1-E1-del-8–11) completely abrogated the exclusive nuclear localization of HA1-E1 (Fig. 3C). In addition, mutation of arginines 10 and 11 to alanines (HA1-E1-R10A,R11A) also abrogated the exclusive nuclear localization (Fig. 4C). Conjugation of this sequence (5PLSKKRRV12) to a non-nuclear protein (BSA) conferred import to the nucleus of digitonin-permeabilized cells (Fig. 5B). This import was both temperature- and ATP-dependent (Fig. 5B). Substitution of 5KKRR with alanines eliminated nuclear import of the substrate (BSA-NLS K12mutant, data not shown). Therefore by all criteria 5PLSKKRRV12 functions as an NLS in E1. However, despite the removal or disruption of this NLS, 5–10% of cells transfected with HA1-E1-del-8–11, HA1-E1-R10A,R11A, and HA2-E1 still show immunofluorescent staining in the nucleus (Ref. 11 and Fig. 4C). This suggests that there may be other functional NLS motifs within the E1 sequence. Indeed, within the C-terminal region there is a putative bipartite NLS (KERLDQPMTEIVSRVSKRK). Bipartite NLS motifs are characterized by two interdependent basic domains separated by 10 intervening “spacer” amino acids (24). Future studies will address the role of this region in nuclear localization of our HA-tagged constructs.

Several proteins have been identified whose rate of import to or retention in the nucleus is enhanced by phosphorylation near a NLS (16). For example, phosphorylation by casein kinase II near the SV40 large T antigen NLS increased the rate of nuclear import (21). Within the N-terminal 11 amino acids of the E1a sequence, there are serines 2, 3, 4, and 7. Serine 7 is a predicted protein kinase C site, and serine 4 can be phosphorylated by Cdc2 (22). Both of these residues are close to the NLS. Thus we defined the phosphorylation state of these serine residues and their potential role in nuclear targeting (Fig. 3). When the first 7 amino acids were deleted (HA1-E1-del-7, including the four serine residues), HA1-E1-del-7 was phosphorylated approximately 60% less than HA1-E1 (Fig. 3B) and was localized predominantly in the nucleus (Fig. 3C). However, approximately 3% of the cells also showed some cytoplasmic localization. One explanation for this observation may be that a certain level of phosphorylation is required for exclusive nuclear localization of HA1-E1. Alternatively, deletion of the first 7 residues includes 5PLS of the putative NLS, and this proline residue may be required for a secondary structural motif necessary for a functional NLS. To address this issue, another construct was generated where the first 4 amino acids were deleted and serine 7 was substituted with an alanine (HA1-E1-del-4,S7A). Phosphorylation of HA1-E1-del-4,S7A was approximately 20% that of HA1-E1 (Fig. 4B) and was...
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localized exclusively in the nucleus (Fig. 4C). Additionally, in our in vitro nuclear import assays substitution of serine 7 for an alanine (BSA-NLS8-7A, Fig. 5) did not alter the efficiency of nuclear import of BSA. Hence, phosphorylation on any of the serine 2, 3, 4, or 7 residues is not essential for nuclear import, whereas it appears that the proline and leucine residues are required for exclusive nuclear targeting of HA1-E1.

The specific phosphorylation level of HA1-E1-del-7 is 60% less than the wild type HA1-E1. This suggests that one or more of the four serine residues within this 7-amino acid region are phosphorylated. Each of these serine residues was substituted individually to an alanine, and their specific phosphorylation compared with the wild type (Fig. 6). HA1-E1-S4A was approximately 50% lower than the wild type. However, the precise level of phosphorylation of each of the constructs varied from experiment to experiment. To delineate exactly which serine was phosphorylated, each of the [32P]labeled constructs were analyzed by two-dimensional gel electrophoresis (Fig. 6C). HA1-E1-S2A, HA1-E1-S3A, HA1-E1-S7A, and the wild type all resolved as four phosphorylated spots; however, HA1-E1-S4A resolved as only three spots (Fig. 6C). This suggests that serine 4 is phosphorylated in HA1-E1/E1a and is consistent with observations made by Nagai et al. (22). Our data also suggest that serine 4 is the site that is phosphorylated most predominantly as removal of this site reduces the level of phosphorylation by 50% of the wild type.

Disruption of the NLS by removal of the KKRR (HA1-E1-del-8–11) or substitution of the arginines for the alanines (HA1-E1-R10A,R11A) results in loss of exclusive nuclear localization of HA1-E1 (Figs. 3C and 4C). However, between 5% and 10% of transfected cells still showed some detectable nuclear localization. In addition, HA1-E1-del-8–11 and HA1-E1-R10A,R11A were phosphorylated to less than 10% of HA1-E1 (Figs. 3 and 4B). This suggests that nuclear localization of HA1-E1 may be required for efficient phosphorylation. Only 3–5% of the total E1b is present in the nucleus (data not shown), and HA2-E1 is phosphorylated approximately 100-fold less than HA1-E1. HA2-E1/E1b also lacks serine 4, which is predominantly phosphorylated in HA1-E1. Thus it appears that the very low levels of phosphorylation of HA2-E1/E1b are due to the absence of serine 4 and its relative abundance outside the nucleus.

Despite these data, which suggest HA1-E1/E1a are phosphorylated in the nucleus, the precise function of E1 phosphorylation is not known. Previous work demonstrated that E1a is phosphorylated in a cell cycle-dependent manner; being maximally phosphorylated in G2 (13). However, no change in the enzymatic activity of E1a was observed with increased phosphorylation. Phosphorylation is not required for nuclear localization of HA1-E1 (Figs. 3 and 4), but may increase the rate of nuclear targeting and/or nuclear retention, neither of which was determined in our in vitro nuclear import assays (Fig. 5). Alternatively, phosphorylation of E1a may be required or important for stable interaction (or complex formation) with specific nuclear E2 proteins. E1 can be phosphorylated by p34cdc2 in vivo and in vitro (22). Pines and Hunter (25) have determined the localization of cyclins A and B, both of which associate with p34cdc2 to form the active kinase. Cyclin B is nuclear from the beginning of mitosis; however, cyclin A is nuclear from S phase onward and may well associate with p34cdc2 to phosphorylate substrates (including E1) in G2.

Many nuclear proteins including transcription factors such as p53, c-Fos, N-Myc, c-Myc (26), and signal transducers such as STAT1 (27) are substrates for the ubiquitin-proteasome pathway. This suggests the requirement for a functional ubiquitin-proteasome system within the nucleus. The proteasome is present in both the cytoplasm and the nucleus (reviewed in Ref. 28), and recent work has identified the functional NLS motifs present within the α subunits (29, 30). Interestingly, proteasomes purified from nuclear fractions were found to contain the mammalian homolog to SUG1 (31). In our current study we present evidence demonstrating that nuclear E1 is highly phosphorylated, whereas the cytoplasmic form is not. The enzymes of the ubiquitin-proteasome pathway present in the nucleus may have modifications that distinguish them from their counterparts within the cytoplasm, perhaps necessary for the different substrate specificities of a nuclear ubiquitin-proteasome system.

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Identification of a Region within the Ubiquitin-activating Enzyme Required for Nuclear Targeting and Phosphorylation

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