The Nucleotidylation of Herpes Simplex Virus 1 Regulatory Protein α22 by Human Casein Kinase II*

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The products of the α genes of herpes simplex virus 1, the infected cells proteins (ICP) 0, 4, 22, and 27 perform regulatory functions, are nucleotidylated, and share the signaling or recognition sequence (RR(A/T)(P/S)R) that correctly predicted the nucleotidylation of viral proteins encoded by U12, U13, U14, and U17 genes expressed later in infection. Extracts from uninfected HeLa cells or casein kinase II purified from sea star nucleotidylated the ICP22 moiety of a glutathione S-transferase-ICP22 (GST22P) fusion protein with [α-32P]ATP or [2-3H]ATP. We report that: (i) Purified HeLa cell casein kinase II specifically labeled a glutathione S-transferase fusion protein containing the amino-terminal 151 amino acids of ICP22 with [2-3H]ATP. (ii) Nucleotidylation of GST-ICP22 by purified enzyme exhibited positive cooperativity (Hill coefficient of 2 and a K₉ of 3.7 μM) but not by ATP γS. (iv) Mutation of the signaling sequence from RRPR to LKAPR abolished nucleotidylation. We conclude that nucleotidylation of proteins by casein kinase II requires the presence of the signaling or recognition sequence, involves the cleavage of the phosphodiester bond between the α and β phosphate, and need not be preceded by phosphorylation.

The 84 known herpes simplex virus 1 (HSV-1) genes form several groups whose expression is coordinately regulated and sequentially ordered in a cascade fashion at both the transcriptional and posttranscriptional levels (1, 2) (for review, see Ref. 3). At least four of the six α genes (the first genes to be expressed after infection), α0, α4, α22, and α27, appear to play a key regulatory role. The products of these genes, designated as infected cell proteins (ICPs) 0, 4, 22, and 27 have been shown to (i) transactivate the transcription of viral genes, both specifically (ICP4) and promiscuously (ICP0) (4–9) and (ii) regulate the processing and levels of mRNA and their corresponding proteins of all (ICP27) or of α0 and a subset of late (γ) genes (ICP22) (10–14). Of particular interest with respect to the function of these proteins is that they are extensively modified posttranslationally. All four are phosphorylated (15) and ICP4 has been shown to be poly(ADP-ribose)ated both in vitro and in infected cells (16, 17). More recently, this laboratory reported that these proteins are also guanylylated and adenylylated (18, 19). This conclusion was based on the observations that these proteins were labeled in isolated nuclei with [α-32P]GTP and [α-32P]ATP, as well as with [2-3H]ATP where the tritium atom is in the purine ring. The four α regulatory proteins contain a common amino acid sequence RR(A/T)(P/S)R designated the alpha protein basic (APB) site. The hypothesis that the APB sequence signals a posttranslational modification was supported by the observation that it accurately predicted the identity of four additional viral proteins labeled by [α-32P]GTP and [α-32P]ATP (20). We should stress that the genes encoding these proteins were mapped by first defining the genomic organization of nucleotidylylated proteins from analyses of proteins specified by HSV-1 X HSV-2 intertypic recombinants containing specific regions of the HSV-2 genome in a HSV-1 background. In the second step, the genes whose protein are nucleotidylylated were identified.

These earlier studies suggested the possibility that ICP22 was nucleotidylylated by a cellular enzyme (19). Consistent with this conclusion, it was shown that an ICP22-GST fusion protein (GST22P) was nucleotidylylated by an enzyme present in a nuclear extract from uninfected HeLa cells (21). Also, the addition of GST22P to the reaction containing [α-32P]ATP and HeLa cell nuclear extracts inhibited the labeling of at least two host cell proteins (H2 and H3) by [α-32P]ATP. The substrate specificity and subcellular localization of the nucleotidylylating activity led us to test the hypothesis that this enzyme might be casein kinase II (CKII). Subsequent studies showed that GST22P could be nucleotidylylated by CKII purified from the Sea Star (21) with [α-32P]ATP, [α-32P]GTP, or [2-3H]ATP but not with [α-32P]CTP.

Casein kinase II is a ubiquitous serine/threonine eukaryotic kinase existing as a heterotetramer of either αββ2, α´β2, or ααβ2 where the subunits have an apparent Mr of 24–28 kDa (β), 40–44 kDa (α), and 37–41 kDa (α´) (22). The α and α´ subunits are encoded by separate but related genes showing 85% homology to each other (23) and are capable of phosphorylase-transfase activity as monomers (24). The significance of the divergence in the α and α´ subunits has yet to be determined. It has been suggested that the β subunit has a regulatory function (25) and that the addition of the β subunit to form the heterotetramer stimulates the phosphorylation activity by at least 10-fold (24). Kinase activity is inhibited by heparin and stimulated by the addition of basic peptides such as polylysine.

The β subunit is normally phosphorylated, but the α subunit may be phosphorylated in the presence of basic substrates (25);
the function of these modifications is unknown. CKII is predominantly a nuclear enzyme (26); it can be co-precipitated with a transcription complex containing ATF1 since it specifically binds to an ATF1-GST fusion protein (27). CKII has been reported to be present in purified virions of HSV-1(F) (28).

The function and posttranslational modifications of CKII have been of interest to this laboratory for several reasons. In earlier studies with a virus (R325) containing a carboxyl-terminal truncation of ICP22, this laboratory showed that ICP22 is required for optimal viral replication in primary human cell strains and in cell lines of rodent origin; in these cells, the virus yields and the production of late (γ) proteins are reduced (13, 14). It is relevant to note that ICP22 is phosphorylated by both the U13 and U3 protein kinases (29) and that the phenotype of viruses lacking the U13 kinase was similar to that of mutants lacking the α22 gene, i.e., phosphorylation of ICP22 by CKII is required for optimal expression of α22 mRNA, ICPO, and a subset of γ proteins (14). However, deletion of both U13 and U3 genes from the viral genome did not affect nucleotidylylation of viral proteins (20).

We now report the following. (i) An activity present in a partially purified uninfected HeLa cell extract that contains CKII (fraction III) was able to specifically label a GST fusion protein containing 151 amino acids from the amino portion of CKII (fraction III) and mixed thoroughly by inversion. Initially, a 20% saturation, dry ammonium sulfate was added directly to the supernatant (fraction I) and the extract was chromatographed at 4°C on a Q-Sepharose column using standard techniques (32). Briefly, after application of fraction II, the column was rinsed with 1.5-column volumes of column buffer, and the volume of the eluate was reduced by concentration against polyethylene glycol flake (Calbiochem). The concentrated protein (fraction II) was filtered through a 0.45-micron polyvinylidene difluoride membrane, and the fractions were concentrated on a Q-Sepharose column as described above. The fractions active for nucleotidylylation were pooled (fraction III).

**CKII Nucleotidylylation of ICP22**

**Materials and Methods**

The HeLa S3 cell line obtained from the American Type Culture Collection was grown in Dulbecco's modified Eagle's medium supplemented with 5% newborn calf serum. HSV-1 strain F is the prototype HSV-1 strain used in this laboratory (30). Subconfluent HeLa S3 cultures containing approximately 4 × 10^6 cells were exposed to 5 plaque-forming units per cell for 1 h and then incubated at 37°C in medium containing 2% newborn calf serum.

**Reagents and Chemicals—**All reagents, inhibitors, tyrosine-agarose, heparin-agarose, nucleotides, and biochemicals, including heparin, were from Sigma. Q-Sepharose fast flow was from Pharmacia Biotech Inc. Protein concentration was determined by the Bradford assay (Bio-Rad). The silver stain kit was purchased from Diachii (Tokyo, Japan). Protein concentration was determined by the Bradford assay (Bio-Rad). Protein concentration was determined by the Bradford assay (Bio-Rad).

**Construction of Plasmids—**All plasmids derived in this study were made by standard procedures described elsewhere (31). Restriction endonucleases were from New England Biolabs and T4 DNA ligase was from U. S. Biochemical Corp. Construction of the GST-ICP22 fusion protein (GST22P) was described elsewhere (21). For construction of the GST-ICP22 fusion protein with a mutated APB site, the expression plasmid pGEX2T was obtained from Pharmacia Biotech Inc.

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**Preparation and Fractionation of Whole Cell Extract—**Suspension cultures of HeLa S3 cells were grown at 37°C in 5% newborn calf serum to a density of approximately 3–5 ml of packed cells per liter. All extraction procedures were at 4°C. The pelletted cells were rinsed three times in 8–10 packed cell volumes of phosphate-buffered saline (140 mM NaCl, 3 mM KCl, 10 mM NaHPO_4, 1.5 mM KHPO_4, pH 7.4), and the cell suspension was lysed by resuspension in 1.5 volumes of 20 mM HEPES (pH 7.8), 520 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM l-1-chloro-3-(4-tosylamido)-4-phosphoryl-2-butanone (TPCK), 10 μM 1-chloro-3-(4-tosylamido)-7-amino-2-heptanoyl-chloride (TLCK), 0.5 mM phenylmethylsulfonyl fluoride, and 0.1% Nonidet P-40 in 25% glycerol followed by rotating the samples for 60 min at 4°C. After pelleting the cellular debris, samples were stored at −80°C. Prior to fractionation, the ammonium sulfate was added directly to the supernatant and mixed thoroughly by inversion. Initially, a 20% saturation was taken and discarded, and a 60% saturation was pelleted, dissolved in lysis buffer, and then dialyzed into column buffer (20 mM ethanalamine (pH 9.5), 50 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.02% Nonidet P-40, 0.5 mM dithiobitol, 0.5 mM phenylmethylsulfonyl fluoride, 10 μM TPCK, 10 μM TLCK, and 25% glycerol). The dialyzed fraction (fraction II) was clarified by centrifugation, filtered through a 0.2 μm cellulose acetate filter (Corning) and then the extract was applied to a Sephadex G-50 column equilibrated with column buffer, and the volume of the eluate was reduced by concentration against polyethylene glycol flakes (Calbiochem). The concentrated protein (fraction II) was filtered through a 0.45-micron polyvinylidene difluoride membrane, and the fractions were concentrated on a Q-Sepharose column as described above. The fractions active for nucleotidylylation were pooled (fraction III).

**Purification of CKII from HeLa Cells—**Preparation of fraction III was as outlined above except the concentration of buffer in the extraction buffer was increased to 50 mM HEPES. Following ammonium sulfate fractionation, the precipitate was resuspended in Q-Sepharose column buffer to 0.5× the starting volume. The suspension was then passed through a Sephadex G-50 column equilibrated with column buffer, and the volume of the eluate was reduced by concentration against polyethylene glycol flake (Calbiochem). The concentrated protein (fraction II) was filtered through a 0.45-micron polyvinylidene difluoride membrane, and the fractions were concentrated on a Q-Sepharose column as described above. The fractions active for nucleotidylylation were pooled (fraction III).

**To concentrate the pooled sample, fraction III was diluted 2-fold with 50 mM Tris-HCl (pH 8.0), 25% glycerol, reapplied to a 5-ml Q-Sepharose column, eluted with a 100-mL gradient as described above, and the active fractions were pooled.**

**Subsequent purification steps were a modification of the procedure by J.S. Sugrera, et al. (33). The Q-Sepharose pool was diluted with an equal volume of 50 mM Tris-HCl (pH 8.0), 1 mM dithiobitol, and 2 mM EDTA (Buffer B) containing 1.6 M (35% saturation) (NH_4)_2SO_4. The extract was stirred over ice for 10 min and then centrifuged at 10,000 × g for 10 min at 4°C. The supernatant was removed and loaded onto a tyrosine-agarose column pre-equilibrated in Buffer B containing 0.8 M (NH_4)_2SO_4. The column was developed with a 200-mL decreasing linear gradient starting with 0.8 M (NH_4)_2SO_4 in Buffer B, ending with Buffer B, and 4-mL fractions were collected. The fractions containing activity that labeled GST22P with [γ-32P]ATP were pooled, diluted 1:3 with Buffer B, and loaded onto a heparin-agarose column pre-equilibrated with Buffer B. The column was rinsed with 10-column volumes of Buffer B containing 50 mM NaCl and then the proteins were eluted with a 100-mL linear gradient from 50 to 800 mM NaCl. The fractions containing nucleotidylylation activity were pooled and assayed for purity using SDS-polyacrylamide gel electrophoresis and silver staining (Diachii, Tokyo, Japan).**

**Purification of Fusion Proteins from Escherichia coli—**The procedure for the growth, induction, and purification of herpesviral fusion proteins in E. coli were a modification of procedures described previously (34). In general, E. coli do not express herpesvirus proteins stably either because of the high guanine/cytosine content of viral DNA or the high content of proline in viral proteins. The following protocol reproducibly generated intact fusion protein to yields as high as 8 mg/liter of bacterial culture. Briefly, E. coli BL21 cells containing plasmids that expressed appropriate fusion proteins were stored in 15% glycerol at −70°C as a freezer stock. One loop full of the freezer stock served as the inoculum for 50 ml of L-broth containing ampicillin (100 μg/ml) that was incubated at 37°C with shaking for 24 h. The 50 mL of L-broth containing ampicillin that was placed in a shaking incubator at 30°C and incubated until an A_600 of 0.8 to 1.2 was reached. At that point, 60 μl of a freshly prepared 20% isopropyl-thio-β-D-galactopyranoside solution was added, and the cells were incubated for an additional 2 h. Pelleted cells were resuspended in 5 ml of ice cold 20 mM Tris-HCl (pH 8.0), 200 mM NaCl (TBS), briefly sonicated
on ice, and 500 μl of Triton X-100 (1/10 volume) was added prior to pelleting the cellular debris at 10,000 rpm for 5 min in a Sorvall SS34 rotor at 4 °C. For GST22PH, the cell extract was prepared as specified above and solubilized in a portion of the ICP22 protein were as follows: line 1, GST22P containing amino acids 49–200; line 4, GST22M containing amino acids 49–200 but with mutations R76L, R77K, R80E, and R81K; and line 5, GST22PH containing amino acids 49–200 plus 6 in frame histidine residues. The filled ovals indicate that the fusion proteins contain intact APB sites, whereas the open box refers to a mutant APB site with the sequence LKAEPER instead of RAPPR at ICP22 amino acids 76–81.

![Diagram](image-url)

**FIG. 2.** Schematic representation of the ICP22-GST fusion proteins. Line 1, the prototype orientation of the HSV genome; the boxes indicate the repeat sequences flanking the unique long (U1) and short (U2) sequences of HSV DNA. Line 2, the c22 gene showing relevant restriction endonuclease sites; the striped box and RRAPR refer to the location of the APB sequence in ICP22. Lines 3–5, the portions of the ICP22 protein fused to the GST protein (oval). The constructs containing a portion of the ICP22 protein were as follows: line 3, GST22PH containing amino acids 49–200; line 4, GST22M containing amino acids 49–200 but with mutations R76L, R77K, R80E, and R81K; line 5, GST22PH containing amino acids 49–200 plus 6 in frame histidine residues. The filled ovals indicate that the fusion proteins contain intact APB sites, whereas the open box refers to a mutant APB site with the sequence LKAEPER instead of RAPPR at ICP22 amino acids 76–81.

**FIG. 3.** Coomassie Blue staining (A) and immune reactivities (B) of the purified GST fusion proteins. The purified fusion proteins were separated on a denaturing 12% polyacrylamide gel and stained with Coomassie Blue, or transferred to nitrocellulose and probed with antibodies to GST as described under “Materials and Methods.”

**Retention of Labeled Proteins on Nitrocellulose Filters—** Autoradiographic images (A) and immunoluminescent reactivities (B) of fractionated proteins labeled with [α-32P]ATP. HeLa cell proteins were isolated, fractionated on Q-Sepharose, labeled at 15 °C with [α-32P]ATP, separated on a denaturing 12% gel, transferred to nitrocellulose, and probed with antibodies to either CKII or Erk-1 using the ECL protocol, as described under “Materials and Methods.” Arrows indicate the positions of CKII, Erk-1, H1, H2, and H3. The labels H1, H2, and H3 refer to the nucleotidylylated host proteins that were identified previously (20, 22). Sodium chloride fractions (15–45) from approximately 0.1–0.4 mM are shown, as well as fraction I (I), fraction II (II), the flow-through (FT), and wash (W).

**Fig. 1.** Autoradiographic images (A) and immunoluminescent reactivities (B and C) of fractionated proteins labeled with [α-32P]ATP. HeLa cell proteins were isolated, fractionated on Q-Sepharose, labeled at 15 °C with [α-32P]ATP, separated on a denaturing 12% gel, transferred to nitrocellulose, and probed with antibodies to either CKII or Erk-1 using the ECL protocol, as described under “Materials and Methods.” Arrows indicate the positions of CKII, Erk-1, H1, H2, and H3. The labels H1, H2, and H3 refer to the nucleotidylylated host proteins that were identified previously (20, 22). Sodium chloride fractions (15–45) from approximately 0.1–0.4 mM are shown, as well as fraction I (I), fraction II (II), the flow-through (FT), and wash (W).
Fraction III of the HeLa Cell Extract, Capable of Nucleotidylylating GST22P, Is Enriched for CKII—Earlier studies from this laboratory have shown that GST22P can be labeled in vitro with uninfected HeLa cell nuclear extract using [$\alpha^{-32}$P]ATP or [$\alpha^{-32}$P]GTP, suggesting that a HeLa nuclear enzyme was required for this modification (21). Furthermore, several lines of evidence suggested that this enzyme had characteristics in common with CKII led to the demonstration that purified Sea Star CKII (Upstate Biotechnology, Lake Placid, NY) could label GST22P with either [$\alpha^{-32}$P]ATP or [2-$^3$H]ATP in a filter binding assay. Because of the high background in the labeling reaction with HeLa cell nuclear extracts, we partially purified the HeLa cell CKII as described under “Materials and Methods” (fraction III). To verify the presence of CKII in fraction III, HeLa cell protein was fractionated on Q-Sepharose, labeled with [$\alpha^{-32}$P]ATP, separated on Q-Sepharose, labeled with [$\alpha^{-32}$P]ATP, separated on a denaturing 12% gel, transferred to nitrocellulose, and probed with antibodies to either CKII or Erk-1 using the ECL technique as described under “Materials and Methods.” The amount of radioactivity present in the purified fraction was determined using liquid scintillation.

RESULTS

Fraction III of the HeLa Cell Extract, Capable of Nucleotidylylating GST22P, Is Enriched for CKII—Earlier studies from this laboratory have shown that GST22P can be labeled in vitro with uninfected HeLa cell nuclear extract using [$\alpha^{-32}$P]ATP or [$\alpha^{-32}$P]GTP, suggesting that a HeLa nuclear enzyme was required for this modification (21). Furthermore, several lines of evidence suggested that this enzyme had characteristics in common with CKII led to the demonstration that purified Sea Star CKII (Upstate Biotechnology, Lake Placid, NY) could label GST22P with either [$\alpha^{-32}$P]ATP or [2-$^3$H]ATP in a filter binding assay. Because of the high background in the labeling reaction with HeLa cell nuclear extracts, we partially purified the HeLa cell CKII as described under “Materials and Methods” (fraction III). To verify the presence of CKII in fraction III, HeLa cell protein was fractionated on Q-Sepharose, labeled with [$\alpha^{-32}$P]ATP, separated on Q-Sepharose, and labeled with either [$\alpha^{-32}$P]ATP or [2-$^3$H]ATP in a filter binding assay. Because of the high background in the labeling reaction with HeLa cell nuclear extracts, we partially purified the HeLa cell CKII as described under “Materials and Methods” (fraction III). To verify the presence of CKII in fraction III, HeLa cell protein was fractionated on Q-Sepharose, labeled with [$\alpha^{-32}$P]ATP, separated on Q-Sepharose, and labeled with either [$\alpha^{-32}$P]ATP or [2-$^3$H]ATP in a filter binding assay. Because of the high background in the labeling reaction with HeLa cell nuclear extracts, we partially purified the HeLa cell CKII as described under “Materials and Methods” (fraction III).

(i) Panel A shows the nucleotidylylation profile of HeLa cell proteins fractionated on a Q-Sepharose column. Bands H2 and H3 predominate in the flow-through, wash, and in the early (low salt) elutions. The majority of the other major labeled bands partition in fractions 33–43. Using a dot-blot apparatus in an in vitro nucleotidylylation assay with GST22P as the substrate, we observed that the activity, which specifically labeled GST22P with [$\alpha^{-32}$P]ATP, peaked between fractions 37 and 41 (data not shown), and the pool of these fractions was termed fraction III.

(ii) Erk-1 (panel C) did not partition with either H2/H3 or the peak of major labeled bands, whereas CKII (panel B) eluted in fractions 33–45 and peaked in fraction 39.

From these results we conclude that CKII, but not Erk-1, is present in the protein fraction (III), which is capable of nucleotidylylating ICP22.

FIG. 4. Tritiated ATP incorporated into the substrate as a function of concentration of fusion protein substrate (A) and ATP (B). The amount of [2-$^3$H]ATP retained on nitrocellulose by fresh GST22P (□) and GST (●) following incubation with fraction III was measured as described under “Materials and Methods.” The amount of radioactivity present on the filters was determined using liquid scintillation.

FIG. 5. Purification of HeLa cell CKII to near homogeneity. The proteins were separated on a denaturing 12% gel and stained using silver stain or transferred to nitrocellulose, and the CKII α subunit was visualized using Antibody 6461D as described under “Materials and Methods.” Lane 1 contains fraction III; lane 2 is blank; lane 3 contains purified CKII; and lane 4 is an immunostaining of the CKII α subunit present in the purified fraction.

Construction of GST-ICP22 Fusion Proteins—To characterize the nucleotidylylation of ICP22, we generated a series of GST fusion proteins (Fig. 2) that contain amino acids 49 to 200 of ICP22. This domain of ICP22 was specifically chosen because earlier studies showed that it is of a sufficient length to be nucleotidylylated by an uninfected cell extract as well as by purified sea star CKII (21). In addition, the amino-terminal fragment of ICP22, expressed in recombinant virus R325, is nucleotidylylated (19). Moreover, earlier studies showed that the ABP site could be used to predict the HSV-1 proteins that would be nucleotidylylated (20), suggesting that this site is either the labeling site or is involved in substrate recognition. To provide a negative control for our in vitro assay, we created GST22M, which has a mutated ABP site with the sequence “LKAPEK” instead of “RRAPRR” at ICP22 amino acids 76–81. In addition, we created a histidine-tagged version of GST22P consisting of the glutathione S-transferase at the amino terminus followed by amino acids 49–200 of ICP22 and 6 histidine residues at the carboxyl terminus (GST22PH). Thus, purification of the tagged fusion protein by affinity chromatography on Ni-NTA-agarose and glutathione-agarose should yield only...
The presence of 1 nucleotidylylation of GST22P by CKII in materials and Methods.” Panel A shows labeling of 1.0 μM GST22P (□), GST22M (□), or casein (△). Panel B shows the effect of nucleotidylylation of GST22P by CKII in the presence of 1 μM [2-3H]ATP and 99 μM ATP (□), ATPγS (■), or ATPαS (○).

Panel A

A

B

Panel A shows initial velocity (V, pmoles/min) versus increasing ATP concentration. Panel B is the double reciprocal plot of the data in panel A.

Fig. 7. Determination of the K_m for ATP. 1 μg of GST22P was labeled at 30 °C using purified CKII and [2-3H]ATP in a filter binding assay and then quantitated by scintillation counting as described under “Materials and Methods.” Panel A shows labeling of 1.0 μM GST22P (□), GST22M (□), Casein (□), or ATPγS (■), or ATPαS (○).

Production and Labeling of GST Fusion Proteins with Fraction III—To show that fraction III was capable of nucleotidylylating GST22P, we first compared the products isolated during purification of GST22P, GST22PH, and GST22M by analysis with denaturing gels and immunoblotting. The purified products were separated in a denaturing gel, electrically transferred to nitrocellulose, and probed with antibody SC-138 (specific for GST) as described under “Materials and Methods.” The results (Fig. 3) were as follows: Coomassie Blue staining shows the bands present in the fraction containing the purified GST fusion proteins (panel A). During the synthesis of the fusion proteins in E. coli BL21 cells, proteolytic degradation occurred as seen in panel A, lanes 1, 2, and 4. Those incomplete fragments that still contained a functional GST moiety bound to the glutathione resin and were purified with the full-length fusion. Despite the double purification, the lane containing the double fusion GST22PH also contains some degradation products (panel A, lane 4), probably due to dimerization of GST (35). Densitometric analysis of the gel represented in panel A (as described under “Materials and Methods”) determined that the top two bands of lane 1 represent 10% of the Coomassie Blue staining protein in that lane, whereas the corresponding area of lane 4 represents 80% of the total (data not shown).

All of the proteins corresponding to the major Coomassie Blue staining bands in the lanes containing GST22P, GST22M, or GST22PH reacted with antibody against GST (panel B, lanes 5, 6, and 8). The observation that the more rapidly migrating bands in lane 8 react with the anti-GST antibody indicate that they are truncated products that may have co-purified due to dimerization of the GST moiety and not unrelated bacterial proteins.

In the second series of experiments, freshly prepared GST22P or GST was incubated with fraction III in the presence of [2-3H]ATP at 30 °C for 30 min and bound to nitrocellulose membranes as described under “Materials and Methods.” Two sets of reaction conditions were analyzed. In the first, the concentration GST22P or GST was varied, whereas the concentration of ATP was held constant. In the second, the amount of GST22P was held constant, and the extent of adenyllylation of this protein was measured as a function of increasing concentrations of ATP.

The results (Fig. 4, panel A) showed that within the limits of the experimental design, the amount of label incorporated into the GST22P by [2-3H]ATP was proportional to the concentration of the fusion protein in the reaction mixture. In contrast, the labeling of GST was at a background level and remained constant, independent of the concentration of the protein. From the data presented in Fig. 4, panel B, an approximate K_m of 35 μM ATP for the activity in fraction III was calculated. This value corresponds approximately to the K_m for ATP previously reported for phosphorylation by CKII (22). This value was also the same as that observed for labeling GST22P with [α-32P]ATP and [α-35P]GTP under identical conditions (data not shown). The labeling of GST22P with [α-35P]ATP was stable to treatment with HCl and NaOH, suggesting that the bond was stable and not a transient intermediate.

Purification of HeLa Cell CKII from Fraction III—To further ascertain that the activity identified in fraction III was due to CKII and not to a contaminating activity, CKII was purified to near homogeneity as described under “Materials and Methods.” The protein in fraction III was concentrated by application to a second Q-Sepharose column, the active fractions were pooled, (NH₄)₂SO₄ was added to 0.8 M, and the sample was fractionated on tyrosine-agarose as a reverse phase column. The fractions containing the peak of activity were pooled, diluted, and fractionated on a heparin-agarose column. The fractions containing

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the peak of activity were pooled, and the proteins were separated by SDS-polyacrylamide gel electrophoresis and visualized by silver staining (Fig. 5, panel A). The identity of the band corresponding to the α subunit of CKII was identified by immunostaining with a rabbit polyclonal antibody generously provided by E. G. Krebs (Fig. 5, panel B). The band with an apparent Mr of 116,000 is enhanced by a gel artifact, and the other bands in lane 3 above 45 kDa represent less than 20% of the stained material as determined by densitometry. The other bands present in lane 3, slightly below those identified as the subunits of CKII, may represent degradation products that do not react with the antibody, as they were present in multiple preparations and could not be removed by further purification steps.

Labeling of GST Fusion Proteins by Purified CKII—In this series of experiments, various substrates were incubated with purified CKII in the presence of [2-3H]ATP. The amount of tritiated nucleotide incorporated was determined using a filter binding assay and scintillation counting as described under “Materials and Methods.” The purified CKII nucleotidylylated GST22P but not GST22M or casein (Fig. 6, panel A), demonstrating that the reaction is specific for the APB site of GST22P and not a nonspecific artifact. In addition, the failure to label GST22M suggests that the sequence RRAPR is required for nucleotidylylation, although the possibility that the mutation alters the folding of the GST22M protein and thereby blocks the reaction cannot be discounted. On the other hand, all three proteins were labeled by purified CKII using [γ-32P]ATP (data not shown) indicating that the kinase was fully functional. The nucleotidylylation reaction could not be stimulated by basic substrates such as polylysine, but as previously demonstrated with Sea Star CKII (21), labeling of GST22P by fraction III with [α-32P]ATP, [γ-32P]ATP, and [2-3H]ATP could be inhibited by the addition of heparin at concentrations below 0.2 μM (data not shown). Panel B shows the ability of CKII to label GST22P with 1 μM [2-3H]ATP in the presence of 99 μM ATP, or the nonhydrolyzable analogs ATPαS and ATPγS. The reaction rate in the presence of ATP and ATPγS were identical, whereas ATPαS completely inhibited the nucleotidylylation activity. This observation indicated that nucleotidylylation proceeds through the hydrolysis of the phosphodiester bond between the α and β phosphate and that the hydrolysis of the β/γ bond is not required. Thus, nucleotidylylation is a unique modification and does not require previous phosphorylation, and it does not proceed through progressive removal of phosphates from the nucleotide.

Determination of the K$_m$ and K$_{cat}$ for Nucleotidylation by Purified CKII—To characterize the nucleotidylylating activity of CKII, the K$_m$ for both of its substrates was determined. First, the GST22P concentration was held constant, and the ATP concentration was varied (Fig. 7, panel A). A double reciprocal plot yielded a K$_m$ of 37.7 μM, a V$_{max}$ of 2.08 and a K$_{cat}$ of 17.1 pmol/min/μg enzyme for ATP. This value is identical to the K$_m$ determined for ATP using fraction III and is completely consistent with the K$_m$ for ATP reported for the kinase activity of CKII (22).

The ATP concentration was next held constant at 100 μM, and the GST22PH concentration was increased. The results shown in Fig. 8 (panel A) were analyzed on a double reciprocal plot and did not yield a linear relationship (Fig. 8, panel B). Repeated efforts consistently yielded a convex line, suggesting a positive cooperativity in nucleotidylylation of GST22PH. To test this hypothesis, a plot of the reciprocal of the initial velocity against the square of the reciprocal of the substrate concentration was made and yielded a linear relationship (Fig. 8, panel C). This result suggests that CKII can nucleotidylylate GST22PH in a positively cooperative manner with an apparent Hill coefficient of 2 and a K$_c$ of 3.7 μM. Further studies will be required to elucidate the nature of this positive cooperativity.

**DISCUSSION**

The salient features and the significance of our results are as follows: (i) HeLa cell nuclear extract (fraction III) containing CKII specifically labeled a GST fusion protein containing 151 amino acids from the amino-terminal portion of ICP22 with [2-3H]ATP. This domain of ICP22 was previously shown to be nucleotidylylated in isolated nuclei, as well as by purified Sea Star CKII (19, 21). CKII, purified to near homogeneity, labeled the substrates GST22P and GST22PH in a manner similar to that described above for fraction III and to that reported earlier for purified Sea Star CKII (21). Our analyses yielded a K$_m$ of 37.7 μM and K$_{cat}$ of 17.1 pmol/min/μg enzyme for ATP. These
values are of the same order of magnitude as those previously determined for the $K_m$ of ATP for phosphorylation by CKII. Adenylylation of the fusion protein GST22M, in which the sequence RRAPRR was replaced with LKAPAEK, was substantially reduced relative to the unmodified polypeptide. These results indicate that CKII nucleotidylylates proteins and that the sequence RRAPRR is necessary for this reaction, suggesting that this site is either required for binding of the enzyme to the substrate or the actual site of the modification.

(ii) The observation that the activity could be inhibited by ATPαS but not by ATPγS suggests that the reaction proceeds through hydrolysis of the $\alpha/\beta$ phosphodiester bond of ATP and does not require hydrolysis of the $\beta/\gamma$ as would be required for phosphorylation.

(iii) Nucleotidylylation of GST22PH shows positive cooperativity. It is conceivable that the the structure of the CKII heterotrimer required for nucleotidylylation is different from that required for protein kinase activity. Elucidation of the interaction between CKII and the substrate nucleotidylylated by this enzyme will require further studies.

Studies on the HSV-1(F) mutant R325 suggest that although host proteins H1, H2, and H3 are labeled in uninfected cells, the nucleotidylylation of viral proteins is a virus-specified process and not a random reaction of a cellular enzyme. R325 is temperature sensitive and at 39.5 °C it expresses primarily the nucleotidylation of viral proteins 0, 4, and 27 may require prior posttranslational modification by gene products expressed later in infection. In addition, recent studies by Hibbard and Sandri-Goldin (36) suggest that the amino acid sequence necessary for the nucleotidylylation of ICP27 is essential for the function of the protein. Thus, deletion of a stretch of 13 amino acids that included the sequence RRAPRT (the ICP27 APB site) is lethal and precludes the expression of late genes. The specific function conferred by the nucleotidylylation of viral proteins remains to be elucidated. It is conceivable that this function differs depending on the protein that is nucleotidylylated.

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