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Identification of a Viral Kinase That Phosphorylates Specific E2Fs and Pocket Proteins

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The transcription factor E2F and its regulation by pRB and related pocket proteins are central to cell cycle control in higher eukaryotes. Much of our knowledge of this regulation has come from studies using immediate-early proteins of DNA tumor viruses. Previously, we reported that the 72-kDa immediate-early region 1 gene product of the human cytomegalovirus, IE72, transactivates the dihydrofolate reductase promoter through the E2F site and that it physically interacts with E2F1 (M. J. Margolis, S. Pajovic, E. L. Wong, M. Wade, R. Jupp, J. A. Nelson, and J. C. Azizkhan, J. Virol. 69:7759–7767, 1995). In this study, we further characterized the mechanism by which IE72 modulates E2F-dependent transcription. In vitro phosphorylation reactions using gel-purified bacterially expressed proteins revealed that IE72 is a kinase that autophosphorylates and phosphorylates E2F1, -2, and -3 (but not E2F4 or -5) and the RB-related pocket proteins p130 and p107 (but not pRB). The region of IE72 spanning amino acids 173 to 197 shows a high level of homology to the ATP binding sites in over 500 kinases. The kinase-negative protein IE72ΔATP, from which this region has been deleted, cannot activate E2F-dependent transcription. The kinase activity of IE72 is also required for its ability to reduce the association of E2F4 with p107 and p130. Taken together, these data suggest that the kinase activity of IE72 is required for E2F-dependent transcriptional activation and that this is likely to result from phosphorylation of specific members of the E2F and pocket protein families by IE72.

E2F was originally identified as a transcription factor required by E1A for activation of the adenovirus E2 promoter. The gene encoding dihydrofolate reductase (DHFR) was the first cellular gene shown to contain a binding site for E2F (6), and transactivation of its promoter by adenovirus E1A was found to be E2F dependent (18). Subsequently, E2F was shown to be involved in the cell cycle regulation of several genes important in cellular growth control (for reviews, see references 23, 24, and 38). E2F activity is regulated by its interaction with the product of the tumor suppressor retinoblastoma susceptibility gene, pRB, and the related pocket proteins, p107 and p130, which are themselves tightly regulated during differentiation (36) and the cell cycle (for a review, see reference 32). With the cloning of E2F1, followed by the cloning of other, related proteins, E2F was shown to consist of a family with five members that heterodimerize with DP1 or DP2, which are members of a related protein family (for a review, see reference 23). The different E2F family members show specificity in their interactions with the pocket proteins; whereas E2F4 is able to interact with all of the pocket proteins, E2F1, -2, and -3 appear to interact only with pRB and E2F5 apparently interacts only with p130 (4, 13, 16, 19, 21, 25, 29). Modulation of transcription from promoters containing E2F sites can be achieved through increased E2F transactivation activity by release of free E2F from E2F-pocket protein complexes and/or by active repression of transcription by pocket proteins brought to the promoter by E2F (for a review, see references 5 and 37). Interaction of E2Fs with the pocket proteins is regulated by phosphorylation of one or both components of the complex. E2F-dependent transcription is activated in late G_1 phase by hyperphosphorylation of pRB, which leads to disruption of the inhibitory pRB-E2F complex. E2Fs and the pocket proteins are differentially expressed and phosphorylated, providing exquisite regulatory potential (12, 45).

The regulation of E2F is a key target for DNA viruses, which have played an important role in the elucidation of the role of E2F in cellular proliferation and the mechanisms of its control (for a review, see reference 30). For example, adenovirus E1A not only led to the original identification of E2F but was also instrumental in the identification of functions of pRB. Binding of pRB and the other pocket proteins by E1A and other viral immediate-early (IE) proteins (e.g., simian virus large T antigen and papillomavirus E7) blocks their ability to associate with E2F and leads to an induction of E2F-dependent transcription (9). This activity is essential for the ability of the viral proteins to transform cells, highlighting the importance of E2F-pocket protein complexes in cell cycle and growth regulation (for a review, see reference 32).

A link between E2F and gene activation by the human cytomegalovirus (HCMV) was established by the demonstration that the major HCMV IE gene products can complement an E1A-deficient adenovirus strain and can transactivate the adenovirus E2 promoter (17, 40, 41). Moreover, E2F sites are present in the promoters of several cellular genes activated by HCMV infection, including those encoding DHFR, DNA polymerase α, and c-Myc (33, 42). These findings implicate E2F as a likely target through which HCMV activates cellular gene expression. Indeed, we have previously determined that HCMV targets E2F via one of its IE proteins, IE72 (28, 43). The data presented here demonstrate that this activation occurs by a novel mechanism among viral IE proteins in that it requires an enzymatic activity of IE72; IE72 was shown to be a protein kinase with selective substrate specificity for different E2F and pocket protein family members, and its kinase activity was found to be required for activation of E2F-dependent transcription.

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MATERIALS AND METHODS

Cell culture and transient transfection. U373-MG cells were grown in monolayers in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum (GIBCO) under an atmosphere of 10% CO_2 and were passaged into 100-mm-diameter plastic tissue culture dishes (3×10^5 cells/dish) the day before transfection. Cells were transfected with double-cesium-banded plasmid DNA by calcium phosphate coprecipitation (10). Cells were harvested by scraping and then lysed by three freeze-thaw cycles. Clarified supernatants were assayed for luciferase activity by use of the Promega luciferase assay kit and a Berthold luminometer. Chloramphenicol acetyltransferase (CAT) activity was measured by a fluor diffusion assay using [^{3}H]acetyl coenzyme A (200 mCi/mmol; NEN) as described previously (31).

Preparation of ^{32}P-labeled IE72 from cells. Cells were incubated for 2 h in phosphate-free medium containing 10% dialyzed fetal calf serum (GIBCO) and then for 2 h in 40 μCi of [^{32}P]orthophosphate per ml of phosphate-free Dulbecco modified Eagle medium supplemented with 10% dialyzed fetal calf serum. Cells were lysed in RIPA buffer (phosphate-buffered saline containing 1% [wt/vol] Nonidet P-40, 0.5% [wt/vol] sodium deoxycholate, and 0.1% [wt/vol] sodium dodecyl sulfate [SDS]) supplemented with 0.1 mM sodium vanadate and 0.1 mM sodium fluoride; IE72 was immunoprecipitated by addition of a polyclonal antibody that specifically recognizes IE72 (α1-2). After being washed four times with RIPA buffer at 20°C, immunoprecipitates were resolved by SDS–8% polyacrylamide gel electrophoresis (PAGE), transferred to polyvinylidene difluoride (PVDF) membranes, autoradiographed, and subjected to Western blotting.

Coupled transcription-translation of CMV IE proteins. IE72, E2Fs, and DP1 were synthesized by coupled transcription-translation, with the Promega TNT reticulocyte lysate kit, from cDNAs cloned into the pBluescriptII KS vector (Stratagene). Briefly, 1 μg of plasmid DNA (1 mg/ml) was added to a 50-μl reaction mixture containing the appropriate reaction buffer, a methionine-free amino acid mixture, and 20 μCi of [^{35}S]methionine (1,000 Ci/mmol) in accordance with the manufacturer's instructions.

In vitro phosphorylation of IE72. His-IE72 and glutathione *S*-transferase (GST)-IE72 were purified from *Escherichia coli* as described previously (28). The purified IE72, bound to the Ni^{2+}-nitrilotriacetic acid (NTA) or glutathione-Sepharose, was incubated in kinase buffer (25 mM HEPES [pH 7.4]–10% glycerol–100 mM NaCl–0.1 mM sodium vanadate–10 mM $MgCl_2$–5 mM $MnCl_2$) in the presence of 5 μCi of [γ-^{32}P]ATP for 30 min at 37°C. Reactions were stopped by addition of EDTA to a final concentration of 10 mM. The beads were washed, and bound proteins were analyzed by SDS–8% PAGE, Coomassie blue staining, and autoradiography.

Phosphoamino acid analysis. Bands containing phosphorylated IE72 were excised from the PVDF membranes and subjected to phosphoamino acid analysis by acid hydrolysis followed by two-dimensional electrophoresis on cellulose microcrystalline plates in the presence of marker phosphoamino acids, as described previously (8). The first dimension of electrophoresis was performed in buffer I (44:156:1,800 formic acid-acetic acid-deionized H_2O [dH_2O], pH 1.9) for 45 min at 1,500 V; the second dimension was performed in buffer II (100:10:1,890 acetic acid-pyridine-dH_2O) for 30 min at 1,300 V. The electrophoresis plate was dried, stained with ninhydrin, and exposed to X-ray film.

Plasmid constructs. cDNAs encoding E2F1 and -2 were provided by K. Helin, E2F3 cDNA was obtained from W. Krek, and E2F4 and -5 cDNAs were provided by R. Bernards. The expression constructs for pRB (pRB60), p107, and p130 were kindly provided by J. Horowitz, M. Ewen, and P. Whyte, respectively. The DHFR reporter constructs used in transient transfection assays contain sequences derived from the hamster DHFR promoter (nucleotide positions −210 to −23 [with ATG at +1]) driving expression of luciferase. The wild-type DHFR promoter construct contains four Sp1 sites and the dyad E2F sites (7). DHFRΔE2F contains a substitution of TA at positions −57 and −56 and does not bind E2F (6).

Construction of IE72 deletion mutants. The GST-IE72 clones expressing only exons 2 and 3 (Δexon4) (exon 1 is not translated) and exon 4 alone (Δexon1–3) were made by PCR from the IE72 cDNA in Bluescript. The cDNA region encoding amino acid residues 1 to 85 was amplified by using primers with the following sequences: 5′-ACGAATTCGGATCCATGGAGTCCTCTGCCAAG AG-3′ (5′ primer) and 5′-ACGAATTCGGATCCTGATTATTCTGCCAGGA CATC-3′ (3′ primer). The 5′ primer used to generate the region encoding exon 4 had the sequence 5′-ACGAATTCGGATCCATGCTCGTCAAACAGATT3′, and the 3′ primer had the sequence 5′-ACGAATTCGGATCCATCCTGATTA GTGGGATCCATAACAGTAAC-3′ (3′ to the stop codon). Clones were verified by sequencing.

The IE72 clone from which the ATP binding site was deleted was generated by two different PCR reactions using the IE72 cDNA. The cDNA region encoding amino acid residues 1 to 173 was amplified by using primers with the sequences 5′-ACGAATTCGGATCCATGGAGTCCTCTGCCAAGAG-3′ (5′ primer) and 5′-CGAATTCCTCCTTAATACA-3′ (3′ primer), and the region encoding amino acid residues 194 to 492 was amplified by using a primer with the sequence 5′-CGAATTCGATGAACTTAGG-3′ (5′ primer) and the aforementioned 3′ primer (3′ to the stop codon). The *Eco*RI sites generated at the ends of the resulting PCR products were cleaved with *Eco*RI and ligated. The resulting larger fragment, containing nucleotides 1 to 492, was cleaved with *Bam*HI and subcloned into the *Bam*HI site of the pGEX2T vector. In this way, the ATP binding site was deleted and codons for two amino acids (Glu and Phe) were inserted. The clone was confirmed by sequencing, and the identity of the protein expressed by the clone was verified by Western blot analysis using the IE72-specific antibody. To make a eukaryotic expression clone, the *Bam*HI fragment from pGEX2T was subcloned in the appropriate orientation into pSG5 (Stratagene).

EMSAs. Electrophoretic mobility shift assays (EMSAs) were carried out as described previously, with a radiolabeled DNA fragment from nucleotide positions −103 to −23 of the hamster DHFR promoter as the probe (28).

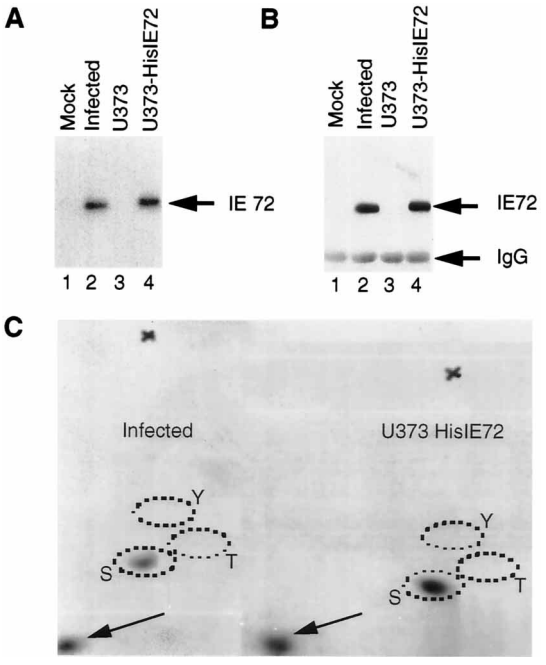

FIG. 1. IE72 is a phosphoprotein which is phosphorylated on serine residues. (A and B) U373 cells were mock infected (lane 1) or infected with the Towne strain of HCMV at a multiplicity of infection of 5 for 17 h (lane 2) and then labeled with [^{32}P]orthophosphate for 2 h. Parental U373 (lane 3) and U373 stably expressing His-IE72 (lane 4) were labeled in the same manner, and then cells were lysed in RIPA buffer and immunoprecipitated with an IE72-specific polyclonal antibody. (A) Immunoprecipitated material was resolved by SDS–8% PAGE, transferred to PVDF membranes, and autoradiographed. (B) Western blot of the same immunoprecipitates probed with IE72-specific antibody and detected by enhanced chemiluminescence. (C) Phosphorylated IE72 (panel A, lanes 2 and 4) bands were excised from the PVDF and subjected to phosphoamino acid analysis followed by two-dimensional electrophoresis as described in Materials and Methods. The positions of marker phosphoamino acids phosphoserine (S), phosphothreonine (T), and phosphotyrosine (Y) are indicated by dotted ellipses. Arrows denote the positions of the free phosphate.

RESULTS

Phosphorylation of IE72 does not require other viral proteins. IE72 has been shown to be phosphorylated in HCMV-infected cells (17, 35). To determine if IE72 phosphorylation required viral infection, phosphorylation of IE72 in U373 cells which were infected with HCMV was compared to that in cells stably expressing His-tagged IE72 (provided by Jay Nelson). Cells were incubated with [^{32}P]orthophosphate and then lysed in RIPA buffer, and IE72 was immunoprecipitated with a specific antibody. IE72 is phosphorylated in these cells (Fig. 1A, lanes 2 and 4). It is unlikely that the phosphoprotein visualized resulted from precipitation of a nonspecific protein, since it comigrated with IE72, as detected by Western blotting (Fig. 1B), and was not detected in mock-infected or control cells (Fig. 1A, lanes 1 and 3). Phosphoamino acid analysis of IE72 isolated from either infected cells or cells expressing His-IE72 detected only phosphoserine, indicating that the majority of the phosphorylation is on serine residues (Fig. 1C). Thus, IE72 is phosphorylated even in the absence of HCMV infection,

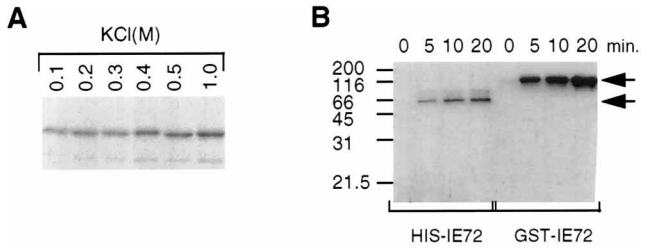

FIG. 2. IE72 is a kinase. (A) His-IE72 was purified from *E. coli* by Ni^{2+}-NTA chromatography as described in Materials and Methods and incubated with the indicated concentration of KCl; this was followed by in vitro kinase reactions with [γ-^{32}P]ATP and resolution by SDS–8% PAGE and autoradiography. (B) HIS-IE72 and GST-IE72 proteins were purified by Ni^{2+}-NTA-agarose and glutathione-Sepharose chromatography, respectively, and then separated by SDS–8% PAGE and stained with Coomassie blue. Gel slices containing the purified proteins were soaked in dH_2O for 2 to 3 h to remove the SDS and then incubated with Ni^{2+}-NTA-agarose or glutathione-Sepharose for 4 to 5 h to elute the protein (ca. 200 ng). Proteins were subjected to in vitro phosphorylation reactions for the indicated periods of time; this was followed by SDS–8% PAGE and then autoradiography for 1 to 24 h. The arrows indicate HIS-IE72 (bottom) and GST-IE72 (top).

indicating that IE72 can be phosphorylated by a cellular kinase and/or that it is a kinase itself.

IE72 is a kinase. To determine if phosphorylation of IE72 could be attributed to kinase activity of the protein itself, His-IE72 was expressed in *E. coli*, purified by Ni-NTA affinity chromatography, and incubated in kinase buffer containing [γ-^{32}P]ATP. His-IE72 was phosphorylated in these in vitro assays (Fig. 2A, lane 1). This phosphorylation appeared to be due to autophosphorylation by IE72 rather than a copurifying kinase, since extraction of His-IE72 with up to 1 M potassium chloride did not affect its kinase activity (Fig. 2A). This was confirmed by demonstrating that both GST-IE72 and His-IE72 retain their autophosphorylation activity following purification by SDS-PAGE and renaturation (Fig. 2B). Phosphorylation resulting from the fortuitous comigration of a bacterial kinase is highly unlikely since His-IE72 and GST-IE72 migrate differently on SDS-PAGE gels. Taken together, these data confirm that IE72 is a protein kinase. Phosphoamino acid analysis of in vitro-phosphorylated His-IE72 from bacteria detected only phosphoserine (data not shown).

Properties of the IE72 kinase. Either magnesium or manganese could support the kinase activity of IE72; in the absence of Mg^{2+}, the minimum concentration of Mn^{2+} required for autophosphorylation was between 5 and 10 mM, whereas the minimum concentration of Mg^{2+} alone was between 10 and 15 mM (Fig. 3, left panel). The kinase could utilize GTP as well as ATP as a phosphate donor (Fig. 3, right panel). The utilization of GTP is a feature of casein kinase II, which can also utilize manganese; however, the kinase activity of IE72 is not inhibited by up to 100 nM heparin sulfate (data not shown), a feature that distinguishes it from casein kinase II (2).

IE72 kinase activity resides in the region encoded by exon 4. Deletion mutants of IE72 were used to identify the region(s) of the protein required for autophosphorylation. The peptide expressed by a construct containing only exons 2 and 3 (Δexon4) had no kinase activity, and deletion of the regions encoded by exons 2 and 3 from IE72 had no apparent effect on its kinase activity (Fig. 4A). Taken together, these data demonstrate that the kinase function is encoded by exon 4. The localization of the kinase activity to exon 4 is consistent with the fact that IE86, which has coding exons 2 and 3 in common with IE72, does not display kinase activity (data not shown). Regions of the amino acid sequence of IE72 in exon 4 conform to features of a kinase (Fig. 4B). There is a potential divalent-cation binding site (46) between amino acid residues 245 and 248 (Fig. 4B). Furthermore, the sequence of the protein between amino acid residues 173 and 197 is homologous to the ATP binding sites of over 500 other kinases.

The putative ATP binding site of IE72 was deleted to determine if it was required for kinase activity and in the hope of generating a kinase-negative IE72 for further functional studies; the resulting construct was designated IE72ΔATP. In vitro kinase reactions performed with the wild type and GST-IE72ΔATP demonstrated that the mutated protein isolated from *E. coli* cannot autophosphorylate (Fig. 5, lane 3), indicating that this region of the protein is required for kinase activity. However, it can serve as a substrate for wild-type IE72 (Fig. 5, lane 5). Therefore, an autophosphorylation site(s) lies outside of the ATP binding site, and IE72 can phosphorylate itself in *trans.*

Specificity of IE72 kinase for E2Fs and pocket proteins. Having identified IE72 as a kinase, we sought to identify other substrates for its kinase activity. Since phosphorylation of the E2Fs and the pRB family of proteins is cell cycle regulated and affects E2F-dependent transcription, we tested whether they could be phosphorylated by IE72. In our in vitro kinase reaction, E2F1, -2, and -3 were phosphorylated by IE72 whereas E2F4 and E2F5 were not (Fig. 6, left panels). These studies also demonstrated that IE72 can phosphorylate p107 and p130 but not pRB (Fig. 6, right panels). Consistent with its inability to autophosphorylate, IE72ΔATP was unable to phosphorylate any of the E2Fs or pocket proteins (data not shown). Thus, IE72 kinase has substrate specificity within the E2F and pocket protein families.

The kinase activity of IE72 is required for its effects on E2F activities. To determine the functional role of the IE72 kinase activity, kinase-deficient IE72 (IE72ΔATP) was tested for its transactivation activity. IE72 or IE72ΔATP was cotransfected into U373 cells with a plasmid containing the luciferase reporter gene under the control of the hamster DHFR promoter. As noted previously (28), activation of the DHFR promoter by IE72 is concentration dependent (Fig. 7). Equivalent expression of IE72 and IE72ΔATP was verified by Western blotting (data not shown); significant activation of transcription by IE72ΔATP was not observed at any concentration (Fig. 7). Therefore, the transactivation effects of IE72 appear to require its kinase activity.

Two repressors of E2F activity, p107 and p130, are phosphorylated by IE72. We therefore examined the effect of IE72 on E2F-p130 and E2F-p107 complexes. ^{35}S-labeled E2F4 associated in vitro with GST-p107 and with GST-p130 (Fig. 8A, lanes 1 and 4). When wild-type IE72 was added to the reaction mixtures, E2F4-p130 and E2F4-p107 complexes were not detected, indicating that IE72 can prevent association of these complexes and/or cause their dissociation (Fig. 8A). In contrast, IE72ΔATP had no effect on the complexes (Fig. 8A). These findings were confirmed by performing EMSAs in which IE72 was added to reaction mixtures containing an E2F DNA

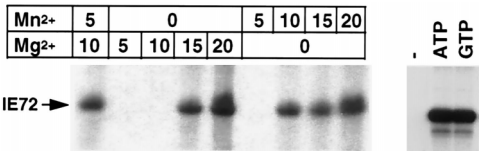

FIG. 3. His-IE72 autophosphorylation can utilize either Mg^{2+} or Mn^{2+} and either ATP or GTP. (Left) Bacterially expressed His-IE72 was affinity purified and then incubated in kinase buffer (25 mM HEPES [pH 7.4]–10% glycerol–100 mM NaCl–0.1 mM sodium vanadate–5 μCi of [γ-^{32}P]ATP) containing the indicated amounts of $MgCl_2$ and/or $MnCl_2$; this was followed by SDS–8% PAGE and then autoradiography. (Right) Purified His-IE72 was incubated in kinase buffer containing 5 mM $MnCl_2$ and 10 mM $MgCl_2$ alone (−) or with 5 μCi of either [γ-^{32}P]ATP or [γ-^{32}P]GTP for 30 min at 37°C; this was followed by SDS–8% PAGE and autoradiography.

FIG. 4. The region of IE72 encoded by exon 4 is sufficient for autophosphorylation. (A) GST-IE72 proteins with the indicated regions of the protein deleted were isolated by glutathione-Sepharose chromatography, band purified, in vitro phosphorylated, and analyzed by SDS–12% PAGE. The left panel shows an autoradiograph of the gel, while the right panel shows the gel after Coomassie blue staining. The sizes of broad-range molecular weight markers (Bio-Rad) are indicated to the left. W.T., wild-type IE72. (B) The amino acid sequence of IE72 (accession no. 00107). The presumptive ATP binding site from amino acid 173 to 196 revealed by PROSITE analysis and the divalent-cation binding site are indicated by the shaded and unshaded boxes, respectively. The N terminus of the region encoded by exon 4 (amino acid residue 86) is indicated by the arrowhead.

binding site probe, in vitro cotranslated E2F4 and DP1, and either GST-p107 or GST-p130. The level of pocket protein-containing complexes was decreased and free E2F DNA binding activity was increased when wild-type IE72 was added, whereas IE72ΔATP did not affect the level of E2F-pocket protein complexes (Fig. 8B). The inability of the kinase-deficient IE72 to affect the complexes in either assay indicates that this activity requires the kinase function. Taken together with the transient-transfection data, these data indicate that the ability of IE72 to activate E2F-dependent transcription may be due, at least in part, to blocked formation and/or dissociation of inhibitory complexes through its kinase activity.

DISCUSSION

We have identified a kinase activity in the HCMV IE72 protein with unique biochemical properties and substrate specificity. IE72 can phosphorylate itself, E2F1 to -3, and the pocket proteins p107 and p130, but not E2F4, E2F5, or pRB. Removal of the putative ATP binding site of IE72 abolishes its kinase activity. This mutation also abolishes its ability to activate E2F-dependent transcription and to reduce the association of E2F4 with p107 and p130, indicating that the phosphorylation of critical substrates by IE72 is required for its E2F-dependent transactivation function.

Phosphorylation events are involved in the regulation of E2F activity, but their precise regulation and effects are not understood. Incubation of E2F with phosphatase inactivates its DNA binding activity (3). Phosphorylation near the DNA binding domain of E2F1 by cyclin A/cdk2 results in decreased binding to DNA (22, 45). Phosphorylation of E2F1 on serines 332 and 337 has been shown to prevent its interaction with pRB and to be a prerequisite for interaction with adenovirus E4 (12). In contrast, phosphorylation of E2F1 on serine 375 has been shown to enhance its affinity for pRB (34). Clearly, these data argue that phosphorylation may be a critical factor in regulation of E2F activity; however, the precise role of individual phosphorylation events remains to be elucidated. The substrate specificity of IE72 kinase is unique and should shed light on the mechanism of transcriptional regulation by E2Fs. Current experiments include precise mapping of the sites where IE72 phosphorylates the E2Fs and pocket proteins in order to determine the effects of this phosphorylation on transcriptional activity.

The substrate specificity of IE72 kinase is particularly interesting in light of the specificity of interactions between the E2Fs and pocket proteins. E2F1 to -3 preferentially interact with pRB, E2F5 interacts preferentially with p130, and E2F4 interacts with all three pocket proteins. Interaction of an E2F with pRB, p107, or p130 inhibits its transcriptional activity. The interaction between an E2F and a pocket protein is dependent on the phosphorylation states of both proteins (12). IE72 does not phosphorylate pRB, and it does not affect E2F1-DP1-pRB complexes under the conditions of our assays (data not shown). In contrast, IE72 phosphorylates p107 and p130 in vitro and dramatically reduces the levels of complexes containing E2F4-DP1 and either p107 or p130. Use of the kinase-deficient IE72 mutant indicates that the effect of IE72 on the E2F-pocket protein interaction is kinase dependent, as are its effects on

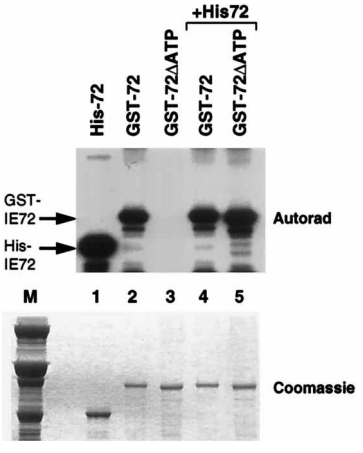

FIG. 5. A mutated version of IE72 with the putative ATP binding site deleted has no kinase activity but can be phosphorylated by wild-type IE72. The indicated fusion proteins, generated as described in Materials and Methods, were expressed in *E. coli*, isolated by Ni^{2+}-NTA affinity chromatography (lane 1) or glutathione-Sepharose chromatography (lanes 2 to 5), and incubated with [γ-^{32}P]ATP. In lanes 4 and 5, His-IE72 protein was added to the immobilized GST proteins. After being washed four times with RIPA buffer, proteins were resolved by SDS–10% PAGE, stained with Coomassie blue (lower panel), and subjected to autoradiography (Autorad; upper panel). (Note: in lanes 4 and 5, phosphorylated His-IE72 is not visualized because only material attached to the glutathione beads was electrophoresed.) Protein molecular weight standards (broad-range markers; Bio-Rad) were loaded in the lane designated M, and the sizes are as indicated in Fig. 4.

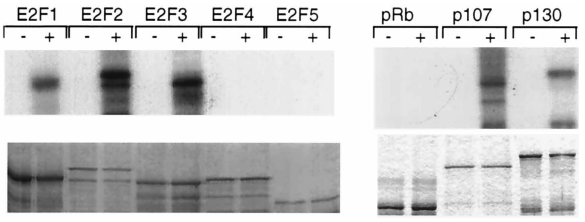

FIG. 6. IE72 can phosphorylate some members of the E2F and pocket protein families. His-IE72 protein was expressed in bacteria, isolated by Ni-NTA chromatography, and gel purified. GST-E2F1 to -5 (left panels) and GST-pRB, GST-p107, and GST-p130 (right panels) were isolated by glutathione-Sepharose chromatography and incubated with [γ-^{32}P]ATP in the absence (−) or presence (+) of purified His-IE72 (100 ng). After being washed, proteins were resolved by SDS–10% PAGE and then stained with Coomassie blue (lower panels) and subjected to autoradiography (upper panels).

E2F-dependent transcription, indicating that reduction of these complexes may be a mechanism of transcriptional activation by IE72. E2F-p130 complexes are most predominant in quiescent G_0 cells, whereas E2F-p107 complexes are most abundant near the G_1/S border (11). IE72 might increase free E2F4 and E2F5 levels through phosphorylation of p130 and p107, which would specifically activate transcription dependent on E2F4 and/or E2F5 and drive cells out of G_0 and toward S phase (39). The presence of different E2F-pocket protein complexes at different points in the cell growth and differentiation cycles, together with the observed substrate specificity of IE72, may enable IE72 to affect E2F-dependent transcription at specific points in the cell cycle. Furthermore, since the roles of individual E2F family members are not clear at present, the substrate specificity of IE72 may provide a useful tool for studying the regulation of individual E2Fs and for elucidating differences in their transcriptional specificities.

Both infection with HCMV and overexpression of E2F1 can induce cells to proliferate (1, 27). Induction of E2F DNA binding activity in cells infected with HCMV (43) and activation of E2F-dependent transcription by IE72 (28) suggest that the E2F-dependent effects of IE72 could be involved in the proliferative response during HCMV infection. The kinase activity of IE72 is essential for its activation of DHFR transcription, suggesting that the phosphorylation of specific substrates by IE72 may play an essential role in the enhanced activity of E2F in cells expressing IE72 and may be a mechanism of HCMV-induced proliferation.

Whereas the IE proteins of several other DNA viruses interact with the pocket proteins, IE72 additionally interacts with the E2Fs (14). Although IE72 cannot interact with pRB (14), it does interact with p107 (35), and IE86 associates with pRB (14). As such, HCMV major IE proteins together may fulfill a role similar to that of the IE proteins of other DNA viruses, and the kinase activity of IE72 appears to be involved.

In addition to kinase function, other factors are involved in transactivation by IE72. The kinase activity of IE72 is apparently not sufficient for transactivation; deletion of exon 3 eliminates transactivation by IE72 (44) but does not prevent interaction with E2F1, autophosphorylation, or phosphorylation of the E2Fs or pocket proteins in vitro (data not shown). Experiments to further elucidate the role of IE72 kinase activity in E2F-dependent transcription and during HCMV infection are under way.

The mechanism by which IE72 activates E2F-dependent transcription is novel among viral proteins in that it requires a

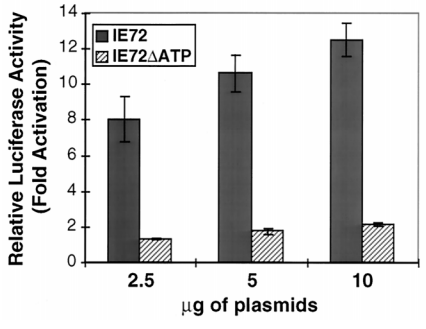

FIG. 7. Deletion of the ATP binding site from IE72 abolishes activation of DHFR transcription. U373 cells were cotransfected with RSV/CAT (0.5 μg), a luciferase reporter vector (10 μg) driven by the wild-type DHFR promoter, and 2.5, 5, or 10 μg of wild-type IE72 or mutant IE72ΔATP and vector DNA to bring the total concentration for each transfection to 20 μg of DNA. Cells were harvested and lysed 48 h posttransfection and assayed for luciferase and CAT activities. Data are presented in units of relative fold activation, which is luciferase activity (in relative light units) divided by CAT activity (in counts per minute) in each dish, with each relative value being divided by that obtained with reporter constructs alone. Error bars represent standard errors of the means for triplicate samples.

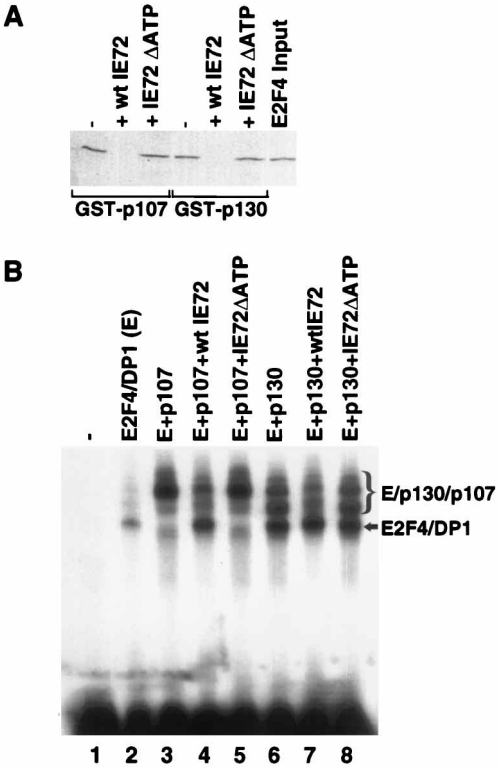

FIG. 8. Deletion of the IE72 ATP binding site abolishes its ability to prevent formation of and/or dissociate E2F4-p107 and E2F4-p130 complexes. (A) ^{35}S-labeled E2F4 was made by in vitro translation. GST-p107 and GST-p130 were isolated by glutathione-Sepharose chromatography and incubated with E2F4 alone (−; lanes 1 and 4) or with E2F4 in the presence of (+) IE72 (lanes 2 and 5) or IE72ΔATP (lanes 3 and 6). Initially, proteins were incubated for 30 min at 37°C in kinase buffer containing 10 mM ATP. Samples were then diluted to 1 mM $MgCl_2$ and incubated for 1 h at 4°C. After extensive washing, samples were resolved by SDS–10% PAGE and exposed to X-ray film for 24 h. wt, wild type. (B) The E2F binding site from the DHFR promoter was labeled with ^{32}P and used as the probe (lane 1, probe alone), and complexes were formed using in vitro-translated E2F4-DP1 heterodimer (E) and GST-p107 (lanes 3 to 5) or GST-p130 (lanes 6 to 8). Approximately 10 ng of wild-type (wt) GST-IE72 (lanes 4 and 7) or GST-IE72ΔATP (lanes 5 and 8) was added to each reaction mixture as indicated. GST-IE72 was phosphorylated in vitro by incubating it in kinase buffer prior to adding it to the reaction mixtures; GST-IE72ΔATP was treated in the same manner. After 20 min of binding to the probe, samples were resolved on 4% polyacrylamide gels and then subjected to autoradiography.

kinase function of IE72 itself. Since viral proteins have been central to the elucidation of the regulation of E2F, the identification of this novel mechanism will hopefully provide a useful tool for the further characterization of E2F regulatory mechanisms. That this may extend to factors other than E2F is indicated by the fact that the activities of some other transcription factors that are activated by IE72 (e.g., AP1 and CTF) are also regulated by phosphorylation (15, 20, 26).

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REFERENCES

1. **Albrecht, T., I. Boldogh, M. Fons, C. H. Lee, S. AbuBakar, J. M. Russell, and W. W. Au.** 1989. Cell-activation responses to cytomegalovirus infection: relationship to the phasing of CMV replication and to the induction of cellular damage. Subcell. Biochem. **15:**157–202.
2. **Allende, J. E., and C. C. Allende.** 1995. Protein kinase CK2: an enzyme with multiple substrates and a puzzling regulation. FASEB J. **9:**313–322.
3. **Bagchi, S., P. Raychaudhuri, and J. R. Nevins.** 1989. Phosphorylation-dependent activation of the adenovirus-inducible E2F transcription factor in a cell-free system. Proc. Natl. Acad. Sci. USA **86:**4352–4356.
4. **Beijersbergen, R. L., R. M. Kerkhoven, L. Zhu, L. Carlee, P. M. Voorhoeve, and R. Bernards.** 1994. E2F-4, a new member of the E2F gene family, has oncogenic activity and associates with p107 in vivo. Genes Dev. **8:**2680–2690.
5. **Black, A. R., and J. C. Azizkhan.** 1996. Transcriptional regulation of growth-related genes by E2F and Sp1. Pezcoller Found. J. **3:**4–16.
6. **Blake, M. C., and J. C. Azizkhan.** 1989. Transcription factor E2F is required for efficient expression of the hamster dihydrofolate reductase gene in vitro and in vivo. Mol. Cell. Biol. **9:**4994–5002.
7. **Blake, M. C., R. C. Jambou, A. G. Swick, J. W. Kahn, and J. C. Azizkhan.** 1990. Transcriptional initiation is controlled by upstream GC-box interactions in a TATAA-less promoter. Mol. Cell. Biol. **10:**6632–6641.
8. **Boyle, W. J., P. van der Geer, and T. Hunter.** 1991. Phosphopeptide mapping and phosphoamino acid analysis by two-dimensional separation on thin-layer cellulose plates. Methods Enzymol. **201:**110–149.
9. **Chellappan, S., V. B. Kraus, B. Kroger, K. Munger, P. M. Howley, W. C. Phelps, and J. R. Nevins.** 1992. Adenovirus E1A, simian virus 40 tumor antigen, and human papillomavirus E7 protein share the capacity to disrupt the interaction between transcription factor E2F and the retinoblastoma gene product. Proc. Natl. Acad. Sci. USA **89:**4549–4553.
10. **Chen, C., and H. Okayama.** 1987. High-efficiency transformation of mammalian cells by plasmid DNA. Mol. Cell. Biol. **7:**2745–2752.
11. **Cobrinik, D., P. Whyte, D. S. Peeper, T. Jacks, and R. A. Weinberg.** 1993. Cell cycle-specific association of E2F with the p130 E1A-binding protein. Genes Dev. **7:**2392–2404.
12. **Fagan, R., K. J. Flint, and N. Jones.** 1994. Phosphorylation of E2F-1 modulates its interaction with the retinoblastoma gene product and the adenoviral E4 19 kDa protein. Cell **78:**799–811.
13. **Ginsberg, D., G. Vairo, T. Chittenden, Z. X. Xiao, G. Xu, K. L. Wydner, J. A. DeCaprio, J. B. Lawrence, and D. M. Livingston.** 1994. E2F-4, a new member of the E2F transcription factor family, interacts with p107. Genes Dev. **8:**2665–2679.
14. **Hagemeier, C., R. Caswell, G. Hayhurst, J. Sinclair, and T. Kouzarides.** 1994. Functional interaction between the HCMV IE2 transactivator and the retinoblastoma protein. EMBO J. **13:**2897–2903.
15. **Hayhurst, G. P., L. A. Bryant, R. C. Caswell, S. M. Walker, and J. H. Sinclair.** 1995. CCAAT box-dependent activation of the TATA-less human DNA polymerase α promoter by the human cytomegalovirus 72-kilodalton major immediate-early protein. J. Virol. **69:**182–188.
16. **Helin, K., J. A. Lees, M. Vidal, N. Dyson, E. Harlow, and A. Fattaey.** 1992. A cDNA encoding a pRB-binding protein with properties of the transcription factor E2F. Cell **70:**337–350.
17. **Hermiston, T. W., C. L. Malone, P. R. Witte, and M. F. Stinski.** 1987. Identification and characterization of the human cytomegalovirus immediate-early region 2 gene that stimulates gene expression from an inducible promoter. J. Virol. **61:**3214–3221.
18. **Hiebert, S. W., M. Blake, J. Azizkhan, and J. R. Nevins.** 1991. Role of E2F transcription factor in E1A-mediated *trans* activation of cellular genes. J. Virol. **65:**3547–3552.
19. **Ivey-Hoyle, M., R. Conroy, H. E. Huber, P. J. Goodhart, A. Oliff, and D. C. Heimbrook.** 1993. Cloning and characterization of E2F-2, a novel protein with the biochemical properties of transcription factor E2F. Mol. Cell. Biol. **13:**7802–7812.
20. **Jackson, S. P.** 1992. Regulating transcription factor activity by phosphorylation. Trends Cell Biol. **2:**104–108.
21. **Kaelin, W. G., W. Krek, W. R. Sellers, J. A. DeCaprio, F. Ajchanbaum, C. S. Fuchs, T. Chittenden, Y. Li, P. J. Farnham, M. A. Blanar, D. M. Livingston, and E. K. Flemington.** 1992. Expression cloning of a cDNA encoding a retinoblastoma binding protein with E2F-like properties. Cell **70:**351–364.
22. **Kitagawa, M., H. Higashi, I. Suzuki-Takahashi, K. Segawa, S. K. Hanks, Y. Taya, S. Nishimura, and A. Okuyama.** 1995. Phosphorylation of E2F-1 by cyclin A-cdk2. Oncogene **10:**229–236.
23. **La Thangue, N. B.** 1994. DRTF1/E2F: an expanding family of heterodimeric transcription factors implicated in cell-cycle control. Trends Biochem. Sci. **19:**108–114.
24. **La Thangue, N. B.** 1996. E2F and the molecular mechanisms of early cell-cycle control. Biochem. Soc. Trans. **24:**54–59.
25. **Lees, J. A., M. Saito, M. Vidal, M. Valentine, T. Look, E. Harlow, N. Dyson, and K. Helin.** 1993. The retinoblastoma protein binds to a family of E2F transcription factors. Mol. Cell. Biol. **13:**7813–7825.
26. **Lukac, D. M., J. R. Manuppello, and J. C. Alwine.** 1994. Transcriptional activation by the human cytomegalovirus immediate-early proteins: requirements for simple promoter structures and interactions with multiple components of the transcription complex. J. Virol. **68:**5184–5193.
27. **Lukas, J., B. O. Petersen, K. Holm, J. Bartek, and K. Helin.** 1996. Deregulated expression of E2F family members induces S-phase entry and overcomes $p16^{INK4A}$-mediated growth suppression. Mol. Cell. Biol. **16:**1047–1057.
28. **Margolis, M. J., S. Pajovic, E. L. Wong, M. Wade, R. Jupp, J. A. Nelson, and J. C. Azizkhan.** 1995. Interaction of the 72-kilodaton human cytomegalovirus IE1 gene product with E2F1 coincides with E2F-dependent activation of dihydrofolate reductase transcription. J. Virol. **69:**7759–7767.
29. **Moberg, K., M. A. Starz, and J. A. Lees.** 1996. E2F-4 switches from p130 to p107 and pRB in response to cell cycle reentry. Mol. Cell. Biol. **16:**1436–1449.
30. **Moran, E.** 1993. DNA tumor virus transforming proteins and the cell cycle. Curr. Opin. Genet. Dev. **3:**63–70.
31. **Neumann, J. R., C. A. Morency, and K. O. Russian.** 1987. A rapid fluor diffusion assay for detection of chloramphenicol acetyltransferase activity. BioTechniques **5:**444–448.
32. **Nevins, J. R.** 1992. E2F: a link between the Rb tumor suppressor protein and viral oncoproteins. Science **258:**424–429.
33. **Pearson, B. E., H.-P. Nasheuer, and T. S.-F. Wang.** 1991. Human DNA polymerase α gene: sequences controlling expression in cycling and serum-stimulated cells. Mol. Cell. Biol. **11:**2081–2095.
34. **Peeper, D. S., P. Keblusek, K. Helin, M. Toebes, A. J. van der Eb, and A. Zantema.** 1995. Phosphorylation of a specific cdk site in E2F-1 affects its electrophoretic mobility and promotes pRB-binding in vitro. Oncogene **10:**39–48.
35. **Poma, E. E., T. F. Kowalik, L. Zhu, J. H. Sinclair, and E.-S. Huang.** 1996. The human cytomegalovirus IE1-72 protein interacts with the cellular p107 protein and relieves p107-mediated transcriptional repression of an E2F-responsive promoter. J. Virol. **70:**7867–7877.
36. **Reichel, R. R.** 1992. Regulation of E2F/cyclin A-containing complex upon retinoic acid-induced differentiation of teratocarcinoma cells. Gene Expr. **2:**259–271.
37. **Sidle, A., C. Palaty, P. Dirks, O. Wiggan, M. Kiess, R. M. Gill, A. K. Wong, and P. A. Hamel.** 1996. Activity of the retinoblastoma family proteins, pRB, p107, and p130, during cellular proliferation and differentiation. Crit. Rev. Biochem. Mol. Biol. **31:**237–271.
38. **Slansky, J. E., and P. J. Farnham.** 1996. Introduction of the E2F family: protein structure and gene regulation. Curr. Top. Microbiol. Immunol. **208:**1–30.
39. **Smith, E. J., and J. R. Nevins.** 1995. The Rb-related p107 protein can suppress E2F function independently of binding to cyclin A/cdk2. Mol. Cell. Biol. **15:**338–344.
40. **Spector, D. J., and M. J. Tevethia.** 1986. Identification of a human cytomegalovirus DNA segment that complements an adenovirus 5 immediate early mutant. Virology **151:**329–338.
41. **Tevethia, M. J., and D. J. Spector.** 1984. Complementation of an adenovirus 5 immediate early mutant by human cytomegalovirus. Virology **137:**428–431.
42. **Thalmeier, K., H. Synovzik, R. Mertz, E. L. Winnacker, and M. Lipp.** 1989. Nuclear factor E2F mediates basic transcription and trans-activation by E1a of the human MYC promoter. Genes Dev. **3:**527–536.
43. **Wade, M., T. F. Kowalik, M. Mudryj, E.-S. Huang, and J. C. Azizkhan.** 1992. E2F mediates dihydrofolate reductase promoter activation and multiprotein complex formation in human cytomegalovirus infection. Mol. Cell. Biol. **12:**4364–4374.
44. **Wong, E., S. Pajovic, and J. C. Azizkhan.** Unpublished data.
45. **Xu, M., K.-A. Sheppard, C.-Y. Peng, A. S. Yee, and H. Piwnica-Worms.** 1994. Cyclin A/CDK2 binds directly to E2F-1 and inhibits the DNA-binding activity of E2F-1/DP-1 by phosphorylation. Mol. Cell. Biol. **14:**8420–8431.
46. **Zheng, J., D. R. Knighton, L. F. T. Eyck, R. Karlsson, N. Yuong, S. S. Taylor, and J. M. Sowadski.** 1993. Crystal structure of the catalytic subunit of cAMP-dependent protein kinase complexed with MgATP and peptide inhibitor. Biochemistry **32:**2154–2161.