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Varicella-Zoster Virus IE63 Protein Phosphorylation by Roscovitine-sensitive Cyclin-dependent Kinases Modulates Its Cellular Localization and Activity*

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During the first stage of Varicella-Zoster virus (VZV) infection, IE63 (immediate early 63 protein) is mostly expressed in the nucleus and also slightly in the cytoplasm, and during latency, IE63 localizes in the cytoplasm quite exclusively. Because phosphorylation is known to regulate various cellular mechanisms, we investigated the impact of phosphorylation by roscovitine-sensitive cyclin-dependent kinase (RSC) on the localization and functional properties of IE63. We demonstrated first that IE63 was phosphorylated on Ser-224 *in vitro* by CDK1 and CDK5 but not by CDK2, CDK7, or CDK9. Furthermore, by using roscovitine and CDK1 inhibitor III (CiIII), we showed that CDK1 phosphorylated IE63 on Ser-224 *in vivo*. By mutagenesis and the use of inhibitors, we demonstrated that phosphorylation on Ser-224 was important for the correct localization of the protein. Indeed, the substitution of these residues by alanine led to an exclusive nuclear localization of the protein, whereas mutations into glutamic acid did not modify its subcellular distribution. When transfected or VZV-infected cells were treated with roscovitine or CiIII, an exclusive nuclear localization of IE63 was also observed. By using a transfection assay, we also showed that phosphorylation on Ser-224 and Thr-222 was essential for the down-regulation of the basal activity of the VZV DNA polymerase gene promoter. Similarly, roscovitine and CiIII impaired these properties of the wild-type form of IE63. These observations clearly demonstrated the importance of CDK1-mediated IE63 phosphorylation for a correct distribution of IE63 between both cellular compartments and for its repressive activity toward the promoter tested.

The Varicella-Zoster virus (VZV)[1] is a human alphaherpesvirus that causes varicella, or chickenpox, during primary infection. Following recovery of the host, VZV establishes latency in the cells of the dorsal root ganglia and reactivates after numerous years to produce herpes zoster, or shingles, usually observed in elderly or immunocompromised patients (1). Molecular events leading to the establishment and the maintenance of the virus latency as well as to its reactivation are still poorly understood. It appears to be the result of a balance between cellular factors, viral proteins, and the host immune system (2).

The viral genome encodes 71 open reading frames (ORFs) (3) that are presumed to be expressed during lytic infection in a sequential cascade composed of three broad kinetic classes as follows: immediate-early (IE), early, and late, a characteristic of all herpesviruses (4, 5). IE gene-encoded proteins are the first to be active because they act as regulators on their own expression, as well as on the transcriptional and post-transcriptional activation of early and late genes. Among the IE proteins, IE62 is the major regulating factor with regard to its transactivation properties on all three classes of viral genes (6–10). During latent infection, only a small subset of VZV genes are expressed. Transcripts from ORF4, -21, -29, -62, -63, and -66 (11–16), and several of the corresponding proteins have been detected in latently infected cells such as IE4, IE62, IE63, ORF21p, and ORF29p (15, 17–19). During latency, the expression of viral proteins that are also present during lytic infection is an original feature that VZV does not share with other alphaherpesvirus (12).

IE63 was the first protein detected in a latency context such as an animal model (17) and human tissue sections (15, 18, 19). This protein is encoded by VZV ORF63 and ORF70 and is the putative homologue of HSV ICP22. It has an apparent molecular mass of 45 kDa and is present in the virion tegument (20). IE63 is abundantly produced at the first stage of infection (17) and is essential for VZV replication (21). Its activity as a potential transcription factor is subject to controversy. It has been shown to exert positive or negative effects on gene transcription, depending on the promoter and the cell type studied (22). However, others claimed that IE63 played only a minor role in the control of VZV gene expression (23). Nevertheless, in our laboratory, IE63 has been shown recently in transient transfection assay to down-regulate the expression of VZV DNA polymerase gene (24).

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[1] The abbreviations used are: VZV, Varicella-Zoster virus; CDK, cyclin-dependent kinase; IE, immediate early; E, early; L, late; CK, casein kinase; CiIII, CDK1 inhibitor III (Ethyl-(6-hydroxy-4-phenylbenzo-[4,5]furo[2,3-b])pyridine-3-carboxylate); RSC, roscovitine-sensitive CDK; GST, glutathione *S*-transferase; CTD, carboxyl-terminal domain of the RNA polymerase II; MOPS, 4-morpholinepropanesulfonic acid; HSV-1, herpes simplex virus, type 1; NLS, nuclear localization signal; FBS, fetal bovine serum; EMEM, essential minimum Eagle's medium; ORFs, open reading frames.

More recently, our laboratory has also demonstrated that IE63 is a transcriptional repressor of some VZV and cellular promoters, and its activity is directed toward the assembly of the transcription preinitiation complex (25).

An interesting feature of the VZV IE63 protein is its cellular localization during the different phases of VZV infection. Indeed, at the first stage of infection, IE63 is predominantly present in the nucleus (17), while during latency, the protein exhibits an exclusive cytoplasmic localization (15). It can be found both in the cytoplasm and the nucleus when reactivation occurs (15). The presence of the IE63 protein and its cellular localization modification might reflect an important role in the latency process.

It has been shown recently that the IE63 protein is phosphorylated by casein kinase-1 and -2 (CK-1 and -2) and by cellular kinases on residues located predominantly at the carboxyl-terminal end of the protein (24, 26, 46). These phosphorylations are required for the regulation activity of IE63 and its correct cellular distribution (24). IE63 has also been shown to be a substrate of the ORF47 product, one of the VZV-encoded kinases (27).

There is more and more evidence that herpesviruses need CDK activity for replication. For example, HSV-1 has been shown to require CDK activity for efficient replication in many cell types, and inhibitors of CDK prevent infection (28). Similarly, it has been demonstrated that the Epstein-Barr virus lytic program promotes specific cell cycle-associated activity involved in the progression from G_1 to S phase (29). Also, VZV needs a CDK activity for replication as inhibitors of the specific kinases are able to prevent VZV replication (30).

Among the inhibitors that impair correct replication of herpesvirus, roscovitine is a purine derivative that inhibits CDK1/cyclin B, CDK2/cyclin A or E, CDK5/p35, CDK7/cyclin H, and CDK9/cyclin T (31). It has been shown that roscovitine prevents VZV replication, results in decreased immediate-early gene expression and abnormal localization of IE63 proteins, and inhibits DNA replication, and it does so without causing cytotoxicity or apoptosis in the host cells (30).

The aim of this study was to analyze the role of IE63 phosphorylation by roscovitine-sensitive cyclin-dependent kinases (RSCs) on its cellular localization and its regulatory properties. RSCs are either implicated in the cell cycle (CDK1, CDK2, and CDK7), transcription (CDK7 and CDK9), or neuronal functions (CDK5). CDK1 (or Cdc2) is a serine/threonine kinase with a molecular mass of 34 kDa, which is responsible for the control of G_2/M progression (32). CDK5 (or neuronal Cdc2-like kinase) is a 33-kDa protein exclusively active in neuronal cells and plays a multifunctional role, including neurite outgrowth, axonal guidance, cytoskeleton assembly, membrane transport, synaptic function, dopamine signaling, and drug addiction (10, 17, 33, 34, 36–41).

For this, we used the CDK inhibitors, roscovitine and CDK1 inhibitor III (CiIII), and a mutational approach to investigate the importance of two potential phosphorylation sites, serine 224 and threonine 222. These two residues were shown to be phosphorylated *in vitro* by CDK1 and CDK5 and *in vivo* by CDK1, and their phosphorylation is important for IE63 cellular distribution and for its gene down-regulation activity in transient transfection assay.

MATERIALS AND METHODS

Plasmids—pcDNA-IE63wt that encodes IE63 under the human cytomegalovirus immediate-early promoter was described previously (24). We also used the pGEX-5x-63wt (24) to transform the *Escherichia coli* JM109 strain to produce GST fusion proteins. pGEX-5x-CTD, which allowed us to produce GST-CTD, was very kindly donated by Steve Hahn (Fred Hutchinson Cancer Research Center and Howard Hughes Medical Institute, Seattle, WA) and Dylan Taatjes (University of Colorado). Mutations were introduced into the *IE63* gene in both the pcDNA-IE63wt and the pGEX-5x-63wt by PCR using the site-directed mutagenesis kit system (Stratagene). For this, several sets of primers were synthesized (Eurogentec). Two types of mutations were generated: substitution of a serine or a threonine by an alanine residue (S224A, T222A, and S224A/T222A) or substitution of a serine or a threonine by a glutamic acid (S224E, T222E, and S224E/T222E). The pcDNA-IE63-FULL, in which all the phosphorylation sites for CK1, CK2, CDK1, and CDK5 were substituted by alanine (S15A, S150A, S157A, S165A, T171A, S173A, S181A, S185A, S186A, S197A, S200A, T201A, S203A, T222A, S224A, and T244A), was generated in our laboratory by S. Bontems using the same protocol. The pcDNA-IE63-REV224S and pcDNA-IE63-REV224S/222T were obtained by reversion of the mutations of Ser-224 and Thr-222 from pcDNA-IE63-FULL. All constructions were subsequently sequenced to verify that no unwanted mutations were introduced during the PCRs. The plasmid pPol-luc, where the luciferase reporter gene was under the control of the VZV DNA polymerase gene promoter, was described previously (24).

Cells—Vero cells (a monkey kidney cell line, ATCC CCL-81) used in this study were grown in essential minimum Eagle's medium (EMEM) (BioWhittaker) supplemented with 10% fetal bovine serum (FBS) (BioWhittaker) and 1% L-glutamine (BioWhittaker). Cell-free virus was prepared from VZV-infected MeWo cells as described previously (24). Supernatant containing cell-free virus was added to Vero cells previously resuspended in EMEM, 10% FBS. Infection of Vero cells was propagated by mixing VZV-infected Vero cells with uninfected cells (1:4). ND7 cells (a donation from Dr. Latchman, Institute of Child Health, University College, London, UK) were obtained from a fusion of murine neuroblastoma cells with primary nerve cells from rat dorsal root ganglia (59). They were grown in RPMI 1640 (BioWhittaker) supplemented with L-glutamine and 5% FBS (Invitrogen).

Immunoprecipitation—IE63 was immunoprecipitated from Vero extracts with a monoclonal mouse antibody raised against the carboxyl-terminal end of the protein (24). CDK1 was immunoprecipitated from nuclear extracts from Vero cells by using an affinity-purified rabbit polyclonal antibody raised against a peptide mapping at the carboxyl terminus of human CDK1 (Santa Cruz Biotechnology). CDK2 was immunoprecipitated from nuclear extracts from Vero cells with rabbit polyclonal antibody raised against the carboxyl terminus of human CDK2 (Calbiochem). CDK5 was immunoprecipitated from either ND7 or nuclear extracts from Vero cells using rabbit polyclonal antibody raised against a peptide mapping at the carboxyl terminus of CDK5 of human origin (Santa Cruz Biotechnology), CDK7 from Vero cells with a mouse monoclonal antibody (BD Biosciences), and CDK9 from Vero cells using a rabbit polyclonal antibody (Santa Cruz Biotechnology). Preparation of total and nuclear cellular extracts was performed as described previously (24). Nuclear extracts were incubated for 2 h with antibodies in the immunoprecipitation buffer (0.5 M HEPES, pH 7.4, 2 M NaCl, 0.5% Triton X-100, 10% glycerol) that included a mixture of phosphatase inhibitors (1 mM Na_3VO_4, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM NaF, and 0.5 mM β-glycerophosphate), and Complete protease inhibitors (Roche Applied Science). 120 μl of Protein A-Sepharose (Amersham Biosciences) were then incubated for an additional 2 h with the complexes kinase-antibodies. The resin was then washed five times with kinase buffer (50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 2 mM dithiothreitol, 1 mM EGTA) supplemented with the protease and phosphatase inhibitors mixture.

In Vitro Kinase Assays—GST-63wt protein was expressed from the pGEX-5x-63wt vector and GST-CTD protein from the pGEX-5x-CTD vector. Proteins were purified on glutathione-Sepharose 4B (Amersham Biosciences) according to the manufacturer's instructions. Mutated GST-63 proteins were expressed and purified following the same protocol. *In vitro* kinase assays were performed by using either nuclear extracts from Vero cells (A) or recombinant (B and D) or immunoprecipitated kinases (C) as follows. A, 50 μg of nuclear extracts from Vero cells were mixed with 30 μl of glutathione-Sepharose 4B-bound GST-63 protein and 10 μCi of [γ-^{32}P]ATP (ICN Pharmaceuticals) in the CDK1 kinase assay buffer (see below). B, several concentrations of recombinant CDK1 were added to 30 μl of glutathione-Sepharose 4B-bound GST-63 protein and 10 μCi of [γ-^{32}P]ATP (ICN Pharmaceuticals) in the CDK1 assay buffer. C, 10 μg of eluted GST-IE63 and 10 μCi of [γ-^{32}P]ATP (ICN Pharmaceuticals) were added to the resin-bound kinases. D, 50 units of recombinant CK1 and CK2 were added to 5 μg of glutathione-Sepharose 4B-bound GST-63 wild-type or GST-IE63-S224A/T222A protein and 10 μCi of [γ-^{32}P]ATP (ICN Pharmaceuticals) in the CK1 and CK2 assay buffer. Roscovitine (Calbiochem), an inhibitor of CDK1, -2, -5, -7, and -9, and CDK1 inhibitor III (Calbiochem), an inhibitor of CDK1, were used in these experiments at 25 and 60 μM,

respectively. Histone H1 (Calbiochem) was used as positive control for the experiments with CDK1, -2, and -5, whereas GST-CTD was a positive control for the experiments with CDK7 and -9. The assay performed with GST alone constituted the negative control (−). All reactions were performed in the appropriate kinase buffer (CDK1: 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM EGTA; CDK2: 100 mM Tris-HCl, pH 7.4, 20 mM $MgCl_2$; CDK5: 20 mM MOPS, pH 7.4, 30 mM $MgCl_2$; CDK7: 10 mM HEPES, pH 7.4, 150 mM NaCl, 10 mM $MgCl_2$; CDK9: 50 mM Tris-HCl, pH 7.5, 4 mM $MgCl_2$, 5 mM $MnCl_2$; CK1: 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$; CK2: 50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 140 mM KCl) and supplemented with the protease and phosphatase inhibitor mixtures. In any case, samples were incubated for 30 min at 30 °C, heat-denatured, and loaded on a 12% SDS-polyacrylamide gel. After migration, the gel was dried, autoradiographed, and analyzed by PhosphorImaging (Amersham Biosciences). The phosphorylation level was quantified by densitometry (%).

In Vivo Kinase Assays—Vero cells were transfected with pcDNA3.1 expressing wild-type or mutated IE63 proteins (IE63wt, IE63-S224A/T222A, IE63-FULL, IE63-REV224S, and IE63-REV224S/222T) or with pcDNA3.1 as control plasmid(−) using the FuGENE 6 transfectant reagent (Roche Applied Science). 6 h post-transfection, $^{32}P_i$ (ICN Pharmaceuticals) was added to the supernatant at a final concentration of 500 μCi/ml for 24 h. Roscovitine and CiIII were used in this experiment at 25 and 60 μM, respectively. IE63 was immunoprecipitated with monoclonal mouse antibody. After extensive washes, samples were heat-denatured and loaded on a 12% SDS-polyacrylamide gel, before PhosphorImager detection. The phosphorylation level was quantified by densitometry and is reported as a percentage relative to wild-type IE63 (%). The expression and immunoprecipitation of IE63 proteins were confirmed by Western blotting (WBα-IE63).

Immunofluorescence—Vero cells were seeded on cover slides into 10-mm dishes and grown in EMEM (BioWhittaker) supplemented with 10% FBS (BioWhittaker) and 1% L-glutamine. The cells were transfected with 2 μg of pcDNA-IE63wt or mutated using FuGENE 6 reagent according to the manufacturer's instructions (Roche Applied Science). VZV-infected Vero cells were also used in these experiments as a positive control. Transfected or infected Vero were either treated or not with 25 μM roscovitine or 60 μM CiIII, 6 h after transfection or infection. 48 h post-transfection or post-infection, the cells were fixed with a solution of acetone/methanol (v/v) for 20 min at −20 °C. After fixation, the nonspecific sites were saturated with a milk-blocking solution (1.5% in PBS) and then incubated with primary antibody. In these experiments, a mouse monoclonal antibody directed against IE63 (9A12) (26) and fluorescein isothiocyanate-conjugated secondary antibody (Dako) were used. The cells were observed by fluorescent microscopy (Nikon) after being previously counterstained with a 1% (v/v) Evans Blue solution. Positive cells were counted and classified according to the IE63 cellular localization: N > C for a major nuclear staining, N = C for an equal distribution of the protein between nucleus (N) and cytoplasm (C), and N < C for a major cytoplasmic staining. Average percentages are the results of at least three independent experiments with 100 positive cells counted per experiment.

Activity Test—Transient transfection studies were carried out with Vero cells seeded into 35-mm diameter 6-well cluster dishes using the FuGENE 6 transfectant reagent (Roche Applied Science). The plasmid pPol-luc was used as reporter vector and pcDNA3.1-, pcDNA-IE63wt, pcDNA-IE63-S224A, pcDNA-IE63-T222A, pcDNA-IE63-S224A/T222A, pcDNA-IE63-S224E, and pcDNA-IE63-S224E/T222E were used as expression vectors. An equimolar ratio of cytomegalovirus promoter was used in each independent experiment. The amounts of DNA were adjusted with herring sperm DNA. Transfected Vero cells were either treated or not with 25 μM roscovitine or 60 μM CiIII, 6 h after transfection. 24 h post-transfection, luciferase assays were performed using the "Luciferase Reporter Gene Assay, High Sensitivity" kit (Roche Applied Science) according to the manufacturer's instructions. For each experiment, the concentration of proteins in each sample was measured to normalize the results. Data from luciferase assays were collected from at least five independent transfection experiments. p values were calculated using the graphpad quickcalcs software (www.graphpad.com). For the comparison of two means, an unpaired t test was chosen. For significantly different values, the p value was <0.05; for not significantly different values, the p value was ≥ 0.05.

RESULTS

Identification and Mutation of Putative Roscovitine-sensitive Cyclin-dependent Kinases-mediated Phosphorylation Sites in IE63—In silico analysis of IE63 amino acid sequence with the PhosphoBase and NetPhos 2.0 prediction tool software (35) revealed the presence of two potential phosphorylatable residues (Thr-222 and Ser-224) by CDK1 in the carboxyl-terminal region of the protein. The probability scores obtained for the phosphorylation by CDK1 were 0.979 and 0.938 for Ser-224 and Thr-222, respectively. In addition, Ser-224 appeared to be potentially phosphorylated by CDK5 with a probability score of 0.809. In order to assay the importance of these residues in IE63 phosphorylation by CDK1 and -5, we decided to substitute them individually (pcDNA-IE63-S224A or pcDNA-IE63-T222A) or in tandem (pcDNA-IE63-S224A/T222A) by alanine residues.

IE63 Is Phosphorylated by Roscovitine-sensitive Cyclin-dependent Kinases in Vitro—As shown previously (30), VZV needs a CDK activity for replication because inhibitors of these specific kinases are able to prevent VZV replication. To determine whether IE63 protein is a phosphorylation target for such kinases, we carried out an *in vitro* kinase assay using nuclear extracts from Vero cells in the presence of roscovitine, which efficiently and selectively inhibited CDK1, -2, -5, -7, and -9 (31). For this, wild-type *IE63*-encoded gene was cloned in pGEX-5x to generate GST fusion protein. The corresponding protein was expressed in *E. coli*, purified, and used as substrate for the assay either in the presence (Fig. 1*A, 2nd lane*) or absence (Fig. 1*A, 1st lane*) of 25 μM roscovitine. The phosphorylation level was quantified by densitometry and reported to IE63 phosphorylation without roscovitine (%). As shown in Fig. 1*A*, GST-IE63 protein is phosphorylated by nuclear extracts from Vero cells (*1st lane*). The use of 25 μM roscovitine reduced but did not totally abolish phosphorylation of IE63 (Fig. 1*A*, *2nd lane, 57.2%*). This basal phosphorylation of IE63 cannot be abolished even if the concentration of roscovitine was increased to 100 μM (data not shown).

IE63 Is Phosphorylated by CDK1 and -5 in Vitro—To determine the relative importance of both potential phosphorylation residues (Ser-224 and Thr-222) and to identify the RSCs responsible for IE63 phosphorylation, wild-type and mutated (pcDNA-IE63-S224A, pcDNA-IE63-T222A, and pcDNA-IE63-S224A/T222A) IE63-encoded genes were cloned in pGEX-5x. GST fusion proteins were used to perform an *in vitro* kinase assay with either recombinant CDK1, CK1, and CK2 or immunoprecipitated CDK1, -2, -5, -7, and -9. As shown in Fig. 1*B*, GST-IE63 is phosphorylated in a dose-dependent manner by recombinant CDK1. Mutation S224A strongly reduced but did not totally abolish CDK1-mediated phosphorylation of IE63. A less pronounced decrease of IE63 phosphorylation was observed with IE63 mutated at Thr-222 (Fig. 1*B*). As expected, double substitution of these two residues completely abolished CDK1-mediated phosphorylation. The use of 25 μM roscovitine completely suppressed CDK1-mediated phosphorylation of IE63 in a similar extent to what was seen with the double mutant type of the protein (Fig. 1*B*). From these experiments, we can conclude that Ser-224 and Thr-222 are the only amino acids phosphorylated *in vitro* by recombinant CDK1. Most surprisingly, in view of the level of phosphorylation of the S224A mutant, we would have expected to reach a higher level of phosphorylation with the T222A mutant. This may be due to a modification in the context of Ser-224 rather than a loss of phosphorylation of Thr-222 itself.

To demonstrate that these observations with recombinant CDK1 can be extended in cellular conditions, CDK1 was immunoprecipitated from nuclear extracts from Vero cells and used in the same conditions. As shown in Fig. 1*C*, IE63 is efficiently phosphorylated by immunoprecipitated CDK1, and as observed with the recombinant enzyme, a single substitution of Ser-224 drastically reduces phosphorylation, whereas only a partial decrease can be detected with T222A mutant.

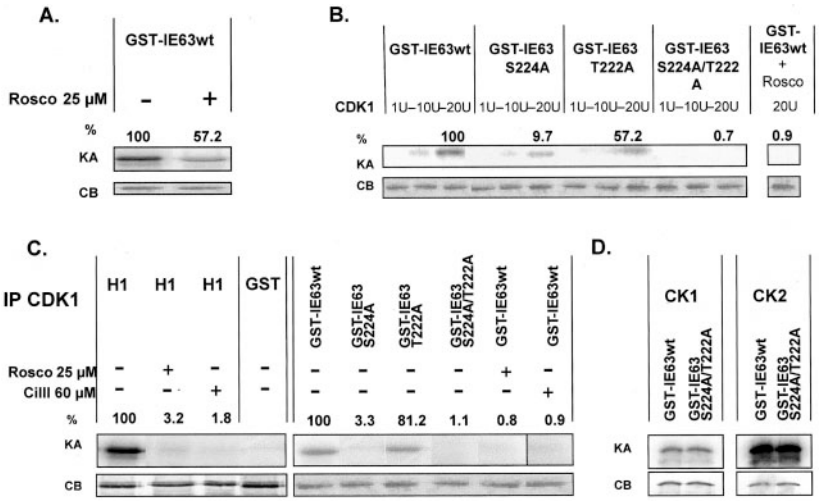


FIG. 1. ***In vitro* phosphorylation of wild-type or mutated IE63.** *A,* we performed a kinase assay using nuclear extracts from Vero cells in the presence of roscovitine. Wild-type *IE63* gene was cloned in pGEX-5x to generate GST fusion protein. 10 μg of the purified fusion protein expressed in *E. coli* was used as substrate for the assay in both the presence (*A, 2nd lane*) and absence (*A, 1st lane*) of 25 μM roscovitine and 10 μCi of [γ-^{32}P]ATP for 30 min at 30 °C. The phosphorylation level was then quantified by densitometry and reported to IE63 without roscovitine and was arbitrarily determined as 100% (%). *B,* 1, 10, and 20 units of recombinant CDK1 were used to phosphorylate (kinase assay, *KA*) 10 μg of either wild-type GST-IE63, GST-IE63-S224A, GST-IE63-T222A, or GST-IE63-S224A/T222A proteins coupled with Sepharose beads in the presence of 10 μCi of [γ-^{32}P]ATP for 30 min at 30 °C. Roscovitine was used in this experiment at a final concentration of 25 μM. *C,* CDK1 was immunoprecipitated (*IP*) from nuclear extracts from Vero cells and incubated with 10 μg of histone H1 or previously eluted wild-type or mutated GST-IE63 in the presence of 10 μCi of [γ-^{32}P]ATP for 30 min at 30 °C. Roscovitine (*Rosco*) and CDK1 inhibitor III (*CiIII*), a specific inhibitor of CDK1, were used in this experiment at 25 and 60 μM, respectively. Histone H1 was used as positive control. The assay performed with GST alone constitutes the negative control (−). *D,* 50 units of recombinant CK1 and CK2 were used to phosphorylate (*KA*) 5 μg of either wild-type GST-IE63 or GST-IE63-S224A/T222A proteins coupled with Sepharose beads in the presence of 10 μCi of [γ-^{32}P]ATP for 30 min at 30 °C. *CB*, Coomassie Blue.

Again, double substitution completely abrogated CDK1-mediated phosphorylation of IE63. The use of roscovitine completely inhibits IE63 phosphorylation as is also the case when CiIII, a specific inhibitor of CDK1, was added. These data confirmed those obtained with recombinant CDK1 and also confirmed that Ser-224 was more prone to phosphorylation by CDK1 than Thr-222 as suggested by *in silico* analysis.

The same assay using immunoprecipitated CDK2, -5, -7, and -9 was then performed to assess the phosphorylation status of GST-IE63. CDK2, -7, and -9 were immunoprecipitated from nuclear extracts from Vero cells using the same protocol and then incubated with GST-IE63 protein (Fig. 2). As observed in Fig. 2, *A*, *C,* and *D*, IE63 protein is not a phosphorylation target for CDK2, -7, and -9 in our conditions. CDK5, a kinase only active in neuronal cells, was immunoprecipitated from either Vero or ND7 cells (Fig. 2*B*). The assay performed with CDK5 immunoprecipitated from nuclear extracts from Vero cells on histone H1 constitutes the negative control because the kinase is not active in these cells. As expected, we saw in Fig. 2*B* that no phosphorylation can be observed on histone H1. On the contrary, CDK5 immunoprecipitated from neuronal ND7 cells is active and phosphorylates histone H1 and IE63 (Fig. 2*B*). Double substitution of Ser-224 and Thr-222 completely abrogated CDK5-mediated phosphorylation of IE63 (Fig. 2*B*). The use of roscovitine but not CiIII completely impaired this phosphorylation, demonstrating the sensibility of CDK5 to roscovitine but not to CiIII.

Finally, we carried out a kinase assay using recombinant CK1 and -2 on GST-IE63 wild-type and GST-IE63-S224A/T222A in order to determine whether these two CDK-1 sites could be recognized by other enzymes known to phosphorylate IE63 at various sites (Fig. 1*D*). It appears that the double substitution S224A/T222A did not modify the phosphorylation level of IE63 by CK1 and CK2. This showed that these two sites were only recognized by CDKs.

Altogether, these results clearly indicate that IE63 is phosphorylated by CDK1 and -5 on Ser-224 and Thr-222. For the following experiments shown in this study, we will only refer to CDK1-mediated phosphorylation of IE63 because CDK5 is not active in Vero cells.

IE63 Is Phosphorylated by CDK1 in Vivo in Vero Cells—We then investigated what happens to IE63 phosphorylation *in vivo*. Vero cells were transfected with plasmids expressing wild-type or mutated IE63 proteins and were metabolically labeled with $^{32}P_i$. IE63 was immunoprecipitated 30 h post-transfection. As shown in Fig. 3, IE63 is phosphorylated *in vivo*. The use of roscovitine or CiIII significantly decreased the level of phosphorylation of the protein, demonstrating the implication of CDK1 in this process. Once all the phosphorylation sites for CK1, CK2, CDK1, and CDK5 (IE63-FULL) were substituted by alanine, no more phosphorylation was detectable. With revertant proteins (IE63-REV224S and IE63-REV224S/222T), about 30% of the phosphorylation level of IE63 was restored to a similar extent as with the simple and double revertant, demonstrating once again the importance of CDK1 phosphorylation and of Ser-224 relative to Thr-222.

IE63 Cellular Localization Is Dependent on CDK1-mediated Phosphorylation in Vero Cells—Because IE63 phosphorylation has already been shown to be important for correct cellular localization (24), we decided to study the importance of the CDK1 phosphorylation sites in this process. As shown previously, wild-type IE63 is predominantly localized in the nucleus and slightly in the cytoplasm of the infected cells (Fig. 4*A*) (24). Quantification analysis (Fig. 4*C*) revealed that the localization was mostly nuclear in about 80% of the cells expressing IE63, whereas an equivalent distribution between the cytoplasm and the nucleus was observed in a much lower fraction (about 20%). Mutation of Ser-224 drastically reduced the distribution of IE63 between both compartments from about 20 to 5% (Fig. 4*B*). Furthermore, we saw in Fig. 4*B* that IE63 localized exclusively in the nucleus in about 95% of the transfected cells. In accordance to what was observed in the kinase assays described above, mutation of Thr-222 also affected IE63 localization but to a much lesser extent (Fig. 4*B*). The abolishment of the two phospho-sites led to a significant increase of the exclusive nuclear localization of IE63 (Fig. 4*B*).

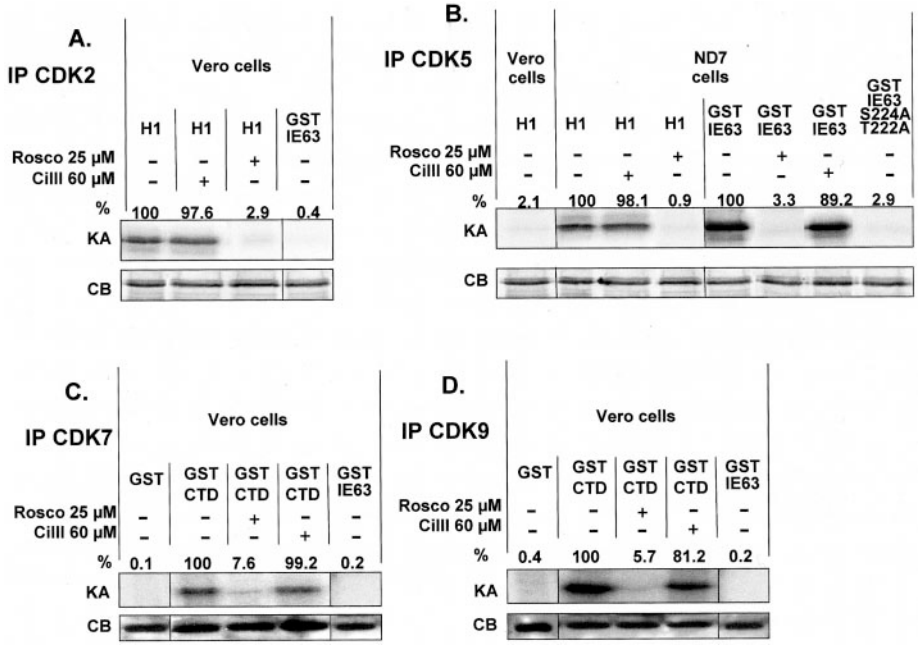


FIG. 2. ***In vitro* phosphorylation of GST-IE63, GST-CTD, and histone H1 by immunoprecipitated CDK2, -5, -7, and -9 from either Vero or ND7 cells.** *A,* CDK2 was immunoprecipitated (*IP*) from nuclear extracts from Vero cells. *B,* CDK5 was immunoprecipitated from nuclear extracts from Vero or ND7 cells; *C,* CDK7 from nuclear extracts from Vero cells; and *D,* CDK9 from nuclear extracts from Vero cells. The immunoprecipitated kinases were then incubated with 10 μg of histone H1 or previously eluted wild-type or mutated GST-IE63 in the presence of 10 μCi of [γ-^{32}P]ATP for 30 min at 30 °C. Roscovitine (*Rosco*) and CiIII were used in this experiment at 25 and 60 μM, respectively. After extensive washes, samples were heat-denatured and loaded on a 12% SDS-PAGE gel, before PhosphorImager analysis. The phosphorylation level was quantified by densitometry (%). Coomassie Blue-stained SDS-PAGE (*CB*) of the GST fusion proteins is shown as loading control. *KA*, kinase assay.

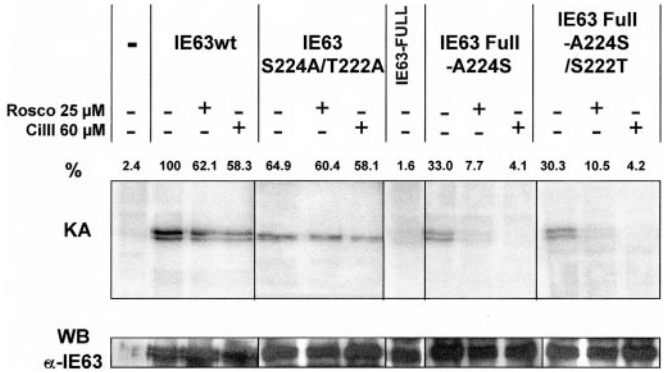
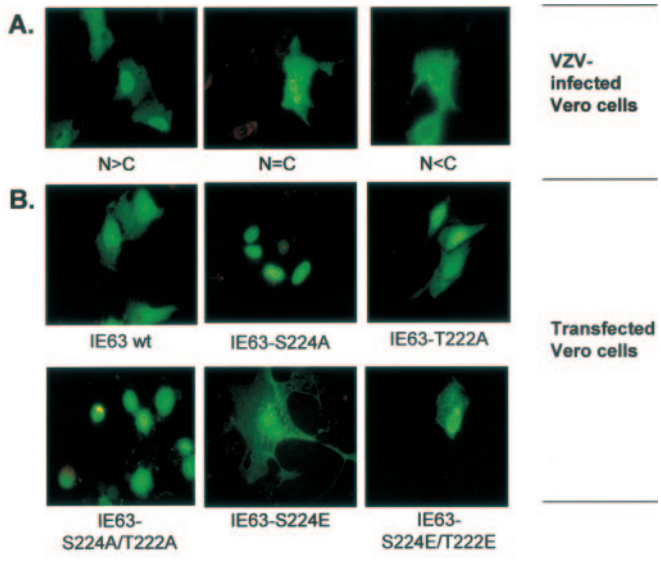


FIG. 3. ***In vivo* phosphorylation of wild-type or mutated IE63.** Vero cells were transfected with pcDNA3.1 expressing wild-type or mutated IE63 proteins (IE63wt, IE63-S224A/T222A, IE63-FULL, IE63-REV224S, and IE63-REV224S/222T) or with pcDNA3.1 as control plasmid (−). After transfection, cells were metabolically labeled with 500 μCi/ml of $^{32}P_i$ for 24 h. Roscovitine (*Rosco*) and CiIII were used in this experiment at 25 and 60 μM, respectively. IE63 was immunoprecipitated with monoclonal mouse antibody (26). After extensive washes, samples were heat-denatured and loaded on a 12% SDS-polyacrylamide gel, before PhosphorImager analysis. The phosphorylation level was quantified by densitometry and is reported as a percentage relative to wild-type IE63 (%). The expression and immunoprecipitation of IE63 proteins was confirmed by Western blotting (*WBα-IE63*). *KA,* kinase assay.

To reinforce the idea that CDK1-mediated phosphorylation was important for the nucleocytoplasmic distribution of IE63, we decided to mimic this phosphorylation by substituting Ser-224 and Thr-222 by glutamic acids. As expected, the single mutation S224E and the double mutation S224E/T222E did not modify the cellular distribution of IE63 (Fig. 4*B*); these mutant proteins were localized as wild-type IE63.

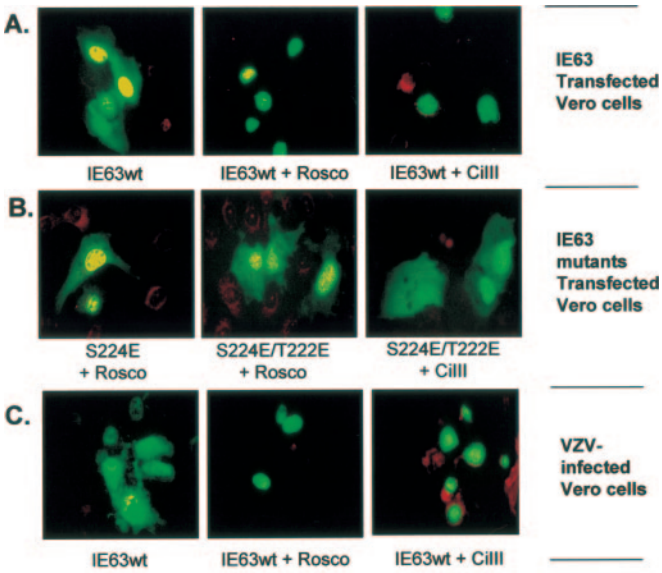
More evidence of the importance of CDK1 in IE63 localization was given by the use of roscovitine and CiIII, the latter inhibiting specifically CDK1. It appears that in our conditions roscovitine and CiIII did not affect the relative number of cells expressing IE63. As shown in Fig. 5, the addition of roscovitine or CiIII led to an important nuclear localization of the protein (Fig. 5, *A* and *D*), to a similar extent as what was observed with the S224A mutant. Furthermore, Fig. 5, *B* and *D,* shows that inhibitors have no effect on the localization of IE63 when the two phosphorylation sites for CDK1 are substituted by glu-



C.

IE63	N>C (%)	N=C (%)	N<C (%)
IE63wt (Infection)	79	19	2
IE63wt	80	18	2
IE63-S224A	95	4	1
IE63-T222A	84	13	3
IE63-S224A/T222A	97	2	1
IE63-S224E	80	18	2
IE63-S224E/T222E	79	17	4

FIG. 4. **Intracellular localization of wild-type and mutated IE63 proteins in VZV-infected or transiently transfected Vero cells.** *A,* Vero cells were infected with cell-free VZV. *B*, transfection was carried out with 2 μg of plasmid expressing wild-type IE63, IE63-S224A, IE63-T222A, IE63-S224A/T222A, IE63-S224E, and IE63-S224E/T222E. 48 h post-transfection, immunostaining analysis was carried out using a monoclonal antibody (9A12) directed against IE63. Secondary antibody used is conjugated with fluorescein isothiocyanate. *C*, positive cells were counted and classified according to the IE63 cellular localization: N > C for a major nuclear staining; N = C for an equal distribution of the protein between nucleus (*N*) and cytoplasm (*C*); and N < C for a major cytoplasmic staining. Average percentages are the results of at least three independent experiments with 100 positive cells counted per experiment.



D.

IE63	N>C (%)	N=C (%)	N<C (%)
IE63wt	79	19	2
IE63 + Rosco	94	6	0
IE63 + CiIII	96	4	0
IE63-S224E + Rosco	82	14	4
IE63-S224E/T222E + Rosco	83	11	6
IE63-S224E/T222E + CiIII	78	17	5
IE63wt (Infection)	80	17	3
IE63wt + Rosco (Infection)	96	3	1
IE63wt + CiIII (Infection)	95	4	1

FIG. 5. **Effect of roscovitine (*Rosco*) and CiIII on the intracellular localization of wild-type IE63 in transfected or infected Vero cells.** *A* and *B*, transfection was carried out with 2 μg of plasmid expressing wild-type IE63 (*A*) and IE63-S224E, IE63-T222E, and IE63-S224E/T222E (*B*). Cells were treated with 25 μM roscovitine 6 h after transfection. CiIII was also used at 60 μM. *C*, Vero cells were infected with cell-free VZV and were treated with 25 μM roscovitine 6 h after infection. CiIII was used at a final concentration of 60 μM. 48 h post-transfection or post-infection, immunostaining analysis was carried out by using a monoclonal antibody directed against IE63. Secondary antibody was conjugated with fluorescein isothiocyanate. *D*, positive cells were counted and classified according to the IE63 cellular localization: N > C for a major nuclear staining; N = C for an equal distribution of the protein between nucleus (*N*) and cytoplasm (*C*); and N < C for a major cytoplasmic staining. Average percentages are the results of at least three independent experiments with 100 positive cells counted per experiment.

tamic acid, demonstrating that the effect of the inhibitors on the localization of the wild-type protein is directly because of an absence of phosphorylation on Ser-224 and Thr-222.

To demonstrate that the role of CDK1-mediated IE63 phosphorylation observed in transiently transfected Vero cells could be verified during VZV infection, infected Vero cells were treated with roscovitine or CiIII and analyzed by immunofluorescence (Fig. 5, *C* and *D*). As expected, IE63 localization was almost exclusively nuclear in about 95% the infected cells in the presence of each inhibitors, demonstrating that the CDK1-mediated phosphorylation is important for the cytoplasmic export and/or nuclear import of IE63 (Fig. 5, *C* and *D*).

CDK1-mediated IE63 Phosphorylation Is Important for Gene Regulation in Vero Cells—Gene regulatory properties of IE63 were shown to be influenced by its phosphorylation level in its carboxyl-terminal region (24). Because the CDK1 phosphorylation sites were also situated in this region, we decided to analyze the activity of IE63 mutated proteins in a transient transfection assay using the luciferase gene controlled by the VZV DNA polymerase promoter. This construct exhibited a rather important constitutive activity in Vero cells, and is thus an accurate tool to measure transcription down-regulation. The basal activity of the VZV DNA polymerase promoter-Luc construct has been measured and is about 200-fold higher than a promoter-less luciferase constructs (data not shown). As shown previously (24), IE63 down-regulated the DNA polymerase promoter in a dose-dependent manner (Fig. 6*A*). In this assay, both S224A and T222A mutants showed a lower down-regulating activity than the wild-type protein (Fig. 6*A*). However, the double mutated protein was obviously less efficient (Fig. 6*A*). Substitution by glutamic acids did not modify the activity of IE63, demonstrating that CDK1 phosphorylation is a crucial event in the regulatory properties of IE63 (Fig. 6*B*).

For further proof of the importance of CDK1-mediated phosphorylation in *IE63* gene regulation, Vero cells were transfected by pcDNA-IE63 and then treated with either roscovitine or CiIII 6 h post-transfection. In the presence of the inhibitors, VZV DNA polymerase promoter down-regulation by IE63 was strongly impaired (Fig. 7, *A* and *B*), although the number of cells expressing IE63 was not modified. The level of inhibition observed in the presence of roscovitine was comparable with what was reached with the S224A/T222A mutant (Fig. 6*A*). This loss of efficiency of repression may have been due to a decrease in the basal activity of the VZV DNA polymerase promoter in the presence of inhibitors. We thus compared this basal activity with either the presence or absence of roscovitine (Fig. 7*C*). It appears that the activity is quite comparable with or without roscovitine.

Furthermore, Fig. 7, *D* and *E,* shows that inhibitors have no effect on the repressive properties of IE63 when the two phosphorylation sites for CDK1 are substituted by glutamic acid, demonstrating once again that the effect of the inhibitors is directly because of an absence of phosphorylation on Ser-224 and Thr-222.

DISCUSSION

Recent data suggest that cell cycle-associated factors, specifically CDKs, play a key role in the life-cycle of many DNA-containing viruses, including members of the herpesvirus family. It has been hypothesized that CDKs regulate the functions of IE proteins to promote either a productive or latent infection. For example, HSV-1 has been shown to require CDKs activity for efficient replication in many cell types, and inhibitors of CDKs prevent infection (28). Jung *et al.* (50) have shown that the repressive activity of hepatitis C virus core protein on the transcription of $p21^{Waf1}$ was regulated by protein kinase A-mediated phosphorylation. Among the inhibitors that impair correct replication of herpesvirus, roscovitine is a purine derivative that inhibits CDK1/cyclin B, CDK2/cyclin A or E, CDK5/p35, CDK7/cyclin H, and CDK9/cyclin T (31). Taylor *et al.* (30) showed that VZV replication may be prevented by the use of this inhibitor. Because IE63 is an essential protein for the replication of the virus, we decided to analyze the phosphorylation status of IE63 by RSCs and its implication in the cellular localization and regulation activity of the protein.

The main results obtained in this study can be summarized as follows. (i) IE63 is phosphorylated *in vitro* by CDK1 and -5 and *in vivo* by CDK1 in Vero cells. Phosphorylation occurred on residue Ser-224 and, to a lesser extent, on residue Thr-222. (ii) This phosphorylation is essential for the correct subcellular localization of IE63. Indeed, the abolition of CDK1-mediated phosphorylation by either a mutational approach or the use of specific inhibitors led to an exclusive nuclear localization of the protein in

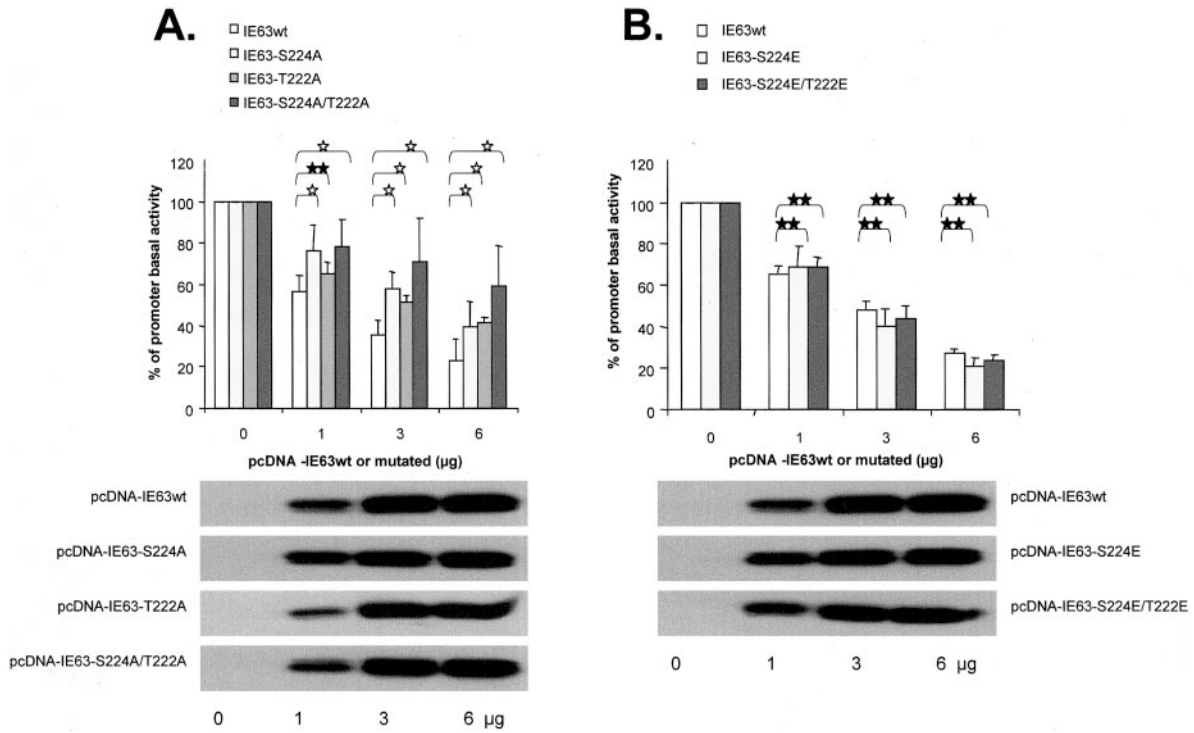


FIG. 6. **Effect of wild-type or mutated IE63 on the VZV DNA polymerase promoter basal expression.** *A* and *B*, Vero cells were transfected with 1 μg of pPol-luc. Increasing concentrations of plasmids expressing wild-type IE63, IE63-S224A, IE63-T222A, IE63-S224A/T222A (*A*) and IE63-S224E, IE63-S224E/T222E (*B*) were added. 24 h post-transfection, cells were harvested, and the reporter gene activity was measured. Results are presented as a percentage of stimulation with respect to the basal expression of the promoter (= 100%). In our experiments, the luciferase activity expressed by the VZV DNA polymerase promoter has been measured and is about 2000 cps relative to a promoter-less luciferase constructs which is about 100 cps. Data from luciferase assays were collected from at least five independent transfection experiments. S.E. of the mean are shown as *error bars*. *, significantly different; **, not significantly different. The expression of wild-type or mutated IE63 protein was confirmed by Western blotting.

transfected or infected cells. (iii) CDK1-mediated phosphorylation of IE63 on Ser-224 and Thr-222 is a crucial event for its repressive properties in transient transfection assay.

In Vero cells, IE63 was solely phosphorylated *in vivo* by CDK1, because CDK5 was not active in this cell type. We showed that the use of roscovitine or CiIII reduced the phosphorylation of IE63 significantly but not completely. The remaining phosphorylation was because of cellular Ser/Thr kinases because it was shown previously that IE63 is a target for CK1 and -2 (24). The decrease that is reported seems to be substantial considering that only one phosphorylation site has been mutated. Therefore, we evaluated the potential secondary effects affecting modification by CK1 and -2. It appeared that the phosphorylation of IE63 by recombinant CK1 and -2 was not modified by the double substitution at Thr-222 and Ser-224. We showed that the substitution of Thr-222 by alanine residue has a minor effect on the phosphorylation status of IE63. This may be due to a modification in the context of Ser-224 rather than a loss of phosphorylation of Thr-222 itself.

Phosphorylation and dephosphorylation events are known to be implicated in many cellular regulatory activities such as cell cycle, signal transduction, or gene expression (33, 43, 44). For example, phosphorylation of VZV IE62 by the viral ORF66 kinase led to its delocalization from the nucleus to the cytoplasm, impairing its transactivation properties (45). It has been shown recently (46) that most of phosphorylation sites in IE63 sequence are essential for infectivity. Furthermore, Cohen *et al.* (47) also demonstrated that phosphorylation of IE63 is a crucial event for a correct replication and an effective establishment of latency *in vivo*. It is possible that phosphorylation of IE63 might affect its incorporation into the tegument, but actually the mechanism of IE63 incorporation into the tegument is still poorly characterized.

Numerous reports indicate that phosphorylation-dependent mechanisms may regulate the subcellular localization of proteins such as those containing a CcN motif (CKII-cdc2-NLS), a short region containing phosphorylation sites for CKII and CDK1 in the proximity of a nuclear localization signal (NLS) (48). For example, the co-chaperone murine stress-inducible protein 1 (mSTI1) possesses a CcN motif that regulates its cellular localization (55). The phosphorylation of this motif by CK-2 favored the nuclear import, whereas phosphorylation by CDK1 favored the cytoplasmic export of the protein. A range of proteins, including p53 (56), lamin (57), and nucleoplasmin (58), exhibit a similar CcN motif. Such a motif might exist in the carboxyl-terminal region of IE63. Indeed, Bontems *et al.* (24) demonstrated the presence of numerous CK-2 phosphorylation sites in this region and an NLS between amino acids 259 and 264. Furthermore, recent results from Baiker *et al.* (46) clearly indicate the presence of a second NLS localized between amino acids 226 and 229. It is possible that the phosphorylation of IE63 by CDK1 favored the cytoplasmic export of the protein. It is interesting to note that preliminary data obtained in our laboratory showed that the export of IE63 was dependent on the nuclear export protein CRM-1. Indeed, leptomycin B, an inhibitor of the nuclear export protein CRM-1, strongly increased the nuclear retention of IE63.[2] An explanation for the nuclear sequestrating of IE63 mutated on CDK1 phosphosite might be that IE63 unphosphorylated on Ser-224 is unable to interact with CRM-1 and remains sequestered in the nucleus by binding to a partner.

IE63 is a tegument protein, which possesses regulatory properties on the viral gene expression as shown previously (22),

[2] S. Bontems, personal communication.

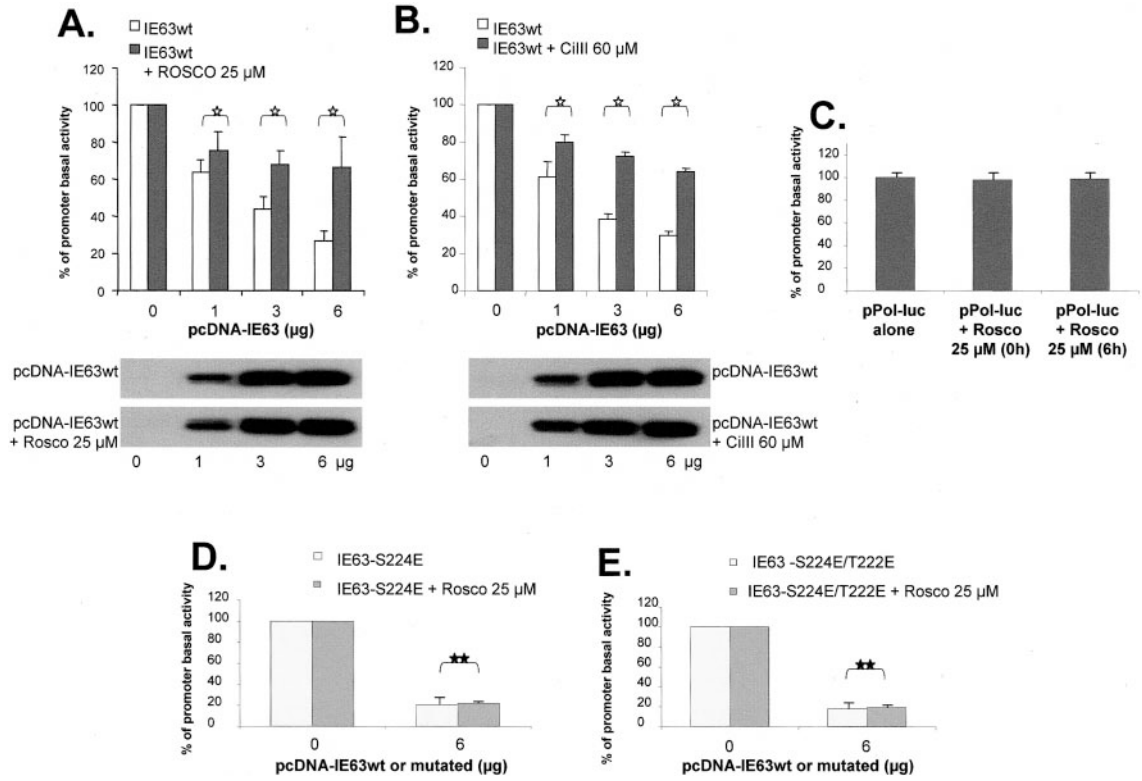


FIG. 7. **Effect of roscovitine (*Rosco*) on the repressive properties of wild-type or mutated IE63 and on the VZV DNA polymerase promoter basal activity.** Vero cells were transfected with 1 μg of pPol-luc. *A*, increasing concentrations of plasmid expressing wild-type IE63 were added. 6 h post-transfection, cells were treated (*IE63wt + Rosco* 25 μM) and untreated (*IE63wt*) with 25 μM roscovitine. *B,* comparison of the VZV DNA polymerase promoter basal activity in the presence or absence of roscovitine. At the time of transfection (0 h) or 6 h later, cells were treated (*pPol-luc+Rosco* 25 μM) or not *(pPol-luc alone*) with 25 μM roscovitine. *C* and *D,* increasing concentrations of plasmid expressing IE63-S224E (*C*) or IE63-S224E/T222E (*D*) were added. 6 h post-transfection, cells were treated (*IE63-S224E* and *IE63-S224E/T222E+Rosco* 25 μM) and untreated (IE63-S224E and IE63-S224E/T222E) with 25 μM roscovitine. *E,* increasing concentrations of plasmid expressing wild-type IE63 were added. 6 h post-transfection, cells were treated (IE63wt+CiIII 60 μM) and untreated (IE63wt) with 60 μM CiIII. 24 h post-transfection, cells were harvested, and the reporter gene activity was measured. Results are presented as a percentage of stimulation with respect to the basal expression of the promoter (= 100%). In our experiments, the luciferase activity expressing the VZV DNA polymerase promoter has been measured and is about 2000 cps relative to a promoter-less luciferase construct which is about 100 cps. Data from luciferase assays were collected from at least five independent transfection experiments. S.E. of the mean are shown as *error bars.* *, significantly different (p value < 0.05); **, not significantly different (p value ≥ 0.05). The expression of wild-type IE63 protein was confirmed by Western blotting.

but these results were disputed (23). The repression or activation observed as an IE63 effect is variable depending on the viral gene promoter being examined and the cell type used in the experiment. Nevertheless, work from our laboratory has shown by transient transfection assay that IE63 down-regulates the expression of the VZV DNA polymerase gene (24). Furthermore, Di Valentin *et al.* (25) have recently demonstrated that IE63 protein is a transcriptional repressor, the activity of which is mediated by disorganizing formation of the preinitiation complex by sequestering one or several general transcription factors (25). Work from another team (49) demonstrated that IE63 is present in a complex that also contains the RNA polymerase II. From the data presented in this work, we can hypothesize that Ser-224 and Thr-222 are important residues that have to be phosphorylated by CDK1 to disorganize the preinitiation complex. Without this post-translational modification, IE63 would be unable to interact with target protein in the preinitiation complex such as TFIIB and TFIIH.

As VZV infects nondividing cells, it must induce the cellular DNA synthesis machinery crucial for the replication of its own genome. For this, the virus utilizes viral and cellular kinases that are essential for pathogenesis. Indeed, Advani *et al.* (51) have observed that CDK1 kinase activity was activated in cells infected with HSV-1 and that the activation is mediated principally by two viral proteins, ICP22 and the kinase encoded by U_L13, the former being the HSV-1 homologue of VZV IE63 (51). Finally, Davido *et al.* (54) have reported that roscovitine reduced transcription of HSV-1 early genes, suggesting that the transactivating or transrepressing functions of IE proteins may require the activities of one or more roscovitine-sensitive CDKs (54). It is relevant to note that, in nondividing neurons, most of the cyclin-dependent kinases are in an inactive state. In the same way, the activities of the IE regulatory proteins are repressed so that the productive phase of viral gene expression does not occur or occurs only transiently, leading to latent infection. After stress, neurons become permissive for viral gene expression again, leading to the production of new infectious virus. The fact that nondividing neurons can support latent as well as productive infection implicates the differential expression and/or activation of cellular proteins in determining whether neuronal infection will be latent or productive. In support of this concept, expression of CDK2, cyclin A, and cyclin E is induced in neurons stressed by a stimulus that induces HSV-1 reactivation (52). Similar to the induction performed by HSV-1, VZV induces CDK4 and CDK2/cyclin E expression and activity in infected fibroblasts (53). Most interestingly, CDK1 is exclusively active in dividing cells such as those supporting a VZV-productive infectious cycle, whereas CDK5 is a prominent kinase in neurons supporting only a latent infection (42).

In summary, these experiments demonstrated for the first time that IE63 is phosphorylated by CDK1 in Vero cells and CDK5 in neuronal cells. This phosphorylation has been shown to be essential for the correct localization of the protein and for

its down-regulating properties, and these functions of IE63 are essential for VZV replication and the establishment of latency.

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