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Phosphorylation of Varicella-Zoster Virus IE63 Protein by Casein Kinases Influences Its Cellular Localization and Gene Regulation Activity

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The abbreviations used are: VZV, varicella-zoster virus; HSV-1, herpes simplex virus type 1; IE, immediate early; E, early; L, late; ORF, open reading frame; CKI, casein kinase I; CKII, casein kinase II; PKC, protein kinase C; NLS, nuclear localization signal; DRB, 5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole; CAT, chloramphenicol acetyltrans-ferase; FITC, fluorescein isothiocyanate; wt, wild type; PBS, phosphate-buffered saline; GST, glutathione S-transferase.

Abstract : During the early phase of varicella-zoster virus (VZV) infection, Immediate Early protein 63 (IE63) is expressed rapidly and abundantly in the nucleus, while during latency, this protein is confined mostly to the cytoplasm. Because phosphorylation is known to regulate many cellular events, we investigated the importance of this modification on the cellular localization of IE63 and on its regulatory properties. We demonstrate here that cellular casein kinases I and II are implicated in the *in vitro* and *in vivo* phosphorylation of IE63. A mutational approach also indicated that phosphoryla-tion of the protein is important for its correct cellular localization in a cell type-dependent fashion. Using an activity test, we demonstrated that IE63 was able to repress the gene expression driven by two VZV promoters and that phosphorylation of the protein was required for its full repressive properties. Finally, we showed that IE63 was capable of exerting its repressive activity in the cytoplasm, as well as in the nucleus, suggesting a regulation at the transcriptional and/or post-transcriptional level.

The varicella-zoster virus (VZV)[1] is a neurotropic virus belonging to the Alphaherpesvirinae subfamily and responsible for two distinct diseases as follows: chicken pox, an illness affecting mostly young children, and shingles, resulting from the virus reactivation in elderly and immunocompromised people (1). Indeed, the characteristics of all members of this viral subfamily are their ability to establish latency in sensory ganglia after primary infection and to reactivate decades later. Molecular events leading to the establishment and maintenance of the virus latency, as well as to its reactivation, are still poorly understood. It appears to be the result of a delicate balance between cellular factors, viral proteins and the host immune system (2). In lytically infected cells, the expression of VZV genes seems to occur in cascade-like events, previously described for herpes simplex virus type 1 (HSV-1) (3). Immediate early (IE) gene-encoded proteins are the first ones to be active because they act as regulators on their own expression, as well as on the transcriptional activation of early (E) and late (L) genes. Among the immediate early proteins, IE62 seems to be the major regulating factor with regard to its transactiva-tion properties on all classes of viral genes (4–8).
Transcripts from several VZV open reading frames (ORFs 4, 21, 29, 62, and 63) have been detected in latently infected human ganglia (9–15). Moreover, several of the corresponding proteins have been detected in latently infected cells (16–18). During latency, the expression of viral proteins also present during the lytic phase of infection is an original feature that VZV does not share with HSV-1. IE63 was the first protein described in a latency context, first in an animal model (19) and second in human tissue sections (16–18). This 45-kDa phos-phoprotein, encoded by two identical ORFs (ORF63 and ORF70), is present in the virion tegument (19). IE63 is abundantly produced during the early phase of infection (20) and is essential for VZV replication (21). Its activity as a potential transcription factor remains unclear. IE63 has been shown to exert positive or negative effects on gene transcription, depending on the type of promoter studied (22). However, others claimed that IE63 played only a minor role in the control of VZV gene expression (23). An interesting feature of the VZV IE63 protein is its cellular localization during the different stages of the viral cycle. Indeed, in the early phase of infection, IE63 is predominantly present in the nucleus of infected cells (20). On the other hand, IE63 exhibits an exclusive cytoplasmic localization during latency and can be found both in the nucleus and the cytoplasm when reactivation occurs (17). The presence of IE63 and its localization modification might reflect an important role in the latency process. Phosphorylation and dephosphorylation events are known to be usually involved in regulation mechanisms such as nuclear and cytoplasmic trans-location, for example. *In vitro* phosphorylation of IE63 may be achieved using recombinant casein kinase II

(CKII), and the protein is also phosphorylated in VZV-transfected Mewo cells (24). The phosphorylation sites used in these *in vivo* and *in vitro* assays are located predominantly at the carboxyl-termi-nal region of the protein. Recently, it has also been shown that IE63 is a substrate for ORF47, one of the VZV-encoded kinases (25). Indeed, using stringent *in vitro* conditions, an extensive phosphorylation of the protein is catalyzed by the ORF47-encoded kinase, which can compete with CKII for the binding to IE63. This viral serine/threonine kinase has often been compared with CKII, notably because they may both use ATP or GTP as phosphate donor (26). These results demonstrate that viral and cellular kinases are capable of phosphorylating IE63. The purpose of this paper was to analyze in further detail the IE63 phosphorylation status. For this, we used a mutational approach to investigate the importance of some of the putative phosphorylation sites on the cellular localization of IE63 and on its activity. Ten potential phosphorylation sites for casein kinase I (CKI), CKII, and protein kinase C (PKC) or Cdc2 were mutated, and *in vitro* kinase assays were carried out using these mutated proteins as substrates. Results indicated that these sites are important in the phosphorylation process of IE63, using either recombinant CKI and CKII or protein extracts from Vero (non-neuronal) or ND7 (neuronal) cells. Im-munofluorescent studies on these neuronal and non-neuronal cell lines transfected with the mutated IE63 proteins indicated that phosphorylation on these residues is required for a suitable cellular localization of the protein in Vero but not in ND7 cells. We also demonstrated that wild-type IE63 protein possesses some repressive properties on two VZV promoters (DNA polymerase and thymidine kinase) and that mutation of the phosphorylation sites strongly impaired its activity. Finally, we also witnessed evidence of an important feature of this protein: IE63 is able to exert its repressive activity both in the nucleus and in the cytoplasm of transfected cells, suggesting that IE63 acts through a transcriptional or post-transcriptional mechanism in the nucleus and/or through a post-transcriptional mechanism in the cytoplasm of transfected cells.

Experimental procedures

Plasmids—The wild-type *IE63* gene was amplified by PCR from the pGEX-63 (20) and cloned in the pcDNA3.1— vector (Invitrogen) to generate the pcDNA63 wt. We also constructed the pcDNA63inv, where the *IE63* gene was cloned in the reverse orientation. This plasmid is used as negative control in some experiments. Mutations were introduced into the *IE63* gene by PCR using the site-directed mutagenesis kit system (Stratagene). For this, several sets of primers were synthesized (Eurogentec). Table I summarizes the strategy used. All mutated genes were cloned in the pGEX-5x (Amersham Biosciences) and transformed in *Escherichia coli* JM109 strain in order to produce GST fusion proteins. All constructions were subsequently sequenced to verify that no additional mutations were introduced during PCR.
To generate pcDNA-63ΔK, the carboxyl-terminal region of IE63 (from amino acids 210-278) was removed by digestion of pcDNA63 wt with *KpnI* and self-ligation. The putative nuclear localization signal (NLS), from amino acids 260-263, was removed by digesting pcDNA63 vector at *Cla* I sites (previously added by PCR) and self-ligation in order to generate pcDNA-63ΔNLS. A plasmid (pPol-luc), where the luciferase reporter gene was under the control of the VZV DNA polymerase gene promoter (pPol), was constructed by inserting a 404-bp-long fragment encompassing the pPol promoter into the pGL3-Basic vector (Promega). This fragment was the result of an *Hin*dIII digestion of the pPol-CAT plasmid (28). ORF62 was also cloned in pcDNA3.1— to generate pcDNA-62. pTkCAT-L, where the VZV thymidine kinase promoter controls the expression of the chloramphenicol acetyltransferase (CAT) reporter gene, has been described previously (28).
Cells, Virus, and Infection Study—Vero cells (a monkey kidney cell line, ATCC CCL-81) were grown in M199 medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen). ND7 cells (ECACC number 92090903) (a gift from Dr. D. Latchman, University College London Medical School) are the result of the fusion of murine neuroblastoma cells with primary nerve cells from rat dorsal root ganglia (27). They were grown in RPMI 1640 (BioWhittaker) supplemented with 5% fetal bovine serum (Invitrogen). Cell-free virus was prepared from VZV-infected Mewo cells, as described previously (29). Briefly, VZV-infected Mewo cells were collected by scraping and resuspended in 1 ml of PGSA buffer supplemented with 10% fetal calf serum (29). Cells were mixed with 1 ml of glass beads (1 mm in diameter) and subjected to mechanical shaking in a mini-BeadBeater (Biospec Products, Bartlesville, OK), at low speed for 10 s. Supernatant containing the cell-free virus was collected by several centrifugations. 150 μl of supernatant was added to Vero and ND7 cells previously grown on cover-slides into 10-mm dishes and then supplemented with fresh medium. Cells were fixed at 24, 48, and 72 h post-infection 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 (15% in PBS) and then

incubated with a monoclonal antibody directed against IE63 (18). The secondary antibody used was coupled with fluorescein isothio-cyanate (FITC) (Dako), and cells previously counterstained with a 1% (v/v) Evans Blue solution were observed by fluorescent microscopy (Nikon).

Total Cellular Protein Extracts—Total cellular extracts from Vero and ND7 cells were obtained by RIPA lysis. Briefly, cells from three confluent 80-cm^2 dishes were harvested, washed in cold PBS (137 mM NaCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl, pH 7.4), and resuspended in 1 ml of RIPA lysis buffer (PBS supplemented with 1% Nonidet P-40, 0.5% Tween 20, 0.1% (w/v) SDS, 5 mM EDTA, 1 mM dithiothreitol, and protease inhibitors (Complete Protease Inhibitors, Roche Molecular Biochemicals)). Cells were allowed to swell on ice for 30 min, vortexed, then transferred on ice for an additional 30 min, and centrifuged 20 min at 20,000 X *g* at 4 °C. Supernatants were kept at -80 °C.

Nuclear and Cytoplasmic Protein Extracts—Cells from three confluent 80-cm^2 dishes were harvested, washed in cold PBS, pelleted, and resuspended in 1.2 ml of cold hypotonic buffer (10 mM HEPES-KOH, pH 7.9, 2 mM $MgCl_2$, 0.1 mM EDTA, 10 mM KCl, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, and protease inhibitors (Complete Protease Inhibitors, Roche Molecular Biochemicals)). Pellets were allowed to swell on ice for 10min. After addition of 0.5% (v/v) Nonidet P-40, cells were vortexed for 2 or 3 s. Suspension was then centrifuged at 20,000 X *g* for 30 s; the supernatant containing the cytoplasmic fraction was kept at -80 °C, and the pellet of nuclei was resuspended in 50 µl of cold saline buffer (50 mM HEPES-KOH, pH 7.9, 2 mM $MgCl_2$, 0.1 mM EDTA, 50 mM KCl, 400 mM NaCl, 10% (v/v) glycerol and protease inhibitors (Complete Protease Inhibitors, Roche Molecular Biochemicals)). Cells were allowed to swell on ice for 30 min. After centrifugation (15 min at 20,000 X *g* at 4 °C), the supernatant containing the nuclear proteins was stored at -80 °C.

For all *in vitro* kinase assays, protein extracts were dialyzed with a high volume (up to 2 liter) of casein kinase II buffer (50 mM Tris-HCl, pH 7.4, 140 mM KCl, and 10 mM $MgCl_2$) for 2 h. Protein concentrations were measured by the Bradford method (Bio-Rad).

Immunoprecipitations—CKI and CKII were immunoprecipitated from Vero and ND7 cellular extracts using polyclonal antibodies raised against CKIδ (R-19: sc-6474, from Santa Cruz Biotechnology, Inc.) and CKIIα (C-18: sc-6479, from Santa Cruz Biotechnology, Inc.), respectively. 40 µl of protein A-Sepharose (Amersham Biosciences) were incubated overnight at 4 °C with 5 µl of antibodies and several concentrations of cellular extracts in the immunoprecipitation buffer (20 mM Tris-HCl, pH 7.5, 200 mM NaCl, 0.5% Nonidet P-40, Complete Protease Inhibitors, Roche Molecular Biochemicals) that includes a mixture of phosphatase inhibitors (1 mM Na_3VO_4, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM NaF, 0.5 mM β-glycerophosphate). The resin was then washed 3 times with cold CKII buffer.

In Vitro Kinase Assays—GST-63 wt protein was expressed from the pGex3X-ORF-63 vector and purified on glutathione-Sepharose 4B (Amersham Biosciences) as described previously (20). Mutant GST-63 proteins were expressed and purified according to the same protocol. GST-IκBα protein was used as positive control and produced as described previously (30). *In vitro* kinase assays were performed with cellular extracts, recombinant kinases, or immunoprecipitated kinases. Several concentrations of cellular extracts or recombinant kinases were added to 20 µl of glutathione-Sepharose 4B-bounded GST-63 protein and with 10 µCi of [γ-^{32}P]ATP (ICN). For the assays using the immunoprecipi-tated kinases, 10 µCi of [γ-^{32}P]ATP (ICN) and 2 µg of GST-63 (eluted from the resin using 10 mM GSH in 50 mM Tris-HCl, pH 8) were added to resin-bound kinases. All reactions were performed in CKII buffer, supplemented with the protease and phosphatase inhibitors mixtures, for 30 min at 30 °C. Resins were washed 3 times with CKII buffer. The samples were loaded on a 10% SDS-PAGE gel. After migration, the gel was dried and autoradiographed on a Fuji x-ray film (General Electrics). Quantifications were carried out by PhosphorImaging (Molecular Dynamics).

For some *in vitro* kinase assays, 5,6-dichloro-1-β-D-ribofuranosylben-zimidazole (DRB) (ICN), which is an inhibitor of casein kinases, was used at several concentrations.

Transient Transfection Studies—Transfection studies were carried out with cells (Vero or ND7) seeded into 35-mm diameter 6-well cluster dishes using the FuGENE 6 transfectant reagent according to the manufacturer's prescriptions (Roche Molecular Biochemicals). In these experiments, pPol-luc or pTkCAT-L was used as reporter vectors and pcDNA 3.1 — , pcDNA62, pcDNA63 wt, pcDNA63-5M, pcDNA63-10M ,pcDNA-63ΔNLS, pcDNA-63ΔK as expression vectors under the control of cytomegalovirus promoter.

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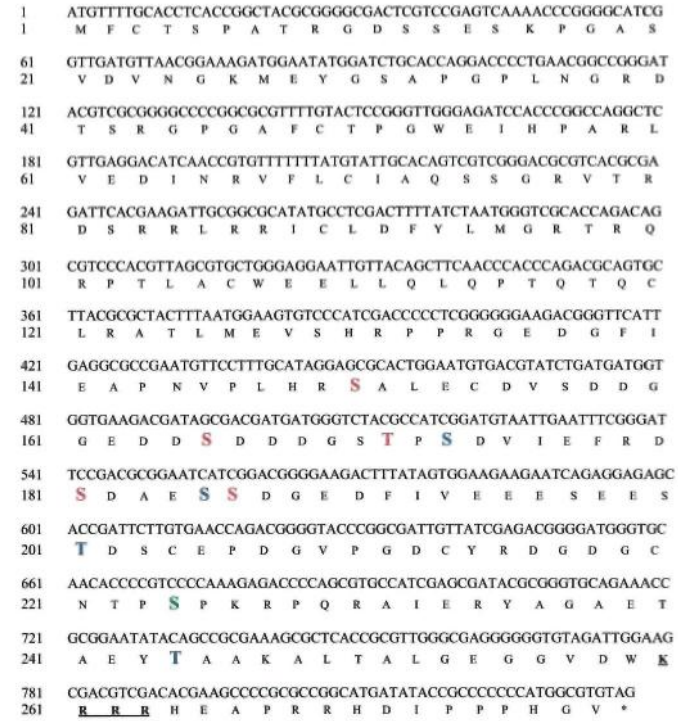

FIG. 1. Complete DNA sequence of the VZV IE63 gene and the predicted amino acid sequence. The putative phosphorylation sites for PKC and/or p34^{cdc2} (in green), CKI (in blue), and CKII (in red) are shown. The bold and underlined sequence represents the putative NLS.

The amounts of DNA were adjusted with herring sperm DNA. Special attention was made to obtain an equimolar ratio of cytomegalovirus promoters in each independent experiment. Luciferase assays were performed using the "Luciferase Reporter Gene Assay, High Sensitivity" kit (Roche Molecular Biochemicals), according to the instructions of the manufacturer. For each experiment, the concentration of proteins in each sample was measured in order to normalize the results. Data from luciferase and CAT assays were collected from at least four independent transfection experiments. CAT activity assays were performed as described previously (28). For Western blot analysis, 15 μg of protein extracts of ND7-transfected cells were loaded on a 10% SDS-PAGE gel. After migration and transfer, detection of IE63 was performed using a monoclonal antibody 9A12, as described previously (18), or a polyclonal antibody (20).

Immunofluorescent Studies—Vero and ND7 cells were grown on cov-erslides into 10-mm dishes and transfected with 1 μg of pcDNA63 wt or mutated forms using 3 μl of FuGENE 6 reagent (Roche Molecular Biochemicals). 48 h post-transfection, the cells were fixed with a solution of acetone/methanol (v/v) during 20 min at -20 °C. After fixation, the nonspecific sites were saturated with a milk-blocking solution (15% in PBS) and then incubated with antibodies directed against IE63. In these experiments, a rabbit polyclonal anti-IE63 serum (20) or a monoclonal antibody directed against IE63 (18) were used. Anti-rabbit or anti-mouse secondary antibodies coupled with fluorescein isothiocya-nate (FITC) (Dako) were then used, and the cells, previously counter-stained with a 1% (v/v) Evans Blue solution, were observed by fluorescent microscopy (Nikon).

Results

Identification and Mutation of the Putative Phosphorylation Sites in the Carboxyl-terminal Region of IE63-–Previous studies suggested that important phosphorylation sites are located in the carboxyl-terminal region (amino-acids 142–278) of the IE63 protein (24). Therefore, we decided to focus our work on this part of the IE63 protein. A computer analysis was carried out to identify putative phosphorylation sites for cellular kinases in the carboxyl-terminal region of IE63. For this, we used the PhosphoBase prediction tool software available on the Center for Biological Sequence Analysis web site (31). As shown in Fig. 1, the analysis revealed the presence of 10 potential phos-phorylatable

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serine or threonine residues in this region. Five of them (Ser-150, Ser-165, Ser-181, Ser-186, and Thr-171) are potential targets for CKII and four others (Ser-173, Ser-185, Thr-201, and Thr-244) for CKI. The Ser-224 was found to be a putative site for PKC and/or p34^{cdc2}-mediated phosphorylation.

In order to assay the importance of these residues in IE63 phosphorylation, we decided to substitute them by alanine residues. The *IE63* gene was cloned first in the pcDNA3.1 — expression vector (Invitrogen) to generate wild-type pcDNA63. We then used a PCR-based mutagenesis strategy to generate mutants. Resulting plasmids are listed in Table I. For example, in pcDNA63-5M, only the five potential phosphorylation sites for CKII were mutated, whereas in pcDNA63 - 10M, all 10 potential sites for CKI, CKII, and PKC/Cdc2-mediated phosphorylation were mutated (Table I).

In Vitro Kinase Assays Using Recombinant CKI and CKII— The mutated *IE63*-encoding genes were cloned in pGEX-5x vector (Amersham Biosciences) to generate GST fusion proteins. The expression of the purified fusion proteins was verified by Coomassie Blue-stained SDS-PAGE gel (Fig. 2, *A* and *B)* and Western blotting analysis (data not shown). In order to improve the impact of the mutations on the phosphorylation rate of the protein, *in vitro* kinase assays were carried out using recombinant CKI and CKII on GST-63-Sepharose-bound proteins. Phosphorylation of wild-type IE63 was observed using increasing concentrations of both CKI and CKII (from 10–50 units) (Fig. 2, *A* and *B).* Mutation of the five putative phosphorylation sites for CKII (GST-IE63-5M) led to a slight decrease of the *in vitro* CKII phosphorylation rate, while, surprisingly, the IE63 phosphorylation rate observed using CKI was somewhat increased. *In vitro* phosphorylation of the GST-63-10M protein, where 10 putative phosphorylation sites for CKI and CKII were mutated into alanine residues, showed an important decrease of the phosphorylation rate for both kinases. These results indicated that the residues aimed in our study are important for the *in vitro* IE63 phosphorylation by CKI and CKII. It also suggested that a few phosphorylation sites for both kinases might still be present elsewhere in the protein because the mutation of the 10 residues did not totally abolish the phosphorylation rate of IE63.

In Vitro Kinase Assays Using Cellular Extracts—To determine whether cellular kinases were able to phosphorylate the IE63 protein, we carried out kinase assays using cellular protein extracts. An *in vitro* kinase assay was then performed on wild-type GST-IE63 using 50 μg of total, cytoplasmic, or nuclear protein extracts from Vero cells previously dialyzed against CKII buffer (Fig. 3*A*). In this experiment GST-IκBα protein was used as a positive control because its CKII-medi-ated phosphorylation was described by several authors (30, 32). An intense phosphorylation of wild-type GST-IE63 using total, cytoplasmic, or nuclear protein extracts from Vero cells was observed. This phosphorylation was strongly reduced when reaction was performed in the presence of 2 mM DRB, an inhibitor of both CKI and CKII (33). This experiment allowed us to conclude that extracts from Vero cells contain casein kinase activities almost equally present in the nucleus and the cytoplasm. In order to confirm the presence and the activity of these kinases in cellular extracts, CKI and CKII were immu-noprecipitated from cytoplasmic and nuclear protein extracts from Vero cells, and *in vitro* kinase assays were performed (Fig. *3B).* A phosphorylation of IE63 was observed with immunopre-cipitated CKI and CKII either from the nucleus or cytoplasm of Vero cells, reinforcing our *in vitro* observations that both cellular CKI and CKII are capable of phosphorylating IE63.

We then decided to compare the relative phosphorylation rate of wild-type GST-IE63 in two different cell lines: Vero cells, which are non-neuronal and permissive cells, and ND7 cells, a neuronal cell line described previously (26) as non-permissive to HSV-1 infection.

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Plasmids developed	Mutated residues	Target vectors	Primers used
pcDNA63 1M	Ser-150	pcDNA63 wt	5′-CCTTTGCATAGG**GC**CGCACTGGAATG-3′ 5′-CATTCCAGTGC**GG**CCCTATGCAAAGG-3′
pcDNA63 2M	Ser-165, Thr-171	pcDNA63 wt	5′-GGTGAAGACGAT**G**CCGACGATGATGGGTCTG**C**GCCATCGGATG-3′ 5′-CATCCGATGGCGCAGACCCATCATCGTCGGC**A**TCGTCTTCACC-3′
pcDNA63–3M	Ser-181, Ser-186	pcDNA63 wt	5′-GAATTTCGGGAT**G**CCGACGCGGAATCAGCGGACGGGGAAG-3′ 5′-CTTCCCCGTCCGCTGATTCCGCGTCGGC**A**TCCCGAAATTC-3′
pcDNA63–4M	Ser-150, Ser-165, Thr-**171**	pcDNA63–1M	5′-GGTGAAGACGAT**G**CCGACGATGGGTCTG**C**GCCATCGGATG-3′ 5′-CATCCGATGGCGCAGACCCATCATCGTCGGC**A**TCGTCTTCACC-3′
pcDNA63–5M	Ser-150, Ser-165, Thr-**171**, Ser-181, Ser-186	pcDNA63–4M	5′-GAATTTCGGGAT**G**CCGACGCGGAATCAGCGGACGGGGAAG-3′ 5′-CTTCCCCGTCCGCTGATTCCGCGTCGGC**A**TCCCGAAATTC-3′
pcDNA63–6M	Ser-150, Ser-165, Thr-**171**, Ser-181, Ser-186, Ser-173	pcDNA63–5M	5′-GGGTCTGCGCCA**GCT**GATGTAATTGAATTTCGGG-3′ 5′-CCCGAAATTCAATTACATC**AGC**TGGCGCAGACCC-3′
pcDNA63 7M	Ser-150, Ser-165, Thr-**171**, Ser-181, Ser-186, Ser-173, Ser-185	pcDNA63–6M	5′-GCCGACGCGGAA**GC**CGCGGACGGGG-3′ 5′-CCCCGTCCGC**GGC**TTCCGCGTCGGC-3′
pcDNA63–8M	Ser-150, Ser-165, Thr-**171**, Ser-181, Ser-186, Ser-173, Ser-185, Ser-224	pcDNA63–7M	5′-GGGTGCAACACCCC**GGC**GCCAAAGAGACCCC-3′ 5′-GGGGTCTCTTTGGCGCCGGGGTGTTGCACCC-3′
pcDNA63 9M	Ser-150, Ser-165, Thr-**171**, Ser-181, Ser-186, Ser-173, Ser-185, Ser-224, Thr-244	pcDNA63 8M	5′-CCGCGGAATAT**GCG**GCCGCGAAAGCGC-3′ 5′-GCGCTTTCGCGGCCGCATATTCCGCGG-3′
pcDNA63–10M	Ser-150, Ser-165, Thr-**171**, Ser-181, Ser-186, Ser-173, Ser-185, Ser-224, Thr-244, Thr-201	pcDNA63–9M	5′-GAGGAGAGCGC**T**GATTCTTGTGAACCAGACGGGG-3′ 5′-CCCCGTCTGGTTCACAAGAATCAGCGCTCTCCTC-3′

TABLE I List of primers and target plasmids used for the construction of the IE63 mutants The 1st column indicates the plasmids generated, and the 2nd column describes the position of mutated residues in the IE63 gene. All mutations were introduced by PCR. The 3rd column resumes plasmids used as target for the PCR strategy, and the primers used are listed in the last column. Boldface letters represent the mutations introduced.

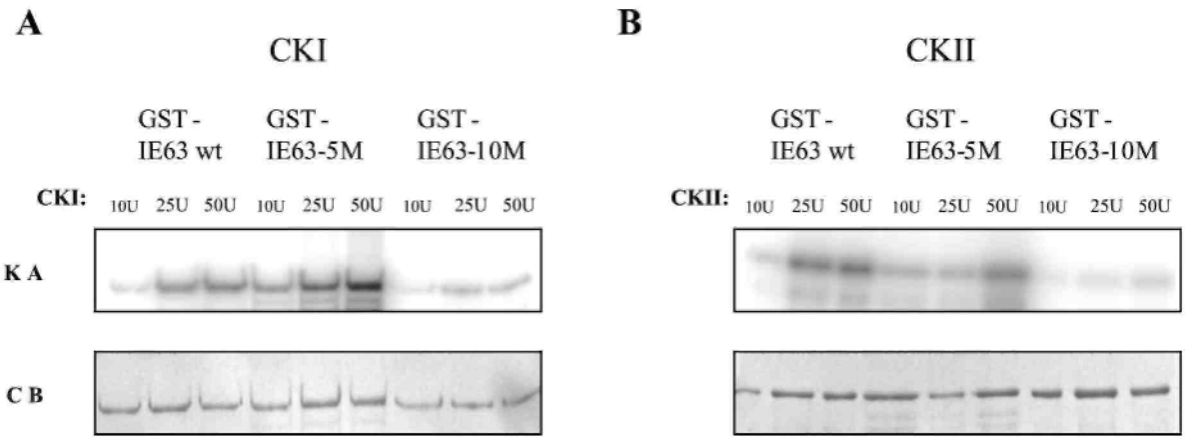

FIG. 2. In vitro phosphorylation of wild-type GST-IE63, GST-IE63-5M, and GST-IE63-10M proteins using recombinant kinases. 10, 25, and 50 units (U) of CKI (A) or CKII (B) were used to phosphorylate (kinase assay, KA) either wild-type GST-63, GST-63-5M, or GST-63-10M coupled with Sepharose beads with [γ—^{32}P]ATP. After extensive washes and SDS buffer treatment, all samples were loaded on a 10% SDS-PAGE gel, and an autoradiography was performed. Coomassie Blue-stained SDS-PAGE gel (CB) of the kinase assays is shown as loading control.

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First, we investigated whether or not ND7 was also non-permissive to VZV infection. For this, Vero and ND7 cells were infected with cell-free virus for 24, 48, and 72 h. An immunofluorescence analysis was then carried out using an anti-IE63 monoclonal antibody. At 48 h post-infection, a few IE63-positive foci were observed in Vero cells, and their numbers largely increased 72 h post-infection (Fig. 4). At identical time points, neither IE63 expression nor cyto-pathic effect can be detected on ND7 cells, suggesting that this cell line is non-permissive to VZV infection.
In vitro kinase assays using total protein extracts from both cell lines were then carried out in the presence or in the absence of DRB. PhosphorImaging and Coomassie Blue-stained SDS-PAGE gel densitometry analyses were used to determine the relative phosphorylation rate of the IE63 protein (Fig. 5).

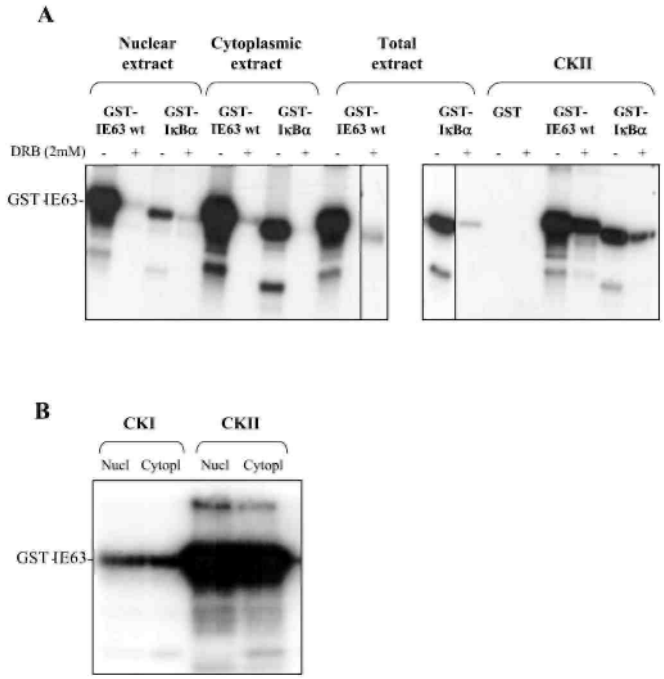

FIG. 3. In vitro phosphorylation of wild-type GST-IE63 using protein extracts from Vero cells, in the presence (+) or absence (—) of DRB (A) or using immunoprecipitated CKI or CKII from Vero cells protein extracts (B). A, GST, wild-type GST-IE63, or GST-IκBα coupled with Sepharose beads were incubated with 50 μg of total, cytoplasmic, or nuclear protein extracts from Vero cells, previously dialyzed in CKII buffer, or with 200 units of CKII in the presence of [γ—^{32}P]ATP. DRB, an inhibitor of CK I and II, was also used in this experiment at a final concentration of 2 mM. B, CKI and CKII were immunoprecipitated from Vero cells protein extracts and incubated with 2 μg of previously eluted wild-type GST-63 in the presence of [γ—^{32}P]ATP. After extensive washes and SDS buffer treatment, all samples were loaded on a 10% SDS-PAGE gel and autoradiographed.

From several independent experiments, we observed that the level of phosphorylation was about 1.5-fold more important using extracts from Vero than ND7 cells. Furthermore, the level of phosphorylation was significantly reduced using 1 M DRB, suggesting the involvement of CKI and/or CKII in both cell types (Fig. *5A). In vitro* kinase assays using cellular protein extracts from Vero and ND7 cells were also carried out with the wild-type GST-IE63 and GST-IE63-10M proteins as substrates (Fig. *5B).* We observed that the mutation of the 10 putative IE63 phosphorylation sites had an impact on the phosphorylation rate of the protein but did not lead to its complete depletion. Again, this observation suggests that cellular kinases might use other phosphorylation sites on IE63 than the one targeted by our study. We also determined the casein kinase activity on wild-type GST-IE63 or GST-IE63-10M in both cell types (Fig. *5C).* For this, an *in vitro* kinase assay was carried out as described above, and the phosphorylation rate obtained for each sample was quantified by phosphorimaging analysis. All values obtained were then plotted *versus* a reference activity curve, established by measuring the phosphorylation rate of wild-type GST-IE63 protein, using decreasing concentrations of CKII. The casein kinase activity determined in Vero cells was 0.66 unit/μg total protein extract and 0.42 unit/μg total protein extract from ND7 cells using wild-type GST-IE63 as

substrate. These data clearly demonstrate that the phosphorylation rate of wild-type IE63 is somewhat less important using protein extracts from ND7 than from Vero cells. The casein kinase activity measured for the GST-63-10M protein was about 0.26 unit/μg total protein extracts from Vero cells and 0.21 unit/μg total protein extracts from ND7 cells, indicating that the mutation of the 10 potential phosphorylations have an important impact on the phosphorylation rate of IE63 but did not prevent it to be phosphorylated by cellular kinases. Results are also given for GST alone as negative control.

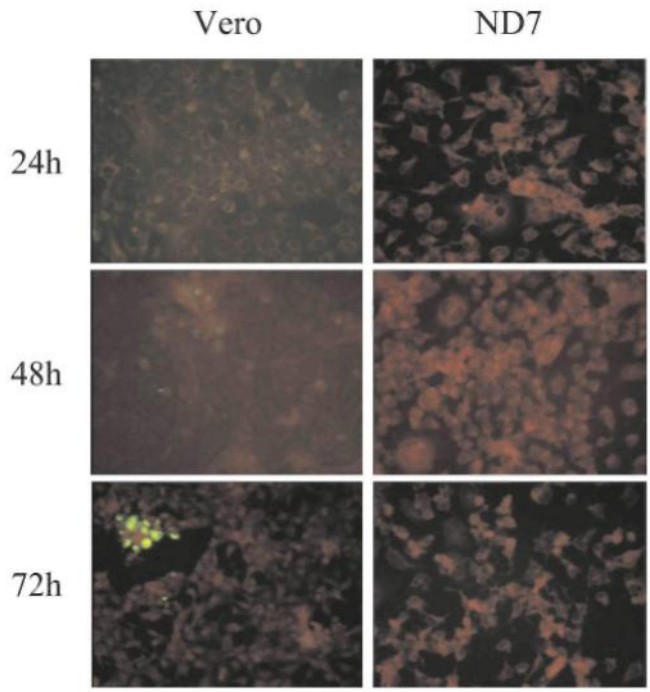

FIG. 4. Infection of Vero and ND7 cells with a cell-free VZV preparation. Vero and ND7 cells were grown on coverslides and then infected with cell-free VZV. Cells were fixed at 24, 48, and 72 h post-transfection. Immunostaining analysis was carried out using a monoclonal antibody (9A12) directed against IE63. Secondary antibody used was coupled with FITC.

IE63 Phosphorylation Influences Its Cellular Localization—We decided to investigate the importance of IE63 phosphorylation on its localization. Plasmids encoding the wild-type and mutated proteins were transfected in both Vero and ND7 cells. Their cellular localization was observed by immuno-staining analysis using either monoclonal or polyclonal antibodies directed against IE63. Quantitative analysis was carried out, and the cellular localization of the proteins in both cell lines was determined, based on at least three independent experiments (Table II). Previous reports (20) revealed that during VZV lytic infection, IE63 is mainly localized in the nucleus of infected cells. When transfected in both Vero and ND7 cells, a prevalent nuclear localization of the protein with a slight cytoplasmic localization was observed (Fig. 6*A*). Indeed, IE63 was present in the nucleus of more than 90% of transfected cells. A positive control for the cytoplasmic localization of IE63 was constructed. Based on a previous report (24), we removed the carboxyl-terminal region of IE63 (amino acids 210–278) necessary for the nuclear localization of the protein, in order to generate pcDNA63-ΔK. We also observed an almost complete cytoplasmic localization of this truncated protein when transfected either in Vero or in ND7 cells (Fig. *6B).* About 82% of Vero cells expressing the protein exhibited a cytoplasmic staining, whereas in ND7 cells, almost all positive cells were stained in the cytoplasm (Table II). This localization of the protein could possibly be explained by the removal of a KRRR region coding for a putative nuclear localization signal (amino acids 260–264). It is also important to note the loss of two putative phosphorylation sites (Ser-224 and Thr-244) for *B, in vitro* phosphorylation of wild-type GST-IE63 or GST-IE63-10M using total protein extracts from either Vero or ND7 cells.

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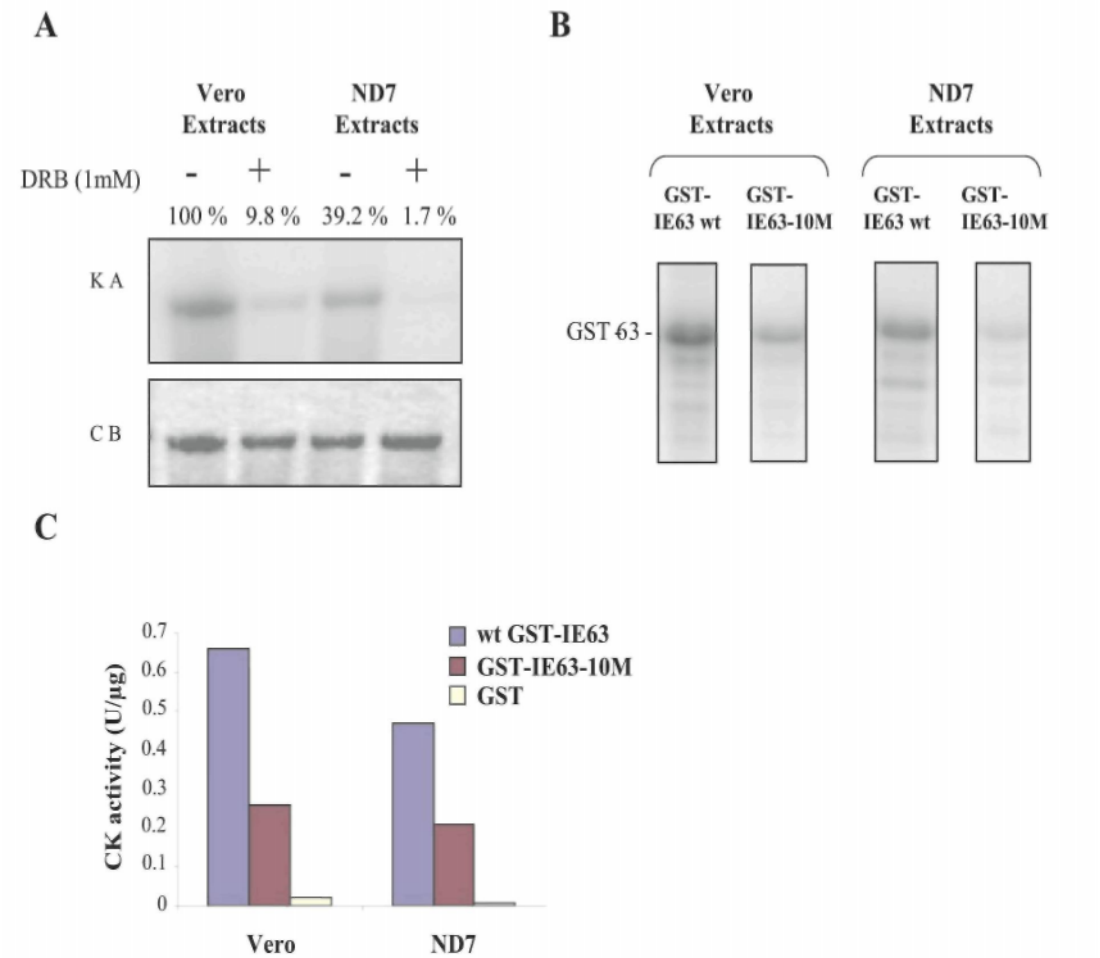

FIG. 5. Comparison of the IE63 phosphorylation rate using either Vero or ND7 cellular protein extracts. A, wild-type GST-IE63 coupled with Sepharose was incubated with 30 µg of total protein extracts from Vero or ND7 cells previously dialyzed and [γ—^{32}P]ATP and in the presence (+) or absence (—) of 1 mMDRB. PhosphorImaging analysis of the autoradiography (KA) and the Coomassie Blue-stained (CB) SDS-PAGE gel allowed us to determine the relative percentage of phosphorylation for each samples.

The kinase assay and the analysis of the results were performed as described above. *C*, the CK-like activity present in Vero and ND7 protein extracts was determined either on wild-type GST-IE63 protein, GST-IE63-10M, or GST. Results are given in units of CK activity per µg of cellular protein extracts.

TABLE II Analysis of the intracellular localization of IE63 after transfection of Vero or ND7 cells with the several constructionconstructions

Vero	N > C	N = C	N <C
pcDNA63-wt	93	7	0
pcDNA63-ΔNLS	0	39	61
pcDNA63-ΔK	0	17.5	82.5
pcDNA63–5M	59	23.5	17.5
pcDNA63–10M	25.5	9	65.5
ND7	N > C	N = C	N< C
pcDNA63-wt	94.5	5.5	0
pcDNA63-ΔNLS	3	13.5	83.5
pcDNA63-ΔK	0	3	97
pcDNA63–5M	95	5	0

pcDNA63–10M	93	7	0

Vero or ND7 cells were transfected with 1 μg of plasmids. 40 hours post-transfection, immunofluorescent staining was carried out. 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 proteins between nucleus and cytoplasm, and N < C for a major cytoplasmic staining. Average percentages are the results of at least three independent experiments. From these experiments, the relative error was estimated at 15-20%.

PKC/Cdc2 and CKI, respectively, in IE63ΔK. We decided then to remove solely the ^{260}KRRR region coding for the putative nuclear localization signal (pcDNA63-ΔNLS). When trans-fected into Vero cells, we observed a major, but not exclusive, localization of this protein in the cytoplasm (Fig. 6*C*); 60% of Vero cells exhibited a cytoplasmic accumulation of IE63, whereas in the other 40%, the protein was evenly distributed between nucleus and cytoplasm (Table II). In ND7, up to 83% of transfected cells was positively stained in the cytoplasm. These results demonstrate that this ^{260}KRRR region is a functional nuclear localization signal for the IE63 protein and is important for its nuclear localization in both cell lines. Meanwhile, as the IE63-ΔNLS protein was not exclusively confined to the cytoplasm of Vero cells, we might postulate that other surrounding residues of IE63 could participate to this process in that cell type.

No major impact on IE63 localization was observed when the first three putative phosphorylation sites for CKII (Ser-150, Ser-165, and Thr-171 into pcDNA63-1M, pcDNA63-2M, pcDNA63-3M, and pcDNA63-4M) were removed (data not shown). However, when Vero cells were transfected with pcDNA-5M, where the five putative phosphorylation sites for CKII (Ser-150, Ser-165, Thr-171, Ser-181, and Ser-186) were removed, a slight delocalization of the protein from the nucleus to the cytoplasm was observed in Vero cells (Fig. *6D).* Indeed, less than 60% of Vero cells was stained predominantly in the nucleus, whereas about 23% of cells exhibited a uniform localization of IE63 between both cellular compartments, and 17% were marked mainly in the cytoplasm (Table II).

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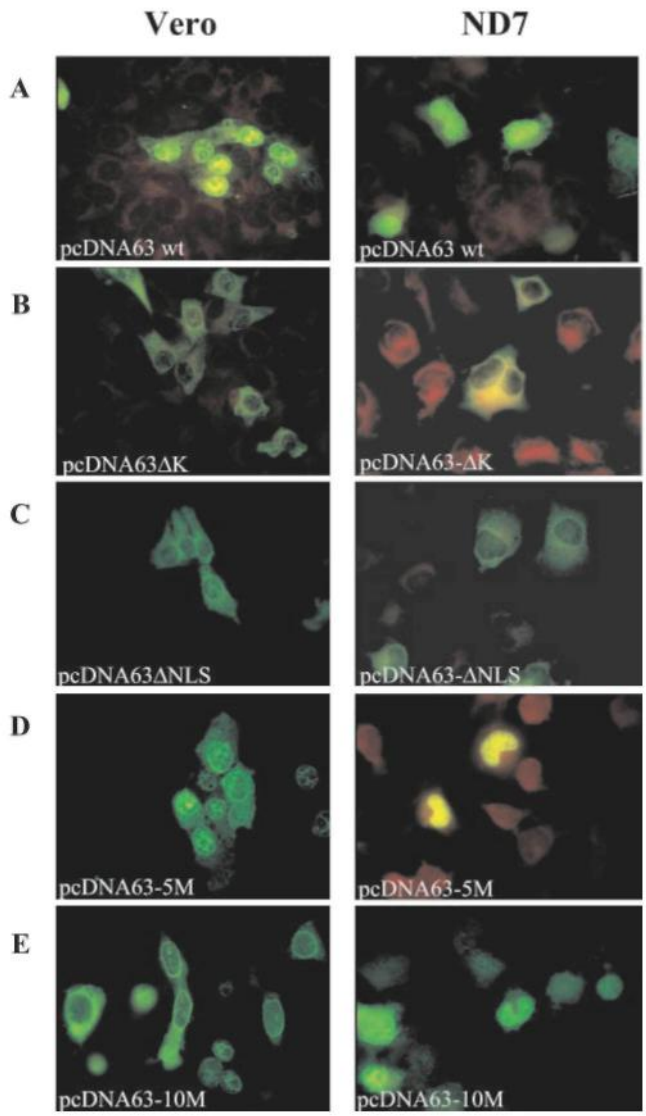

FIG. 6. Intracellular localization of wild-type IE63 or mutated in Vero and ND7 cells. Vero and ND7 cells were transfected with 1 μg of plasmid expressing wild-type IE63 (A), IE63-ΔK(B), IE63-ΔNLS (C), IE63-5M (D), and IE63-10M (E). 40 h post-transfection, immuno-staining analysis was carried out using a monoclonal antibody (9A12) directed against IE63 (A, D, and E). Polyclonal antibodies were also used (B and C), as the monoclonal antibody did not recognize the truncated IE63 proteins. Secondary antibodies used were coupled with FITC.

In contrast, the IE63-5M protein was mainly localized in the nucleus of ND7 cells as the wild-type protein (Fig. 6*D*).
When mutations for the CKI and PKC/Cdc2 putative phos-phorylation sites were also included (pcDNA63-10M), we observed an increasing delocalization of the IE63 protein from the nucleus to the cytoplasm of Vero cells (Fig. 6*E*). We also observed a striking accumulation of the protein at the nuclear envelope, just as if the protein had been excluded from the nucleus and was concentrating in the perinuclear space. In this case, more than 65% of the positive Vero cells exhibited a predominant cytoplasmic localization (Table II). Again, no major impact of these mutations was observed on the localization of the protein when transfected in ND7 cells (Fig. 6*E*). These results indicated an important role of the phosphorylation event in the cellular localization of the IE63 protein in Vero cells.

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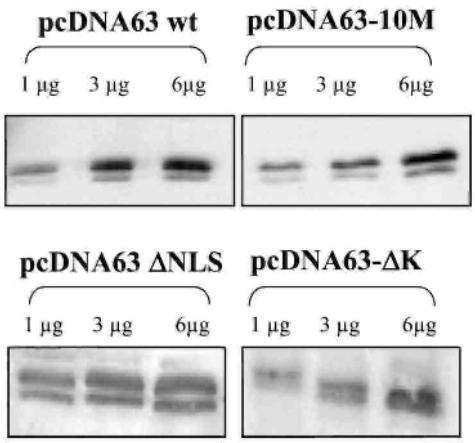

FIG. 7. Control of the expression of wild-type and mutated IE63 in transfected cells. Protein extracts from ND7 cells previously transfected with 1 µg of pPol-luc plasmid and increasing concentrations of wild-type pcDNA63 (A), pcDNA63-10M (B), pcDNA63-ΔNLS (C) or pcDNA63-ΔK (D) were loaded on a 10% SDS-PAGE gel. After migration and transfer, IE63 proteins were detected using either monoclonal (A and B) or polyclonal (C and D) antibodies. Secondary antibodies used were coupled to peroxidase.

In the neuronal cell line we tested, however, phosphorylation of these residues did not play an important role for the correct protein localization.

Phosphorylation Influences IE63 Regulating Properties— Regulatory properties of IE63 are still unclear at this time. It was shown previously (22) that this protein can either up-regulate the thymidine kinase gene or down-regulate the *ORF62* gene, but these results were questioned by a later report suggesting a limited regulatory activity of IE63 on VZV gene expression (23). In this context, we decided to investigate in more detail the activity of IE63 and to observe the impact of phosphorylation events on gene regulation. The regulatory properties of the wild-type or mutated IE63 protein were assayed on the basal expression of the VZV DNA polymerase gene promoter (pPol). This promoter is an accurate tool for repression studies, as it exhibits a rather important constitutive activity. For this, transient transfections were carried out using a luciferase reporter gene under the control of the VZV pPol (pPol-luc). Vero and ND7 cells were transfected with 1 µg of this plasmid and with increasing concentrations of wild-type or mutated pcDNA63. Each independent condition tested was carried out using the same equimolar ratio of promoters. Results were expressed in percentage of stimulation with respect to the basal activity obtained with the pPol-luc alone (= 100%). First, the efficient expression of wild-type or mutated IE63 proteins was verified by Western blotting analysis (Fig. 7). In Vero or in ND7 cells, we observed a significant decrease of the basal luciferase activity when increasing concentrations of wild-type pcDNA-63 were added (Fig. 8*A*). The observed repression was about 70% in both cell types at 6 µg of wild-type pcDNA63. In order to validate our reporter system, we tested the ability of IE63 to repress another VZV promoter. For this, we used the thymidine kinase promoter controlling the expression of the chloramphenicol acetyltransferase (CAT) reporter gene (Fig. *8B).* We observed in this system that IE63 is also able, in both cell lines, to repress the basal activity of the thymidine kinase promoter to a similar level to what was observed with the VZV DNA polymerase promoter. All following experiments were then conducted using this pPol-luc reporter system. It is also important to note that a construct, where the wild-type *IE63* gene had been cloned in the reverse orientation, was used as a negative control (pcDNA63inv).

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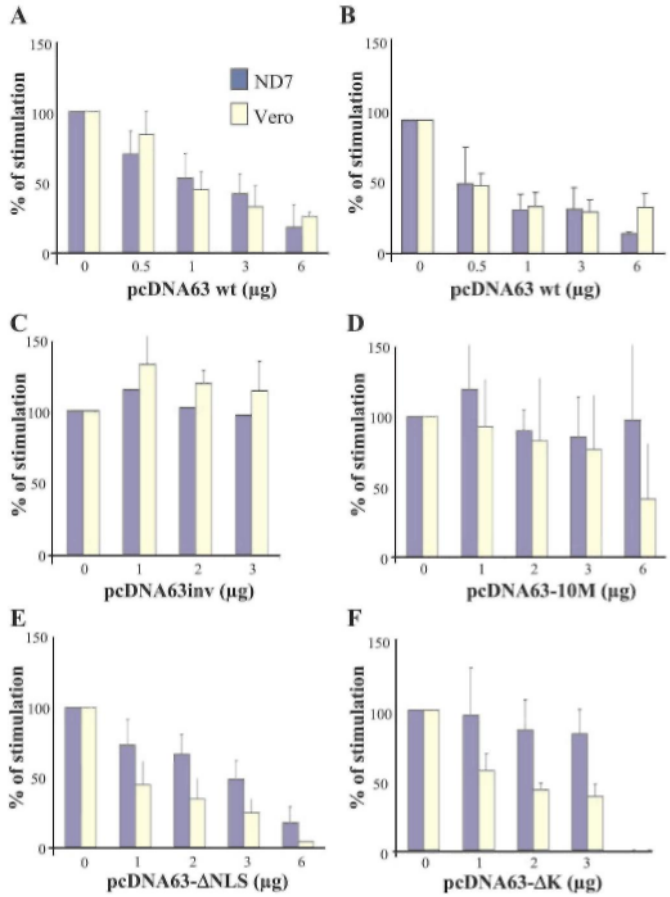

FIG. 8. Effect of wild-type or mutated IE63 on the VZV promoter basal expression. Vero and ND7 cells were transfected with 1 µg of pPol-luc (A and C—F) or with 1 µg of pTK-CAT (B). Increasing concentrations of plasmids expressing wild-type IE63 (A and B), IE63 inv (C), IE63-10M (D), IE63-ΔK (E), and IE63-ΔNLS (F) were added. Forty hours post-transfection, cells were harvested, and the reporter gene activity was measured. Results are presented in percentage of stimulation with respect to the basal expression of the promoter (= 100%).

With this plasmid transfected in identical conditions, as described above, no repression on the basal activity of the VZV DNA polymerase promoter was observed in both cell lines (Fig. 8*C*), demonstrating that the repressive activity of IE63 was due to the expression of a correctly expressed IE63.

When this experiment was performed using the IE63 protein mutated on the 10 potential phosphorylation sites (IE63-10M), no significant repression of the luciferase activity was observed in ND7 cells, whereas in Vero cells, this activity was greatly reduced (Fig. *8D).* These results suggest the importance of the phosphorylation events for a complete and functional activity of IE63. This loss of activity seemed to be dependent on the phosphorylation mechanism and was not due to a change in the cellular localization in Vero cells, because experiments carried out with the pcDNA-63ΔNLS (Fig. 8*E*) revealed that IE63-ΔNLS, mainly localized in the cytoplasm, was still able to repress the basal activity of the promoter. The repression property observed with the IE63-ΔNLS protein was similar to that observed with the wild-type protein in both cell types, indicating that the ^{260}KRRR region was not required for the activity of IE63 on this promoter. These results also suggest that IE63 is able to exert its activity by two independent ways, through a transcriptional or a post-transcriptional mechanism in the nucleus and/or a post-transcriptional mechanism in the cytoplasm. This phosphorylation-dependent regulation mechanism is not cell type-dependent because it was observed in both ND7 and Vero cells. Interestingly, we also observed that IE63, deleted from its carboxyl-terminal region (pcDNA-63 ΔK), was still able to act as a repressor on the luciferase reporter gene when transfected in Vero cells but not in ND7 cells (Fig. 8*F*). These data suggest that, in ND7 cells, the repressive activity of IE63 is dependent on the carboxyl-terminal region (amino acids 210–278). This region contains a NLS and also two putative phosphorylation sites for PKC/Cdc2 or CKI. As experiments carried out with pcDNA63-ΔNLS showed that the NLS was not required for the activity of the protein, we might

suspect that the presence of these two putative phosphorylation sites are important for the repressive activity of IE63 in ND7 cells but not crucial in Vero cells.
We also decided to evaluate the regulatory properties of wild-type or mutated IE63 when the expression of the pPol-luc promoter was stimulated by IE62, a VZV-encoded transcription factor. Indeed, it has been shown previously that IE62 was able to activate the VZV DNA polymerase promoter when trans-fected in cells (34). For this, Vero and ND7 cells were trans-fected with 1 μg of pPol-luc. Stimulation was achieved using 0.5 μg of pcDNA-IE62, and increasing concentrations of wild-type pcDNA63 or pcDNA63-10M were added. Results were presented in percentage of stimulation with respect to the stimulation obtained with 0.5 μg of pcDNA62. As expected, IE62 was able to stimulate the expression of the reporter gene up to 20-fold in Vero cells and about 10-fold in ND7 cells (data not shown). The difference in the level of pPol-luc stimulation between both cell lines could be explained by the fact that the level of basal expression of the VZV DNA polymerase promoter was higher in ND7 than in Vero cells. This stimulation was greatly reduced when increasing concentrations of wild-type IE63 were added, in both cell lines (Fig. 9*A*). Indeed, using 6 μg of wild-type pcDNA63, the stimulation of the promoter was about 30% that observed without IE63 in both cell types. Again, when the 10 putative phosphorylation sites of IE63 had been mutated, no repressive activity of the protein was observed in ND7 cells, whereas in Vero cells, this IE63-10M protein still exhibited some repressive properties (Fig. 9*B*).

Discussion

The viral tegument is an amorphous structure located between the capsid and the external envelope of herpesviruses. Four different proteins, ORF10, IE4, IE62 and IE63, have been identified within the VZV tegument (35). They have all been described previously as regulatory proteins; ORF10, the homo-logue of HSV-1 VP16 protein, is able to transactivate the VZV *IE62* promoter (36), whereas IE4 and IE62 are phosphoproteins known for their ability to stimulate the expression of all three classes of VZV genes (IE, E, and L) (4–8, 28). The activity of IE63 is not so well defined. This tegument protein possesses regulatory properties on the viral gene expression as shown previously (22), but these results were disputed (23).
The first part of this report was devoted to the analysis of the important phosphorylation sites of IE63. We showed that cellular kinases from both Vero and ND7 cells were able to phos-phorylate this protein and that the phosphorylation rate of IE63 was higher in the permissive and non-neuronal cell line (Vero), compared with the neuronal but not permissive ND7 cells. Immunoprecipitation experiments and the use of a CKI and CKII inhibitor (DRB) in an *in vitro* kinase assay allowed us to conclude that those cellular kinases were implicated in IE63 phosphorylation. CKI and CKII are ubiquitous proteins involved in many cellular events; CKII is known to be implicated in various biological processes such as signal transduction, cell cycle, apoptosis, and cell transformation, and it can phospho-rylate more than 160 proteins including many viral proteins (37). CKI was also described as a pleiotropic kinase with a large number of substrates (38).

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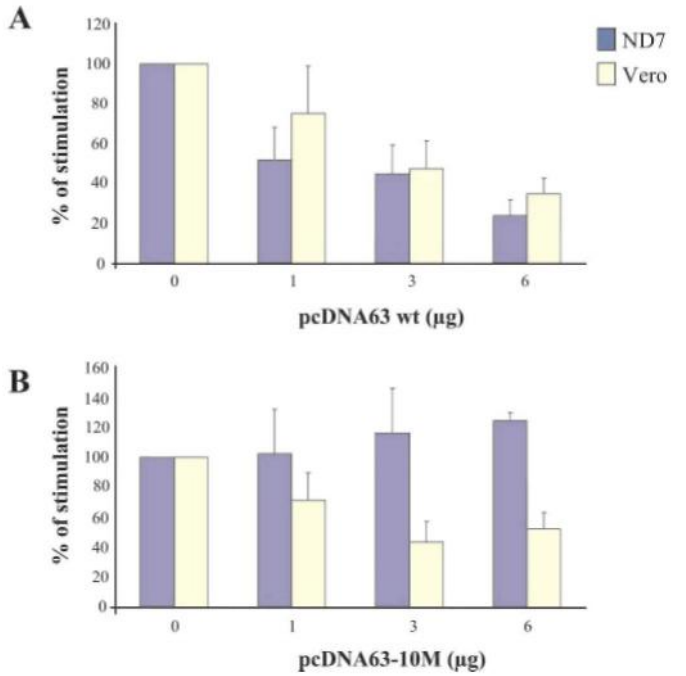

FIG. 9. Effect of wild-type or mutated IE63 on the expression of the VZV DNA polymerase promoter stimulated by IE62. Vero and ND7 cells were transfected with 1 µg of pPol-luc and 0.5 µg of pcDNA-IE62. Increasing concentrations of plasmid encoding wild-type IE63 (A) or IE63-10M (B) were added. 40 h post-transfection, cells were harvested, and the luciferase activity was measured. Results are presented as a percentage of stimulation with respect to the stimulation obtained with 0.5 µg of pcDNA62 (= 100%).

Phosphorylation of IE63 in Mewo cells, or using recombinant CKII, was described previously (24), and potential phosphorylation sites were predicted in the carboxyl-terminal region of the protein (between residues 142 and 278). A site-directed mutagenesis strategy allowed us to show that phosphorylation of IE63 was not restricted to the five serine or threonine residues targeted by CKII in this region (Ser-150, Ser-165, Thr-171, Ser-181, and Ser-186). Indeed, the IE63-5M protein, where all these potential CKII sites are mutated into alanine residues, may still be phosphorylated using recombinant CKII. These results suggest that CKII might also use target sites located elsewhere in the protein. Analysis of the amino acid sequence of the protein revealed the presence of two other putative phosphorylation sites for CKII in the amino-terminal (Thr-8) and in the central (Thr-124) parts of the protein. Using two other prediction programs (NetPhos 2.0 (39) and Scansite (40)) Ser-197 can also be identified as a residue with a high probability of being phosphorylated. According to the scansite program Ser-197 might be a potential target for CKII. The relevance and the implication of these sites in the phosphorylation of IE63 remain to be elucidated. We also observed a slightly higher CKI phosphorylation rate of IE63-5M in comparison with the wild-type protein. We suspect that mutations introduced in this mutant might modify the accessibility and/or the affinity of CKI for its target sites. Four potential sites for CKI (Ser-173, Ser-185, Thr-201, and Thr-244) and 1 site for PKC/Cdc2 (Ser-224) are present in the carboxyl-terminal region of IE63. The impact of their mutation, in combination with those for CKII, was investigated (IE63-10M). These results indicated that CKI was implicated in the phosphorylation process of IE63. Indeed, we showed that wild-type IE63 may be phosphorylated *in vitro* using recombinant CKI and that the mutation of the four phosphorylation sites largely abrogated its phosphorylation by CKI. Netphos and Scansite analyses also revealed the presence of two other sites of high probability for being phosphorylated by CKI, Ser-15 and Ser-203. These residues might be responsible for the slight phosphorylation remaining in the IE63-10M mutated protein. Interestingly, we also noticed a lower CKII phosphorylation rate of the IE63-10M protein in comparison to what was observed with the IE63-5M. We postulate that the last mutations introduced in the IE63-10M protein (and especially the mutation of the residue 201) could have modified Ser-197 accessibility to CKII. Kinase assays performed using cellular protein extracts and measure of the casein kinase activity revealed an important decrease (60–70% using Vero and ND7 protein extracts, respectively) of the phosphorylation rate of IE63-10M in comparison with the wild-type protein. This observation has been made regardless of the nature of the cell type, even if the phosphorylation rate of IE63 was more

important using protein extracts from Vero than from ND7 cells. These results obtained with the neuronal and non-neuronal cellular models suggested that members of the casein kinase family are implicated in the phosphorylation of IE63.

Phosphorylation and dephosphorylation events are implicated in many cellular regulatory activities such as cell cycle, signal transduction, or gene expression (41–43). Many transcription factors are common targets for cellular kinases and are regulated by phosphorylation mechanisms (41, 42). Indeed, one of the strategies developed by cells to regulate the expression of cellular genes is the control of transcription factors localization and their shuttling through the nucleus. For example, phosphorylation of IE62 by the viral ORF66 kinase led to its delocalization from the nucleus to the cytoplasm, impairing its transactivation properties (44). In this context, we investigate the impact of phosphorylation event on the cellular localization of IE63. The use of two different cell lines allowed us to compare the behavior of IE63 in a non-neuronal cell line permissive to VZV (Vero) and in neuronal ND7 cells, previously shown to be of low permissivity to HSV-1 infection (26). We demonstrated here that VZV does not replicate in this cell type. The wild-type IE63 protein exhibits a major nuclear localization in Vero- and ND7-transfected cells, as observed previously (20) in infection studies. The removal of the four amino acids located between residues 260 and 264 (KRRR) led to a predominant cytoplasmic localization of the protein in both cell lines, suggesting that this short basic sequence acts as an effective, but not exclusive, nuclear localization signal for IE63. The removal of a large part of the carboxyl-terminal region of the protein (IE63-ΔK) induced a cytoplasmic delocalization of the protein when transfected in Vero and in ND7 cells, confirming the observations of others in Mewo cells (24). The mutation of the five potential phosphorylation sites for CKII located in the carboxyl-terminal region of IE63 led to a slight delocalization of the protein from the nucleus to the cytoplasm of Vero cells. Experiments carried out with mutants IE63-1M, IE63-2M, IE63-3M, and IE63-4M, where only some of the CKII phosphorylation sites were mutated, did not shed light on determining the CKII site responsible for this slight delocalization. Furthermore, the mutation of all CKI, CKII, and PKC/Cdc2 phosphorylation sites located in this area (IE63-10M) considerably alters the localization of IE63 in Vero cells, as more than 65% of them exhibited a major cytoplasmic staining. In this case, we also observed a striking localization of the protein at the nuclear membrane, suggesting that the absence of phosphorylation impaired its transport into the nucleus. Results obtained with several intermediate constructs, such as IE63-6M, IE63-7M, IE63-8M, and IE63-9M, did not allow us to identify a key site in the process (data not shown). Our observations suggest that the correct import of IE63 into the nucleus requires a complete phosphorylation of the carboxyl-terminal region of the protein, rather than the phosphorylation of one or two particular sites. However, it should be pointed out that Ser-224 could also be considered as a potential target site for $p34^{cdc2}$, a member of the cyclin-dependent serine/threonine kinase family involved in the G_2/M transition of the cell cycle (45). It has been shown previously that, during HSV-1 infection, this cellular kinase is required for the expression of a subset of late viral proteins and that its own expression is induced by UL13 and ICP22, the latter being the HSV-1 homologue of IE63 (46). Furthermore, several reports (46–49) also indicated that Cdc2 could modulate the cellular localization and the function of several transcription factors. In this context, it would be interesting to define the potential role that this kinase could play on the localization and/or activity of IE63. An interesting observation was also made when the cellular localization of IE63 mutated in some of its phosphorylation sites was investigated in ND7 cells. Indeed, in this neuronal cell line, no impact of the mutations of the phosphorylation sites was observed; the IE63-5M and IE63-10M proteins exhibited a nuclear localization like the wild-type protein, and after deletion of its NLS, the protein remains in the cytoplasm of transfected cells. These results indicate that in permissive cells (Vero), phosphorylation of the IE63 protein is important for its accurate localization, whereas in cells that do not support VZV infection (ND7), the phosphorylation rate of IE63 does not seem to be a critical factor for its subcellular localization. This suggests that, depending on the cell type, IE63 might use different transport mechanisms that could be more or less sensitive to its phosphorylation status. Numerous reports indicate that phospho-rylation-dependent mechanisms may regulate the subcellular localization of proteins such as those containing a CcN motif, a short region containing phosphorylation sites for CKII and Cdc2 in the vicinity of a nuclear localization signal (50–54).

To understand the relevance of the phosphorylation-depend-ent localization of IE63, we developed an activity assay with the wild-type protein and then examined the behavior of the mutated proteins in this context. Because the trans-repressive properties of IE63 on IE promoters have been questioned (23), we investigated the activity of IE63 on the regulation of the gene encoding the DNA polymerase, a putative early protein (ORF28). Results indicated that the wild-type IE63 protein was able, in a dose-dependent way, to down-regulate the expression of the luciferase reporter gene driven by this promoter (pPol) in both cell lines tested. This repressive activity was clearly observed either in the basal activity

of the pPol or after its stimulation by IE62. This down-regulating property might reflect an important role played by IE63 in the infectious cycle. An important feature of IE63 was observed when experiments were carried out with the 63ΔNLS protein. Indeed, despite its cytoplasmic localization, this protein was still capable of repressing the basal expression of the pPol. This property was established in neuronal and in non-neuronal cells with an efficiency similar to the wild-type protein. IE63 thus acts as a repressor either in the nucleus or in the cytoplasm of trans-fected cells, and this viral protein could exert its activity by two independent ways, through transcriptional and/or post-tran-scriptional mechanisms. Experiments carried out with the IE63-10M protein indicated that the phosphorylation status of IE63 is important for its activity. Indeed, in neuronal cells we observed a complete loss of the pPol repression when phosphorylation sites of the protein were mutated. These observations were made when pPol was stimulated by IE62 and on its basal activity. In Vero cells, the repression property of IE63 on the basal expression of the promoter was also slightly reduced.
Hence, the phosphorylation status of IE63, rather than its cellular localization, is critical for the fulfillment of its repressive activity, and casein kinases are involved in the regulatory process. Several reports (37, 55, 56) indicate that members of the CK family possess regulatory properties and are involved in the control of cellular or viral gene expression.
Taken together, these results indicate that in the models studied, cellular CKI and CKII are important for the correct nuclear localization of IE63 and for its repressive properties in a cell type-dependent way. Furthermore, we showed that IE63 exerts its repressive properties in the nucleus as well as in the cytoplasm of transfected cell, suggesting its implication in tran-scriptional and/or post-transcriptional mechanism(s). In order to determine the biological relevance of our observations and to evaluate them in an infectious context, we intend to develop a VZV recombinant virus carrying mutations in the phosphoryl-ation sites of *IE63* genes. The replication of this recombinant virus will be tested in cultured cells, and its infectivity and its ability to establish latency in dorsal root ganglias will be addressed in our rat model. Recent studies (25) indicated that ORF47, one of the VZV-encoded kinase, is able to use IE63 as substrate *in vitro*. This Ser/Thr kinase has often been compared with CKII and is not essential for viral replication in cultured cells (57–60). Meanwhile, a study developed in a SCID-hu mice model indicated that ORF47 is required for viral growth in human T cells and skin (61). Although this suggested that in the differentiated cells studied endogenous CKII is unable to compensate the lack of ORF47, it will be necessary to clarify the ability of ORF47 to phosphorylate *in vivo* IE63 and to estimate the impact on its localization and activity before inferring a role for cellular kinases in the infection process.

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