Published in : Journal of biological chemistry (2000), vol. 275, iss. 42, pp. 32822-32832
Status : Postprint (Author's version)

Gene Activation by Varicella-Zoster Virus IE4 Protein Requires Its Dimerization and Involves Both the Arginine-rich Sequence, the Central Part, and the Carboxyl-terminal Cysteine-rich Region.

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Varicella-zoster virus (VZV) open reading frame 4-en-coded protein (IE4) possesses transactivating properties for VZV genes as well as for those of heterologous viruses. Since most transcription factors act as dimers, IE4 dimerization was studied using the mammalian two-hybrid system. Introduction of mutations in the IE4 open reading frame demonstrated that both the central region and the carboxyl-terminal cysteine-rich domain were important for efficient dimerization. Within the carboxyl-terminal domain, substitution of amino acids encompassing residues 443–447 totally abolished dimer-ization. Gene activation by IE4 was studied by transient transfection with an IE4 expression plasmid and a reporter gene under the control of either the human immunodeficiency virus, type 1, long terminal repeat or the VZV thymidine kinase promoter. Regions of IE4 important for dimerization were also shown to be crucial for transactivation. In addition, the arginine-rich domains Rb and Rc of the amino-terminal region were also demonstrated to be important for transactivation, whereas the Ra domain as well as an acidic and bZIP-containing regions were shown to be dispensable for gene transactivation. A nucleocytoplasmic shuttling of IE4 has also been characterized, involving a nuclear localization signal identified within the Rb domain and a nuclear export mechanism partially depending on Crm-1.

The abbreviations used are: VZV, varicella-zoster virus; HSV-1, herpes simplex virus, type 1; NLS, nuclear localization signals; LUC, luciferase; PCR, polymerase chain reaction; GST, glutathione *S*-trans-ferase; CAT, chloramphenicol acetyltransferase; LTR, long terminal repeat; PAGE, polyacrylamide gel electrophoresis; wt, wild type; NES, nuclear export signal; hnRNP, heterogeneous nuclear ribonucleopro-tein; LMB, leptomycin B; HIV-1, human immunodeficiency virus, type 1; TK, thymidine kinase; TBP, TATA-binding protein.

Varicella-zoster virus (VZV)[1] is an α-herpesvirus that causes two distinct diseases in man, chicken pox and shingles. Shortly after entry into the cells, VZV genes are expressed in a temporal cascade. The immediate-early genes are expressed first; these stimulate early gene expression, providing most of the proteins necessary for viral DNA replication. After DNA synthesis has occurred, genes of the late class, which mainly encode structural proteins, are expressed. This orderly pattern of expression has been proposed mainly by comparison with herpes simplex virus, type 1 (HSV-1) (1), another α-herpesvirus that has been intensively studied. The use of transient trans-fection assays has clearly shown that several VZV proteins, *i.e.* those encoded by open reading frames 4, 10, 29, 61, 62, and 63, possess regulatory properties (2–8). Three of these polypeptides, encoded by open reading frames 4, 62, and 63, are expressed during the immediate-early phase of lytic infection (9–11) and are thus referred to as IE4, IE62, and IE63. Therefore, VZV immediate-early proteins contribute to the control of the viral cycle progression as in other α-herpesviruses.
The IE4 protein is a transactivator of gene expression whose regulatory properties are not yet fully understood (2, 4, 11–14). IE4 stimulates VZV gene expression regardless of the cell type envisaged, *i.e.* monkey fibroblasts or human T lymphocytes (2, 4, 11, 12). It also appears that IE4 is capable of heterologous transactivation (11–13). The available data suggest that IE4 could exert its functions through transcriptional and post-transcriptional mechanisms (11–13). VZV IE4 is a 452-amino acid long protein that shares considerable amino acid sequence homology with HSV-1 IE protein ICP27, especially in the car-boxyl terminus and in the central part of the protein (13, 15). The carboxyl-terminal region of ICP27 that is rich in cysteine and histidine residues has been shown to bind zinc (16) and be required for multimerization (17). Whereas the carboxyl-termi-nal region of IE4 also contains cysteine and histidine residues, it is not known whether this region also binds zinc nor whether it forms a potential zinc finger domain. A rather large part of ICP-27 spanning amino acids 260–434 was shown to be critical for gene activation (18, 19). Mutations in this activation domain exhibited a transdominant

negative phenotype (18). The amino-terminal regions of these two proteins have a more limited amino acid homology; however, both are highly acidic (14). Sixteen of the first 66 amino acids of the amino-terminal region of VZV IE4 are either aspartic or glutamic acid, and seven residues are serine which, if phosphorylated, may be negatively charged. Net acidity is characteristic of several transcriptional activators; however, other critical structural features are also required for transactivating activity (for review, see Ref. 20). Close to the amino-terminal region, ICP27 possesses a sequence that resembles an RGG box, an RNA-binding motif found in a number of cellular proteins involved in mRNA and rRNA metabolism. The RGG box sequence is composed of 15 consecutive arginine and glycine residues and is required for ICP27 nucleolar localization, possibly reflecting an *in vivo* RNA binding activity (19–22). VZV IE4 does not bear an RGG box but instead has three arginine-rich regions (Ra, Rb, and Rc) and three potential bZIP sequences that are fused to the amino-terminal side of the Rb region. The function of these repeats is still unknown. ICP27 contains multiple nuclear localization signals (NLS) that function with differing efficiencies. A strong NLS maps to residues 110–137; it bears similarity to the bipartite NLS found in *Xenopus laevis* nucleoplasmin. Weak NLS(s) map to the central and/or carboxyl-terminal portion of the protein (22). No equivalent to this strong NLS has been yet found in VZV IE4. However, area encompassing the putative bZIP domain shares some homology with the NLS of the HIV-1 Tat protein. Recently, ICP27 has been shown to shuttle between the nucleus and cytoplasm through a leucine-rich nuclear export signal in the amino terminus (23, 25). Since it has been demonstrated that ICP27 can bind seven intronless HSV-1 transcripts and that the export of these transcripts to the cytoplasm is substantially reduced during infection with 27-LacZ virus, where ICP27 is not expressed, it may be suggested that export of intronless mRNAs by ICP27 comprises at least part of its function as an essential regulator of viral gene expression. Whereas VZV IE4 exhibits a main cytoplasmic localization, no potential NES site can be located within its amino-terminal portion.
The purpose of this report was to clarify the molecular mechanisms of IE4-mediated gene activation. Our results indicate that IE4 homodimerization mainly occurred through the central and the carboxyl-terminal region of the protein. Amino acid substitution within the carboxyl-terminal domain showed that a GKYFKC peptide was crucial for dimerization. Regions of IE4 important for dimerization were also shown to be necessary for transactivation. In addition, the arginine-rich domains Rb and Rc of the amino-terminal region of IE4 were also demonstrated to be important for transactivation, whereas the first Ra domain as well as an acidic and bZIP-containing regions were shown to be dispensable for gene transactivation. A nucleocy-toplasmic shuttling of IE4 has also been characterized. It likely involved a nuclear localization signal identified within the Rb domain. In addition, we demonstrated that IE4 shuttled between the nucleus and the cytoplasm partly via a Crm1-de-pendent mechanism. Both the central and carboxyl-terminal regions are involved in the nuclear export of IE4.

Materials and methods

Cells and Transfections—The HeLa human cervical epithelioid carcinoma cell line was grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Transfections were carried out on cells seeded into 35-mm diameter six-well cluster dishes using the FUGENE 6reagent (Roche Molecular Biochemicals). To determine CAT activities, whole-cell extracts were prepared by the freeze-thaw method as described previously (12). LUC assays were performed using the "Luciferase Reporter Gene Assay, high sensitivity" kit (Roche Molecular Biochemicals), according to the instructions of the manufacturer. Data from CAT and LUC assays were collected from at least four independent transfection experiments.
Plasmids—Plasmids pHIV-1-CAT and pTK-CAT were described previously (12, 26). In these constructs, the wild-type LTR of HIV-1 or the VZV thymidine kinase gene promoter, respectively, is cloned upstream of the CAT gene. The reporter construct p$(gal4)_5$SV40-LUC was a gift from Dr. M. Müller (University of Liege, Belgium) and contained the LUC reporter gene under the control of five copies of the Gal4 DNA-binding sites upstream of the SV40 promoter.
Plasmids pM and pVP16 (CLONTECH) harbored the SV40 promoter driving the Gal4 DNA-binding domain or the HSV-1 VP16 activation domain, respectively. The constructs pM4 and pVP16-IE4 were made by insertion of the *IE4* gene into the *Eco*RI site of pM or pVP16 in frame with the Gal4 DNA-binding domain or the HSV-1 VP16 activation domain coding sequence, respectively. The IE4 coding sequence was amplified by PCR using oligonucleotides carrying an *Eco*RI site at 5'.
Base substitutions or deletion into the *IE4* gene were generated by PCR using mismatching primers that created a new restriction site in the vector. The PCR mixture consisted of 25 mM KCl, 10 mM Tris-HCl (pH 8.8), 5 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.8 mM dNTP mix, 2.5 units of *Pwo* DNA polymerase (Roche Molecular Biochemicals), 1 μM each primer, and 200 ng of pM4 in a total volume

of 100 μl. The amplification procedure started with a 2-min preheating step at 94 °C followed by 35 cycles, each consisting of a 94 °C denaturation segment for 15 s, a 50-60 °C annealing segment for 30 s (depending on the set of primers used), and a 72 °C extension segment for 4 min; the whole set was followed by a final extension at 72 °C for 7 min. After amplification, the PCR products were resolved on a 0.8% agarose gel electrophoresis, and the fragment corresponding to the size of the linearized vector was recovered using the Bio-Rad gel extraction kit (Bio-Rad). After phosphorylation of the 5'-end of the fragment with the T4 polynucleotide kinase (Roche Molecular Biochemicals), the plasmid was recircularized by the T4 DNA ligase (Roche Molecular Biochemicals), transformed into *Escherichia coli* DH5α, and then analyzed for the presence of the new restriction sites introduced during the PCR. By this procedure, plas-mids harboring mutations in the carboxyl-terminal region of IE4 were obtained as follows: pM4-G442, -K443, -Y444, -FK445, -C447, -ST448, -FN450, and-C452 (the numbers refer to the position of the mutation in relation to the first methionine residue of the IE4 protein). Substitutions within the amino-terminal region of IE4 were also created by PCR. These plasmids were named pM4-Rb, pM4-Rc, pM4-Rb + Rc, pM4-bZIP, pM4-bZIP + Rb + Rc. The corresponding mutations are detailed in Fig. 4. Deletion mutants in the *IE4* gene were obtained by a similar procedure: pM4-D-(1–65), pM4-D-(66-110), pM4-D-(111–181), pM4-D-(111-150), pM4-D-(150-182), pM4-D-(183-390), pM4-D-(182-231), pM4-D-(266-302), pM4-D-(314-385), pM4-D-(403-452), pM4-D-(427-452), and pM4-D-(444-452); the positions of the deleted amino acids are indicated in parentheses.
To create pC4 wt and mutated, the wild-type IE4 sequence as well as both mutated sequences were excised from pM4 wt or mutated by digestion with *EcoRI.* The 1359-base pair fragments were then cloned into the polycloning site of pCDNA3.1.- (Invitrogen, Inc., Leek, The Netherlands) under the control of the cytomegalovirus promoter-enhancer or the T7 promoter. The nomenclature used for the description of these mutated pC4 plasmids was the same as detailed above.
Immunofluorescence—HeLa cells seeded into 10-mm dishes were transfected with 2 μg of pC4 or its derivatives using the FUGENE 6 reagent (Roche Molecular Biochemicals). To perform immunofluorescence studies, transfected cells grown on coverslips were treated as described previously (27). In order to detect wild-type IE4 or mutated forms, a rabbit polyclonal antiserum that was raised against a GST-IE4 fusion protein was prepared and used as described (10). In some experiments, leptomycin B (LMB, provided by B. Wolff, Novartis, Vienna, Austria) was added to the culture medium 48 h post-transfection at a concentration of 10 nM. Six hours later, cells were fixed and treated for indirect immunofluorescence.
In Vitro Analysis of Protein-Protein Interactions—The various constructs (GST, GST-TK, GST-p50, and GST-TFIIB (29)) were expressed in *E. coli* following classical induction with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside for 3 h at 37 °C. Lysates were prepared as described (29), and proteins were then purified on glutathione-Sepharose 4B affinity beads (Amersham Pharmacia Biotech) in phosphate-buffered saline/Triton 1% (v/v), following extensive washing in phosphate-buffered saline. IE4 proteins were expressed from pC4 and its derivatives and labeled with [^{35}S]methionine (ICN, Brussels, Belgium) using the *in vitro* TNT-T7-coupled reticulocyte lysate system (Promega Inc., Madison, WI). 5 μl of ^{35}S-labeled proteins were incubated with 30 μl of protein-coupled Sepharose beads in 400 μl of NETN (20 mM Tris-HCl (pH 8), 100 mM NaCl, 1 mM EDTA, 1.5% (v/v) Nonidet P-40). Binding reactions were allowed to take place for 3 h at 4 °C, and the beads were then washed six times in NETN. Bound proteins were eluated by boiling for 2 min in 1X SDS sample buffer, followed by loading on 10% SDS-PAGE. Gels were subsequently dried and autoradiographed.

Results

IE4 Homodimerization Is Required for Transactivation— Since we previously showed that IE4-activated gene expression under the control of the HIV-1 LTR through interactions with members of the NF-κB family and factors of the basal transcription machinery (29), we decided to investigate whether gene activation by IE4 requires its homodimerization. Therefore, to study IE4 dimerization, we used a mammalian two-hybrid system (30) based on the construction of two chimeric proteins between IE4 and either the Gal4 DNA-binding domain or the VP16 activation domain.

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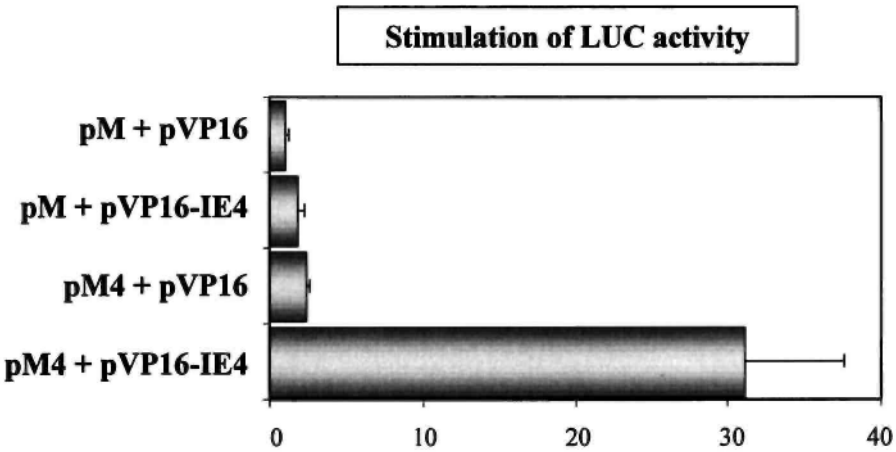

FIG. 1. IE4 homodimerization in the mammalian two-hybrid system. HeLa cells were co-transfected with 1 μg of the reporter plasmid p(gal4)$_5$SV40-LUC and 2 μg of pM or 2 μg of pM4 together with 2 μg of pVP16 or pVP16-IE4 as indicated. LUC assays were carried out 48 h post-transfection. Fold stimulation of LUC activity was calculated relative to the basal level of the reporter plasmid in the presence of pM and pVP16, arbitrarily set to 1. Data from four independent experiments are shown with standard errors of the means.

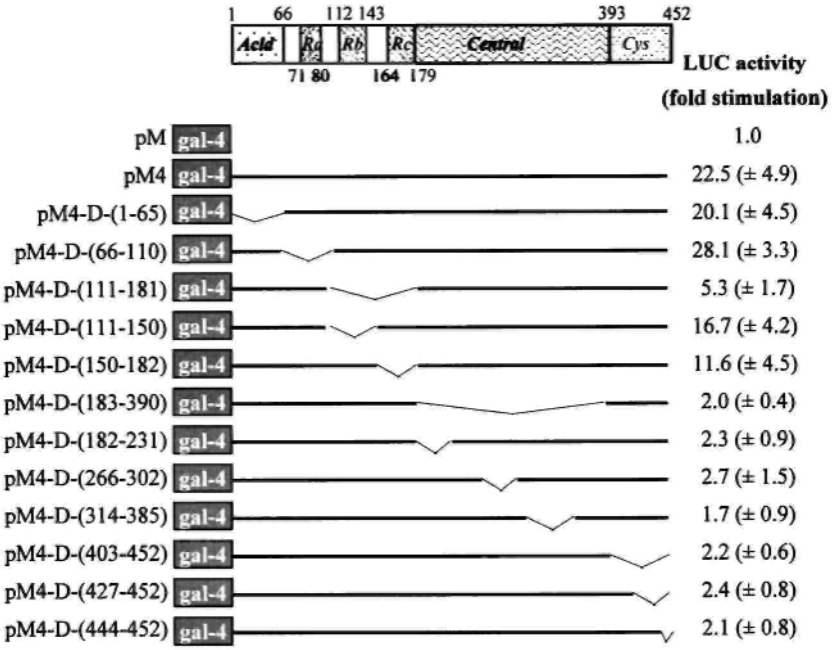

FIG. 2. Homodimerization properties of IE4 deletion mutants. A schematic representation of the 452-amino acid coding region of IE4 is illustrated. The acidic (Acid) and the arginine-rich regions (Ra, Rb, and Rc), as well as the central part (Central) and the cysteine-rich carboxyl-terminal domain (Cys) are shown. The fusion constructs used to measure IE4 homodimerization are depicted with the Gal4 DNA-binding domain fused to various deleted IE4 proteins (lines and dotted lines). The deleted amino acids are represented by dotted lines and are indicated in parentheses. HeLa cells were co-transfected with 1 μg of p(gal4)$_5$SV40-LUC, 2 μg of pVP16-IE4, and 2 μg of the various constructs as indicated. Fold stimulation of LUC activity was calculated relative to the basal level of reporter plasmid in the presence of pM and pVP16-IE4, arbitrarily set to 1. Results from at least four independent experiments are presented with standard errors of the means.

The unmodified vectors or each of the individual fusion genes displayed low background LUC activity when co-transfected with reporter plasmid p(gal4)$_5$-SV40-LUC into HeLa cells, whereas transfection

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with a combination of plasmids encoding IE4-Gal4 DNA-binding and IE4-VP16 fusion proteins (pM4 and pVP16-IE4) resulted in a 32fold increase in LUC gene expression (Fig. 1). These data indicate that IE4 homodimerization is readily detectable in mammalian cells and is required for gene transactivation of an artificial construct.

The Central and Carboxyl-terminal Regions of IE4 Are Important for Homodimerization—In order to delineate the regions of IE4 that are important for dimerization, we first introduced deletions into the *IE4* gene fused to the Gal4 DNA-binding domain (Fig. 2). Deletion of either the acidic domain (Fig. 2, *pM4-D-(1–65)*) or the first arginine-rich region (Ra) (Fig. 2, *pM4-D-(66–110)*) within the amino-terminal region did not affect homodimerization. Individual deletion of the argin-ine-rich regions b (Rb) and c (Rc) only partially affected ho-modimerization (Fig. 2, *pM4-D-(111–150)* and *pM4-D-(150– 182)*), whereas the removal of both Rb and Rc decreased homodimerization by about 75% (Fig. 2, *pM4-D-(111–181)*). It should be noted that this deletion was rather large and re-moved 70 amino acids. On the other hand, deletion of the central region of IE4 comprised between amino acids 182 and 385 yielded an important reduction of homodimerization (about 90% reduction) (Fig. 2, *pM4-D-(183-390)).* Smaller deletions (between 49 and 71 amino acids into pM4-D-(182–231), -D-(266–302), and -D-(314–385)) were introduced within the central domain, and all led to a complete loss of homodimerization. Reintroduction of a irrelevant VZV sequence within the deleted *IE4* gene (pM4-D- (266–302)) did not allow recovery of efficient homodimerization (data not shown), demonstrating that the central region of IE4 itself is involved in the homodimerization process. Deletions within the cysteine-rich domain of IE4 demonstrated that the carboxyl-terminal region is important for homodimerization (Fig. 2, *pM4-D-(403-452)).* Indeed, deletion of either the complete cysteine-rich region (amino acids 393-452) or part of it (amino acids 444–452) drastically reduced homodimerization, demonstrating that the carboxyl-terminal regions of IE4 is also important for homodimerization.

The GKYFKC Sequence within the Carboxyl-terminal Region Is Crucial for Homodimerization—Since the carboxyl-terminal region of IE4 is rather conserved among α-herpesviruses (Fig. 3*A*), we decided to substitute several amino acids comprised between residues 442 and 452 (Fig. *3B).* As mentioned above, deletion of the last 10 amino acids led to a 90% drop in LUC activity (Fig. *3B, pM4-D-(444-452)).* Mutation of the Gly-442 reduced homodimerization to about 30% of the initial value, and either single or double substitution within the KYFKC sequence completely abolished its activity.

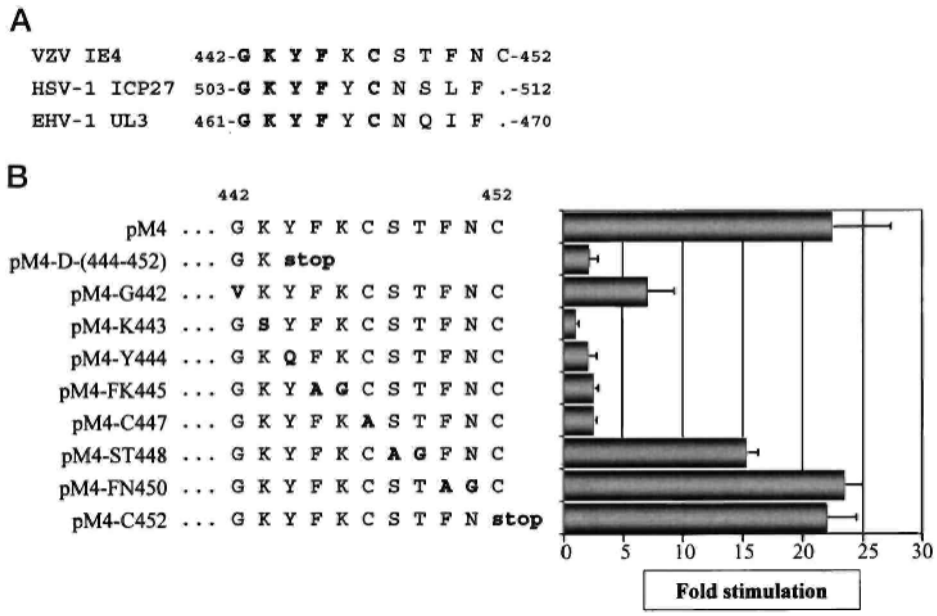

FIG. 3. Role of the carboxyl-termi-nal region of IE4 in homodimeriza-tion. A, alignment of the amino acids within the carboxyl-terminal region of IE4 with the corresponding regions found in other α-herpesvirus homologs (ICP27 from HSV-1 and UL3 from EHV-1). Numbers indicate the boundaries of the sequences in the context of the native proteins. B, relative homodimerization activity of the Gal4-IE4 mutants bearing various amino acid substitutions. The boldface letters indicate amino acid substitutions. HeLa cells were co-transfected with 1 μg of p(gal4)$_5$SV40-LUC, 2 μg of pVP16-IE4, and 2 μg of the various fusion Gal4 constructs as indicated. Fold stimulation of LUC activity was calculated relative to the basal level of the reporter plasmid in the presence of pM and pVP16-IE4, arbitrarily set to 1. Results from at least four independent experiments are shown with standard errors

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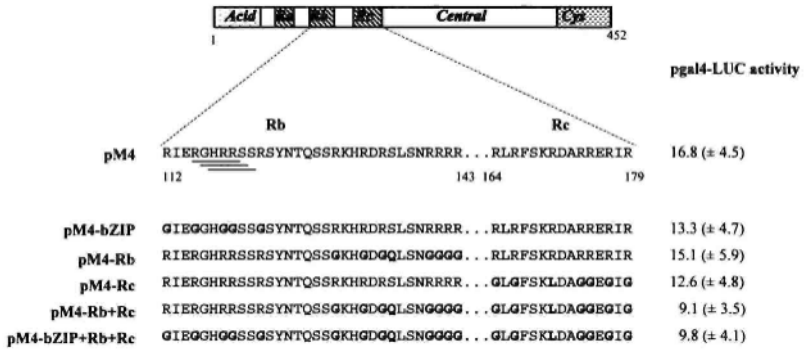

FIG. 4. Role of the arginine-rich regions Rb and Rc in IE4 homodimer-ization. The schematic representation of IE4 protein is described in the legend to Fig. 2. The sequences of the mutated Rb and Rc arginine-rich regions are represented with the letters in boldface corresponding to the substituted amino acids, as well as the three bZIP-like sequences that are underlined. The transfection of HeLa cells and the LUC activity were determined as described in the legend to Fig. 2.

On the other hand, substitution of the last five amino acids (from 448 to 452) did not affect reporter gene stimulation (Fig. *3B),* showing that the hexapeptide GKYFKC within the carboxyl-terminal region played an essential role in IE4 homodimerization.

Arginine-rich Repeats Rb and Rc Are Not Essential for IE4 Homodimerization—Since deletion of both arginine-rich regions Rb and Rc decreased IE4 homodimerization, amino acid substitutions were introduced within either the bZIP domain situated at the amino-terminal part of Rb, in Rb, in Rc, or in both Rb and Rc, or in the bZIP, Rb, and Rc (Fig. 4). Substitution of arginine residues with the bZIP sequence (amino acids 112-122) did not affect dimerization (Fig. 4, pM4-bZIP). Substitution of individual arginine residues by glycines within Rb or Rc did not modify either the level of IE4 homodimerization (Fig. 4, *pM4-Rb* and *pM4-Rc).* Finally, substitutions of all arginine residues in Rb and Rc (Fig. 4, *pM4-Rb + Rc)* or within the bZIP sequence, Rb and Rc *(pM4-bZIP + Rb + Rc),* only slightly affected LUC activity, showing that IE4 homodimerization, in this system, depends only on the integrity of both the central and carboxyl-terminal regions.

Regions of IE4 Important for Dimerization Are Also Important for Transactivation—IE4 was shown to activate expression of VZV genes as well as those of other viruses such as HIV-1 (2, 4, 11–14). In order to clarify the mechanism of gene activation by IE4 on VZV promoters and on heterologous promoters, we used transient transfection assays of HeLa cells with an IE4 expression plasmid (pC4) and a reporter gene construct under the control of either the HIV-1 LTR (pHIV-1-CAT) or the VZV TK promoter (pTK-CAT) (12, 26). IE4 expression led to a dose-dependent increase in CAT activity under the control of both promoters (data not shown) (12). Transfection of 2 μg of pC4 gave rise to 15- and 7-fold increases in CAT expression under the control of the HIV-1 LTR and VZV TK promoter, respectively (Fig. 5). In order to delineate regions of IE4 important for activation of either the VZV TK or HIV-1 promoter, base deletion and base substitutions were introduced into the *IE4* gene. Deletion of amino acids 1–65 of the amino-terminal region of IE4 (pC4-D-(1–65)) did not modify transac-tivation of either reporter construct, demonstrating that this acidic stretch was not required for gene activation. Removal of the first arginine-rich region Ra (pC4-D-(66–110)) did not modify reporter gene activation under the control of the HIV-1 LTR, whereas a slightly decreased activation was observed with the VZV TK promoter (Fig. 5). On the other hand, tandem deletion of arginine-rich regions Rb and Rc (pC4-D-(111–181)) significantly reduced transactivation of the two promoters. Interestingly, Rb turned out to be more important than Rc in the activation of the two promoters. Individual deletion of Rb strongly abolished gene activation by IE4 (pC4-D-(111–150)), whereas removal of Rc (pC4-D-(150–182)) only had a partial effect on promoter transactivation (Fig. 5). Base substitutions within these regions revealed that the bZIP within Rb did not participate significantly in gene activation, whereas mutations of the positively charged amino acids within Rb lowered the efficiency of transactivation (Table I). As we had previously observed with the deletion mutants, the removal of the positive charges in Rc reduced transactivation to a lesser extent than in HeLa cells were co-transfected with 0.5 μg of pTK-CAT or 0.2 *μg* of pHIV-1-CAT and 2 *μg* of IE4 expression vectors expressing either the wild-type or mutated IE4 proteins, as indicated. CAT activities were determined 48 h post-transfection.

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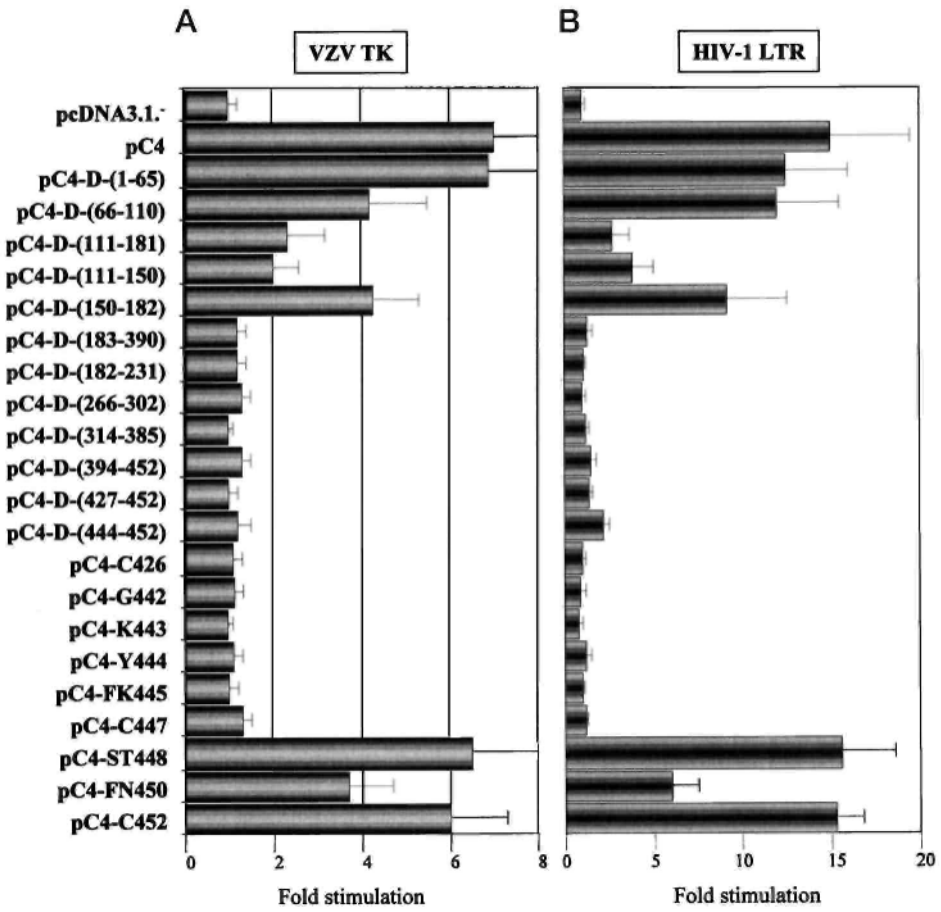

FIG. 5. Regulatory properties of mutated IE4 proteins on the VZV TK promoter (A) or the HIV-1 LTR (B).

Fold stimulation of CAT activity was calculated relative to the basal level of reporter plasmid in the presence of control plasmid alone (pCDNA3.1-), arbitrarily set to 1. The results shown are representative of five independent experiments, and *error bars* represent standard errors.

Rb (Table I). These data demonstrated that Rb and, to a lesser extent Rc, were involved in the transactivating properties of IE4, whereas these regions appeared dispensable for IE4 homodimerization.

As expected, the regions of IE4 that were involved in its dimerization were required for gene activation. Each individual deletion of the central part of IE4 abolished the reporter gene activation controlled by the HIV-1 LTR or the VZV TK promoter (Fig. *5,pC4-D-(183-390), -D-(182-231), -D-(266-302), -D-(314-385)).* Identical results were obtained when the car-boxyl-terminal region was removed (Fig. 5, *pC4-D-(394-452)* and *pC4-D-(444-452)).* When substitutions were introduced into the carboxyl-terminal part of IE4, they revealed the importance of cysteine 426 (Fig. 5, *pC4-C426),* as described previously (13, 29). As observed for dimerization, mutations within the GKYFKC peptide (pC4-G442, -K443, -Y444, -FK445, and -C447) strongly reduced transactivation, whereas mutations of the last five amino acids, STFNC, did not give to an abolished gene activation process (Fig. 5, *pC4-ST448, -FN450,* and *-C452).*

Arginine-rich Regions Rb and Rc Interact with Transcription Factor IIB and p50—It has been shown that VZV IE4 acts, at least, by transcriptional activation and can interact with different components of the basal transcription complex such as TBP and TFIIB as well as with p50 and p65 NF-κB subunit (29). To determine which regions of IE4 are involved in these interactions, *in vitro* protein-protein interaction experiments were made using a fusion protein between GST and p50 or TFIIB coupled to glutathione-Sepharose beads. A GST-TK fusion protein that carries VZV thymidine kinase was purified according to the same procedure and used as a negative control in addition to GST alone.

Equal amounts of *in vitro* translated ^{35}S-labeled IE4 or mutated IE4 were incubated with GST-,

TABLE I *Transactivation properties of IE4 proteins modified in the arginine-rich regions* The

expression vectors used were derived from the plasmids described in Fig. 4 and contain the same amino acid substitutions in the Rb and Rc domains. Mutant IE4 proteins were tested as described in the legend to
GST-TK-, GST-p50-, or GST-TFIIB-coupled Sepharose beads.

Fig. 5. Means and S.D. were calculated from at least five independent transfections.

	Fold stimulation on VZV TK	Fold stimulation on HIV-1 LTR
pC4	7.1 (±2.1)	11.3 (±4.1)
pC4-bZIP	4.6 (±1.5)	8.1 (±3.2)
pC4-Rb	2.2 (±0.5)	2.0 (±0.7)
pC4-Rc	3.4 (±1.7)	5.8 (±2.0)
pC4-Rb + Rc	2.7 (±1.0)	1.3 (±0.4)
pC4-bZIP + Rb + Rc	3.3 (±1.5)	1.1 (±0.3)

After extensive washing, bound proteins were eluted and analyzed by SDS-PAGE. Most of the IE4 specifically interacted with GST-p50 and GST-TFIIB (Fig. 6), and there was no IE4 retained by the GST protein alone and the GST-TK-coupled Sepharose beads (data not shown) as expected. Deletions into the central and carboxyl-terminal parts of IE4 did not affect the interactions because IE4-D-(182–231), -D-(266–302), -D-(314– 385), and -D-(444–452) were retained by GST-p50 and GST-TFIIB as efficiently as the IE4 protein (Fig. 6) and not by GST and GST-TK (data not shown). In contrast, an IE4 mutant protein that lacks the arginine-rich regions Rb and Rc, IE4-D-(111–181), failed to interact with all GST-p50 and GST-TFIIB fusion proteins (Fig. 6). Therefore, IE4 protein deleted individually of Rb or Rc region were tested in the GST-pull-down assay. Each of the *in vitro* translated IE4-D-(111–150) and IE4-D-(150–182) proteins were found capable of binding to GST-p50 and GST-TFIIB with similar affinity than IE4 (Fig. 6).

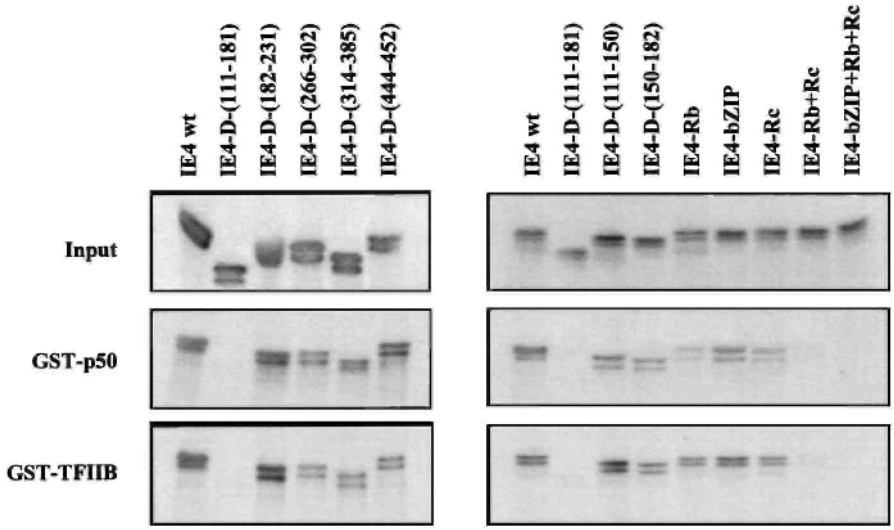

FIG. 6. Role of the arginine-rich regions Rb and Rc in their interactions with the transcription factor IIB and the NF-κB protein p50. Fusion proteins GST-p50 and GST-TFIIB, coupled to Sepharose beads, were incubated with in vitro translated [^{35}S]methionine-labeled IE4 wild-type and mutated as indicated at the top of each panel. The beads were extensively washed in NETN and eluted in SDS sample buffer before proteins were resolved on a 10% SDS-PAGE. Experiments were repeated three times.

Similar results were obtained with IE4 mutant proteins containing base substitutions within these regions. As shown in Fig. 6, substitution of arginine residues into the bZIP, Rb, or Rc (IE4-bZIP, IE4-Rb, and IE4-Rc) did not significantly affect interaction with GST-p50 and GST-TFIIB, whereas mutations of the positively charged amino acids within Rb and Rc (IE4 — Rb + Rc, IE4 — bZIP + Rb + Rc) completely disrupted these properties. These data demonstrated that Rb together with Rc, which were involved in the transactivating properties of IE4, are implicated in multiple protein-protein interactions with transcriptional factors such as p50 and TFIIB.

Published in : Journal of biological chemistry (2000), vol. 275, iss. 42, pp. 32822-32832
Status : Postprint (Author's version)

The Central and Carboxyl-terminal Domains Are Important for the Cytoplasmic Localization of IE4— In order to demonstrate that the loss in gene activation observed with amino acid-substituted or -deleted IE4 was not due to protein instability and to analyze the intracellular localization of the mutated IE4 proteins, immunofluorescence was carried out on HeLa cells transfected with the various mutated constructs. As shown in Fig. 7*A*, transfection with pC4 revealed a classical distribution of IE4 (27), *e.g.* a predominant distribution of the protein within the cytoplasm and a mixed distribution within the cytoplasm and the nucleus. Deletion within the amino-terminal part of the protein (amino acids 66–110), including the acidic and Ra domains, did not modify IE4 localization (data not shown). Deletions or base substitutions within the arginine-rich domains Rb gave a predominant distribution of the protein within the cytoplasm of transfected cells (Fig. *7B* and data not shown), whereas mutations into the Rc domain did not seem to affect IE4 localization. Deletions within the central region of IE4 revealed that all of the mutated IE4 proteins were preferentially localized within the nucleus; a punctated nuclear distribution of IE4 was even observed when amino acids 314–385 were deleted (Fig. 7*C*). A similar nuclear distribution was also observed with deletion and several amino acid substitutions in the carboxyl-terminal part of IE4. Individual mutations of the GKYFKC sequence led to a nuclear distribution of the protein as observed in cells transfected with pC4-D-(444–452) and pC4-Y444 (Fig. *7D* and data not shown), whereas mutations of amino acids 448–452 gave rise to both nuclear and cytoplasmic forms of the molecule as observed with the pC4-C452 expression vector. Because deletion of amino acids 427–452 gave rise to a nuclear distribution of the mutated protein, we also introduced deletion of the Rb or Rc domains together with deletion of amino acids 427–452 in order to analyze whether these sequences were involved in nuclear distribution of the protein. Immunofluorescence studies on cells transfected with pC4-D-(111–150) -(427–452) or pC4-D-(150–182) -(427–452) exhibited a predominant nuclear localization, although more cytoplasmic forms were observed in comparison with the pC4-D-(427–452) (Fig. *7D* and data not shown), indicating that Rb and Rc argi-nine regions only played a partial role in the nuclear localization of the protein. Similar observations were made when the arginine residues of the bZIP sequences were substituted tan-demly with the deletion of amino acids 444–452 (data not shown). These results demonstrated that the arginine residues of the bZIP-Rb-Rc region could only be partially involved in the nuclear localization of IE4 or acted only as a weak NLS.

Crm1 Is Involved in IE4 Nuclear Export—Because IE4 was predominantly found in the cytoplasm of transfected cells and some mutated IE4 proteins were exclusively found in the nucleus, we analyzed whether IE4 could utilize Crm1 as a cofactor for nuclear export. Some RNA export proteins use the exportin Crm1, a Ran-GTP-dependent transporter, to shuttle their cargo from the nucleus to the cytoplasm (31). Recently, it has been shown that ICP27 also mediated the export of some viral RNAs via a Crm1-dependent pathway, whereas other viral mRNAs are exported via another pathway (32). To determine whether nuclear export was indeed required for cytoplasmic localization of IE4, cells transfected by pC4 were treated with leptomycin B (LMB), a specific inhibitor of Crm1 that acts by blocking the formation of the NES-Crm1-Ran-GTP complex. The localization of IE4 was then analyzed by indirect immunofluorescence. Fig. 8*A* demonstrates that LMB blocks the cytoplasmic accumulation of IE4. The distribution of IE4-specific fluorescence was determined by counting immunofluorescence-positive cells and ranking them in one of the following categories: cells exhibiting exclusively nuclear or cytoplasmic staining or cells exhibiting both nuclear and cytoplasmic staining with cytoplasmic fluorescence either higher than, equal to, or lower than the nuclear fluorescence. Without LMB treatment, IE4 staining was predominantly cytoplasmic or cytoplasmic and nuclear, with staining in the nucleus weaker than in the cytoplasm (Fig. *8B)* as described previously (27). After 6 h of incubation in the presence of LMB, IE4 became predominantly nuclear or simultaneously nuclear and cytoplasmic with nuclear staining greater than or equal to the cytoplasmic staining (Fig. *8B).* To eliminate any new IE4 synthesis, cycloheximide was also included with LMB, allowing us to monitor the movement of pre-existing protein. The distribution of IE4 into trans-fected cells was similar to that observed without cycloheximide (data not shown). The fact that cytoplasmic retention of IE4 protein is partially disrupted by LMB suggests that cytoplasmic localization of IE4 requires at least nuclear export mediated by Crm1.

Published in : Journal of biological chemistry (2000), vol. 275, iss. 42, pp. 32822-32832
Status : Postprint (Author's version)

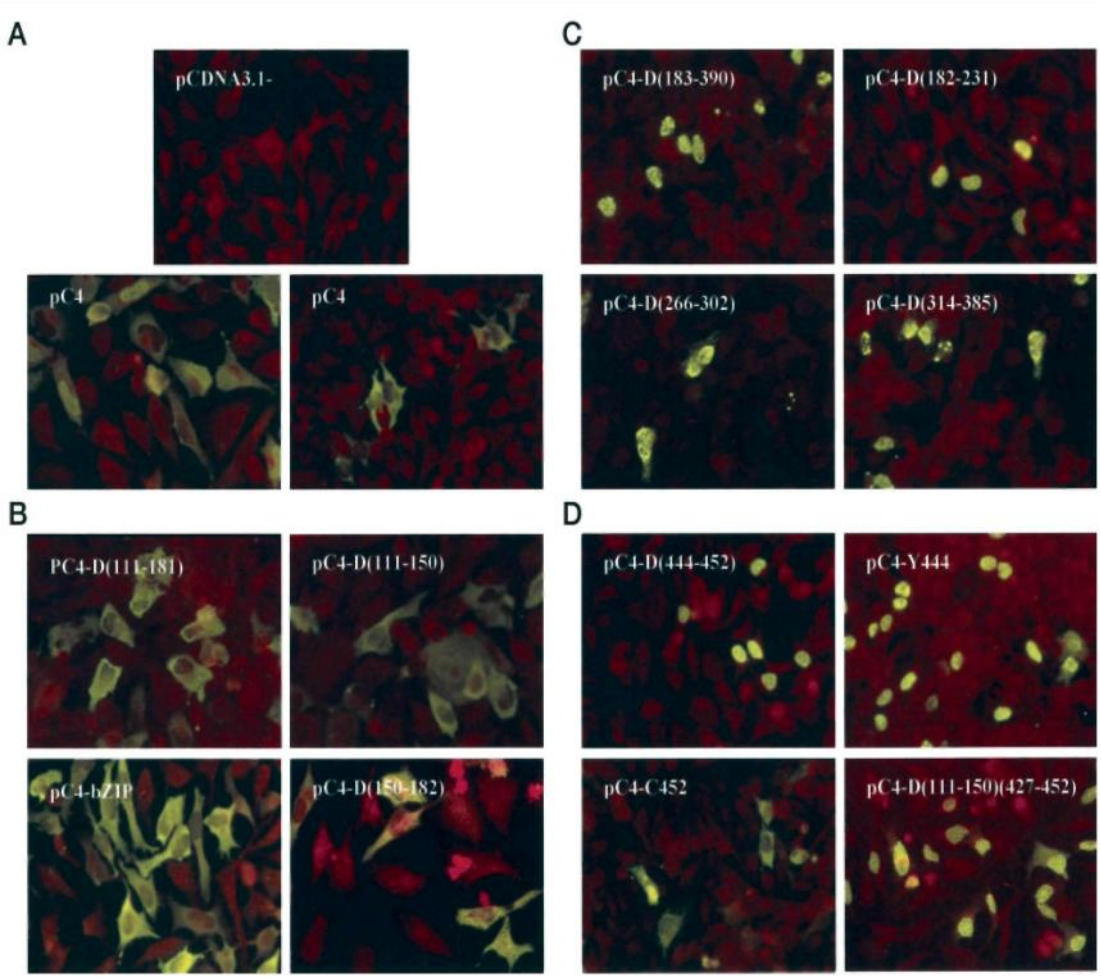

FIG. 7. Localization of wild-type and mutated IE4 proteins in HeLa cells. Cells were transfected with 2 μg of the control vector pCDNA.3.1.- or the expression plasmids encoding IE4 wt (A), amino-terminal- (B), central- (C), and (D) carboxyl-terminal-mutated IE4 proteins, as indicated on the panels, and reacted with a polyclonal antibody raised against IE4 before immunofluorescence detection.

Unexpectedly, LMB treatment did not alter the cytoplasmic localization of the deleted proteins IE4-D-(111–181) and IE4-D-(111–150) (Fig. 8*A*). In contrast, exposure to LMB of cells transfected by pC4-D-(150–182) resulted in a shift in the distribution of the deleted protein from a predominantly cytoplasmic to a nuclear localization as observed with the IE4 protein (Fig. 8*A*). Similar results were obtained with the substituted mutants into Rb or Rc regions; LMB did not modify the cyto-plasmic localization of the IE4-Rb protein, whereas it partially blocked the IE4-Rc into the nucleus as observed with the IE4 protein. These results confirm that IE4 mutated into the Rb region was not blocked by LMB because it did not reach the nucleus and that a nuclear localization signal is likely located into the Rb domain.

Discussion

Despite structural similarities (15), VZV IE4 and HSV-1 ICP27 cannot complement each other (14, 33) and act differently in transient transfection assays. IE4 transactivates a wide variety of target constructs whether expressed alone or in synergy with VZV IE62 (2–4, 12, 13, 33). In contrast, ICP27 alone has little effect, if any, and acts as a transrepressor or transactivator when co-transfected with transcriptional activators such as ICP4 and ICP0 (19, 34–37). Studies on ICP27 have shown that its expression is required for the switch from early to late virus gene expression, lately they have highlighted the multifunctional nature of this protein that acts both at the transcriptional and post-transcriptional levels (reviewed in Ref. 38). Although the molecular mechanisms underlying VZV IE4 regulatory properties are still greatly misunderstood, the available data suggest that IE4 could also exert

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Status : Postprint (Author's version)

its functions through transcriptional and post-transcriptional mechanisms (11–13, 29). Based on amino acid sequence homologies, different regions of VZV IE4 can be mapped as follows: (i) an acidic region located at the amino-terminal part of the protein; (ii) an arginine-rich region, also located near the amino terminus, having limited amino acid homology with other herpesvirus family members; (iii) a central region; and (iv) a zinc finger-like sequence located close to the carboxyl terminus with the last two regions sharing considerable amino acid conservation.

In the present study, we have attempted to dissect the functional domains of the VZV IE4 protein that are important for gene activation. By using the mammalian two-hybrid system, we found that VZV IE4 is capable of homodimerization. We have shown that an intact carboxyl-terminal cysteine-rich region as well as the central portion of the protein are required for this interaction. Moreover, these two regions seem involved in the correct intracellular localization of the protein. Previous studies have demonstrated that IE4 may have several mechanisms of action. Activation of VZV genes encoding the thymi-dine kinase or IE62 seems to occur partly by a post-transcrip-tional mechanism, whereas stimulation of a heterologous promoter such as HIV-1 LTR or the cytomegalovirus promoter seems to involve a transcriptional mechanism (11, 13).

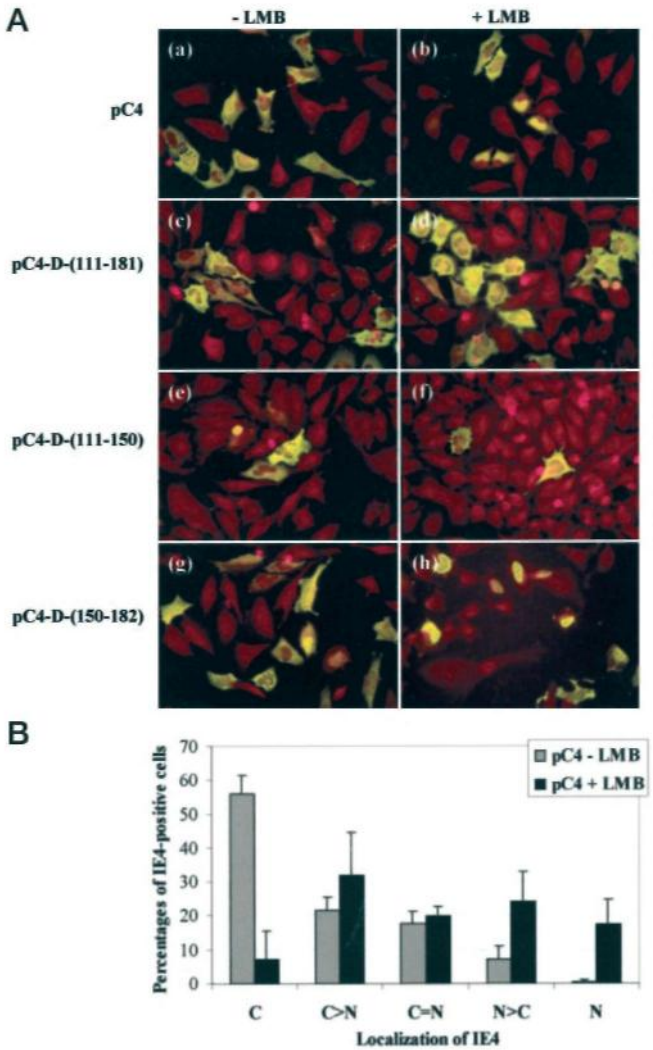

FIG. 8. Shuttling of IE4 is partially mediated by the Crm1-de-pendent nuclear export pathway. A, HeLa cells were transfected with 2 – g of the expression plasmids encoding IE4 wt (a and b), IE4-D-(11l-181) (c and d), IE4-D-(111-150) (e andf), or IE4-D-(150-182) (g and h) as indicated. 48 h post-transfection, cells were incubated with (+ LMB) (b, d, f, and h) or without (- LMB) (a, c, e, andg) LMB for 6 h. Cells were then fixed, and the intracellular distribution of IE4 was examined with an antibody specific for IE4 and a fluorescein isothio-cyanate-labeled secondary antibody. B, percentage of IE4-positive cells in each localization pattern after transfection of HeLa cells by pC4 treated (pC4 + LMB) or not (pC4 — LMB) with LMB. Immunofluores-cence-positive cells were counted and categorized in one of five groups as follows: cells that exhibited only cytoplasmic staining (C); cytoplasmic staining

higher (ON), equal (C=N), or lower (N>C) than nuclear staining; or nuclear staining only (N). The average percentage of cells belonging to each category was calculated from three independent transfection experiments, and error bars represent standard errors.

In order to analyze the domains of the protein implicated in these two mechanisms, we have tested the ability of a variety of mutants to transactivate the VZV TK promoter and the HIV-1 LTR in transient expression experiments. We showed that the domains turned out to be similar for stimulation of the two promoters and that, in addition to the two regions needed for dimerization, the arginine-rich region is also required.

The acidic region at the amino terminus of the protein is not essential for dimerization or for transactivation of the two promoters as we expected from our previous study, which demonstrated that this region was not involved in dimerization nor in HIV-1 LTR transactivation (29). This is in accordance with a previous report proposing that this region was not required for activation of a reporter gene carrying an efficient polyadenyl-ation signal, whereas it was essential for transactivation of a reporter gene carrying a minimal polyadenylation signal (14). Our work also confirmed that the acidic amino-terminal region was not involved in the proper addressing of the protein. Although the amino-terminal regions of VZV IE4, HSV-1 ICP27 (14, 15), equine herpesvirus 1 (EHV-1) UL3 (39), and their γ-herpesvirus homologs (40, 41) share little amino acid homology, they are all acidic. In contrast to the acidic amino-terminal region of VZV IE4, the corresponding region of ICP27 has previously been shown to be important for trans-regulatory functions in transient expression assays (14, 42), as well as for full viral replication (19, 42–44). Whereas these IE4 and ICP27 regions can functionally substitute for each other in transient expression assays, the IE4 acidic region cannot efficiently complement an HSV-1 mutant virus expressing ICP27 lacking this domain (14). Recent data have revealed that ICP27 shuttles between the nucleus and the cytoplasm at late times postinfection through a leucine-rich nuclear export signal located between residues 7 and 15 (23, 25). It should be pointed out that no similar sequence has been found in the amino-terminal extremity of the IE4 protein.

Previous studies have shown that the basic domain located just next to the acidic domain of VZV IE4 could be implicated in dimerization and transactivation properties (14, 29). This domain has been divided into three sequences rich in arginine residues called Ra, Rb, and Rc located between amino acids 71–80, 112–143, and 164–179, respectively. Our present data suggest that Ra, which has no counterpart in the ICP27 sequence, does not seem to play any role in transactivation nor in dimerization, as previously shown (29). The Rb sequence does not appear to be implicated in dimerization, whereas Rc may have a partial effect in this property. Computer analysis has revealed the presence of three bZIP-like domains between residues 115 and 122 in the Rb region (29). However, our results showed that these motives were not essential for the dimeriza-tion of IE4, even though they have been shown to be required for the dimerization of many transcription factors (45). On the other hand, Rb and, to a lesser extent Rc, seem to be needed for a full transactivation of the VZV TK promoter and the HIV-1 LTR. ICP27 also contains an arginine-rich domain that is divided into two sequences called R1 and R2, which showed some similarities to the IE4 Rb sequence, and that was essential for the regulatory properties of the HSV-1 protein (24). ICP27 is able to mediate export of viral intronless mRNAs and to bind *in vivo* to RNA requiring the R1 sequence, an arginine-glycine-rich region that resembles an RGG box (23). Moreover, it was shown recently that ICP27 could interact with two cellular proteins, the heterogenous nuclear ribonucleoprotein K (hnRNP K) and the B subunit of casein kinase 2 (46). The ICP27 region required for these interactions did not include the RGG box (R1) domain but the adjacent arginine-rich R2 sequence. Because the IE4 Rb sequence seems to be essential for regulatory properties, we can postulate that this sequence could be implicated in protein-protein interactions. This hypothesis is also supported by a previous report showing that IE4 could interact with the TATA-binding protein and transcription factor IIB of the basal complex of transcription as well as with the p50 and p65 NF-κB subunits (29). The pull-down experiments presented above confirm that the interactions with TFIIB and p50 could be mediated at least by the arginine-rich domain encompassing the Rb and Rc domains. Although protein-protein interactions through the arginine-rich domain are not usual in gene regulation, it was recently demonstrated that interactions between two proteins important in the outcome of Fanconi anemia occurred in the cell nucleus through an unusual arginine-rich interaction domain (47).

The carboxyl-terminal region of VZV IE4 seems to be crucial for dimerization, transactivation, and correct cellular localization as shown with the various deletion mutants tested in this study. In particular, substitutions within the carboxyl-terminal domain showed that a GKYFKC peptide located between residues 442 and 447 was crucial for dimerization and transacti-vation. Mutation of the Lys residue (residue 504) in the hom-olog region in ICP27 led to the loss of interaction with the small

nuclear ribonucleoprotein particles (48), demonstrating the role of this hexapeptide region in protein-protein interactions. Although this carboxyl-terminal region of VZV IE4 contains cysteine and histidine residues, it is not known whether this region binds zinc or whether it forms a potential zinc finger domain. Recent studies have revealed that ICP27 self-associates *in vivo* and that the carboxyl-terminal region beginning at residues past 480 and extending to position 508 must be intact for multimerization to occur, although the internal region encompassing amino acids 288–444 may have partial effects on dimerization, as demonstrated by co-immunoprecipitation assays (17).

In this study, we have unambiguously demonstrated that the central region of VZV IE4 was also important for dimerization, as well as for proper intracellular localization and gene activation. Reintroduction of a irrelevant VZV sequence within the deleted VZV*IE4* (pM4-D-(266–302)) gene did not allow recovery of efficient homodimerization (data not shown), demonstrating that the central region of IE4 was involved in the homodimerization process and that the size of the deletion would not have affected the distance between functional domains of VZV IE4. Recently, computer analysis of the central and carboxyl-terminal sequences of ICP27 revealed three KH-like RNA binding motifs, as well as an SM protein-protein interaction motif, which are very well conserved among ho-mologs from other α-herpesviruses (32). The KH motifs were first identified in the human heterogeneous nuclear ribonucle-oprotein (hnRNP) K protein as a triple repeat (49). Mutations of the KH-like motifs into ICP27 resulted in lethal phenotypes, and the authors (32) established that substitution into the KH3 domain affected RNA binding *in vivo* as well as nuclear export of ICP27. They proposed a model in which KH1 and KH3 domains interact with each other. These interactions could be important to form a structure that can interact with RNA, and RNA binding could be a prerequisite for nuclear export of ICP27. Alternatively, the KH domains might interact with other proteins, as snRNPs (48) or casein kinase 2 (46). Moreover, the central region of ICP27 (amino acids 179–406 encompassing KH1- and KH3-like motifs), without the zinc fingerlike domain, has also been directly implicated in the interaction with ICP4, an essential regulatory protein of HSV-1 (50), confirming that this region could be implicated in multiple protein-protein interactions. Deletions introduced into the central part or the carboxyl terminus of IE4 overlap some potential KH-like motifs. The various deletions introduced into IE4, *i.e.* IE4-D-(182–231), IE4-D-(314–385), and IE4-D0(394–452), disrupt the KH1-, KH2-, or KH3-like motifs localized between residues 181–258, 303–360, and 392–452, respectively (32). Therefore, it is obvious that these motifs could play an essential role in the IE4 dimerization property as well as in the proper intracellular localization of the protein.

On the other hand, the proper localization of the IE4 protein seems to be crucial for the regulatory properties because mutations into the Rb regions gave rise to a cytoplasmic distribution of the mutated proteins and mutations into the central and carboxyl-terminal parts also led to a nuclear distribution, with a loss of the regulatory properties for all of these mutants. We thus hypothesized that the regulatory properties of IE4 could be mediated by the shuttling of the protein between the cytoplasm and the nucleus. The fact that cytoplasmic retention of IE4 protein is partially disrupted by LMB suggests that cyto-plasmic localization of IE4 requires at least nuclear export mediated by Crm1, but another transport pathway cannot be excluded. Protein trafficking into and out of the nucleus normally occurs through direct interaction between a transport signal on the protein and the transport receptors that mediated passage through the nuclear pore complex. In many cases, nucleocytoplasmic shuttling is accomplished by the combined actions of an NLS for import and a distinct NES for export (for reviews see Refs. 51 and 52). The observation that the VZV IE4 deleted in either the central or the carboxyl-terminal regions was localized into the nucleus argues that VZV IE4 could possess a nuclear localization signal. This potential signal for nuclear localization could be located within Rb region, since mutation within this domain seemed to exclude the protein from the nucleus. Experiments with LMB have confirmed that an NLS is located into the Rb domain because the proteins mutated into the Rb region remains cytoplasmic even in the presence of LMB. Classical NLSs consist usually of one or more clusters of basic amino acids (51). Therefore, the sequence RKHRDRRSLSNRRRR into the IE4 Rb domain could be a good candidate for NLS. Unexpectedly, only partial relocalization of carboxyl-terminally truncated VZV IE4 within the cytoplasm was observed when the Rb region has been deleted, indicating that IE4 contains at least another nuclear localization signal. On the other hand, we cannot exclude the possibility that another mechanism of import exists for the nuclear mutated IE4 proteins. Because these mutants have also lost their dimer-ization property, we can postulate that they reach the nucleus by passive diffusion, as is the case for the mitogen-activated protein kinase, in which dimeric and monomeric forms enter the nucleus by active transport and passive diffusion mechanisms, respectively (53).

In this paper, we have shown that IE4 utilizes, at least, Crm1 as a cofactor for nuclear export. Some proteins, especially some RNA export proteins, use the exportin Crm1, a Ran-GTP-dependent transporter, to shuttle their cargo from the nucleus to the cytoplasm through a leucine-rich NES (31).

Recently, it has been shown that ICP27 also mediated the export of some virus RNAs via a Crm1-dependent pathway, whereas other virus mRNAs are exported via another pathway (32). The shuttling of ICP27 between the nucleus and the cytoplasm at late times post-infection occurs through a leucine-rich NES located in the amino-terminal part (23, 25) but also requires the central and carboxyl-terminal regions that have been demonstrated to interact with RNA, a prerequisite for nuclear export (32). No NES has been found in the amino-terminal part, but a putative sequence rich in leucine residues (^{321}LLEN-LKLKLG^{330}) was found into the KH2-like motif in the central part of the protein and could be correspond to an NES. However, shuttling can also be controlled by an emerging class of transport signals known as nucleocytoplasmic shuttling signals that can direct both nuclear import and export (for review see Ref. 54). All proteins currently known to contain this type of signal also associate with mRNA. An example of this is the hnRNP A1 protein that, by virtue of its M9 domain, is actively exported from the nucleus and imported into the nucleus via a novel pathway mediated by the transportin protein. The hnRNPK protein contains also a nucleocytoplasmic shuttling signal of 24 residues, called KNS, in addition to a classical bipartite-basic NLS (55). This signal is localized between the KH2 and KH3 domains implicated in RNA-binding activity described above. Moreover, KNS appears to mediate export via a Crm1-independent pathway (28). Curiously this sequence exhibits some similarities with a sequence mapped between the KH2- and KH3-like motifs found into IE4, which is very well conserved among α-herpesvirus homolog proteins. Particularly the tetrapeptide SADE is perfectly conserved between KNS and IE4, and specific serines and acidic residues seem to be necessary for the KNS activity (28). We can thus postulate that IE4 also uses this type of newly described nucleocytoplasmic shuttling signal that confers bi-directional transport across the nuclear envelope and which is insensitive to LMB treatment. On the other hand, because deletions into the central and carboxyl-terminal parts of the protein affect the nuclear export, we cannot exclude the possibility that RNA binding of IE4 could also be a prerequisite for nuclear export, as is the case for ICP27. Moreover, these mutations also affect the dimerization property of the protein, allowing us to postulate that IE4 could bind RNA as a dimer before being exported into the cytoplasm. Future work will be necessary to confirm this hypothesis and also to test the RNA binding property of IE4.
VZV IE4 has been shown to be a multifunctional protein. Here, we have presented strong biochemical evidence demonstrating that IE4 self-associates *in vivo* and that IE4 ho-modimerization mainly occurs through the central and the carboxyl-terminal regions beginning at residues past 182 and extending to position 447. Amino acid substitution within the carboxyl-terminal domain showed that a GKYFKC peptide was crucial for self-interaction. Regions of IE4 important for dimer-ization were also shown to be crucial for transactivation and for proper intracellular localization of IE4. In addition, the argin-ine-rich domains Rb and, to a lesser extent, Rc were also demonstrated to be important for transactivation but not for homodimerization, whereas the amino-terminal sequence encompassing the acidic sequence, the first arginine-rich domain Ra, and the bZIP-like sequences were shown to be dispensable for gene transactivation. A Crm1-dependent nuclear export mechanism has also been shown to be important for the cellular localization of IE4.

Acknowledgments

We thank U. Siebenlist (National Institutes of Health, Bethesda), E. Verdin (Gladstone Institute, San Francisco), and M. Mu¨ller (University of Liege) for providing plasmids.

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